Studies on the genetic transformation of pigeonpea by biolistic and *Agrobacterium*-mediated gene transfer

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CERTIFICATE

This is to certify that the thesis entitled **Studies on the genetic transformation of pigeonpea by biolistic and *Agrobacterium* - mediated gene transfer,** submitted in partial fulfillment of the requirements for the Degree of Master of Technology is a record of the bonafide research work carried out by **Mr. R. V. RAMCHANDAR,** under our guidance. The results embodied in this dissertation have not been submitted to any other university or institution for the award of any degree or diploma.

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CONTENTS

1. INTRODUCTION

When farmers began harvesting the first domesticated plants in about 8000 B.C. the human population was estimated to be four million (Kung, 1993). Presently released United Nations report of world population in future estimated that at the end of 1998 world population was 5900 million (590 crore). It will cross 900 crore by the year 2050 A.D. 1080 Crore and 1100 crore at the end of 2150 A.D. and 2200 A.D. respectively (U.N.Report, 1999). Food production in world is increasing at arithmetic progression, while population is growing at geometric progression. We will have to produce as much food in the first half of the 21st century as was produced over the past 10000 years. Techniques for selecting high yielding varieties have been developed over centuries of human history since river valley civilizations to modern Green Revolution. Conventional plant breeding techniques have resulted in dramatic crop improvement (Borlaug, 1983, Goodman *et al.,* 1987) and will continue in the future. By the late 1960s use of chemical fertilizers, pesticides, agriculture machinery, irrigation facilities, intercropping, multiple cropping, land management, green manuring and production of high yielding varieties by breeding were responsible for the first Green Revolution.

The first Green Revolution which began in the sixties doubled food grain production. Steady progress in crop yields is expected from current plant breeding procedures. However, advances are not sufficient to meet future food demands of ever growing population.

It is true that a great deal has been and will continue to be achieved by conventional breeding programmes. In general, the breeder aims to introduce a desirable gene into a plant which already has many attractive attributes. This is done by crossing that plant with another which contains the desired gene, but is in many other respects less than perfect. The hope is that the progeny of such a cross will combine the good features of the first plant with the desired gene of the second. Unfortunately, the chances of such a perfect combination of genes occuring tend to be extremely small. On the contrary, there is just as much likelihood of obtaining progeny with only the undesirable features of each parent. Consequently the breeder

has to select the progeny that contains the desired gene and have also retained many of the good features of the first parent. These progeny plants should be back crossed with the first parent (elite plant) to remove unwanted traits. Moreover a typical breeding scheme involves numerous back crosses lasting at least 10-15 years, even when strategies are used to stack the odds in breeder's favour. The most rapid progress can be made when the plants are already very similar, since this will reduce the number of back crosses needed (Trevan *et al.*,1998).

However the most useful genes tend to be found in different plants or organisms. Unfortunately, fertilization can only be achieved between few species or types in most of the cases. Hence, classical breeding is limited by sexual compatibility (Kung, 1993). This results in a very limited gene pool (when compared to the total gene pool of all different organisms) being available for conventional plant breeding involving sexual crosses.

The Green Revolution is considered to have resulted, at least in part, from the application of Mendelian genetics to crop improvement. During the 1960's, it was realized that the production of grains of the Green Revolution would be over come by the increase in the world population with in a few decades. There fore, the development of alternate strategies for increasing plant productivity were considered to be essential.

The advances made in biotechnology facilitate the transfer of cloned and well defined genes into plant cells, the identification and characterization of regulatory sequences present in plant genes and the regeneration of intact and fertile plants from a single transformed cell.

Discovery of genetic laws (Mendel, 1864), transformation (Griffith 1928), nature of transforming principle (Avery *et al.*, 1944), elucidation of DNA structure (Watson and Crick ,1953), discovery of plasmid (Akiba and Ochiai, 1959), restriction enzymes (Smith and Nathans, 1970), production of recombinant DNA (Cohen, *et al.*, 1973), discovery of Ti plasmid (Schell, 1974) and production of transgenic plants (Schell, 1983) were milestones of scientific and technological developments which are essential to the advancement from the first Green Revolution to the anticipated second Green Revolution or Gene Revolution.

Since cloned genes can be transferred directly or indirectly to the plant cells by different methods, there is no limitation with respect to the gene pool available. Additionally, well defined small groups of genes encoding defined and desired traits can be transferred. This can enable the elite plant to keep all its good characteristics intact. Moreover, this may not involve back crosses to remove undesired characteristics and may not require years of time, labour consuming efforts. Genetic engineering is responsible for crop improvements at the molecular level, produces plants with greater ranges of genetic variabilities.

Foreign genes were first introduced into tobacco and petunia in 1983 (Bevan *et al.*,1983, Herrera - Estrella *et al.*, 1983 Fraley *et al.*, 1983). Scores of plants that include fruit trees, vegetables and grain crops have since, been transformed in order to bring about change in the genetic make up, that could spawn a biological revolution (Narayanswamy, 1994). The genetically transformed crops acquire new traits such as pest resistance and tolerance to herbicides. More exciting possibilities would be the addition of protein staples like corn, changing the type of oil produced by soybean, producing naturally decaffeinated coffee beans etc. Plants could be vaccinated against diseases by the introduction of viral genes so that chemical insecticides could be eliminated or less relied upon (Narayanaswamy 1994). "Bio-engineering is intended to be more a boon than a bane to mankind " (Nash 1990).

Pigeonpea is an important pulse crop in the semi arid tropics and ranks fifth among the edible legumes. Pigeonpea is a high protein grain legume and caters the protein requirement of the majority of the population in the Indian subcontinent (George *et al.*, 1994). It is a source of protein for 80% vegetarians in India.

Improvement of this crop by classical breeding techniques has already achieved. Steady progress in crop yields as expected from current plant breeding experiments. These advances in breeding cannot meet future demands. An integrated approach of genetic manipulation of crop along with breeding programmes are needed for enhancement of pigeonpea germplasm. Various techniques such as *Agrobacterium*- mediated gene transfer, viral vectors, direct gene transfer to protoplasts, biolistics or particle gun and microinjection into zygotic and microspore derived proembryos, pollen transformation, pollentube

pathway, macro-injection, electroporation, liposome fusion, liposome injection and microlaser were used to generate transgenic plants (Potrykus, 1991). Of the various approaches to integrative transformation *Agrobacterium*-mediated gene transfer and direct gene transfer to protoplasts are routine and efficient methods (Potrykus, 1991). *Agrobacterium* mediated gene transfer and particle bombardment into multiple shoot initials are two approaches of genetic transformation expected to be applied to pigeonpea in the present studies. Various potential means for genetic engineering of pigeonpea include herbicide tolerance (Comai *et al.*, 1985) and insect resistance (Chakrabarti *et al.*, 1996, Kumar *et al.*,1996), bacterial resistance (Anzai *et al.*, 1989), water stress tolerance, salt tolerance, cold sensitivity, and improvement of its overall nutritional quality such as increasing contents of methionine and lysine, other sulfur containing amino acids (Altenbach *et al.*, 1989).

Engineering pigeonpea plants with insecticidal crystal protein genes from *Bacillus thuringiensis* (Bt) will be very active against pod borers. Genetically modified plants with Bt gene will complement the existing pest control strategies and may generate substantial economic and ecological benefits. Many bruchids are storage pests. They cause extensive damage during storage. In some cases damage is also done even in fields by cowpea weevil (*Callosobruchus chinensis)*. Transfer of bruchid resistance from the common bean to pigeonpea with the alpha-amylase inhibitor gene by genetic engineering protects plants from the attack of old world bruchids (*Bruchus pisorum, Callosobruchus maculatus* etc) (Ishimoto *et al.*, 1996, Chrispeels, 1997).

Serine protease inhibitors are toxic to *Helicoverpa armigera* (pod-borer) (Johnston *et al.*,1993). Transgenic pigeonpea plants expressing protease inhibitors will be protected from the *pod-borer* (Hilder *et al.*, 1987, Johnson *et al.*, 1989).

In the present study an attempt was made to transform and regenerate pigeonpea from multiple shoot initials by using *Agrobacterium* and biolistic mediated approaches. This type of studies forms the basis for the future developments in pigeonpea transformation.

2. OBJECTIVES

The present studies were carried out with the following objectives:

1. Production of transgenic pigeonpea plants expressing β- glucuronidase (GUS) and hygromycin phosphotransferase (*HPT*) genes by *Agrobacterium* - mediated transformation.
2. Isolation of plasmids from *Agrobacterium* and bombarding multiple shoot initials with plasmids containing marker genes by using the biolistic device.
3. Determining the dose of hygromycin required to select transformants.
4. Analysis of putative transformants with GUS histochemical assay.
5. Determining the suitable DNA isolation method from pigeonpea.

3. REVIEW OF LITERATURE

3.1 Pigeonpea

Food legumes are important economically and a rich source of protein, calories, vitamins and minerals in the diet of many individuals in developed and developing countries. *'Cajanus cajan'* also known as pigeonpea or red gram or arhar, belongs to a monotypic genus of the subtribe cajaninae of tribe phaseolae in the family fabaceae (Bentham and Hooker, 1865). Pigeonpea is an important pulse crop in the semi-arid tropics and ranks fifth in area grown among the edible legumes of the world (Nene *et al.,* 1990). Pigeonpea is grown in about fifty countries of Asia, Africa and America, the major growers among them are India, Kenya, Malawi, Uganda, Thailand, Indonesia, Philippines (Kamble *et al.,* 1998). India alone contributes over 90% of the world pigeonpea production. Pigeonpea is a major staple grain legume of India grown on 3.44 million ha with production of 2.72 million tonnes in 1993-94 (Perane, *1997).*

Remains of pigeonpea were discovered in Egyptian tombs which belonged to 2200-2400 B.C. It is assumed that traders carried it to India. The earliest mention of pigeonpea as tuwari is in the text "gatha saptasati" written by Hala in second century A.D. This implies that pigeonpea cultivation was practiced in India even 2100 years back. Joseph Hooker in his flora of British India (1872-97) reported pigeonpea as cultivated upto elevations of 2000 meter in the Himalayas.

Pigeonpea can withstand drought due to its deep and extensive root system, which provides access to water stored deep in the soil profile (Sheldrake *et al.,* 1979). It can also endure periods of water stress as a result of relatively high levels of desiccation to the tolerance and osmotic adjustment (Flower *et al.,* 1987). Pigeonpea is important crop in semi-arid tropics because of its draught tolerance and production of high yields in years when other crops fail. Pigeonpea ranks fifth among the most important legume crops of the world after beans, peas, chick peas,

broad beans and is second most important food legume in India. It is recognized to produce heavy crops of protein rich seeds even on low fertility soils and offers a vast potential over a wide range of climatic and soil conditions in the tropics and performs best on areas where annual rainfall ranges from 500-1500 mm/yr, and where the soils are drained.

Pigeonpea is grown in a number of traditional cropping systems in India. The majority of the production is based on inter crop systems with a cereal (sorghum or millet), where pigeonpea grows on residual soil moisture and is harvested after the cereal main crop some 7-11 months after sowing. Pigeonpea is generally grown as a relatively long season crop with or without inter-cropping, although short duration varieties have also been developed.

Pigeonpea contains 17-28% protein and therefore plays a major role in supplementing the diets of the majority of the population in areas where low protein roots and cereal crops constitute the major part of the diet. Pigeonpea also helps in improving soil fertility by fixing atmospheric nitrogen and cycling nutrients and contributing to sustainability. Dry seeds are used widely in India as dry split pea for dahl preparation for human consumption. In the Carabbean region, the pea is consumed as green vegetable (Nene *et al.*, 1990).

Various specific applications of the pigeonpea crop are as follows

1. Soil ameliorant : Pigeonpea is a leguminous crop which fixes atmospheric nitrogen into available nitrogen in the soil. This process benefits the crop which is inter-cropped with pigeonpea and the subsequent crops. N_2 fixation ability in pigeonpea is estimated as 40 Kg of nitrogen per hectare (Kumar Rao *et al.*, 1981).

2. Human food : Pigeonpea seeds can be processed into dahl, which is most acceptable form of pigeonpea. Whole seeds of pigeonpea are used in various traditional foods and as a snack in Eastern Africa, Western India and Indonesia. Other food items such as tempe, ketchup and noodles can be used as vegetables. Tender pods can be consumed as salads (Joseph and Saxena, 1996).

3. Animal feed : Pigeonpea produces about 20-25 tonnes per ha of edible forage at a time of the year when there is a fodder deficit for animals. The remaining

portion after milling seeds, consists of broken seeds, unfilled seeds, seed powder, seed bran which is a good supplement for protein(Nene *et al.*, 1990).

4. Other plant products : Dried stem can be used as a fuel for rural poor. The energy value of this fuel is about one half that of same weight of coal (Panikar 1950). Lac and silk are produced by using pigeonpea. Its leaves are fed to silkworm (*Boroceras cajani*) in souhern Madagaskar to produce silk (Watt, 1908). Pigeonpea plants are used to cultivate scale insects (*Laccifera lacca*) in Philippines and China and India which produce lac. Various parts of pigeonpea plant are used as folk medicines (Morton, 1976) and as medicine for patients, who are suffering from sickle cell anaemia (Ekeke and Shade 1985).

5. Good crop for eroded soil on slopy lands : Eroded soils with very poor organicmatter and nutrients are not suitable to raise most crops. Pigeonpea performs well in this type by reducing soil erosion, bind up the soils and provide yields ranging from 0.5 – 0.8 / ha. (Joseph and Saxena, 1996).

6 Good crop for draught regions : Pigeonpea is a draught tolerant crop, deep root system penetrates the hard pan of the soil which allow water uptake from deeper regions (Joseph and Saxena, 1996, Sheldrake *et al.*, 1979).

The major biotic constraints in reducing the yields of pigeonpea are pod-borers, plume moths and agromyzed fly maggots. One of the major constraint identified is gram pod borer (*Helicoverpa armigera*), as there is not a single variety which can resist the fore said pest incidence in rainy season (Reddy *et al.*, 1998). Of the various factors responsible for the low yield of crop in the country the pod-borer complex is of significance. In peninsular India, early and medium maturing pigeonpea varieties suffer 55% pod damage solely due to the attack of pod-borer complex (Anonymous, 1974). According to Rajagopalan and Kumar (1985) early and medium maturing varieties suffer 45 and 55% pod damage and 23 and 30% seed damage due to the pod-borer respectively.

The pod-borer complex infesting early maturing varieties of pigeonpea and its reproductive phase reported to bring significant reduction in grain yield upto the extent of 60-90% under different agro-ecological conditions (Anonymous, 1974). Among the pod-borers, gram pod-borer (*Helicovepa armigera*), plume moth

(*Exealastis atmosa)* and pod fly (*Melanogromyza abtusa malloch)* the turpod bug (*Clavigralla gibbosa spinola*) referred to as pod borer complex and mainly responsible for the loss of yield (Chaudhary *et al.*,1980, Sing and Rai, 1985 Thakur *et al.,* 1983). Sehgal and Ujagar (1985) reported cowpea pod borer (*Maruca testulasis)* though a sporadic pest causes heavy loss in seed yield to early maturing varieties of pigeonpea.

Engineering pigeonpea plants with *Bacillus thuriengiensis* crystal protein genes (Bt) will be very effective against pod-borers. Bt transgenic plants can complement with existing pest control strategies and may generate substantial economic and ecological benefits to farmers. Many bruchids are storage pests and cause extensive damage during storage. In some cases damage is also done even in fields by cowpea weevil (*Callosobruchus chinensis).* Transfer of bruchid resistance from the common bean to pigeonpea with the alpha-amylase inhibitor gene by genetic engineering protects plants from the attack of old world bruchids (*Bruchus pisorum, Callosobruchus maculatus* etc), (Ishimoto *et al.,* 1996, Chrispeels, 1997).

Serine protease inhibitors are toxic to *Helicoverpa armigera* (Johnston *et al.,*1993). There have also been several studies aimed at creating trangenic plants with increased resistance to insect pests. Hilder *et al.*, (1987) transformed tobacco with a gene that encodes a cowpea trypsin inhibitor and reported that transgenic plants showed resistance to tobacco bud worm *Heliothis virescens,* and to corn earth worm *Helicoverpa zea.* Zhao *et al.,* (1996) produced transgenic tobacco plants expressing CPTI and toxin gene from *B. thuringiensis.* Bio assays of transgenic plants containing both genes had chanced toxicity to *Helicoverpa armigira* larvae compared to plants that had either of the individual genes (Reek, 1997). Pigeonpea plants expressing SBTI or other serine protease inhibitors is expected to be protected from the damage caused by *Helicoverpa.*

3.2. Pigeonpea tissue culture

Crop improvement in pigeonpea is possible using biotechnological/genetic engineering techniques. Successful regeneration and transformation protocols are a prerequisite for genetic manipulation. Construction of an ideal and suitable regeneration system is first step in this process. Regeneration without

transformation and transformation without regeneration does not have any value in transgenic plant technology. Pigeon pea is one of the most important legume crop and is notoriously recalcitrant to regenerate from tissue culture (George *et al.*, 1994, Geeta *et al.*, 1998). Much effort has to be devoted to develop and optimize efficient in vitro regeneration systems to facilitate a variety of technologies including transformation of pigeonpea with particle bombardment and *Agrobacterium*-mediated genetic transformation.

Shama Rao and Narayanswamy (1975) reported regeneration of pigeon pea from callus cultures of hypocotyls obtained from gamma irradiated seeds, but failed to observe in unirradiated controls. Cultural conditions for regeneration from callus of leaves and cotyledons were first reported by Kumar *et al.*, (1980) with an emphasis on creating genetic diversity in this crop. They reported production of 5-18 shoot buds from excised cotyledons of pigeonpea when cultured on BAP (2.25 mg/l) containing medium.

Mehta and Mohan Ram (1983) reported formation of 5-35 shoot buds from the surface of cotyledones of the seedlings raised on Gamborg B5 medium (Gamborg *et al.*, 1968) supplemented with BAP (2.4 mg /l). They observed that when the seeds were cultured on B5 media with BAP (2.4 mg/l), the cotyledons expanded, initiation of callusing was observed in 3 to 4 weeks. In 70 to 80% of the cultures, small green dome like structures appeared from the cotyledonary surface at several loci. These developed into shoot buds. These were elongated and subsequently rooting was induced by transferring it to B5 medium with NAA (1 mg/l).

George and Eapen (1994) reported formation of embryos from immature cotyledons of MS media supplemented with 2-4 D, picloram or NAA (5mg /l). These were later transferred to MS containing 3% mannitol. Mannitol favoured development. They obtained plantlets on subsequent transfer to MS media containing zeatin (0.1 mg /l), G.A. (1 mg/l), ABA (0.25mg/l) and silver nitrate (10mg/l). They reported the formation of shoot buds either singly or in clusters from the abaxial surface at the distal end of mature cotyledons on MS media supplemented with BA (1 mg/l) and IAA (0.1 mg/l). These buds are after 2-3 subculture produced a mean of 6 shoots per explant. They also reported formation

of callus from primary leaf segment of 8-10 old seedlings on BA (2 or 5 mg/l) and IAA (0.1 mg/l). However numerous shoots were originated from submerged region of callus in 36% of the cultures after 4 weeks. Subculture of root bearing callus to fresh MS medum with 1 mg/l BA resulted in development of about 8 well developed shoots per culture. They reported that mature seeds on MS medium containing 10 or 15 mg/l BA resulted in production of shoot primordia (30-35 per explant) from the nodal region of 80-85% of the seedings. However upon transfer to MS with BA (1mg/l- and IAA (0.1 mg/l) only upto 11 shoots were obtained from single explant.

Prakash *et al.*, (1994) reported formation of multiple shoots from cotyledonary node when seeds were placed on MS medium containing 3% sucrose and BAP (1-10 mg/l). The seeds placed on MS medium containing 5 mg/l BAP produced maximum (45.7) mean number of shoots. The multiple shoots were excised from cotyledons and transferred after 12 days to MS media containing 2 mg/l BAP. Then the mass of shoot initials was transferred to MS basal medium for elongation. After a week, the shoots (5-6 cm long) were excised and cultured on MS medium supplemented with IBA (0.1- 5 mg/l) for rooting. Then they were transferred to soil. Plants showed normal morphological character and growth. Complete regeneration of plants under invitro conditions took only 50 days.

Chintapalli *et al.*, (1994) reported organogenesis from 6 day old seedling cotyledons. Cotyledons were placed on L2 medium supplemented with 2mg/l of BA. Multiple shoots produced were subcultured at 3 week intervals on maintenance medium L2, supplemented with 0.1mg/l BA and 2% sucrose, 1% activated charcoal. Transfer of these elongated shoots to hormone free L2 medium produced roots.

Eapen and George (1993) reported plant regeneration from leaf discs (10-12) day old seeding). They placed on MS media supplemented with BA (1.13 mg/l) either alone or in combination with IAA, IAA aspartic acid. They reported 14 shoot buds for regenerating plants per explant. The callus cultures with shoot buds were grown for 2-3 passages on medium with 1.13 mg/l BA well developed shoot buds were rooted on half strength MS medium with 0.2mg/l NAA and about 25 plants were transplanted to soil.

Geeta *et al.,* (1998) cultured seeds on MS basal agar and placed cotyledonory nodes, epicotyl, hypocotyl, leaf cotyledons on MS media augmented with BAP or kinetin (from 7 day old seedling). Cotyledonary node explants showed highest shoot bud generation than epicotyl, hypocotyl segments. BAP at concentration of 2 mg/l produced highest number of shoot buds (93.2%). Kinetin at 2 mg/l produced highest number of multiple shoots (75.4%). These multiple shoots were later transferred to MS medium containing BAP and low levels of NAA (0.01-1mg/l). This medium enhanced multiple shoots formation and elongation of shoots. Optimum concentration of NAA that produced multiple shoots was 0.01mg/l. The optimal BAP concentration for elongation of shoots is 1 mg/l. Elongated plants were rooted on subsequent transfer to rooting media, MS containing IAA, NAA, IBA. MS media supplemented with 0.2 mg/l IBA showed best and responsible for 92% of rooting.

Mohan and Krishna murthy (1998) reported de novo organogenesis from the distal half of cotyledon explants that lack pre-existing meristems. They placed cotyledon explants on MS basal media and EC6, B5 basal media supplemented with 5 mg/l BAP, 0.5 mg/l kinetin and 7.97 mg/l adenine sulfate (3 weeks). The shoot buds, with the explants attached were then transferred to EC6/B5/MS basal media supplemented with 0.5mg/l BAP 0.05 mg/l kinetin and 0.79 mg/l adenine sulphate. The shoot buds produced well developed shoots after 2-3 sub cultures of MS medium supplemented with NAA alone (0.5 mg/l) or 0.1 mg/l NAA in combination with BAP (0.1 mg/l - 0.5 mg/l). The shoots elongated further are transferred to rooting medium, half strength MS with 1 mg/l IBA for rooting. This protocol fulfills the requirements of genetic transformation by *Agrobacterium.*

Sreenivasu *et al.,* (1998) reported regeneration of somatic embryogenesis in 609, 852, 855, 856 H-86-25 varieties cotyledon and leaf explants from 10 day old seedlings produced embryogenic callus and somatic embryos when cultured on MS medium supplemented with 2.2 mg/l thiodiazuron. Subsequent withdrawl and growth of TDZ from the induction medium resulted in the maturation and growth of the embryos into plantlets on MS basal medium.

Pigeonpea and *Agrobacterium*

Although members of *Agrobacterium* are considered wild host range pathogens, the ability of the bacterium to produce a compatible reaction varies widely among host species (Byrne *et al.*, 1987). Genotype variation in compatible reactions between host plant and *Agrobacterium* strains has also been reported in *Cicer arietinum* (Islam and Riazuddin, 1994), *Pisum sativum* (Hobbs *et al.*, 1989).

Rathore *et al.*, (1997) reported in vitro transformation of pigeon pea by wild strains of *Agrobacterium tumefaciens*. Aseptically grown seedlings of five pigeon pea genotypes (Bahar, Pant A 106, H82-1, UPAS 120, ICPL 151) were inoculated with *Agrobacterium tumefaciens* strains A 281, A281, A6, T37 by wounding the basal part of epicotyl with sterile syringe. These seedlings incubated as 25 centigrade under 16 hours light and 8 hours darks cycles. Tumour formation occurred and scored after 25-30 days. Strain A281 induced tumour formation in all cultivars T37, A6 in four cultivars, and T37 in three cultivars. Bahar and pant-A 106 showed highest response to agro infection.

3.3 Agrobacterium – a transformation vector

bird's eye view

More than nine decades ago Smith and Townsend (1907) published an article in that they presented that gram positive bacterium which is now called *Agrobacterium tumefaciens* is the causative agent of the wide spread neoplastic plant disease crown gall. Since then a huge number of biologists through out the world have focused their attention on this organism in order to analyse the molecular mechanism involved in the phenomenon of crown gall production. It was driven by the thought that the study of these plant tumors might reveal mechanisms relevant to animal neoplacia / cancer. However this expectation was not met. Although *A.tumefaciens* and crown gall proved to be intrinsic interest, because the tumerous growth was shown to result from the expression of genes on a DNA segment of bacterial origin that was transferred and stably integrated into a plant chromosome. The demonstration of relationship between the presence of bacterial DNA in the plant genome and tumorigenesis suggested that *Agrobacterium* might be exploited to

deliver genetic material into plants to produce transgenic plants possessing novel traits.

Agrobacterium tumefaciens and *A.rhizogenes* are soil bacteria which induce crown gall and hairy root disease respectively at wound sites on dicotyledonous plants and in few monocotyledonous plants. Crown gall and hairy root cells can grow in vitro in the absence of plant growth regulators in contrast to normal plant cells (Tepfer, 1984, Braun, 1958). This is due to expression in the plant cells of *onc* (oncogenicity) genes that are present on the T (transferred)-DNA (Binns *et al.*, 1988). The T- DNA segment of the Tiplasmid contain genes called *ipt* for an isopentenyl transferase, *iaaM* for a tryphtophan monooxygenase and *iaaH* for on indole acetamide hydrolase. These genes thus determines pathways for the production of the plant hormones isopentenyl-AMP (a cytokinin) and IAA (an auxin). In Riplasmid the genes called *rol* have been identified which are responsible for the transformation of normal plant cells into hairy roots (Capone *et al.*, 1989, Schmulling *et al.*, 1988).

Besides *onc* genes the T-DNA contain genes which code for the production of enzymes involved in production of certain tumor specific metabolites and are condensates of aminoacid, sugar derivatives known collectively as opines. The *Agrobacterium* responsible for tumor formation selectively catabolizes the opine whose synthesis it has induced, using it as a source of carbon and nitrogen. Since these has inducible opine catabolic genes on Ti or Ri plasmid (reviewed Hooykaas, 1992). Interesting opines induce not only these catabolic genes but also the conjugative transfer (*tra*) genes on these plasmids over the bacterial population present in the tumor tissue.

The type of opines formed by crown gall cells depends on the infecting *Agrobacterium* strains (reviewed Hooykaas, 1992). Thus *Agrobacterium* strains can be classified according to the typical opines present in tumors as octopine, nopaline, luecinopine and succinamopine. *A. rhizogenes* strains are classified as agropine and mannopine types (Hooykaas, 1992).

Ti plasmids of *Agrobacterium tumefaciens*

Ti plasmids, found in all pathogenic and non-pathogenic strains of *A.tumefaciens* are around 100 to 250kb nucleotides. These plasmids have a molecular weight of 120 to $160x10^6$ daltons. Ti plasmids found in different strains of *Agrobacterium* have four regions of homology. The T-DNA and the *vir* (virulence) region are associated with tumour formation, whereas other two are involved with conjugative transfer and the replicative maintenance of the plasmid within *Agrobacterium* (Draper *et al., 1988*).

During infection by *Agrobacterium* a piece of DNA is transferred from the bacterium to the plant cell. The piece of DNA is a copy of a segment called the T-DNA (transferred DNA). The T-DNA is stable within the plant genome and hybridization of a Ti plasmid specific probe to tumor DNA has shown that the T-DNA found in plant cell is colinear with the T-DNA found in the Ti plasmid of the *Agrobacterium*, indicating that no major re-arrangements of the sequence take place during establishment of the tumour. The site of integration of T-DNA into plant DNA is random. One or more copies of the T-DNA can be present in the plant DNA, although multiple T-DNA copies can occur in tandem. The T-DNA is delimited by flanking 25bp direct repeats called borders (Wang *et al.*, 1984, Zupan *et al.,1997*). Border sequences are the only cis elements necessary for T-DNA processing. So any DNA between these borders can serve as substrate for transfer to a plant cell. (Zupan *et al.*, 1997).

Vir region: 40 kb non T-DNA linked region that mediate processing and transfer of T-DNA (Stachel and Nester, 1986). The *vir* region of octopine Ti plasmid consists of eight operans from *vir A* to *vir H*. These *vir* genes, whose products are directly involved in T-DNA processing and transfer, are tightly regulated so the expression occurs only in the presence of wounded plant cells, the targets of infection. Control of gene expression is mediated by the *vir A* and *vir G* proteins a two component regulatory system (reviewed Winans, 1992). *vir A* detects the small phenolic compounds released by wounded plants resulting in autophosphorylation (Jin *et al.*, 1990). The *vir A* then phosphorylates the regulator *vir G*, which activates the transcription of the remainder of the *vir* genes (Jin *et al.*, 1990).

Following induction and subsequent expression of the *vir* region, some of the *vir* proteins function in the production of a transfer intermediate as a single standard copy of the T-DNA called the T-Strand (Stachel *et al.*, 1986, Zupan *et al.*, 1995). *vir D1* and *vir D2* are essential for this process (Filichkin and Gelvin, 1993). Both Vir D1 and Vir D2 recognize the 25 bp border sequence and produce an SS endonucleolytic cleavage in the bottom strand of each border. These nicks (Wang *et al.*, 1987) serve as the initiation and termination sites for T-Strand production. A single molecule of Vir D2, remains covalently associated with the 5' end of the T-Strand after nicking (Ward and Barnes, 1988, Zupan, 1995) giving the nascent T-Strand a polar character, that may ensure, in some subsequent steps in the transfer process. The 5' end is the leading end. T- Strand production is thought to result from the displacement of the bottom strand of the T-DNA between the nicks.

The T strand must travel through numerous membranes and cellular spaces of both prokaryotic and eukaryotic membranes before its arrival in the plant nucleus. Thus to preserve its integrity T-strand likely travels as an single standard DNA protein complex. VirE2 product is an inducible single stranded (SS) nucleic acid binding protein encoded by the Vir E locus that binds without sequence specificity (Citovsky *et al.*, 1988, Christie *et al.*, 1988), Vir E2 binds tightly and cooperatively, which means that a T-strand would be completely coated. Consequently degradation by nucleases would be prevented and indeed, invitro binding of Vir E2 is responsible for protection of T-strand from nucleolytic degradation. Finally the binding of Vir E2 unfolds and extends ss DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T strand along with Vir D2 and Vir E2 are termed the T-complex (Zupan *et al.*, 1995).

Subsequently the T-complex must exit the bacterial cell, passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane. Once inside the plant cell, the T complex targets to the plant cell nucleus and crosses the nuclear membrane, then which the T-strand becomes integrated into a plant chromosome. The role of Vir D4 and Vir B products are not clear during the transport of T-complex from *Agrobacterium* to plant cell. A *cvB* gene product (a Chrosomal gene product from nopaline strains of *Agrobacterium*) has been proposed to participate in T - complex formation

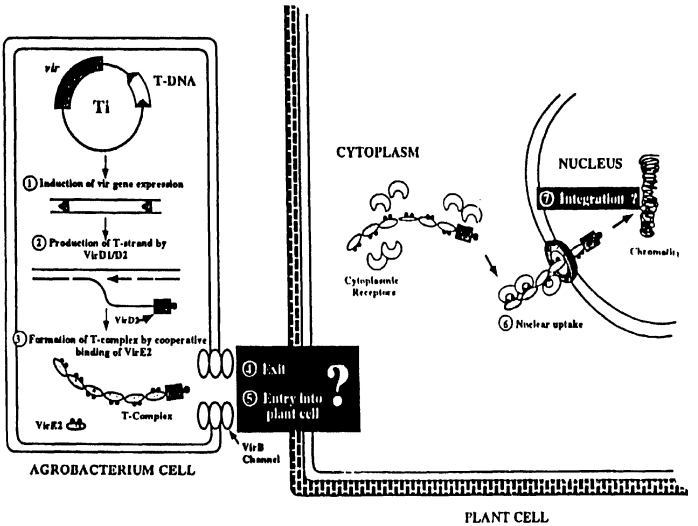


Figure: 3.3(a) : Basic steps in the transformation of plant cells by A.tumefaciens. (Source: Transfer of T-DNA from Agrobacterium to the plant cell, J.R. Zupán and P.Zambryski (1995). Plant Physiol. 107:1041-1047)

(reviewed Zupan, and Zambryski, 1997). This sequence non-specific SS DNA binding protein forms a complex with T-Strand along with Vir D2 and Vir E2.

Agrobacterium plasmids as transformation vectors

The ability of natural plant genetic engineer, *Agrobacterium* to transfer desired sequences of DNA into the plant genome has been exploited in the development of a variety of plant transformation vectors. The efficient use of *Agrobacterium* as a transformation vector required the suppression of the oncogenes. Infection of plant cells with wild type *Agrobacterium* resulted in the formation of crown gall tumours, that precluded the regeneration of plants. Analysis of strains with transposon insertions in the oncogenes showed that the strains were still capable of delivering the DNA into the host cell genome. The oncgenes encoded by the Ti plasmid are neither required for the transfer of the T-DNA to the plant cell nor its integration into the nuclear DNA. Hence, these genes can be replaced not only allowing the insertion of foreign DNA, but also removing the *onc* functions. Another important milestone was the discovery that the *vir* gene products can also function in trans. Utilizing above observations two types of systems were developed.

(1) Ti plasmid itself was engineered by replacing the *onc*genes with the marker gene and any other gene of interest (Zambryski *et al.*, 1983) (2) In the second system, the transferred region (T-region) was maintained on a second plasmid that functioned in trans in the binary system (Hoekema *et al.*, 1983). Binary system is the one of the most commonly used system today for plant transformation.

Cis vectors/cointegration vectors:

These are derivatives of wild type Ti plasmid in which the T-DNA *onc* genes have been removed and in some cases replaced by a specific piece of DNA which has a region of homology to a small cloning vector, that can replicate in *E.coli*. This vector strategy depends on cointegration in *A.tumefaciens* between homologous regions on the modified Ti plasmid (*Vir* helper) and a small *E.coli* cloning vector (Intermediate vector) which contains a selectable marker gene that will function in plant cells and unique sites for the insertion of foreign DNA.

The intermediate vector containing foreign DNA sequences is normally introduced into the *A. tumefaciens* by conjugation and using appropriate selection, Trans conjugants can be obtained in which the foreign DNA has been stabilized within the T-DNA as a result of homologous recombination.

To give one example, PGV 3850 (Zambryski *et al.*,1983) is a cis vector in which the onc genes of a nopaline type Ti plasmid (C58) have been deleted and replaced in PBR 322 can be introduced into PGV 3850 by a two step process of transfer into the *Agrobacterium* via conjugation followed by recombination. In order to carry this out PBR 322 containing the gene sequence to be transferred to the plant cell and a resistance marker which allows selection in *Agrobacterium*(kanamycin or streptomycin/spectinomycin) is introduced into an *E.coli* strain (GJ 28) containing two helper plasmids PGJ 28 and PR 64 drd 11. These plasmids provide col E1 helper functions which allow transfer of all three plasmids via conjugation into *Agrobacterium*. However PBR 322 is unable to replicate in *Agrobacterium* and hence will not be maintained. Neverthless recombination take place between PBR 322 and PGV 3850 which results in the transfer of gene sequence of interest into the Ti plasmid. Trans-conjugants can be selected by the resistance encoded by the plasmid and resistance to rifampicin which is encoded by the chromosome of *Agrobacterium*.

Trans vectors / binary vectors.

Trans or binary vectors are based on plasmids that can replicate in both *E.coli* and *Agrobacterium* and contain the T-DNA border sequences. These can be designed so that border sequences flank multiple cloning sites which allow insertion of foreign DNA, and markers that allow direct selection of transformed plant cells. The plasmids can be manipulated in *E.Coli* and transferred via conjugation or by direct transformation to *Agrobacterium* strains which contain a Ti plasmid which bear a *vir* region but lacks T-DNA and 25 bp repeat sequence. The interaction of two compatible plasmids one containing the *vir* region and other carrying foreign DNA (cloning vector) is responsible for transfer of desired DNA to the plant cells. In another words transfer of the foreign DNA on the cloning vector to the plant cell can be mediated by *vir* region functioning in trans.

Selectable/screenable marker genes for the identification of transformed plant cells

Several selectable and screenable genes are widely available today for plant transformation several requirements must be considered in the development of a truly useful selectable marker system. It is most critical that the selective agent must be inhibitory to plant cells. However not all compounds toxic to plant cells are necessarily useful as selective agents. The best selective agents are compounds that arrest growth of non-transformed cells or slowly kill them.

SELECTABLE MARKER GENES

Enzymes encoded	Selective agent
Neomycin phospho-transferase	Kanamycin
Hygromycin phosphotransferase	Hygromycin
Dihydrofolate reductase	Methotrexate
Gentamycin acetyl-transferase	Gentamycin
Bleomycin resistance	Bleomycin
Phosphinothricine acetyl transferase	Phosphinothricin

Screenable marker (reporter) genes

Some genes need to be tagged with selectable marker genes, whose expression is easily detected through highly sensitive enzyme assays. These genes were called as screenable marker genes or reporter genes.

Nopaline synthase
β - Glucuronidase
Streptomycin phosphotransferase
Fire fly luciferase
Bacterial luciferase
Green flourescent protein gene.

3.4 Biolistic mediated gene transformation

Particle bombardment is an efficient method for delivering DNA into plant cells. This method is especially beneficial for those plants which appear to be a poor interaction with *Agrobacterium*, which is a natural vector used for gene transfer to plants (reviewed Weising *et al.*,1988). It also offer other advantages (Gray and Finer, 1993) over *Agrobacterium*–mediated transformation such as the use of more simplified plasmid constructions, elimination of false positives due to *Agrobacterium* persistence in the host tissue and simplified transformation protocols. Since the development of the first particle delivery system (Klein *et al.*, 1987), several different types of bombardment devices have been developed, including an electrically triggered discharge gun(Mccabe *et al.*, 1988, Mccabe and Christou, 1993) pneumatic particle guns(Oard *et al.*, 1990, Seki *et al.*,1991, Kikkert,1993, Oard,1993) helium, nitrogen and carbon dioxide powered devices (Finer *et al.*,1992, Vain *et al.*,1993) and a microtargeting gun (Sautter *et al.*,1993). These devices have been developed toward the same goals more simplicity safety, accuracy, and a lower cost for DNA delivery.

In this method, 1-2 μm tungsten or gold particles coated with the DNA to be used for transformation, are accelerated to velocities which enable their entry into plant cells/nuclei. Particle acceleration is achieved by using a device which varies considerably in design and function. The most successful device accelerates particles in one of the two ways.

(1) by using pressurized helium gas

(2) by the electrostatic energy released by a droplet of water exposed to a high voltage.

The main components of a helium pressure device are gas acceleration tube, rupture disc, stopping screen, micro carrier carrying particles coated with DNA and target cells. These components are enclosed in a chamber to enable creation of partial vaccum which facilitates particle acceleration and reduces damages to plant cells. After creation of partial vaccum sufficiently pressurized helium gas is released in the acceleration tube to break the repture disc. This generates helium shock waves which accelerates the macroprojectile to which DNA coated microprojectiles are attached. The macroprojectile is stopped by a stopping screen, and the micro projectile pass through this screen. Generally a 1000 psi of helium pressure is used for acceleration.

The macrocarrier is a 2.5 cm diameter, 0.06mm thick plastic membrane which is used only once. The light mass of macroprojectile offers certain advantages, including rapid acceleration. The microprojectiles vary in diameter from 0.5 to 2.0 μm. The average size of 1.0 μm is commonly used. Tungsten particles are cheaper, but are irregular shape and size, may be toxic to certain cell types and show surface oxidation which may lead to precipitation of DNA. In addition they tend to form agglomerates after addition of DNA which reduces particle dispersion. In comparison gold particles are more uniform in size (1-3 μm) and shape and show much lower toxicity but they are much costlier and show variable coating with DNA. Biolistic technique, a means for direct gene transfer has enabled plant biologists to transform soybean (Mccabe *et al.*,1988, Christou *et al.*,1990), corn (fromm *et al.*,1990, Gordon-Kamm *et al.*,1990), rice (Christou *et al.*, 1991), cotton (Finer and Mcmullen, 1990) and peanut (Livingston, 1995).

The particle bombardment technique allows to transform those plants, which cannot be transformed by using conventional *Agrobacterium* or electroporation techniques. In addition the technique by passes the need for time consuming and labour intensive tissue culture manipulations involving protoplast or callus cultures.

4. MATERIALS AND METHODS

4.1. Direct regeneration of pigeonpea from cotyledonary node

Pigeonpea (*Cajanus cajan L*) var ICPL 88039 seeds were used for regeneration. All media consisted of MS salts and vitamins (Murashige and Skoog, 1962), 0.8% agar (difco), BAP, 3% sucrose and pH was adjusted to 5.8 and autoclaved at 121°C for 20 minutes .

Seeds were surface sterilized with 70% ethanol for two minutes. Later seeds were washed with 0.1% mercuric chloride and 0.1ml of tween- 20 for 8 minutes with vigorous shaking on a gyratory shaker. Then seeds were washed with sterilized distilled water for five times in laminar flow. Then seed coats were removed aseptically and placed in test tubes containing MS medium with 5 mg/l (22.23 µmol) benzyle amino purine (BAP) and 3% sucrose with 0.8% agar. Two seeds were placed in each culture tube containing 10 ml of medium.

Multiple shoot initials generated on cotyledonary node were excised from 12 day old seedlings and transferred to MS medium with 5 mg/l BAP for induction of shoots continuously. The mass of shoot initials were later transferred on to MS medium containing 2 mg/l BAP and then to MS medium containing 1 mg/l BAP for further elongation.

4.2. Determination of minimum lethal concentration (LD 50 or MLD) of hygromycin for selection of transformants.

The sublethal concentration of hygromycin for non-transformed multiple shoot initials of pigeonpea determined in this experiment can be used to select transformants. 12-15 day old multiple shoot initials produced on MS medium containing 3 mg/l sucrose and 5 mg/l BAP, 0.8% agar were excised from cotyledons and placed on same induction medium containing different concentrations(2 mg/l, 5 mg/l, 8 mg/l, 10 mg/l, 15 mg/l) of hygromycin. The effect of hygromycin was studied and the minimum lethal concentration was determined.

4.3. Transformation with *Agrobacterium tumefaciens*

4.3.1 Sterilization and culture of pigeonpea :

Pigeonpea(ICPL 88039) seeds were first treated with 70% alcohol for two minutes. Later these were treated with 0.1% $HgCl_2$ with two drops of tween-20 for 8 minutes with vigorous shaking on shaker. Then seeds were washed with sterilized distilled water to remove traces of $Hgcl_2$ for five times in laminar flow. Then seed coats were removed aseptically and seeds were placed in test tubes containing MS medium with 5 mg/l BAP (22.23 μM). After12 days multiple shoot initials were separated from cotyledonary node and were cocultivated with *Agrobacterium* strains (pCAMBIA1301, pJB 90GI).

4.3.2 Preparation of *Agrobacterium* strains :

Agrobacterium C58 strains harboring pCAMBIA1301 and pJB90GI were streaked freshly on YEB media (An *et al.*,1988)containing appropriate antibiotics and incubated at 28°c overnight. Later from those plates *Agrobacterium* C58 strain harboring pCAMBIA1301 plasmid was inoculated into 25ml of YEB media containing 50 μg/ml. kanamycin. *Agrobacterium* C 58 strain harbouring pJB90GI was inoculated into 25 ml of YEB medium containing 100 ug/ml spectinomycin. These were incubated on shaker at 150 rpm at 28°c overnight. 5 ml of above cultures were centrifuged at 4°c 4000 rpm for 5 minutes and pellets were suspended in 10 ml of half strength MS medium. Again these cultures were centrifuged and the pellets suspended in 10 ml of half strength MS medium.

4.3.3 **Cocultivation of Multiple shoot initials with *Agrobacterium* :**

12 day old multiple shoot initials induced on MS media containing 5 mg/l BAP were excised from cotyledanary node and were dipped in the *Agrobacterium* strains along the cut area. The explants were placed on sterile filter paper to remove excess amount of bacteria. Later these explants were co-cultivated with *Agrobacterium* suspension on MS medium containing 5 mg/l BAP and 3% sucrose 0.8% agar for 3 days. The explants were cultured on MS medium containing 5mg/l BAPwith 250 mg/L cefotaxime for 15-20 days to induce new multiple shoot initial continuously from explants and to arrest *Agrobacterium* gr

4.3.4 Selection of transformed shoots and its elongation:

The multiple shoots were transferred to MS media containing 2 mg/l BAP, 250 mg/l cefotaxime and 5 mg/l hygromycin to select for putative transformants. In subsequent subcultures hygromycin concentration in MS medium was increased up to 8 mg/l for selection. For elongation of shoots BAP concentration in MS medium was reduced to 1 mg/l.

4.3.5 Histochemical assay :

Sections of multiple shoots and leaves were incubated overnight in 200 µl of x – gluc (5 bromo – 4 chloro – 3 indolyl β glucuronide) assay mixture in eppendorf tubes at 37^0c. Subsequently the assay mixture was removed by sequential changes in 70-100% ethanol until tissue had no chlorophyll. The tissue was mounted in glycerol and observed under a microscope and photographed.

4.4 Transformation with Biolistic device

4.4.1 Isolation of plasmids from *Agrobacterium* strains:

Plasmids of pCAMBIA1301 and pJB90GI were isolated from *Agrobacterium* strains by alkaline lysis method (Birnboim and Doly 1979). *Agrobacterium* C 58 strains harbouring pCAMBIA1301 and pJB90GI were streaked freshly on YEB media containing 50 µg/ml kanamycin and 100 µg/ml spectinomycin respectively and incubated at 28°c overnight. From these plates *Agrobacterium* C-58 strain harboring pCAMBIA1301 plasmid and PJB90GI were inoculated into 20 ml of YEB liquid medium containing 50 µg/ml kanamycin and 100 µg/ml spectinomycin respectively. These were incubated overnight on a shaker. About 200 µl of liquid culture from the above cultures were transferred to 20 ml of liquid LB medium (appendix-II) containing suitable antibiotics and incubated on shaker for 5 to 6 hours. From this 10 ml of culture was taken into centrifuge tubes and centrifuged at 5000 rpm at 4°C for 5 minutes. The supernatant was poured off and cells were re-suspended thoroughly in 200 µl of GTE solution and incubated on ice for 2-5 minutes. Then 400 µl of alkaline SDS solution was added and the contents were mixed by inverting the tubes rapidly five times and stored in ice for 3

minutes. 300 µl of ice cold solution of 5 M potassium acetate(pH 5.2) was added and the tubes were gently inverted for10 seconds, to disperse solution III and stored in ice for 3-5 minutes followed by centrifugation at 12000 rpm for 5 minutes to remove cell debris. Proteins were removed by adding equal volume of phenol : chloroform : isoamyl alchohol(25:24:1) mixture. After centrifugation at 12000 g for 2 minutes the aqueous layer was transferred to a fresh tube. The DNA was precipitated by adding 0.8 volumes of isopropanol at room temperature and centrifuged at 12000 g for 5 min to pellet down the DNA. The pellet was rinsed with 1 ml of 70% ethanol at 4 °C to remove the salts. DNA was air dried and dissolved in 50 µl of 10mM tris (pH 8.0) and stored at -20 °C.

4.4.2. Microcarrier preparation:

60 mg of tungsten particles of 1.0 µm diameter were weighed in a 1.5 ml microfuge tube. To this 1 ml Of freshly prepared 70% ethanol was added. The eppendorf tube was vortexed on a platform vortexer for 3-5 minutes. The tungsten-ethanol mixture was incubated for 15 minutes and the micro projectiles were pelleted by spinning for 5 seconds in a microfuge. Alcohol was removed and one ml of sterile water was added to tungsten particles followed by vortexing for one minute. The particles were allowed to settle for one minute and pelleted by spinning for 2 seconds in a microfuge and the liquid was removed. This step was repeated thrice. To microparticles 1 ml of sterile 50% glycerol was added to the particles to bring the microparticle concentration at 60 mg/ml.

4.4.3.Coating DNA on to microcarriers

The microcarriers prepared in 50% glycerol (60 mg/ml) were vortexed for 5 minutes on platform vortexer to resuspended agglomerated particles. From this 50µl (3 mg) of micro carriers was taken to a 1.5 ml microfuge tube. This tube was vigorously vortexed and during vortexing, 5 µl DNA, 50 µl $cacl_2$ (2.5 M) 20 µl spermidine (0.1 M) was added in above sequential order. Then vortexing was continued for 2-3 minutes. Then microcarriers were pelleted down by spinning for 2 seconds in a microfuge tube. Then the liquid was removed and the pellet was

washed with 140 µl of 70% ethanol. Again the pellet was washed with 70% ethanol. Then pellet was re-suspended in 48 µl of 100% ethanol.

4.4.4 Bombardment of multiple shoots with microcarrier plasmid system.

12-day old multiple shoots were excised from pigeonpea seedlings germinated on MS medium containing 5 mg/l BAP. These were placed in the middle of the petriplates containing MS medium with 5 mg/l BAP. Biorad biolistic bombardment system(PDS-1000/He) was used to bombard multiple shoot initials. 6 µl of microcarriers was coated on macrocarrier. This was fixed to biolistic gun along with rupture discs(1200psi) and stopping screens. Then petriplates containing multiple shoot explants were placed under the projectile path and bombarded at 1200 psi of helium in evacuated environment.

4.4.5 Assays of transient expression of reporter gene

Bombarded tissues were incubated in the dark at 28° C for 24-36 hours between bombardment and transient gene expression assays. Sections of multiple shoots were incubated in GUS histochemical assay buffer with 1.0 mg/ml of the substrate 5 bromo - 4 chloro-3 indolyl β Glucuronide (x-gluc) overnight at 37°C. Subsequently the chlorophyll was extracted with 70% ethanol and the development of blue color in the tissues due to beta–glucuronidase activity was visualized under a microscope.

4.4.6 Cultivation of bombarded explants

The bombarded explants were cultivated in MS medium containing 5 mg/l BAP to induce transformed shoots. 15 days later the explants with developing shoots were shifted to MS medium containing 2 mg/l BAP and 5 mg/l hygromycin to select for transformed shoots. In subsequent subculture these shoots were transferred to MS medium containing 1 mg/l BAP and 8 mg/l hygromycin for further development of shoots.

4.5 Isolation of pigeonpea genomic DNA, Restriction and Southern hybridization

4.5.1 Isolation of pigeonpea genomic DNA by without liquid nitrogen method :

Pigeonpea variety plants were grown in glass house. The leaves were collected and used to isolate DNA by different methods. Good amount of DNA was isolated by DNA miniprep protocol after some modifications (Fulton *et al.*, 1995). 500 mg of plant tissue was homogenized in a mortar with pestle in the presence of 600 µl of freshly prepared DNA extraction buffer. Another aliquot 400 µl of buffer was added and transferred to a centrifuge tube and incubated at 65°C in water bath for 45 minutes. The tubes were filled with 700 µl chloroform and mixed well by vortexing and centrifuged at 10000 rpm for 5 minutes. The aqueous phase was pipeted out into new microfuge tube and 0.8 volumes of isopropanol was added and inverted to mix the contents until DNA precipitated. DNA was fished out and placed in an eppendorf tube and washed with 70% ethanol and dried upside down on paper towels. The DNA was re-suspended in 100 µl TE and 10 µl was loaded in 0.8% agarose gel for verification. 2 µl of DNA free RNase was added to degrade RNA. Then DNA was purified by adding equal volume of phenol:chloroform extractions and precipitated with ethanol and dissolved in TE and stored at -20 °C.

4.5.2 Extraction of plant genomic DNA by CTAB Method.

500 mg of freshly harvested pigeonpea leaves were weighed and were crushed in the mortar and pestle using liquid nitrogen. 10 ml of hot 2x CTAB buffer (65 °C) was added. Contents were transferred to centrifuge tubes. 10ml of $CHCl_3$ + Isoamyl alcohol(24:1)mixture was added to remove chlorophyll and cell debris. This was mixed thoroughly and centrifuged for 10 minutes as 1000 rpm and supernatant was collected into fresh tubes. To this 1/10 volume of 10% CTAB (prepared in 0.7 M NaCl) was added and mixed. Then equal volume of chloroform: isoamyl alcohol was added and mixed and centrifuged at 1000 rpm for 5 minutes. The aqueous layer was collected and equal volume of CTAB

precipitation buffer was added and mixed gently. Then it was centrifuged for 15 minutes at 10000 rpm and supernatant was discarded To dissolve the pellet high salt TE buffer was added and to 2 volumes of ice cold absolute ethanol was added to precipitate DNA followed by centrifugation for 15 minutes at 10000 rpm to obtain DNA pellet. The pellet was dried and dissolved in 100 µl of 10 mM tris. 10µl of DNA was loaded in 0.8% agarose gel for verification. Later its volume was increased upto 500 µl and 2 µl of DNA free RNase was added to degrade RNA. The DNA was purified by phenol-chloroform extractions, precipitated with ethanol and dissolved in TE and stored at -20 C.

4.5.3 DNA estimation

DNA isolated by above methods was estimated at 260mm and its purity was determined by measuring O.D ratio at 260/280. The DNA isolated by CTAB method showed the 260/280 ratio of 1.812.

4.5.4 DNA Restriction and Southern hybridization

To equal amount of the DNA extracted by different methods 1µl of Hind III was added along with 3 µl of reaction buffer. Then the tubes were incubated at 37°C for overnight. The reaction was stopped by adding 0.7 µl of 0.5 M EDTA to a final concentration of 10 mM. Then the tubes were incubated at 37°C for overnight. Then these were loaded in slots of 0.8% agarose gel and electrophoresed at 50v. The gels were observed on U.V Trans-illuminator and photographed with a polaroid camera.

The gel was placed in a tray and soaked in 2-3 volumes of depurinating solution for 10 minutes on a shaker. The solution was decanted and rinsed with distilled water and soaked in 2-3 gel volumes of denaturing solution for 20 minutes with agitation. The denaturing solution was decanted, and gel was rinsed with distilled water and soaked in 2-3 gel volumes of neutralizing solution for 10 minutes. A tray of the blotting apparatus was filled with 20x SSC buffer and the gel was placed on a glass plate. Filter paper wicks connected to supply the buffer to gel. Dry hybond-N^+ membrane (9x6 cm) was placed on the gel. Then 2 layers of fitter paper cut to the size of the gel were placed above the hybond-N^+ membrane and

were wetted with 20x SSC. Paper towels were placed on the filter papers to apply capillary action. To compress paper towels weight was placed in the center and blot was left for overnight. On next day hybond-N$^+$ membrane was removed and washed with 2x SSC and DNA was cross linked with exposure to U.V light on transilluminater.

4.5.5. Hybridization of Southern blotted DNA :

(1) Preparation of labeled probe

Single standard DNA, immobilized on the hybond-N filter was challenged with single standard, non-radio labeled *cox* probe. 10µl of the *cox* DNA (10 ng/µl) to be labeled was taken in a microfuge tube and denatured by heating for 8 minutes in the boiling waterbath followed by rapidly cooling on ice for 5 minutes to avoid renaturation. To the denatured DNA 10 µl of reaction buffer was added and mixed thoroughly and kept on ice for few minutes. Then 2 µl of labeling reagent (0.1 w/v sodium azide) was added and mixed. Later to above mixture 10 µl of the cross linker working solution (0.94% v/v formaldehyde) was added and mixed. The tube was spinned briefly in a microcentrifuge to collect the contents at the bottom of the tube. Then it was incubated for 30 minutes at 37°C. The labeled probe was used immediately for hybridization.

2. Hybridization :

20 ml of alkophos direct hybridization buffer was heated to 55°C. The blot which was placed in a bag of saran wrap, was pre hybridized with hybridization buffer for 15 minutes at 55°C in water bath to avoid non-specific binding of probe to membrane. Then labeled probe 10 µl was added to the buffer used for the pre-hybridization buffer. Then blot was incubated overnight at 55°C in a water bath.

4.5.6 Post hybridization washes:

The blot was removed carefully from hybridization solution and was washed with primary wash buffer (55°C) for 10 minutes at 55°C with gentle agitation. This step was repeated. Then blot was placed in a clean container and excess of

secondary wash buffer was added. Another wash was also done in fresh secondary wash buffer at room temparature for 5 minutes.

4.5.7 Signal generation with CDP star:

The signal detection was carried out with the CDP star kit from Amersham. The secondary wash buffer was drained out from the blot. Then blot was placed on a clean non absorbent surface. Then CDP star detection reagent was applied on the blot and left for 2-5 minutes. The excess detection reagent was drained off. The blot was wrapped in a saran wrap and was placed DNA side up in the film cassette. Then lights was switched off and an auto radiography film was placed on the top of the blot and cassette was closed and exposed for 30 minutes at room temparature. Later the film was removed and developed.

4.5.8 Detection of DNA by color reaction:

The blot after CDP signal generation was washed with buffer 3 and it was kept in a saran wrap. To the 10ml of color detection buffer 80µl of (Boehringer–Mannheim) NBT-BCIP mixture was added. This detection reagent was added to the blot and incubated upto color development. The blot was rinsed with distilled water and blot was photographed.

5. RESULTS

5.1. Direct regeneration of pigeonpea from cotyledonary node.

Seeds of pigeonpea responded to benzyl amino purine (BAP) in the induction medium swelled at the cotyledonary node and exhibited inhibition of growth of the primary root. Many shoot initials were seen arising from the swollen nodal portion at the position of axial buds. The number of shoots reached peak stage at 12-16 days. Continued production of the shoot initials was obtained when the cotyledonary node explant was excised and cultured again in induction medium.

5.1.a Morphogenetic response of pigeonpea seeds on MS medium containing 5mg/l BAP after 3 weeks.

Genotype	Conc of BAP	Germination percentage	Percentage of germinated seedlings showing multiple shoots	Average number of shoots per explant.
ICPL 88039	5 mg/L	75%	90%	32
ICPL 88039	0	75%	0	0

5.1b Response of multiple shoot initials after excision from cotyledonary node and culture on MS medium containing 5 mg/l BAP after 3 weeks of proliferation.

Concentration of BAP	Number of Shoots per explant
5 mg/L	45

The multiple shoot initials showed elongation on transfer of the explant to MS medium containing 2 mg/l BAP and later these are transferred to MS medium containing 1 mg/l BAP for further elongation.

5.2 Determination of minimum lethal concentration(LD 50 or M.L.D) of hygromycin for selection of transformed plants.

12-15 day old multiple shoot initials produced on MS medium containing 5mg/l BAP were excised from cotyledons and placed on same induction medium containing different concentrations of hygromycin. Half of the multiple shoots survived on MS mediumcontaining 5 mg/l hygromycin after three weeks. Hence 5 mg/l hygromycin was selected to screen transforments in subsequent experiments.

5.2a Effect of hygromycin on multiple shoot initials after three weeks:

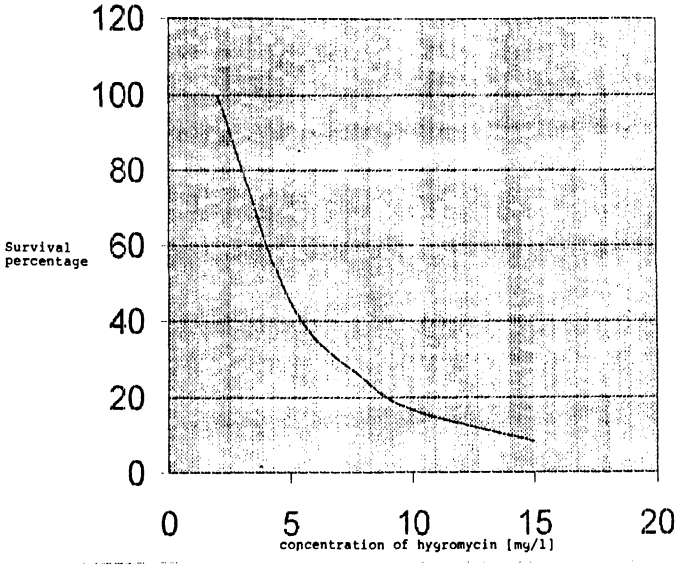
Conc of hygromycin	No. of explants placed	No. of explants survived
2 mg/l	20	20
5 mg/l	20	9
8 mg/l	12	3
10 mg/l	12	2
15 mg/l	12	1

5.3. *Agrobacterium*-mediated genetic transformation

Multiple shoot initials of pigeon pea transformed with *Agrobacterium* strains were cocultured for 3 days and later placed on MS medium with 5 mg/l BAP and 250 mg/l cefotaxime to arrest the growth of ***Agrobacterium*** and enhance the induction of multiple shoots for 15 to 20 days. Eventually multiple shoots were elongated in MS2B media supplemented with 5 mg/l hygromycin and 250 mg/l cefotaxime. Survived multiple shoots were further screened by elongating them in MS medium with 1 mg/L BAP and 8mg/l hygromycin.

5.3a Number of Multiple shoots recovered after cocultivation and subsequent selection on selection medium. Multiple shoots survived in selection media (5 mg/l hygromycin) were counted after three weeks.

Agrobacterium with plasmid	Number of multiple shoots transformed	Number of explants growing on selection medium (5 mg/l, hygromycin	Transformation frequency in the presence of hygromycin
PCAMBIA1301	48	22	45.8%
pJB90GI	48	23	49%



Figre: 5.2(a) The graph showing effect of hygromycin on multiple shoot initials of pigeonpea. [M.L.D of hygromycin calculated from the graph as 5 mg/l].

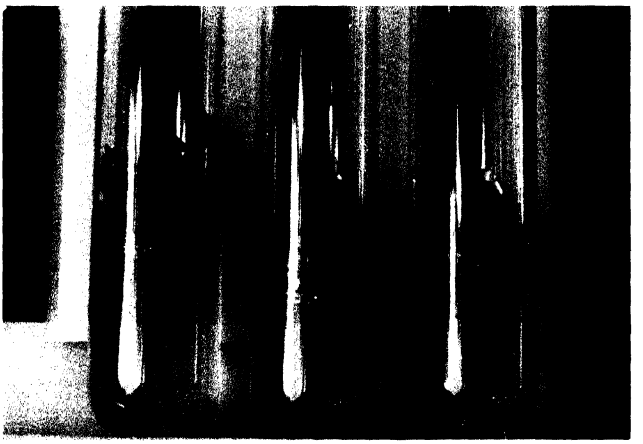


Figure 5.3(a): In vitro produced tobacco plants growing on MS 4 media containing 8mg /l hygromycin.

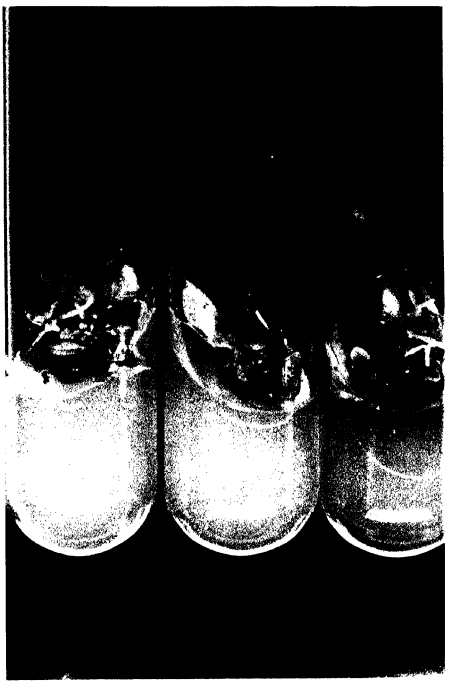


Figure 5.3(b): Multipleshoots of pigeonpea growing on selection medium containing 8mg/l hygromycin.

5.4 GUS histochemical assay of putative transforments.

The leaves from the elongated shoots growing in the selection medium were incubated overnight in x-gluc assay solution at 37^{0}C. After incubation Chlorophyll was extracted out by placing leaves in ethyl alcohol. The number of leaves showing blue color were counted.

5.4a GUS histochemical assay

Plasmid	**Number of Plants Tested**	**Number of plants showing GUS activity**
PCAMBIA 1301	22	8
pJB 90 GI	14	5

36% of putative transforments growing on the selection media (MS medium containing 1 mg/l BAP and 8 mg/l hygromycin) showed positive Gus activity. β-glucuronidase expression frequency was calculated by formula,

$$\frac{\text{number of transforments showing β-glucuronidase activity}}{\text{total number of explants transformed with } \textit{Agrobacterium}} \times 100$$

The GUS expression frequencies obtained for the plasmids pCAMABIA1301, pJB90GI transformed lanes were 16.66% and 10.4% respectively.

Figure 5.4(a): A leaf from transformed plant growing on selection medium, showing beta-glucuronidase positive loci after incubation in x-gluc assay mixture and subsequent clearing with ethanol.

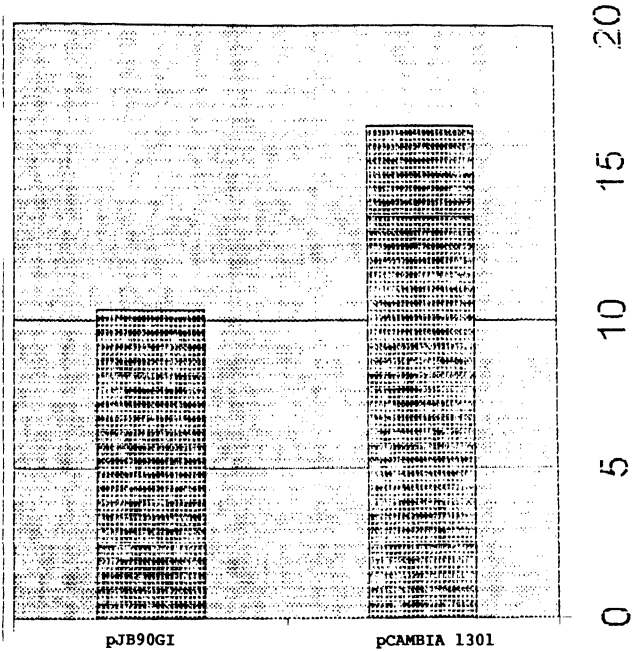


Figure:5.4(b): Percentage of beta-glucuronidase expression frequency for Agrobacterium strains.

5.5 Electrophoresis of Restricted plasmid DNA

The pJB 90 GI, pCAMBIA 1301 plasmids isolated by alkaline lysis method were restricted with suitable restriction endonucleases and electrophoresed on 0.8% agarose gel at 60 Volts. The gel was stained in a sulution of IX TAE Containing 0.5 μg/ml ethidium bromide for 30 minutes. The gel was visualized on U.V. trans illuminater.

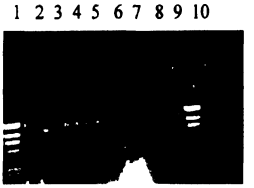


Fig. 5.5a. Electrophoresis of restricted plasmids.

Lane 1 and 10: Lambda phage DNA restricted with Hind III enzyme. This DNA ladder consists of 6 fragments possessing 23.1, 9.4, 6.7, 4.4, 2.3, 2.0.K.b pairs.

Lane 2 - Unrestricted pCAMBIA1301

Lane 3 - pCAMBIA1301 digested with Xho I.

Showing 10.843kb large fragment. 1.0 kb Smaller fragment is invisible.

Lane 4 - pCAMBIA 1301 digested with Bgl II showing linearized 11.837kb plasmid

Lane 5 - pCAMBIA 1301 digested with NCO I showing linearized 11.837 kb plasmid.

Lane 6 - Un digested PJB 90 GI plasmid.

Lane 7 - pJB 90 GI restricted with Sal I showing linearized 12.7 kb fragment.

Lane 8 - pJB 90 GI restricted with Hind III showing 10.1 kb large fragment and small 2.6 kb fragment.

Lane 9 - PJB 90 GI restricted with Xho I.

5.6. TRANSIENT GUS EXPRESSION STUDIES AFTER BOMBARDMENT

After 36 hours of bombardment, sections of the multiple shoot initials were incubated in x-gluc assay solution for overnight. After chlorophyll extraction with ethanol Beta - glucuronidase positive explants were observed and counted. Sections from every one explant out of 3 multiple shoot initials showed transient Gus expression. (Transient Gus expression frequency was 33.33%).

Figure 5.6(a): A section through multiple shoot initial showing transient beta-glucuronidase expression(magnified), 36 hr after bombardment with plasmid and subsequent overnight incubation in x – gluc assay mixture and after clearing with ethanol.

5.7 The isolation and electrophoresis of pigeonpea genomic DNA.

Pigeon pea genomic DNA was isolated by DNA miniprep protocol (without liquid nitrogen method modified from (Fulton et al.,1995) and modified DNA mini prep protocol using liquid nitrogen and CTAB Rogers *et al.*, 1986) methods. DNA isolated by above methods was estimated at 260 nm and its purity was determined by measuring O.D. ratio at 260/280. The DNA isolated by CTAB method showed the 260/280 ratio of 1.812. The genomic DNA isolated by DNA mini prep protocol modified with using liquid nitrogen also showed 260/280 ratio of 1.8537. DNA isolated by these methods was later electrophoresed on 0.8% agarose gel. DNA isolated by DNA miniprep protocol without liquid nitrogen showed shearing. However the DNA isolated by same protocol with using liquid nitrogen, and CTAB method showed distinct bands with out shearing.

5.8 Restriction and Southern hybridization

Pigeonpea genomic DNA isolated by various protocols was estimated and equal amount of DNA was restricted with Hind III enzyme. Later these were electrophoresed on 0.8% agarose gel(Fig. 5.8a). After Southern blotting the hybond-N^+ membrane was hybridized with *cox* probe labeled with a single digoxigenin labeled dideoxyuridene triphosphate (DIG dd UTP). Dig labeled DNA was detected by using antidigoxigenin-alkaline phosphatase conjugate (Anti DiG–AP). Subsequent addition of CDP star detection reagent {aqueous solution of < 1.5% disodium 2–chloro 5 (4 methoxy spiro (1,2–dioxetane 3, 2' (5 chloro)–tricyclo (3, 3, 1, 3, 7, decan)–4 yl phenyl phosphate} generated signals. This signals were detected on X-Ray film.

Detection of DNA by color reaction

The blot after CDP signal generation was washed with buffer-3 to remove substrate. Then DNA was detected by adding NBT–(Nitroblue tetrazolium salt) BCIP (5 bromo 4 chloro 3 indolyl phosphate). Alkaline phosphotase was reacted with BCIP and produced an insoluble blue precipitate and was visualized as distinct bonds(fig. 5.8b).

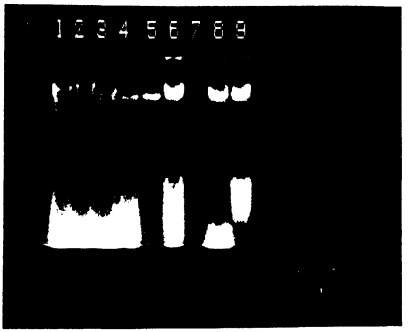


Figure 5.7(a): Electrophoresis of pigeonpea genomic DNA.
Lanes 1,2. Pigeonpea genomic DNA isolated by DNA miniprep protocol (Fulton's method).
Lanes 3,4. Pigeonpea genomic DNA isolated by DNA miniprep protocol (Fulton's method) by using liquid nitrogen.
Lanes 5,6,7,8,9. Pigeonpea genomic DNA isolated by CTAB method (Rogers *et al.*, 1986).

Table 5.7(a): Estimation of pigeonpea genomic DNA.

S.No.	Method	A1(260)	A2(280)	A1-A2	A1/A2
1	DNA mini prep protocol W/o liquid nitrogen	0.030	0.017	0.013	1.75
2	DNA mini prep protocol using liquid nitrogen	0.037	0.020	0.017	1.85
3	CTAB method	0.029	0.016	0.013	1.81

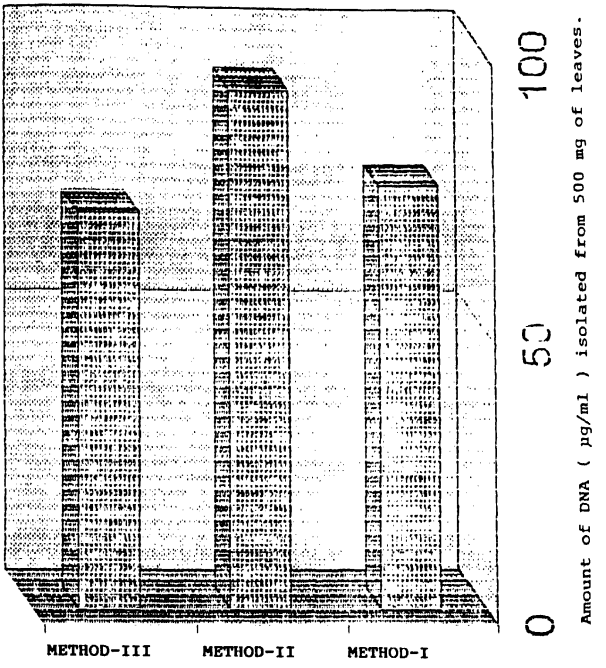


Figure:5.7(b): The graph showing amount of DNA isolated from 500mg leaves from pigeonpea by using three different methods.

METHOD-I : DNA miniprep protocol without liquid nitrogen (Fulton, 1995).

METHOD-II : DNA miniprep protocol modified by using liquid nitrogen (Fulton, 1995).

METHOD-III : CTAB method (Rogers et al.,1986).

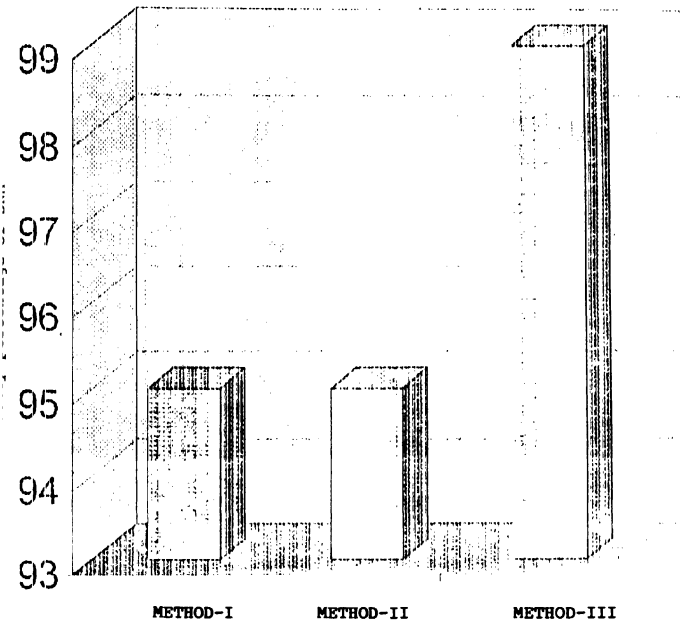


Figure: 5.7(c): The graph showing purity/quality percentage of DNA, isolated by three different methods from pigeonpea leaves.

METHOD-I : DNA miniprep protocol without liquid nitrogen (Fulton, 1995).

METHOD-II : DNA miniprep protocol modified by using liquid nitrogen (Fulton, 1995).

METHOD-III : CTAB method (Rogers et al.,1986)

Figure. 5.8 (a): Electrophoresis of Pigeonpea genomic DNA after restriction with Hind III.

Lane 1: Pigeonpea genomic DNA isolated by DNA mini prep protocol (W/O liquid nitrogen method, Fulton, 1995).

Lane 2: Pigeonpea genomic DNA isolated by DNA mini prep protocol using liquid nitrogen.

Lane 3,4,5, Pigeonpea genomic DNA isolated by CTAB method (Rogers *et al.*, 1986).

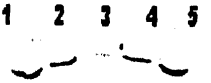


Figure 5.8(b): Southern blotting and subsequent hybridization with non-radio labeled *cox* probe.
Lane1. Pigeonpea genomic DNA isolated by DNA mini prep protocol(W/O liquid nitrogen method, Fulton, 1995).
Lane2. Pigeonpea genomic DNA isolated by DNA mini prep protocol using liquid nitrogen.
Lanes 3,4,5. Pigeonpea genomic DNA isolated by CTAB method(Rogers *et al.*, 1986)

6. DISCUSSION

Efficient 'in vitro' regeneration system is a pre-requesite for the production of transformed plants. There have been several reports of plant regeneration via organogenesis in pigeonpea using different explants. In the present study we used MS medium with 5mg/lBAP to induce multiple shoots from cotyledonarynode. Mehta and Mohan Ram (1983) reported formation of 5-35 shoot buds from the surface of cotylendons of the seedling raised in Gamborg (Gamborg *et al.*,1968) B5 medium supplimented with BAP (2.4mg/l). Kumar *et al.*,(1983), reported production of 5-18 shoot buds from excised cotyledons of pigeonpea when cultured on BAP containing media (2.5 mg/l). Prakash *et al.*,(1994) reported formation of multiple shoots from cotyledonary node, by placing seeds on MS medium containing BAP(1-10 mg/l). They reported seeds placed on MS medium containing 5 mg/l BAP produced a maximum (45.7) mean number of shoots. In our study ICPL 88039 seeds placed on MS medium containing 5 mg/l BAP produced a maximum of 32 shoots. Continued production of shoot initials were obtained by placing excised multiple shoots on MS medium containing 5 mg/l BAP. The multiple shoot initials showed elongation on transfer to the MS medium suplimented with 2mg/l BAP followed by MS medium with 1 mg/l BAP.

Hygromycin is anticyclitol antibiotic that inhibits protein synthesis in prokaryotic and eukaryotic cells. A gene from a bacterial resistance (R) factor that encodes a hygromycin phosphotransferase (HPT) has been used as selective marker gene. Half of the multiple shoots survived on media containing 5 mg/l hygromycin after 3 weeks. Hence 5 mg/l hygromycin was considered as minimum lethal dose.5 mg/l hygromycin was employed in media to select transforments. In subsequent subcultures 8 mg/l hygromycin was used in media. However untransformed (glucuronidase negative) plants also found in 8 mg/l hygromycin containing medum.

Negative results from untransformed control plants for beta glucuronidase test and positive β-glucuronidase expression in transformed plants(which are transformed with intron-gus reporter systems) are suitable assays to analyse gene products(Birch, 1997). In general, Gus activity staining can be used effectively only if appropriate negative control is employed in specific and stringent assay conditions. In our study

we found that, β-glucuronidase expression frequency calculated for the *Agrobacterium* mediated process was 13.5%.

Transient GUS expression frequency observed for particle bombardment after 36 hours incubation was 33.33%. Sections from every one explant out of 3 multiple shoot initials showed transient GUS expression.

The isolation of nucleic acids is a fundamental requirement for the identification of genes for plant genetic engineering as well as for the study of gene expression at the molecular level in the transformed plants. A critical factor in the isolation of plant DNA is the efficient disruption of the plant cell wall. Unfortunately, many techniques for breaking open cells also shear DNA and thus any method must be a compromise between DNA length and yield. We observed that the DNA isolated from pigeonpea by DNA miniprep protocol without liquid nitrogen(Fulton *et al.*,1995)showed shearing. While the DNA isolated by the above same method with using liquid nitrogen and CTAB protocol produced DNA without shearing. Among these two methods CTAB method produced good quality DNA with 260/280 ratio of 1.812.

7. CONCLUSIONS

Seeds of pigeonpea provide a leading source of plant proteins for people of South asian countries. In India 80% of the total proteins in food consumption is derived from pigeon pea seeds. Germplasm improvement in pigeonpea is possible only by genetic engineering techniques. Breakthroughs in pigeonpea transformation by *Agrobacterium* and particle bombardment techniques laid the foundation for future engineering of pigeonpea plants, to develop novel agronomically useful plants possessing new traits.

One of the major biotic constraints in pigeonpea cultivation is *Helicoverpa armigera* Manipulating pigeonpea plants with Bt crystal protein genes is very effective strategy to resist boll worm complex. Pigeonpea plants engineered with soybean trypsin inhibitor (SBTI) or with other serine protease inhibitors shall be protected from Helicoverpa. Transforming pigeon pea with alpha amylase inhibitor gene by genetic engineering protects it seeds after harvesting during storage at least from old world bruchids. Glyphosate or other herbicide tolerant traits are expected to improve pigeonpea plants in general. For example transforming pigeonpea with 5–enol pyruvyl shikimate–3–phosphate synthase gene from a mutated prokaryote that is less sensitive to glyphosate or by over expression of EPSP synthase produce glyphosate tolerate plants. An integrated approach, utilizing breeding techniques along with genetic engineering is required to produce higher yields of this legume.

The need for the second green revolution is urgent. The existing conventional strategies during nineties experienced a very checkered growth of agricultural production. Transgenic plants could be a possible approach to combat with these problems. Already eight countries have adopted transgenic crops commercially. In 1998, the area under these crops globally exceeded 27.8 million hectare. The first country to commercialize biotechnology was China, and the first transgenic crop was tobacco. That was in early 1990s. But the US soon took the lead. It now accounts for 74% of global area under transgenic crops. The other countries in order of importance are Argentina, Canada, Australia, Mexico, Spain, France and South Africa. Transgenic crops, which have so far been grown on commercial scale include

soybean, corn, maize, cotton, rapeseed and potato. The use of transgenic crops is spreading across the globe. Several countries including India, are ready for commercialization of transgenic crops. The sooner we go into it the better

The following results were drawn from the above study.

(1) Production of transgenic pigeonpea plants were is possible both by *Agrobacterium* and biolistic-mediated processes.

(2) Direct regeneration of pigeonpea via multiple shoot initials from cotyledonary node could be successfully used to generate transgenic plants.

(3) The stable β-glucuronidase expression frequency calculated for *Agrobacterium*-mediated gene transfer process to multiple shoot initials was 13.5%.

(4) For the isolation of plant DNA from pigeonpea CTAB method (Rogers *et al.*,1986) and modified miniprep protocol using liquid nitrogen(Fulton, 1995) were efficient methods.

APPENDICES

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APPENDIX – II

1. L.B.MEDIUM

Bacto–peptone	10 grams.
Yeast extract	5 grams.
Sodium chloride	10 grams.
Distilled water	1000 ml.
pH–7.0	

2. YEB Medium

Bacto–Peptone	5 grams.
Yeast extract	1 gram.
Beet extract	5 grams.
Sucrose	5 grams.
Magnesium sulfate - hepta hydrate	0.5 grams
Distilled water	1000 ml.

3. 50 x TAE 1 Ltr.

Tris base –	242 grams.
Glacial Acetic acid	57.1 ml.
0.5 M EDTA	100 ml.

4. GTE Solution
(25 mm Tris-HCl pH 8.0, 10mm EDTA, 0.5M Glucose for 100 ml.)

0.2M Tris–HCl (pH 8.0)	12.5 ml.
0.5 M EDTA	2.0ML.
Glucose	9.01gm.

Make volume with Distilled Water 200ml. This should be autoclaved and stored at room temperature.

5. 3M Potassium acetate (pH 5.2)
Dissolve 117.78 gms. Of Potassium acetate in 200ml of distilled water. Titrate to pH 5.2 with glacial acetic acid. Make the volume to 400 ml with distilled water and store at room temperature.

6. NaOH – SDS (Lysis buffer)
0.2 N NaOH
1% W/V SDS
add 100ml 2N NaOH and 100 µl 10% SDS to 800 µl water. (Prepare fresh).

7. TE (10mm Tris. HCl (pH 7.5), 1 mM EDTA pH 7.5)

2M tris–Hcl(pH 7.5)	0.5 ml.
0.5 Ml. EDTA	0.2 ml

make to volume with distilled water autoclave and store at room temperature.

8. RNase:
Dissolve 10mg RNase in one ml water and boil in boiling water bath for 10 minutes.

9. 2 x C TAB buffer
2% CTAB (w/v)
100mm Tris (pH 8.0)
20mM EDTA (pH 8.0)
1.4m M NaCl.

10. 10% CTAB Solution
    10% CTAB
    50m M Tris (pH 8.0)
    10m M EDTA (pH 8.0)
11. High Salt TE buffer
    10m M Tris (pH 8.0)
    1 m M EDTA (pH 8.0)
    1 M NaCl.
12. Cefotaxime (Alkem laboratories Limited) sterile cefotaxime in vial (250mg) dissolved with sterile distilled water and used. Cefotaxime will degrade in the light in culture medium after approximately 3 weeks.
13. Hygromycin (Sigma) Dissolved in distilled water 50mg/ml and sterilized by filtration stored at -20°C.
14. Buffer 3
    0.1 M Tris HCl.
    0.1 M NaCl.
    50m M $MgCl_2$
    pH 9.5
15. DNA Extraction buffer
    5 m M EDTA pH 7.5
    0.35 M Sorbitol
    0.1 M tris–base
    For 100 ml

Sorbitol	-	6.3 gms.
5M Tris base	-	10 ml.
0.5 M EDTA	-	1 ml.

    First add tris and EDTA in water slowly add Sarbitol with gentle shaking (avoid air bubbles) and finally make up volume to 100 ml.
16. Nuclei lysis buffer-0.2M Tris, 0.05 M EDTA, 2μ M Nacl, 2% CTAB, Sarkosyl 5%.
    For 100 ml.

1 M Tris	20 ml
0.5 M EDTA	10 ml
10% CTAB	20ml
10% Sarkosyl	50ml
50m M NaCl	4 ml.

17. Micro prep buffer–2.5 parts DNA extraction buffer, 2.5 parts nucleolysis buffer, 1.0 part 10% Sarkosyl+1.0 part of 10% CTAB for pigeonpea add 1.0 gms. Sodium bisulfate/100 ml buffer immediately before use (Sodium bisulfate can be increased to avoid color in final product).
18. Hybridization buffer
    To the 12% w/v of Urea solution add NaCl to give a concentration of 0.5 M. add blocking reagent to a final concentration of 4%. Mix for 1-2 hours on a magnetic stirrer store at–15°C to–30°C.

19. Primary wash buffer (1 litre)
(Urea 2M, SDS 0.1%, Sodium phosphate 50mM, 1m M Magnesium Chloride 0.2% blocking reagent.)

Urea	120gms.	2 M
SDS	1gm.	0.1%
0.5M Sodium phosphate (pH 7.0)	100ml	50m M
1.0 M Magnesium chloride	1 ml	1m M
Blocking reagent	2 gms.	0.2%

0.5M Sodium phosphate can be made by using Sodium dihydrogen phosphate (mono basic) and adjusting the pH to 7.0 with NaoH.

20. Secondary wash buffer–20x stock.

Tris base	121 gms.	1 M
Nacl.	172 gms.	2 M

Adjust pH to 10 make up to 1 litre with water. This can be kept for upto 4 months in refrigerator at 2-8°C.

21. Secondary wash buffer–working Solution
Dilute stock 1:20 and add 2 ml /l. of 1 M magnesium chloride to give a final concentration of 2m M magnesium chloride in the buffer.

22. Cross linker solution:
4.7% v/v formaldehyde.

23. Labeling reagent
0.1% w/v Sodium azide.

24. Depurinating solution (0.25 M HCl) for 1 litre.

HCl (12M)	20.83 ml.
Distilled water	979.27 ml.

25. Denaturing solution (0.5 M NaoH, 1.5m NaCl) for 1 litre

NaOH	20 gms.
5 M NaCl	300 ml.

Make up volume to 1 litre with distilled water

26. Neutralizing solution (3.0M Nacl, 0.5M Tris – HCl, pH 7.4) for 1 litre.
5M NaCl–600ml (or 175.3gms of NaCl)
2M tris – HCl (pH 7.4)–250.0 ml.
Distilled water to volume.

27. 20 x SSC
(3.0 M NaCl, 0.3M Sodium citrate, pH 7.0) for One litre.

NaCl	175.3 gms.
Sodium citrate	88.2 gms.

Dissolve in 800ml of distilled water and then make to volume. The pH of the solution does not usually need adjusting.

28. 2 x SSC for one litre.

20 x SSC	100 ml.
Distilled water	900ml.

29. NBT – BCIP stock solution (Boerhinger–Mannheim)
One ml NBT / BCIP stock solution, in 67% v/v dimethyl sulfoxide(DMSO.).

30. Colour – substrate solution (Freshly prepared)
200 micro litre NBT / BCIP Stock Solution are added to 10 ml buffer 3.

APPENDIX-III

MURASHIGE AND SKOOG (1962) MEDIUM

SALT	CONC. (mg/l)	STOCK (per l)	USE (per l)
MAJOR SALT (X 50)			
NH_4NO_3	1650	33.0 g/200ml	10 ml
KNO_3	1900	38.0 g/400ml	20 ml
KH_2PO_4	170	3.40 g/200ml	10 ml
$CaCl_2$	440	8.80 g/200ml	10 ml
$MgSO_4.7H_2O$	370	7.40 g/200ml	10 ml
MINOR SALTS (x 100)			
H_3BO_3	6.20	6.20 mg/l	)
KI	0.83	83.0 mg/l	)
$MnSO_4.4H_2O$	22.3	2230 mg/l	)
$ZnSO_4.7H_2O$	8.6	860 mg/l	) 5.0 ml
$Na_2MoO_4.2H_2O$	0.25	25 mg/l	)
$CuSO_4.5H_2O$	0.025	2.5 mg/l	)
$CoCl_2.6H_2O$	0.025	2.5 mg/l	)
IRON (X 100)			
$Na_2EDTA.2H_2O$	37.3	3.73 g/l	)
$FeSO_4.7H_2O$	27.8	2.78 g/l	) 10 ml
OR			
FE NA_2 EDTA	40	2.0 G/500 ml	10 ml
ORGANICS (X 100)			
GLYCINE	2.0	200 mg/l	) 10 ml
NICOTINIC ACID	0.5	50 mg/l	)
THIAMINE HCl	0.1	100 mg/l	)
PYRIDOXINE HCl	0.5	50 mg/l	)
m-Inositol	100	5.0 g/500 ml	10 ml

GUS HIST

ASSAY MIXTURE
(for 5 ml)

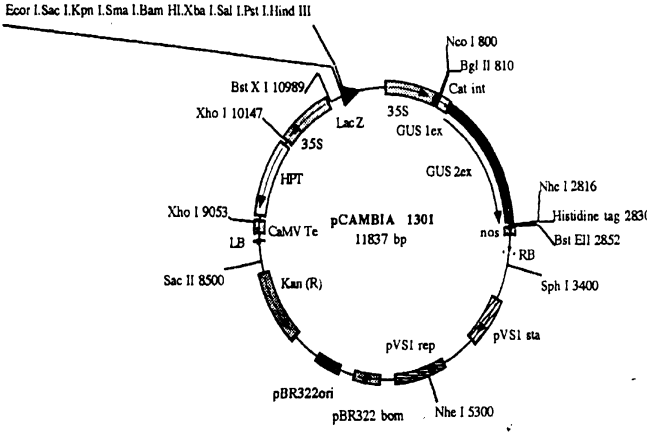
1. Dissolve 5 mg X-gluc in 50 µL Dimethyl formamide and ADD
2. 5 ml Phosphate buffer (0.05M, pH 7.0)
3. 1 mM Potassium ferrocyanide (25 µL of 200 mM stock)
4. 1 mM Potassium ferricyanide (25 µL of 200 mM stock)
5. 10 mM Na_2EDTA (50 µL of 1 M stock)
6. 0.1% Triton-X (100 µL of 1:10 diluted stock)
7. 0.2% Sodium azide (200 µL of 50 mg/ml stock)

Note: The assay mixture can be stored for several months at 0 ^0C in a dark bottle. Usually there is no need to add items 3,4, and 7.

ASSAY

Add about 200 µL (or sufficient to dip the tissue) of assay mixture to tissue sample (sections or discs or strips) and vaccuum infilterate for 3-5 minutes. Incubate the sample at 37 ^0C for 3-24 hours in dark. Remove the assay mixture and clear the tissue of chlorophyll by sequential changes in 70-100% ethanol untill tissue has no chlorophyll. Alternatively, for difficult to clear tissue add 75% lactic acid and autoclave for 15 minutes. This gives a very good image for photography. Mount the tissue in glycerol and observe under a microscope.

APPENDIX-V



Plasmid name: pCAMBIA 1301

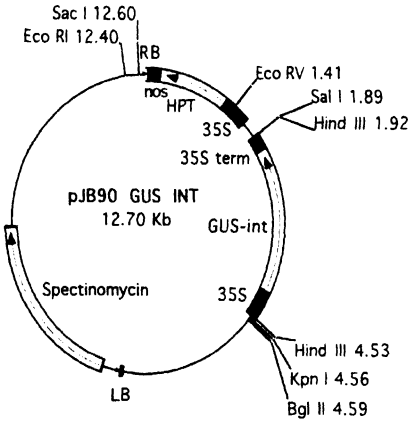
Plasmid size: 11837 bp

Constructed by: Richard Jefferson, CAMBIA

Construction date: 1996

Comments/References: General purpose Binary vector with GUS-Int and HPT genes. Has a multicloning site in the Lac Z alfa for cloning novel genes. Kanamycin resistance is used for bacterial selection. Histidine tag enables the "HexaHis" column purification of GUS protein.

APPENDIX-VI



Plasmid name: pJB90 GUS INT
Plasmid size: 12.70 kb
Constructed by: Deepak Pental, TERI, New Delhi
Construction date: ?
Comments/References: pJB 90 modified from pGSFR 780A