Chapter 5

TRANSFORMATION OF PLANTS FOR SALINE TOLERANCE

5.1 Strategies of generating transgenic plants

The following are the major strategies for creating transgenic plants:

5.1.1 Electroporation:

In this technique, protoplasts are incubated in buffer solution containing DNA and subjected to high-voltage electrical pulses. DNA migrates through pores in the membrane and integrates into the plant genome (Sorokin et al., 2000)

Advantages- This technique is a Simple, fast and inexpensive way for transient and stable transformation of different tissues.
Disadvantages- It has a low efficiency and requires careful optimization, also the range of tissues that can be transformed is narrow as compared to particle bombardment.

5.1.2 Microprojectile Bombardment:

In this technique Tungsten or gold particles are coated with DNA and accelerated towards target plant tissues using a special apparatus called a particle gun. These accelerated particles punch holes in the plant cell wall and usually penetrate only1-2 cell layers. The DNA-coated particles goes either near or in the nucleus, where the DNA comes off the particles and integrates into plant chromosomal DNA (Klein et al., 1987).

Advantages- It is one of the most versatile and effective technique. It is also environmentally friendly.

Disadvantages-low efficiency of transformation, low survival of bombarded cells caused by mechanical damages, DNA is not protected during bombardment, high number of transgene copies, random intracellular target.

5.1.3 Microinjection:

Using a micromanipulator and an inverted microscope, DNA is injected into cells or protoplasts by a syringe. (Holm, 2000)

Advantages-Possibility for optimization of amount of DNA delivered to a single plant cell, precision and predictability of the DNA delivery place.

Disadvantages - A single cell receives DNA during a single injection event. It is an expensive technique and requires exceptional manual capabilities.

The above methods have been used widely in plants but have the following disadvantages:

1. A relatively low efficiency of transformation
2. Integration of many transgene copies into the plant genome
3. Rearrangements of the transgene copies and chromosomes
4. Transgene silencing

5.1.4 *Agrobacterium tumefaciens* mediated transformation:

This has become the method of choice for plant molecular biologists. In this method, co-cultivation of plants or plant explants with *Agrobacterium* is done and the gene of interest is transferred by T-DNA mediated transfer from bacterial cell to the plant genome.

The phytopathogenic bacterium *Agrobacterium tumefaciens* genetically transforms plants by transferring a portion of its resident Ti plasmid, the T-

DNA (Transferred DNA) to the plant cells (Gelvin, 2000). It naturally infects wounded plant tissues and causes the formation of crown gall tumors (de la Riva, 1998). Virulent strains of *A.tumefaciens* harbor large plasmids of size 140-235 kb (Zaenen et al., 1974)

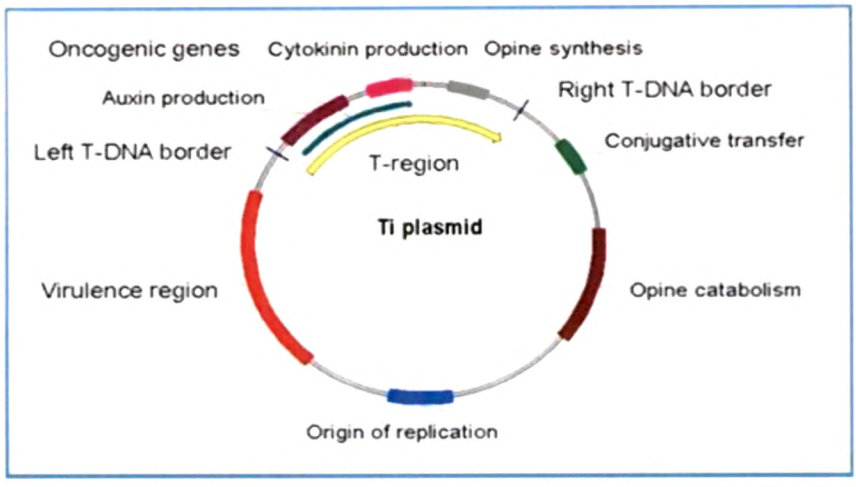


Fig. 5.1: Ti Plasmid (Chilton MD, 1978)

5.1.4.1 Ti Plasmid

The Ti plasmid consists of the following regions:

1) Right T-DNA border sequence and Left T-DNA border sequence. These are imperfect direct repeats flanking the T-DNA region. They are not transferred to the plant genome but are essential components in the transfer process.

2) Origin of replication which lies outside the T-DNA and allows the Ti plasmid to be stably maintained in *A. tumefaciens*.

3) Gene for opine catabolism which lies outside the T-DNA region. Opines are unique and unusual condensation products of an amino acid and keto acid.They are synthesized within the crown gall and then secreted. They can be used as a carbon and nitrogen source by an *A. tumefaciens* carrying a Ti plasmid bearing the gene for catabolism of that particular opine. This is therefore a unique mechanism that has evolved where each strain of *A.*

tumefaciens genetically transforms only those plant cells which produce a compound that it alone is able to utilize (Glick, 2003).

4) T-DNA region which consists of oncogenic genes namely those for auxin and cytokinin production. These phytohormones are responsible for the tumorous growth which results due to a disturbance in the levels of the phytohormones. It also contains gene for opine synthesis. All genes of the T-DNA have promoter sites and polyadenylation sites that are eukaryotic in nature (Ooms et al., 1981).

5) The vir (virulence) genes that lie outside the T-DNA region encodes virulence (Vir) proteins process the T-DNA region from the Ti-plasmid, producing a 'T-strand'. After the bacterium attaches to a plant cell, the T-strand and several types of Vir proteins are transferred to the plant through a transport channel. Inside the plant cell, the Vir proteins interact with the T-strand, forming a T-complex. This complex targets the nucleus, allowing the T-DNA to integrate into the plant genome and express the encoded genes as shown below.

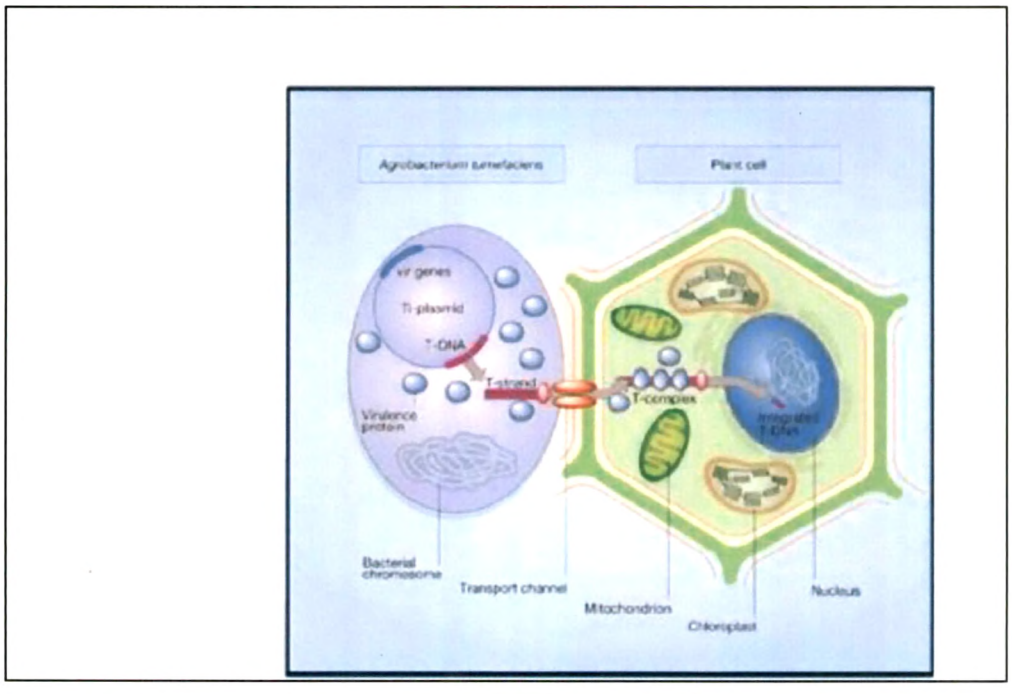


Fig. 5.2: Transformation of plant cells by Agrobacterium (Gelvin, 2005)

As the T-DNA was found to be the only part of the Ti plasmid to be transferred to plant cells, it was proposed that Ti plasmids could be used as a cloning vector for introducing foreign genes to plants where the gene of interest could be inserted in the T-DNA region and thus use this plasmid for transferring the gene of interest to plant cells. However there were certain limitations noticed when the natural Ti plasmid was used as a vector which are as follows:

1) The natural Ti plasmids being large molecules are extremely difficult to handle.
2) The natural Ti plasmid cannot replicate in *E. coli* as it lacks an *E. coli* replication origin and cannot be manipulated in *E. coli*
3) This plasmid lacks unique restriction endonuclease sites which are a prerequisite for any cloning experiment.
4) The production of phytohormones by transformed plant cells which is undesirable as it prevents transformed plant cells from being regenerated into mature plants.

Taking into consideration these limitations, following modifications were made in the Ti plasmid so that it became suitable as a vector:

1) The genes for synthesis of phytohormones were deleted so that tumors could not be generated in transformed plant cells and caused a decrease in the size of the plasmid.
2) Other dispensable regions were also removed causing a considerable decrease in the size of the plasmid.
3) An *E. coli* replication origin was introduced outside the T-DNA.
4) A bacterial selectable marker gene was added outside the T-DNA.
5) A multiple cloning site (MCS) was added in the T-DNA region to facilitate cloning the gene of interest into the T-DNA region.
6) A plant selectable marker gene was introduced in the T-DNA region so that it facilitated selection of transformed plant cells.

These modifications did not prevent the T-DNA transfer to the plant cells.
When the T-DNA of a Ti plasmid is deleted, it is termed as a disarmed Ti plasmid. In order to use the modified Ti plasmid as a vector two different types of vector systems were developed:

5.1.4.2 Co integrative vector

Cointegrative vector system was developed where it consisted of the cointegrate vector which contained the *E. coli* replication origin, a bacterial selectable marker gene operative in both *E. coli* and *A. tumefaciens*, the cloned gene of interest, the right border sequence, plant selectable marker gene and a region homologous to a segment of the disarmed plasmid contained in *A. tumefaciens*. The disarmed plasmid contained the *A. tumefaciens* replication origin, the left border sequence and the vir gene cluster. When the cointegrate vector was transformed in an *A. tumefaciens* bearing the disarmed plasmid, recombination occurred between the two plasmids to form the recombinant Ti plasmid (cointegrated plasmid). This was now capable of transferring the gene of interest to the plant cells when infected as the gene of interest was now located between the left and right border sequences. In this case, the T-DNA was cis with respect to the vir genes. The cointegrate vector could not replicate in *A. tumefaciens* as it lacked *A. tumefaciens* replication origin (Gelvin, 2003)

5.1.4.3 Binary vector

Binary vector system was a further advancement. It was constructed on the basis that there was no special advantage in retaining the T-DNA cis with respect to the vir genes (Hoekema et al., 1983). The observation that the T-DNA need not be physically linked to the vir genes for the transfer of the T-DNA into plant cells resulted in the development of the binary vector system (de Framond et al., 1983). In this system, the binary cloning vector contained both E. coli and *A. tumefaciens* replication origins outside the T-DNA making it a shuttle vector, a bacterial selectable marker gene operative

both in E. coli and *A. tumefaciens*, a plant selectable marker gene and the gene of interest cloned between the left and right border sequences. Cloning of the gene was performed in *E. coli*. The cloned plasmid was transferred to an *A. tumefaciens* strain bearing a disarmed Ti plasmid. The disarmed Ti plasmid provided the Vir proteins in order to enable the T-DNA of the binary vector to be transferred to plant cells (Gelvin, 2003).

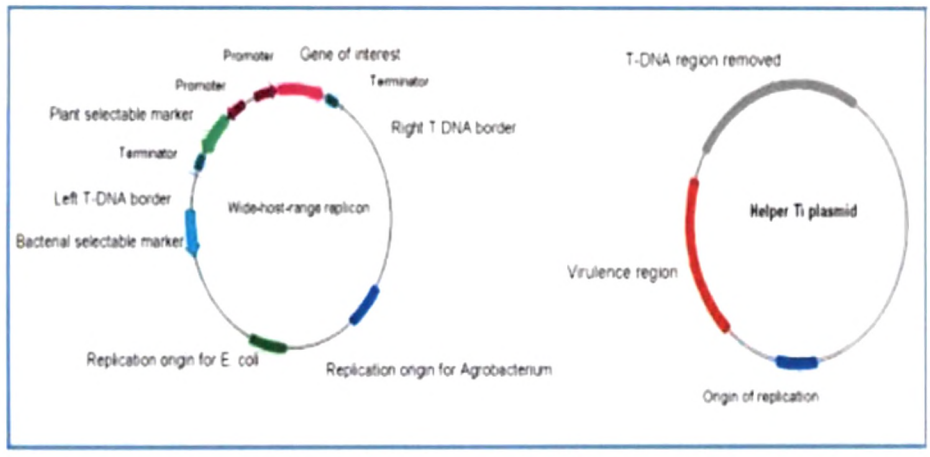


Fig. 5.3: Binary Vector System (Hoekema *et al.*1983),

The binary vector system has advantages over the cointegrative vector system which are as follows:

1) Use of binary vector system obviates the need for in vivo recombination.
2) The binary vector exists as a separate replicon and its copy number is not linked strictly to the copy number of the Ti plasmid.
3) Use of binary vector system is more efficient and quicker (Walkerpeach and Velten, 1994).

5.2 Materials and Methods

Agrobacterium mediated transformation of groundnut.

5.2.1 Plant Material, culture initiation and maintenance

Ground nut var. GG-20 obtained from Junagadh Agricultural University was used as the source of planting material.

The mature seeds were washed under running tap water for 25 min., treated with a solution of detergent Extran for 10 min, then subjected to treatment of

antifungal agent Bavistin (2%) for 10 min and finally washed thoroughly in sterile double distilled water.

Further procedures were carried out in Laminar Air Flow under sterile condition. Seeds were rinsed in 70% iso propyl alcohol (1 min) followed by 3 to 4 washes with double distilled water. The explants were then treated with 0.1% $HgCl_2$ for 7.5 min followed by three washes of sterile double distilled water.

Experiments were conducted on MS (Murashige and Skoog, 1962) medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose.
The media were solidified with 0.65% agar (colloids). The pH was adjusted to 5.8 before autoclaving at 121 C for 20 min. The cultures were maintained at 28 ± 1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

Transformation was attempted using three different explants and regeneration protocol as explained below.

Mature dry seeds were imbibed in sterile double distilled water for 6 h after surface sterilization and kept for germination on autoclaved filter paper bridges in sterile double distilled water in a test tubes. In case of embryoid axis and cotyledon explants, the explants were collected from 3-days-old seedlings. While in case of young immature leaves the explants were taken from 7 days old seedlings.

In case of embryo axis, the seed coat and the radical were removed and the cotyledonary nodes were excised by cutting both epicotyls and hypocotyls approximately 2-3 mm above and below the nodal region and the embryo axis was bisected along the longitudinal plane. The meristematic region present in the nodal region was macerated by 6-8 diagonal shallow cuts by a sterile surgical blade. From each seed, two explants were obtained.

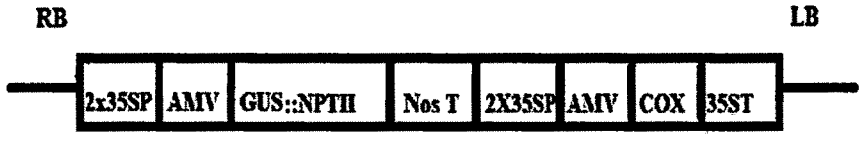
In case of cotyledon explants the embryos were surgically removed from the cotyledons and two explants were obtained.

In case of young green immature leaflet explants, seven days pre-incubated seeds on MS medium with 2.22 µM BAP were taken out aseptically, elongated radical was cut apart and the green young leaflets were separated and used as explants.

5.2.2 Agrobacterium strains and plasmid vectors

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) harboring the binary plasmid pHS724 kindly provided by Dr. Gopalan Selvaraj, Plant Biotech Inst., NRCC, Canada was used as a vector system for transformation. The uidA or GUS (8 glucuronidase) gene driven by the CaMV 35S promoter and Nos T, nopaline synthase gene terminator sequences served as reporter gene. The neomycin phosphotransferase II (nptII) gene driven by the nopaline synthase (NOS) promoter and Nos T, nopaline synthase gene terminator sequences was used as the selectable marker gene. Plasmid pHS724 contains the gene of interest - *cox* for choline oxidase gene and kanamycin resistance gene for bacterial selection. Plasmid pHS724 carries gene of interest driven by a double 35S promoter, and terminated by 35S poly A signal and the *nptII* and *uidA* genes.

5.2.3 Vector Map:



pHS724

Fig. 5.4 : Vector map

The vector contains Right border and Left border sequences which can specifically recombine with the right and left border of the Ti plasmid. The *cox* gene is under the control of viral 35S promoter. GUS represents the eukaryotic reporter gene while in case of primary selection, the transformants can be screened by kanamycin resistance. The size is 15.6 Kb.

5.2.4 Co-cultivation

5.2.4.1 Preparation of *Agrobacterium* suspension for co-cultivation

A single colony of *Agrobacterium* strain was incubated in 50 ml of YEP medium and grown overnight on a shaker at 200 rpm at 28°C to an OD at 600 of 1.4–1.6. The bacterial culture was centrifuged at 5,000 rpm and the pellet was resuspended in 50 ml of liquid suspension medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 100 mg/l myo-inositol, 30 g/l glucose and 100 µM acetosyringone. The suspension was kept at 28°C for 30 min and used for explant infection.

Different explants as explained above were infected with *A. tumefaciens* suspension culture for 20 min at room temperature under continuous shaking and transferred on to co-cultivation medium for 5 days at 21°C in the dark. The co-cultivation medium for different explants was different as mentioned in the table-5.1.

5.2.4.2 PROTOCOL USED FOR *AGROBACTRIUM* MEDIATED TRANSFORMATION

Isolate single Colony of *Agrobacterium* strain

↓

Incubate in 50ml YEP medium and keep over night/2 days on shaker @ 200 rpm at 28 °C

↓

Growth of the bacterial suspension should be to an OD 600 of 1.4-1.6

↓

Centrifuge the suspension at 5000 rpm and re-suspend pellet in liquid MS (MS salts + B_5 vitamins + 100 mg/L myo-inositol + 100 µM acetosyringone + 2% sucrose)

↓

Keep suspension at 28 °C for about half an hour and use it for explant infection

↓

Infect the surface sterilized explants with above suspension for about 20 min at RT, continuously shaking

↓

Transfer explants ascetically on to co-cultivation medium and incubate at 28 °C for 2-5 days in dark.
(MS salts + B5 vitamins + 100 mg/L myo-inositol + PGR + 3% sucrose)

↓

After co-cultivation, rinse explants 5-6 times with sterile DD water, remove excess bacterial suspension by blotting and inoculate in induction medium with the distal end in direct contact of media and incubate for 3-4 weeks.

The cotyledonary explants and the embryo axis with cotyledon were placed in such a way that adaxial surface of the cotyledon were in direct contact with the medium. The immature leaf explants were given superficial cuts on the margin and placed on the medium.

After 5 days of incubation the *Agrobacterium* infection was conspicuously seen in ring fashion on cotyledons and the leaf explants (Figure-5.5). After co-cultivation for 5 days, the explants were rinsed 5–6 times with sterile water separately under laminar hood. All explants were blotted on sterile paper to remove excess bacterial suspension and further placed on different shoot induction medium as shown in table 5.1. Cefotaxime (Alkem, India) (200 mg/l) was added in all shoot induction medium to eliminate overgrowth of *A. tumefaciens*. All the cultures were maintained at 28±1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

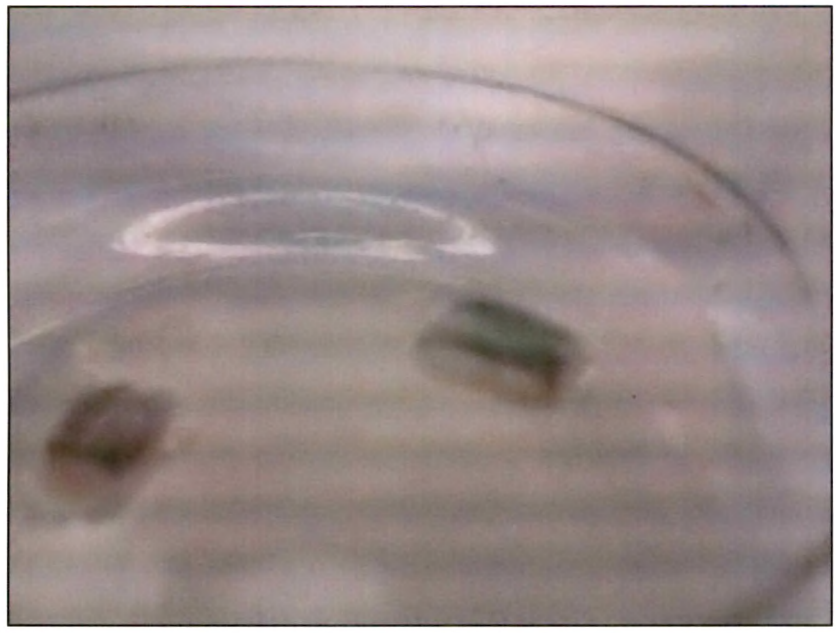


Fig. – 5.5A

Fig. 5.5B

Fig.5.5A: Infection of leaf explants after 5 day co-cultivation with *Agrobacterium*.
Fig.5.5B:Infection of cotyledon explants after 5 day co-cultivation with *Agrobacterium*.

Explants	**Embryo axis**	**Cotyledon explants**	**Young leaves**
Co-cultivation medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP and 0.53 µM NAA	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP and 2.68 µM NAA	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA
Shoot induction medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP, 0.53 µM NAA , 200 mg/l cefotaxime	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP, 2.68 µM NAA , 200 mg/l cefotaxime	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA, 200 mg/l cefotaxime
Shoot multiplication and Selection medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 8.88 µM BAP, 0.53 µM NAA, 200 mg/l cefotaxime 60 mg/l kanamycin	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 8.88 µM BAP, 200 mg/l cefotaxime 60 mg/l kanamycin	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA, 200 mg/l cefotaxime 60mg/l kanamycin
Rooting medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,

Table – 5.1: MS medium with different combinations used during different stages of production of transformed plants using three different explants of groundnut var. GG20.

5.2.5 Selection of transformants, multiplication and growth conditions

After 4 weeks of incubation on shoot induction medium, shoot forming buds were observed growing directly on embryo axis explants as well as proximal end of the cotyledons explants. However, immature young leaf explants

could not grow properly on the selection medium. The bud forming region from the embryo axis explants and cotyledon explants were transferred to respective shoot multiplication and selection medium consisting of MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 8.88 µM BAP, 200 mg/l cefotaxime and different concentrations of kanamycin (Macleods, India) for both the type of explants.

Two subcultures of 4 week intervals were done and multiple shoot buds were subcultured onto respective fresh medium. After two cycles of continuous kanamycin selection the shoots that attained > 2 cm length were excised and transferred onto MS medium supplemented with 4.30 µM α-naphthalene acetic acid to induce roots.

5.2.6 GUS analysis (Jefferson et al., 1987)

The ß -glucuronidase (GUS) histochemical assay was used as a rapid way to detect the presence of the *uidA* gene (GUS) in the putative transformants as described by Jefferson *et al.*1987 using leaf segments in regenerated shoots from explants. GUS assay was carried out by incubating the whole transformed plant tissues in GUS assay solution overnight at 37°C.

5.2.6.1 Preparation of GUS assay solution.

X-gluc solution to assay for GUS activity:

1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) ß-D-glucuronic acid was prepared in 0.1% of 50 mM Na_2HPO_4 at pH 7 along with Triton X-100. The aliquots of the solution were stored in the dark in a refrigerator. 0.01 g of X-Gluc was weighed out and dissolved in about 15 ml of sterile distilled water. 1 ml of 1 M sodium phosphate stock solution and 0.02 ml of the detergent Trition X-100 was added and after proper mixing the total volume made to 100 ml with distilled water. The solution was stored in the dark in the refrigerator.

1M sodium phosphate stock solution:

14.2 g of Na2HPO4 (dibasic sodium phosphate) and 13.8 g of NaH2PO4.H2O (monobasic sodium phosphate) was dissolved in approximately 90 ml of sterile, distilled water. pH was adjusted to 7.0 using NaOH. And the total volume was adjusted to 100 ml with distilled water.

5.3 Results and Discussion:

Explants were co-cultured with *Agrobacterium* strain LBA4404 carrying the binary vector pHS724 and then transferred to respective shoot induction medium to select for transformed shoots. Co-cultivated explants swelled and developed shoot buds with little callus after 3 weeks on selective shoot regeneration medium (Fig. 5.6). Shoot clumps which survived this selection step were sub-cultured to shoot multiplication medium. Differences in explant response on transformation frequency were apparent in culture (Table 5.2). Two weeks after culturing in selective shoot multiplication medium, the green shoot buds along with yellowish-white shoots were transferred to fresh medium with 60 mg/l kanamycin sulfate along with respective controls. The yellowish white coloration of shoot and leaves might be due to extra genetic load of the gene of interest along with uidA gene and nptII gene present and indicate the positively transformed shoots. There was significant reduction in qualitative growth performance of control plants compared to transgenic plants on Kanamycin rich MS medium (Fig. 5.7). The kanamycin resistant transformed elongated shoots were rooted in respective medium. Root induction was observed within 3 weeks of culture. The transformation frequency in terms of number of explants producing kanamycin resistant shoots was better using co-cultivated embryo axis explants (32%) than for cotyledons explants (21%). Different factors which affected the transformation frequency were studied.

Fig 5.6A

Fig 5.6B

Fig –5.6 : Cotyledon explants after 3 weeks of incubation on regeneration medium
A: Swelling of explants with growing buds and callus at the proximal end **cotyledon explants**.
B: Shoot bud producing plants from the proximal end of cotyledon explants.

Co-cultivation period Without kanamycin (No of days)	Shoot bud regeneration frequency* (mean + SD)		
	Embryo axis	Cotyledon explants	Young leaves
1	5.50 ± 1.08	5.20 ± 1.03	3.10 ± 1.37
2	13.50 ± 2.37	10.20 ± 1.23	-
3	18.40 ± 1.07	12.60 ± 1.84	-
4	26.10 ± 3.07	18.60 ± 1.51	-
5	32.20 ± 2.94	21.10 ± 2.69	-

Table 5.2. Effect of co-cultivation period on shoot bud regeneration on three explants of groundnut on shoot induction medium with 60 mg/l kanamycin sulfate.

* 30-35 explants were cultured per experiment and replicated three times.

5.3.1 Effect of Explant type on transformation

Co-cultivated explants showed different responses upon inoculation with *Agrobacterium*. The efficiency of shoot bud formation from embryo axis or cotyledon explants was not affected by *Agrobacterium* infection when compare with uninfected explants. On respective shoot induction medium, 50% embryo axis explants showed multiple shoot bud induction while 30% explants showed shoot bud formation in case of cotyledon explants at the proximal end. In case of young leaves explants shoot bud formation could not be achieved even after four weeks of incubation on the medium. Subsequent transfer of leaves explants also did not yield any bud formation. This might be because the leaves were too tender and young when co-cultivated with agrobacterium. These young leaves explants were transferred to the respective medium as per experimental design till rooting.

Fig.5.7 A

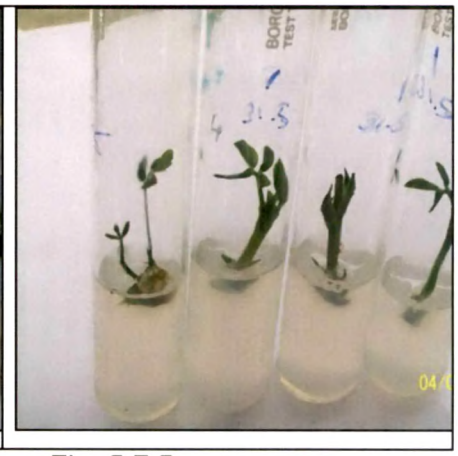


Fig. 5.7 B

Fig. -5.7 C

Fig. 5.7 Comparison of growth performance of transformed and control plants in kanamycin rich MS medium.

A: Untransformed plants in MS medium without kanamycin.

B: Untransformed plants in MS medium with 60 mg/l kanamycin.

C: Transformed plants in MS medium with 60 mg/l kanamycin.

5.3.2 Effect of Induction medium.

The excised multiple shoot buds from cotyledon and embryo axis explants were sub-cultured for two to three cycles on respective MS medium as shown in table 5.1. The transformation experiment was conducted with 30 explants each. The induction medium has profound effect on the number of shoots buds development and subsequent regeneration. Out of various combinations of BAP and NAA used for all the kinds of explants, the concentrations mentioned in table 5.1 could only yielded considerable number of shoots. Following two cycles of kanamycin selection, 32 shoots were recovered from the embryo axis explants while 20 shoots were recovered from cotyledon explants.

5.3.3 Effect of co-cultivation periods on transformation

Co-cultivation period had influence on the transformation frequency of different explants. Transformation frequencies of embryo axis and cotyledon explants co-cultivated on respective shoot regeneration medium for different periods are shown in Table 5.1. After two cycle of sub culture, the maximum transformation frequency occurred in embryo axis explants (32.20%) followed by cotyledon explants (21.10%) after 5 days of co-cultivation than after 1, 2, and 3 days of co-cultivation. Thus five days of co-cultivation resulted in the highest percentage of shoot regeneration on selection medium. These differences could be explained by the fact that the concentration of *Agrobacterium* was higher after 5 days of cultivation and this could increase considerably the possibility of gene transfer in groundnut. Similar observations were made in alfalfa by Chabaud *et al* (1988). Similar results were also observed in cowpea (Muthukumar *et al.* 1996) and in pea (DeKathen and Jacobsen 1990, Lulsdorf *et al.,* 1991). Co-cultivation for 48 h has been generally adopted, although longer periods have been reported to enhance the transformation efficiency in other legumes (Chabaud *et al.,* 1988, Geetha *et al.,* 1997).

Fig.5.8: Transformed multiple shoot from embryo axis explants.

However, extended co-cultivation period for more than 2 days for young leaf led to explant abortion caused by bacterial contamination. Thus young leaf explants could not yield any transformed shoots. These results do not agree with that of Eapen et al. (1994) who obtained an average of 6.7% of shoot regeneration on selection medium containing 50 mg/l kanamycin. Cheng et al. (1996) reported that the frequency of transformed fertile plants was 0.2% to 0.3% of the leaf explants inoculated. The difference in results could be explained by the fact that they used mature leaf segments while here we used tender young immature leaves which are fragile comparatively.

5.3.4 Effect of kanamycin concentration in selection:

To find out optimum concentration of Kanamycin and its effect on different explants, various concentration of kanamycin were added to respective medium as shown in table 5.3 and their effect were observed prior to *Agrobacterium* transformation. At 80 mg/l, kanamycin caused necrosis in all explants. Concentrations of 80 mg/l and 100 mg/l kanamycin completely inhibited shoot bud formation. Kanamycin concentration of 100 mg/l caused all explants to become necrotic within two weeks of incubation of culture and killed almost all the type of explants. At 60 mg/l, kanamycin

level produced highest transformation efficiency (45% and 40 % for embryo axis and cotyledon explants respectively) with normal growth of explants without any deleterious effect on both embryo axis as well as cotyledon explants, while in case of young leaves even 40-60 mg/l kanamycin also caused chlorosis and death of the explants. Thus, transformation efficiency was decreased when there was an increase in the kanamycin level beyond 60 mg/l. Thus, higher concentration of kanamycin caused necrosis of explants. Accordingly, in all the experiments with transformation kanamycin concentration of 60 mg/l was used in multiplication and selection medium for the initial selection of transformants. A concentration of 40 mg/l kanamycin was used for selecting transformants in subsequent subculture to prevent possible escapes. Similar findings were also reported by Kar et al. (1996) in chickpea and Venkatchalam et al. (1998).

Explant type	Kanamycin sulfate (mg/l)	No. of explants Co-cultured	No. of explants producing shoots	Transformation frequency (%)
Embryo axis	40	35	19	54.29
	60	35	16	45.71
	80	35	14	40.00
	100	35	8	22.86
Cotyledon	40	35	18	51.43
	60	35	14	40.00
	80	35	13	37.14
	100	35	3	8.57
Young leaves	40	35	11	31.43
	60	35	6	17.14
	80	35	4	11.43
	100	35	0	0.00

Table 5.3: Transformation frequency of groundnut var. GG20 as influenced by explant type and level of kanamycin sulfate.

5.3.5 Confirmation of transformants

5.3.5.1 Phenotypic Expression:

Shoot growth was determined to assess the stress tolerance of the transgenic plants. There was a significant reduction in the growth of transformed plants even under non-stress conditions compared to control plants (Fig.5.9), but this might be due to kanamycin-containing agar medium to select for only transgenic plants. Transformed plants which survived the selection pressure for two subcultures were inoculated in MS medium containing 100mM NaCl along with the control plants and incubated under standard growth room condition. All transgenics and their corresponding untransformed controls suffered a growth reduction under conditions of salinity (100mM NaCl). This correlates with the results obtained in case of *Brassica napus* and *Arabidopsis* by Huang et al. (2000). Growth reduction due to salinity was less severe in the transformed plants than in the untransformed control (Fig-5.11) in presence of 100 mM NaCl in medium.

After 12 days of incubation it was observed that the control plants which were healthy and were green in color started developing leaf burning and curling from the leaf margins while the transformed plants did not show such sign of burning of leaf edges and remained healthy and continued to grow. This indicates the accumulation of glycine betain as a consequence of expression of COX gene in the transformed plants increased saline tolerance up to 100 mM level. Hayashi et al. 1998 also reported development of different level of saline tolerance in transgenic *Arabidopsis* transformed with the cholin oxidase gene from *Arthrobacter globiformis* (CODA).

Fig. 5.9 Comparison of transformed plant with the control plants growing on MS medium without stress.

Fig. 5.10 Transformed plants which survived 2 cycle selection under kanamycin rich medium growing on MS medium with 100 mM NaCl .

Fig. 5.11 Comparison of performance of transformed plants with control plants.
L: control plants in MS medium without NaCl
M: control plants in MS medium with 100 mM NaCl
R: Transgenic plant in MS medium with 100 mM NaCl.

The whole regenerated plants along with cotyledons were subjected to *in situ* GUS assay. The expression of uidA gene was verified by histochemical staining of the leaf of the transgenic plants. The GUS positive regenerants showed the typical indigo blue colouration of X-Gluc treatment, while the control did not. Young leaves were more densely stained than other tissue of the plant and showed various GUS spots and GUS positive sectors on different leaf, shoot and cotyledon parts (Fig. 5.12). Both leaves and cotyledon parts of the transformants showed dense GUS positive sectors and not only GUS spots which indicate uniform transgene integration.

Fig. 5.12B Blue colored X – gluc staining in the leaves of transformed plants.

Fig. 5.12A Comparison of X – gluc staining in the leaves of control & transformed plants.

L : control plant

R : Transformed plant

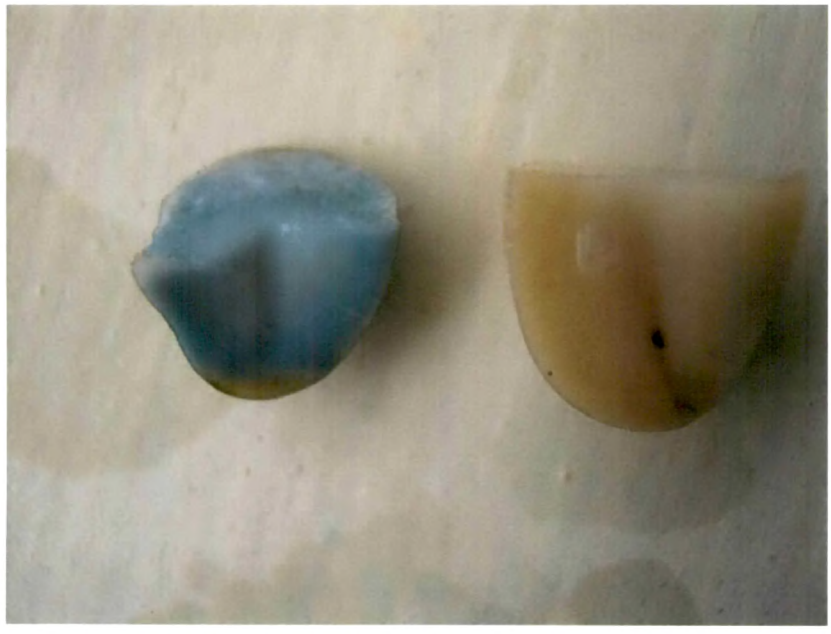


Fig. 5.13 Comparison of X – gluc staining in the cotyledon tissues of control & transformed plants

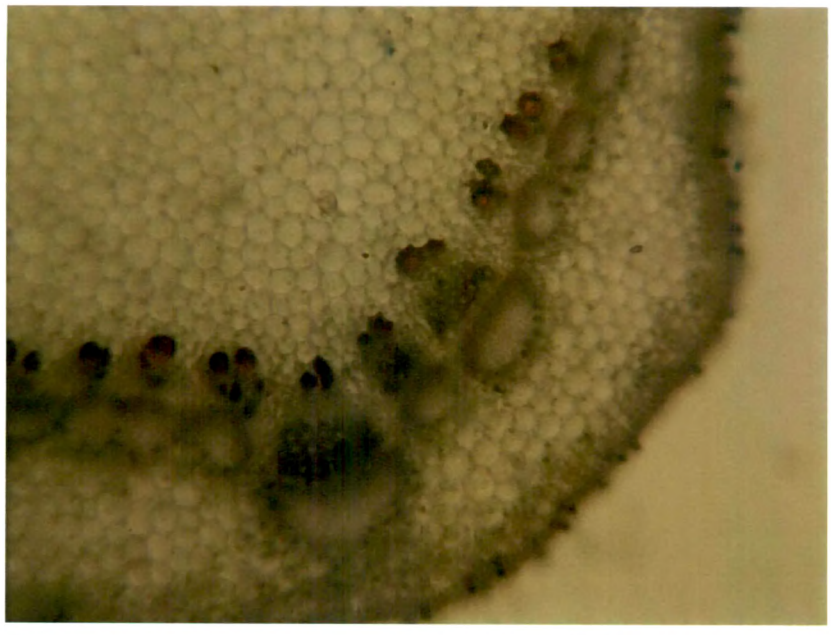


Fig.5.14 Blue spots of X – gluc staining in the cells of stems

5.3.5.2 GUS Expression:

This is a typical expression pattern of the CaMV 35S promoter regulated uidA gene in young tissues. Leaf tissues from untransformed control plants did not show GUS activity. These results and the extensive GUS expression in tissues, clearly demonstrate the stability of the inserted genes in the transformed plants. GUS expression has been reported previously by many workers in the groundnut (Eapen and George, 1994; Cheng et al., 1996).

However, further acclimation of these transgenic plants and growth performance under different salt concentrations in pots or soil is required to be performed.