IN PLANTA TRANSFORMATION STUDIES IN CHICK PEA (*Cicer arietinum* L.)

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

I. INTRODUCTION

Pulses play an important role in rainfed and irrigated agriculture by improving physical, chemical and biological properties of soil and are considered excellent crops for natural resource management, environmental security, crop diversification and consequently for viable agriculture. Indian population is predominantly vegetarian and protein requirement for the growth and development of the human being is mostly met with pulses.

Among the grain legumes, chickpea (*Cicer arietinum* L.) is the third most important in the world after dry beans and dry peas. Chickpea cultivation is mainly confined to Asia with 90 per cent of the global area and production (Bahl and Salimath, 1996). India has the distinction of being the largest chickpea producer and accounts for over 64 and 68 per cent of the total area and production in the world. In India, chickpea occupies 7.1 million hectare area with a production of 5.8 million tonnes annually. With respect to productivity, Andhra Pradesh, Bihar, Uttar Pradesh, West Bengal and Himachal Pradesh recorded an average yield of 1038 kg per hectare, while Karnataka recorded an average yield of 333 kg/ha (Bahl and Salimath, 1996).

Despite being the largest producer in the world, the country is in short supply of pulses. During 2004-05, the pulses production in the country was 13.3 million tonnes from 22.47 million hectare area. This is below the domestic requirement leading to import of pulses to the tune of 1.47 million tonnes (Ali, 2006). The major constraints that limit the realization of potential yield of chickpea are well known. These include biotic and abiotic stresses prevalent in chickpea growing areas besides socio-economic factors. Out of these, susceptibility of existing varieties to *Fusarium* wilt (*Fusarium oxysporum* f.sp. *cicer*) and pod borer (*Helicoverpa armigera* Hubner) have been major bottlenecks.

Chickpea is attacked by about 57 insect species in India, half a dozen of which are considered to be of economic significance. However, about 80-90 per cent of total pest damage is due to a single pest that is gram pod borer, *H. armigera* (Srivastava *et al*., 1996). More than 90 per cent pod damage has been recorded in some farmers' fields in India (Nene and Kanwar, 1988). The use of chemical insecticides has traditionally been the primary management option for *Helicoverpa* control on chickpea (Lateef, 1985 and Reed *et al*., 1987).

Chickpea crop is attacked by a variety of diseases among which wilt (*Fusarium oxysporum sp.,* ciceri) is a major disease causing losses up to 60-70 per cent. The extent of damage varies with temperature. This is most prevalent in dry areas where the crop is generally grown. There is no effective and economical chemical control for wide spread diseases like wilt. Management of soil borne pathogens causing wilt with fungicides is almost impractical (William, 1982) Five races of *Fusarium oxysporum sp.,* ciceri were identified for which resistant genes are dispersed in different wild relatives (Bahl and Salimath, 1996). So breeding for the disease is a difficult task in chickpea.

Over dependence on chemical pesticides has become untenable in view of the health hazards and environmental pollution leading to elimination of several non target beneficial fauna like natural enemies, bees, pollinators, birds, reptiles and small mammals. In recent years, however, the development of insecticide resistance in *H. armigera* (Gunning *et al*., 1984; Daly and Murray, 1988; Forrester *et al*., 1993) and renewed emphasis on sustainable environment-friendly crop protection practices has highlighted the need to develop alternative pest management technology that is viable, socially just and environmentally safe ensuring maintenance of resource base. Both economically and ecologically, breeding chickpea cultivars having resistance to the pest is the most important component of integrated pest management. As the screening of cultivated genotypes or germplasm has not identified inherent resistance (Sharma and Ortiz, 2000; Van Der Have, 1970), breeders are turning towards annual *Cicer* species as a possible source of desired traits. Unfortunately, interspecific hybridization in chickpea has been largely unsuccessful (Ahmad *et al*., 1988) and the wild species have not responded well to introgression through conventional breeding techniques for yield improvement (Van Rheenen *et al*., 1993). Genetic transformation provides a complementary means for the betterment of field crops. Research on transgenic crops, as in the case with conventional plant breeding aims to alter selectively

by adding or removing a character of choice in a crop plant, bearing in mind the regional needs and opportunities.

Resistance to insects has been demonstrated in transgenic plants expressing genes for δ-endotoxins from *Bacillus thuringiensis*, protease inhibitors, chitinases, secondary plant metabolites and plant lectins. *B. thuringiensis* (Bt) a gram negative bacterium produces a variety of insecticidal crystal proteins highly toxic to lepidopteran, dipteran and coleopteran insects. Genes responsible for crystal proteins (cry) have been identified, cloned and transferred into plant system. They are characterized by their potency and specificity towards specific insect pests and their relative safety to non-target insect species and vertebrates, particularly humans (Zhang *et al*., 1998). There are a variety of *cry* genes that have been used in transgenic crop plant development. Cotton (*cry1Ab, cry1Ac*) (Perlak *et al*., 1990), potato (*cry 3a*) (Perlak *et al*., 1993), maize (*cry1Ab*) (Koziel *et al*., 1993), japonica rice (*cry1Ab*) (Fujimjato *et al.,* 1993), tomato (*cry 1Ac*) (Mandaokar *et al.,* 1999), tobacco (*cry 1Ab*) (Perlak *et al*., 1991) tomato (*cry 1Ab*) (Perlak *et al*., 1991) are some of the transgenic crop plants developed with Bt toxins. Among the many cry genes, c*ryIAc*, which is effective against *H. armigera*, is preferred for chickpea transformation (Kar *et al*., 1996 and Krishnamurthy *et al*., 2000).

Almost all plant species synthesize some proteins with antimicrobial properties and their role in inhibiting bacterial and fungal growth has been demonstrated (Stuart and Harris, 1942). To date, a number of antimicrobial peptides have been isolated from many plant species (Broekaert *et al.,* 1997). Many of these peptides are being employed to obtain disease resistant varieties by cloning and transferring the genes encoding such peptides from cultivated or wild species and observed the inhibition of pathogens after infection (Carmona *et al.,* 1993; Birch, 1997). In a variety of transgenic dicot plants, constitutive expression of a single defense gene has strengthened the plant's defense against fungal infection (Alexander *et al.,* 1991). Two defense genes (*rafp1* and *rafp2*) were cloned from *Raphanus sativus,* and expressed in tobacco. The crude protein extract of the expressed gene in pET28a+ caused reduction in the growth of *Scierotium rolfsii, Rhizactonia bataticola* and *Fusarium solani* (Kulakarni, 2006).

Several methods are available for genetic transformation of plants including microprojectile bombardment, electroporation, sonication, *Agrobacterium*-mediated transformation, *etc*. The *Agrobacterium*-mediated genetic transformation is more efficient as it results in integration of well defined DNA sequence, potentially low copy number, high co-expression of the introduced genes and preferential integration into actively transcribed regions (Birch, 1997; Gheysen *et al*., 1998).

Many methods of plant transformation are tissue culture based, requiring regeneration of whole plants from transformed cells. The utility of the techniques greatly depends on the establishment of tissue culture procedures in the species (Birch, 1997). Chickpea, like other large-seeded legumes, is recalcitrant to *in vitro* regeneration and genetic transformation. The lack of reproducibility of regeneration protocols and highly problematic rooting and subsequent transplantation of the *in vitro* regenerated shoots is a major limiting factor for obtaining complete transgenic plants and their progeny. Tissue culture is labour intensive and can be difficult to master. In addition, even under optimal transformation and regeneration conditions, tissue culture can result in somaclonal variation, morphological abnormalities, changes in chromosome number, and loss of fertility (Larkin and Scowcroft, 1986; Feldmann and Marks, 1986; Lioyd *et al* 1986; Valvekens *et al* 1988).

Developing tissue culture-independent transformation system is of great interest because such a system would avoid constraints imposed by genotype specificity in transformation and regeneration and avoid tissue culture induced genetic variation. In addition, transgenic plants would be produced inexpensively and rapidly. With these points in view, the present investigation was carried out with the following objectives.

1. To standardize *in planta Agrobacterium* mediated gene transformation.

2. To confirm trasgenics through PCR.

2. REVIEW OF LITERATURE

Conventional plant breeding, combined with improved agricultural practices and modern technology, has contributed to dramatic crop improvement over the past several years and will continue to provide future benefits. However, there is an intense pressure to produce further improvements in crop quality and quantity. Conventional plant breeders and related scientists have worked diligently and skillfully to upgrade quality and raise yields by employing various crop improvement techniques and have obtained commendable results.

Biotechnology, based on recombinant DNA technology, is being advocated as an important adjunct to conventional plant breeding for sustainable development in agriculture which permits introduction of useful traits encoded by transgenes (Estruch, 1997). The tools and techniques of recombinant DNA technology have widened the definition of "gene pool" in plant breeding because it is now possible to mobilize candidate genes of interest into plants from hitherto inaccessible bioresources. Encouraged by these developments, a wider role of biotechnology in ecotechnology based precision farming of future especially for developing countries like India has been envisaged. In recent years, the practical utility of alien genes through transgenesis has been extensively demonstrated and transgenic plants harbouring genes for insect pest resistance, herbicide tolerance, improved post harvest shelf life, and for quality have been developed in a number of crop plants and are being grown commercially in both developed and developing countries. The past two decades have seen major advances in plant transformation, and a wide range of species can now be genetically transformed (Christou, 1995; Siemens and Schieder, 1996; Tarek *et al.,* 2002). These technologies have had considerable impact both on basic scientific research, where they have enabled advances in understanding plant processes and on production of economically viable agricultural products like transgenic plants. Transgenic plants are being used as an assay system for the modification of endogenous metabolism or gene inactivation (Newel, 2000).

2.1 *In planta* transformation

Genetic transformation of plants occurs naturally (Hooykas and Schilperoort, 1992). Scientists have been able to carryout controlled plant transformation with specific genes since the mid-1970s although many different techniques have been tested for gene delivery to plant cells, *Agrobacterium* mediated transformation has been extensively employed. The first transgenic plant of *Nicotinana tobaccum* was produced via *Agrobacterium* mediated transformation (Horsch *et al*., 1984). With this success, many crop plants were transformed via *Agrobacterium*. This is the simplest method now available for transferring genes into intact plant tissue.

Genetic transformation of plants can be transient or stable and transformed cells may or may not give rise to gametes that carry genetic material on to subsequent generations (Hooykas and Schilperoot, 1992). The generation of genetically homogeneous lines carrying the transformation event in all cells has typically presented two separate hurdles: transformability and regenerability of intact reproductively competent plants from transformed cells (Birch, 1997;Hansen and Wright, 1999). In most of the cases, transgenic plants are produced by methods which include the transformation of individual plant cells followed by regeneration of whole plants from those transformed cells (Christou, 1995; Fraley *et al*., 1983; Potrykus, 1991). Although these approaches work well for some species, in others they have been proven difficult to regenerate whole plants from those tissues susceptible to transformation. Even if the crop of interest is amenable to *in vitro* regeneration, it is unfortunate that plant regeneration from single transformed cells often produces mutations ranging from single base changes to loss of the entire chromosomes. In addition, significant epigenetic changes (like DNA methylation) can also occur (Phillips *et al*., 1994). Hence, the efforts were made to develop protocols for *in planta* transformation where there is no need of *in vitro* regeneration. There are only a few species such as *Arabidopsis*, tomato, soybean, *Beta vulgaris*, cotton, rice for which transformation systems avoiding tissue culture based regeneration are available (Anthony *et al*., 2000) and literature pertaining to this is reviewed herein.

2.2 Transformation methods that avoid tissue culture

A number of laboratories have pursued plant transformation methods that avoid tissue culture. In many cases these methods have targeted meristem or other tissues that will ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997). The same is true of popular tissue culture based transformation methods for corn, rice, wheat and soybean, which target young apical meristem for transformation (Birch, 1997). For non-tissue culture approaches, both *Agrobacterium* and tungsten particles have been used in a number of species to transform tissues or apical meristem cells that are subsequently allowed to grow into plants and produce seeds (Chee and Slighton, 1995; Birch, 1997). However, transformed sectors did not persist into gametes at reasonable frequencies or the methods were difficult to reproduce (Birch, 1997). Electroporation mediated gene transfer into intact meristem in *in planta* and a variety of pollen transformation procedures have also been reported (Chowrira *et al*., 1995; Touraev *et al*., 1997). However, most of these methods have been difficult to reproduce and have not gained widespread acceptance.

2.1.1 Transformation procedures targeting reproductive tissues

Germ-line transformation is the common feature that allows avoidance of tissue culture and regeneration in vacuum infiltration, seed transformation and floral dip methods (Feldmann and Marks, 1987; Bechtold *et al*., 1993; Chang *et al*., 1994; Katavic *et al*., 1994; Clough and Bent, 1998). This simple and inexpensive method was especially useful for plants that are not capable of regeneration and cannot pass through a tissue culture cycle (Tse and Chan, 2000 and Martinez and Herrero, 1998).

Injection of naked DNA into ovaries has been reported to produce transformed progeny (Zhou *et al*., 1983). Similarly, a mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers (Langridge, 1992). These procedures proved to be impractical because of their low reproducibility.

Some of the laboratories have succeeded in generating transformed *Arabidopsis* lines by "clip ’n squirt" methods (Chang *et al*., 1994; Katavic *et al*., 1994). Reproductive inflorescence were clipped off, *Agrobacterium* was applied to the center of the plant rosette, new inflorescence formed a few days later were again removed, *Agrobacterium* was reapplied, and plants were then allowed to develop and set seed. Transformants were obtained more reliably than with the seed treatment method, but the methods were only marginally more productive than traditional tissue culture approaches in *Arabidopsis* (Valvekens *et al*., 1988).

Direct access to the gamete is an option that can be achieved by the delivery of DNA into the pollen (Hu and Wang, 1999). Subsequent pollination with the bombarded pollen led to the recovery of transgenic plants (Touraev *et al*., 1997). However, this apparently clear strategy might, in fact be complicated by a variety of factors including the presence of nucleases and methylases in pollen (Oakelet *et al*., 1997), pollen survival and polyploidy (Leitch and Bennett, 1997).

Crucial stage of the revolution in *Arabidopsis* transformation came when Bechtold *et al.* (1993) reported success of transformation by "vacuum infiltration" method, which eliminated the *in vitro* steps. Transformation rates often exceeded 1 per cent of the seeds tested. *Arabidopsis* plants at the early stages of flowering were uprooted and placed *en masse* into a bell jar in a solution of *Agrobacterium*. A vacuum was applied and then released, causing air trapped within the plant to bubble off and be replaced with the *Agrobacterium* solution. Plants were transplanted back to soil and grown to seed. In the next generation, stably transformed lines could be selected using an appropriate antibiotic or herbicide. Transformation rates often exceeded 1 percent of the seeds tested. Variations of this extremely simple new method have been widely adopted by *Arabidopsis* researchers. Tissue culture and plant regeneration are no longer necessary and the associated high rates of mutation are avoided. Significant findings resulting from these efforts included the discoveries that (a) plant did not need to be uprooted, treated with *Agrobacterium* and replanted and that transformants could be obtained by treating only the protruding inflorescence (b) inclusion of

Silvet L-77, a strong surfactant that shows relatively low toxicity to plants, often enhanced transformation reliability (Clough and Bent, 1998); and (c) many different *Arabidopsis* ecotypes were transformable and many different *Agrobacterium* strains could be used, although notable difference in efficiency did exist (Anon., 2004).

Rapid and simple *in planta* transformation methods have been developed for the model legume *Medicago truncatula*. The approach involved infiltrating flowering plants with a suspension of *Agrobacterium* in which transformation frequency ranged from 4.7 to 76 per cent. The transformed plants were genetically stable and the analysis of T_2 generation indicated that the transgenes were inherited in a Mendelian fashion (Anthony *et al*., 2000).

Although the benefits of vacuum infiltration and floral-dip transformation methods are evident, efforts to apply these methods to other plant species have generally been unsuccessful. However, reports of success with *Brassica campestris* subsp. *Chinensi* and *Medicago truncatula* suggest that the method is not uniquely restricted to *Arabidopsis* (Liu *et al*., 1998).

Most important, the popular name “vacuum infiltration” was superceded when a number of groups found that plants could be transformed when dipped in *Agrobacterium* solution with no vacuum infiltration (Feldmann, 1997). Some workers subsequently moved to spray application of *Agrobacterium* rather than dipping. A number of other mechanistic clues and procedural tips were shared (Anon., 2004; Clough and Bent, 1998). Transformation by infiltration of adult plants with *Agrobacterium* has also been reported in Pakchoi (Liu *et al*., 1998) and has been informally reported for other brassicaceae beyond Pakchoi and Arabidopsis. Siemens and Schiedar (1996) reported that application of the *Arabidopsis* flower infiltration to *Brassica napus* and *Beta vulgaris*, was unsuccessful.

For the *Arabidopsis* seed transformation and vacuum infiltration methods, it was shown early that most primary transformants carry hemizygous T-DNA insertion events (Feldmann, 1992; Bechtold *et al*., 1993). The presence of the T-DNA on only one of the two homologous chromosomes implies that productive transformation occurs later in floral development after the divergence of male and female gametocytes. *Arabidopsis* is self pollinated, and if transformation occurs earlier, self fertilization is expected to give rise to some homozygous transformants due to the presence of some T-DNA insert in pollen and embryo sac cells. Transformation target was further defined in that transformants obtained from a given plant usually carried independent T-DNA insertion events (Feldmann, 1992; Bachtold *et al*., 1993). This suggests that transformation occurred after the divergence of individual pollen or egg cell lineages within a flower. A developmental end point for the typical target of transformation can also be postulated. Although the result is not as well established, typical primary transformants apparently carry the transgene in all parts of the plant, suggesting that transformation occurred before the cell division in a fertilized embryo. Hence, transformation seems to occur in developing flowers after individual gametophyte cell lineage formed but before extensive development of the embryo.

2.1.1.1 Pollen Tube Pathway (PTP) transformation

Use of pollen tube pathway to deliver foreign DNA into embryo was first reported in cotton (Zhou *et al*., 1983). This technique has been used to introduce total exogenous genomic DNA or plasmid DNA into other crops, including cotton (Ni *et al*., 1998; Zhou *et al*., 1983,), rice (Duan and Chen, 1985; Luo and Wu, 1989), soybean (Lei *et al*., 1994, 1995; Liu *et al*., 1992, 1997; Zhao *et al.,* 1995) and wheat (Yu *et al*., 1999; Zhen *et al*., 1998).

Initially, total genomic DNA was used for transformation. In these studies, treated plants were monitored and compared with untreated controls for variations in plant morphology, fertility, pest resistance, seed composition and peroxidase isozyme expression patterns (Lei *et al*., 1994, 1995; Liu *et al*., 1992, 1997; Zhao *et al*., 1995). Moore *et al.* (1996) reported failure to achieve transgenic soybean and cotton plants using a similar approach.

Different methods of pollen tube mediated transformation was reported by Chen *et al.* (1998) in watermelon like placing a droplet of solution with DNA on the surface of the style from which the stigma was excised following pollination, or injection of DNA into the ovary

following pollination. Treatments included circular and linearized plasmid DNA at 24 and 48 hours post pollination.

Lu *et al.* (2002) introduced cellulose-synthesizing genes derived from *Acetobacter xylinum* into cultivated cotton plants by using PTP. Seeds obtained from putative transenic plants were germinated on media containing hygromycin and phosphinothricin through PCR amplification. It was noticed that, five seedlings out of 934 seeds contained all four foreign genes by PCR. Three *Bt* transgenic lines, 95-1, RIOI and R-19 have been indigenously developed in China through PTP transformation. The stability of *Bt* gene expression and field efficacy has been confirmed (Narendra and Singh, 2000).

Herbicide tolerant and aphid-resistant soybean lines were obtained by introducing plasmid DNA carrying the *bar* and *gha* (*Galanthusnivas agglutinin*) gene via this technique (Liu, 1997). But, Huixia *et al.* (2002) reported from a detailed study that soybean pollen tube pathway transformation was not reproducible. Van der *et al.* (2004) obtained transformed lily plants with *npt II* gene following pollination with bombarded pollen. However, segregation analysis showed that transmission of the genes to the F_1 was not Mendelian.

Ellen and Randy (2004), reported direct transformation of higher plants by using PTP method, in which solutions containing donor DNA and various concentrations of the plant growth regulators such as NAA, GA_3 and DMSO were applied directly to the wound site of severed styles which have been previously pollinated with mentor pollen, allowing for the formation of pollen tubes extending from the style into the ovule. The recipient can be any flowering plant and be with any construct.

Integration of T-DNA into the maize genome as a result of treatment of silks with *Agrobacterium* cells, containing activated *vir* genes, was demonstrated. *In planta* treatment of maize (*Zea mays* L.) was performed during flowering in field. Cell suspension of *A. tumefaciens* line GV3101 (pTd33), carrying activated *vir* genes, was applied onto the previously isolated silks, which were afterwards pollinated with the pollen of the same cultivar. Integration of T-DNA into maize genome was confirmed by PCR (the *npt*II and *gus* reporter genes) and histochemical staining of the seedling tissues, obtained from the transformed seeds. Amplification of the *npt*II gene showed the presence of about 60.3% of PCR-positive plants out of the total number of kanamycin-resistant seedlings examined, or 6.8% of the total of number of seedlings (Chumakov *et al*., 2006).

2.1.1.2 Ovules as the primary target site for transformation

Given that transformation can occur by mere dipping of flowers in *Agrobacterium* solution and that anthers and pollen are exposed whereas ovules are not, it seemed likely that the male germ-line would be the target of transformation Ye *et al.* (1999) reported frequent GUS staining of ovules and pollen and showed ovules were the primary target for transformation. Ovule transformation was convincingly demonstrated when constructs containing a GUS marker gene were used to document sites of delivery of T-DNA (Ye *et al*., 1999; Desfeux *et al*., 2000). Further, Desfeux *et al.* (2000) showed that ovules were the target for transformation through a simple experiment. In their study, no transformants were observed among more than 14,000 seeds produced following inoculation of the pollen donor, but 71 transformants were recovered out of roughly 14,800 seeds produced following inoculation of the pollen recipient. These findings seemingly rule out transformation of pollen as it develops within anthers, but do not preclude the possibility that pollen is transformed after it germinates on the stigmatic surface of the pollen recipient. Interestingly, Desfeux *et al.* (2000) and Bechtold *et al.* (2000) did not observe GUS staining of anthers or pollen and concluded that pollen transformation is not common. However, Bechtold *et al.* (2000) did not observe staining of ovules or embryos in their work.

Genetic linkage analysis with a marked chromosome demonstrated that most transformants (25 of 26 tested) carried T-DNA on the maternally derived chromosome set (Bechtold *et al*., 2000). Only in one of 26 events most likely origin was due to integration of T-DNA within the paternal set.

2.1.2 Transformation targeting meristem and embryos

Direct transformation protocol targeting meristem and embryos without an *in vitro* phase is fast and beneficial by reducing the effort needed to obtain a reasonable number of transformed plants (3 months instead of 9 months). Feldmann and Marks (1987) carried out early stages of the revolution that transformed *Arabidopsis* transformation. They applied *Agrobacterium* to *Arabidopsis* seeds, grew plants to maturity in absence of any selection, then collected progeny seeds and germinated them on antibiotic containing media to identify transformed plants (Feldmann and Marks, 1987; Feldmann, 1992). Although the procedure was difficult to reproduce consistently, successful rounds produced transformants at a high rate that thousands of transformed lines were produced in a matter of few years.

In model legume *Medicago truncatula*, gene transfer by infiltration of young seedling with *Agrobacterium* has given 2.9 to 27.6 per cent transformation frequency. The transformed plants were genetically stable and the analysis of T_2 generation indicated that the transgenes were inherited in a Mendelian fashion (Anthony *et al*., 2000).

The transfer of genetic material into soybean tissue was accomplished by infecting germinating seeds with *Agrobacterium tumafaciens* (Chee *et al*., 1989). Graves and Goldman (1986) reported that transformed plant tissues could be obtained via *Agrobacterium* mediated infection of the mesocotyl region of germinating corn seeds.

In three important recalcitrant crops viz., sunflower, safflower and peanut embryos with one cotyledon were infected with *Agrobacterium* and allowing the seedlings to grow into a mature plant. Plants were screened in T_o and T_1 generations. This method is not only tissue culture independent but also genotype independent and permits screening of a large number of transformants in a short span of time (Rohini and Rao, 2002). Similarly, Yaye *et al.* (2004) produced transgenic *Hibiscus sabdariffa* plants by using embryonic axes of mature seeds with one cotyledon and infected by immersion in a suspension of *Agrobacterium* LBA 4404 strain.

Kojima *et al.* (2000) developed a simple and efficient method for transformation of buckwheat (*Fagopyrum esculentum* M.) plants in which apical meristems of seedlings were inoculated with *Agrobacterium tumefaciens*. Ping *et al,* (2003) developed *in planta* transformation of mulbary trees (*Morus alba* L.) in which meristems of axillary buds of young plants were inoculated with *A. tumefaciens*. For kenaf (*Hibiscus canabinus* L.) either the apical meristems or meristems of axillary buds of young plants were inoculated with *A. tumefaciens* (Kojima *et al.,* 2004).

In another study, seeds of rice were soaked in water for 2 days. Thereafter, the embryo containing an apical meristem was inoculated with *A. tumefaciens* by piercing a site of the husk overlying the embryonic apical meristem with a needle that had been dipped in an *A. tumefaciens* inoculum. The inoculated seeds were then grown to maturity (T_o plants) and allowed to pollinate naturally to set seeds (T_1 seeds) in pots under non sterile conditions. The transformation efficiency of T_1 plants was estimated to be 40% and 43% by PCR and a histochemical assay of ß-glucuronidase respectively (Supartana *et al*., 2005). By following same procedure Supartana *et al*., (2006) developed *in planta* transformation method for wheat.

Ahmad and Mirza (2005), developed a new transformation protocol for rough lemon (*Citrus jambhiri* Lush.), a major rootstock used in the citrus growing regions of Pakistan. *A. tumefaciens* carrying the binary vector p35*GUS*INT, containing *nptII* and *GUS* genes, was used. The transformation method was based on injection of *Agrobacterium* into citrus fruits followed by histochemical assay of *GUS* activity in different tissues. Different tissues of mature fruits exhibited significantly different percentages of transient *GUS* expression: in rind (76%), spongy tissue (92%), juice vesicles (0%) and seeds (83%) (P<0.01). The incubation period after injecting the *Agrobacterium* culture also showed a significant (P<0.01) effect on the transient expression of *GUS* in these tissues. An incubation period of 48 h was found to be the best (72%) for transformation of whole fruit, followed by 72 h (67%) and 96 h 49%). Transient *GUS* expression also varied significantly (P<0.01) in juice vesicles and seeds as fruit matured. Juice vesicles from mature fruits showed no transient *GUS* expression, while those from immature fruits showed 50% expression. Furthermore, transformation of seeds

had no effect on their germination capability. Germinating seeds from mature fruits injected with *Agrobacterium* culture showed tolerance to kanamycin (100 mg/L), which varied with the incubation period (55% at 48 h, 25% at 72 h and 23% at 96 h).

2.3 Factors influencing the rate of transformation

The rate of transformation influenced by many factors like plant genotype, *Agrobacterium* strain, plasmid vectors, temperature, surfactant etc.

In *Hibiscus sabdariffa*, Hooykaas and Schilperoort (1992) reported that the rate of *in planta* transformation indicated a strong interaction between its plant genotype and the strain. In numerous plant transformation systems, the choice of host genotype and/or *Agrobacterium* genotype has been an important parameter (Birch, 1997). But, Rohini and Rao (2002) reported that transformation through embryo picking is genotype independent in groundnut, safflower and sunflower. A better understanding of T-DNA transfer and other aspects of *Agrobacterium* plant interactions (Hooykas and Schilperoort, 1992; Mysore *et al*., 2000) may also allow engineering of better host/bacteria combinations.

A temperature of 22^0C was found to be optimal for T-DNA delivery in tobacco (Dillen *et al.,* 1997). In *Medicago truncatula,* Trieu *et al*. (2000) obtained transformants only if seedlings were subjected to a 4°C/14d vernalization treatment that induced earlier flowering.

Clogh, and Bent, (1998) have found that optical density (OD) of bacteria doesn't make much of a difference, that vacuum doesn't even make much of a difference as long as a decent amount of surfactant is present.

In *Arabidopsis,* silique that gave transformants were all from flowers inoculated 5 to 10 days prior to anthesis, a time when the locule is open. GUS staining of ovules occurred only in flowers that were inoculated 5 or more day prior to anthesis (Desfeux *et al*., 2000).

Despite production of fertile transgenic plants through transformation mediated by *A. tumefaciens,* transformation efficiency is still low. Apart from plant genotype, *Agrobacterium* strains, plasmid vectors, virulence gene inducing compounds, some other factors are becoming important for improving transformation efficiency of plant species. The use of wounded tobacco leaf extract increased the transformation efficiency in embryo transformation of sunflower, safflower, and groundnut (Rohini and Rao, 2002). Inclusion of thiol compounds, L-cysteine, dithiothreitol and sodium thiosulphste in co-cultivation medium increased transformation efficiency as high as 16.4% in soybean (Opabode, 2006).

2.4 Screening and/or confirmation of transformation events

2.4.1 Selectable markers and screening procedures

The genetic transformation of plants requires 'marker' genes that allow the recognition of the transformed cells in the background of untransformed ones. These genes are dominant, usually of microbial origin and placed under the control of strong, constitute, eukaryotic promoters, often of viral origin (Birch, 1997). The most popular selectable marker genes used in plant transformation vectors include constructs providing resistance to antibiotics such as kanamycin and hygromycin and genes that allow growth in the presence of herbicides such as phosphinotricin, glyphosate, bialaphos and several other chemicals.

Identification of transgenic plants immediately after the transformation process and the inheritance of transgenes in their progeny, usually involves time consuming, laborious and often expensive procedures, such as southern or northern hybridization, dot blot analysis, enzymatic assays or PCR. Although PCR is a fast and sensitive method, it is susceptible to cross contamination and the reaction conditions often need to be carefully optimized. Direct *in planta* assays for selectable marker gene activity, such as spraying whole plants or leaf painting with herbicide (Datta *et al.,* 1992) or germination of seeds on selective media (Hiei *et al.,* 1994) are both simple and direct methods. However, simple leaf painting is not effective for detecting plants transformed using antibiotic resistant genes and germination tests are not suitable for early identification of initially regenerated transgenic plants. Wang and

Waterhouse (1997), gave a very simple leaf assay that rapidly and reliably identifies transgenic plants expressing the hygromycin resistant gene, *hph* or the phosphinothricin resistant gene, *bar*. Leaf tips were cut from plants propagated either in the glasshouse or in tissue culture and cut surface embedded in solid medium containing the appropriate selective agent. Non-transgenic barley or rice leaf tips had noticeable symptoms of either bleaching or necrosis after 3 days on the medium and were completely bleached or necrotic after one week. Transgenic leaf remained green and healthy over this period. This gave unambiguous discrimination between transgenic and non- transgenic plants. The leaf assay was also effective for dicot plants tested (tobacco and peas).

2.4.2 Kanamycin screening

Kanamycin resistance is the most widely used selection criterion for higher plant transformation. The gene *npt II* was first established as a useful dominant selectable marker for higher plants (Bevans *et al*., 1983; Fraley *et al*., 1983; Herrera-Estrella *et al*., 1983). Since then its usefulness has been demonstrated with a broad group of plants (Steinbiss and Davidson, 1989).

Kanamycin resistance is conferred by transgenic expression of neomycin phosphotransferase, the product of the *npt II* gene from the bacterial transposon Tn5. The enzyme neomycin phosphotransferase transfers a phosphate from ATP to the aminoglycoside and thereby inactivates it. The *npt II* gene is effective both in nuclear and plastid genomes (Carrer *et al*., 1993).

Kanamycin has proven to be the most widely applicable selective agent (Curtis *et al*., 1996) but the concentration is species specific. Species like *Cicer arietinum* (Sarmah *et al*., 2004; Fontana *et al*., 1993; Sanyal *et al*., 2003). *Lycopersicum esculentum* (Fillatti *et al*., 1987), *Brassica napus* (Moloney *et al*., 1989) are selected at low concentration of kanamycin (15-100 mg/L) whereas *Betal vulgaris* needs relatively high concentration of kanamycin (400 mg/L).

Recently, Zapata *et al.* (1999) reported a new technique to screen the T_1 plants generated by selfing the T_0 plants. The T_1 progeny were germinated in the greenhouse and selected for the expression of T-DNA marker gene encoding *npt II* by painting 2 per cent kanamycin paste on the leaves. The plants that survived were analyzed by DNA blots. Evidence for integration of the GUS gene was observed in two successive generations from the regenerates (T_0).

Chengbin *et al.* (1999) reported seedling based selection procedure for identifying the transgenic arabidopsis seedlings, in which seedlings were sprayed for 2 days with 100 mg/l kanamycin followed by 2 days with 200 mg/l kanamycin, followed by 1 day with 500 mg/l kanamycin. They found that kanamycin resistant transformants were effectively selected by this method.

Yaye *et al.,*(2004) adopted a new screening procedure for *npt*II gene in *Hibiscus sabdariffa*. After 24 hour co-cultivation with *Agrobacterium* strain and decantication with cefotaxime, embryos were grown on soilrite containing MS medium added with a killer concentration of kanamycin(100µg/ml) during 4 weeks at room conditions and thereafter transferred to greenhouse. 54.3% of the seedlings grew well on selective medium; 68% of the explants excised from putative transformed plants were found to be GUS positive.

Although the use of kanamycin as a selectable marker in transformation experiments has been reported to be safe (Fuchs *et al*., 1993; Kuiper *et al*., 2001) the use of antibiotics as selectable marker in general is being more and more restricted by various regulatory organizations concerned with biosafety. Several strategies have been proposed to eliminate the use of antibiotic selection marker genes in transgenic plants (Yoder and Goldsbrough, 1994; Hohn *et al*., 2001). These strategies include the use of selective agents, such as herbicides (De Block *et al*., 1987) and other agents including amino acids, via altered metabolism or the total elimination of marker genes by co-transformation (Komari *et al*., 1996), site specific recombination (Dale and Ow, 1991), use of transposable elements (Yoder and Goldsbrough, 1994) and intra chromosomal recombination (Zubko *et al*., 2000).

Kanamycin has been used for selection in most of the chickpea transformation studies reported (Fontana *et al*., 1993; Kar *et al*., 1996, 1997; Krishamurthy *et al*., 2000). However recently Tewari *et al.* (2004) used a desensitized aspartate kinase (AK) gene as a non-antibiotic selection marker for production of transgenic chickpea, which was found to be a better selection marker than kanamycin as transgenic plants could be identified more easily and rapidly. AK, the first enzyme of the aspartate family biosynthetic pathway consisting of several isozymes that are feedback inhibited by its end products, lysine and threonine. This is due to the complete inhibition of AK activity by those two amino acids, which results in methionine starvation. Thus, plants transformed with densensitized AK are not expected to be feedback inhibited and they survive lycine and threonine treatments. Thus antibiotic free selection systems can be more commonly used in future in other legumes as well.

2.4.3 GUS staining

GUS activity can be quantified using either fluorometric or spectrometric assays, both of which are reasonably cheap and simple. Localization of GUS expression is possible histochemically also. However, the histochemical assay is expensive and needs destructive sampling. β-glucuronidase (GUS) activity is histochemically detected by incubating the tissues in GUS buffer with specified time. After staining, plant tissues are cleared in 70 per cent ethonol before observation.

Ovule transformation was convincingly demonstrated when constructs containing a GUS marker gene were used to document sites of dilivery of T-DNA (Ye *et al*., 1999; Desfeux *et al*., 2000).

35S and other standard promoter are poorly expressed in gametophytic tissues; so additional promoters used for GUS fusions were *Arabidopsis* ACT II (Desfeux *et al*., 2000), an oilseed rape skp1-like promoter (Bechtold *et al*., 2000), or a figwort mosaic virus promoter (Ye *et al*., 1999). Staining was observed in ovules in mature flowers and in younger flowers that had not yet reached pollination (Ye *et al*., 1999; Desfeux *et al*., 2000).

GUS reporter is widely used for studies on transformation in chickpea (Sanyal *et al*., 2003; Ramana *et al*., 1996; Fontana *et al*., 1993; Kar *et al*., 1996: Chakrabarty *et al.,*2000)

2.4.4 Molecular and genetic characterization of transgenic plants

The transgenic plants need to be confirmed for the presence and expression of the introduced gene by molecular methods followed by genetic characterization (Birch, 1997). The integration of the target gene is confirmed through ‘polymerase chain reaction’ routinely, which screens putative transgenics and classify either as positive or negative. The next technique could be used to detect the presence of a given sequence of DNA or RNA in the non-fractionated (not subjected to electrophoresis) DNA is ‘Dot blot’, where sample DNA’s from several individual can be tested in a single test run. Dot blots are useful in detecting presence of the sequence being transferred in a number of suspected transgenic individuals and the presence of specific mRNA in several such individuals or in different tissues of a single individual.

Southern blotting or southern hybridization is used to demonstrate the presence of the gene in question in transgenics, where detection of DNA fragments, which are complementary to given DNA, is critical. This is the common method to confirm the stable integration of DNA in the genome and also to know the number of copies integrated. Few examples of use of southern blotting to know the presence of transgene are available in chickpea (Krishnamurthy *et al*., 2000; Kar *et al*., 1996; Tewari-Singh *et al*., 2004 and Sarmah *et al*., 2004), pigeonpea (Geetha *et al*., 1999; Lawrence and Koundal, 2001) and groundnut (Mckently *et al*., 1995; Venkatachalam *et al*., 2000). The number of copies of a transgene construct inserted is variable for all transformation methods. The integration of a single T-DNA copy is common, but high numbers are also observed. Data from several different transgenic dicotyledonous species showed an average of three T-DNA inserts, with occasionally up to 20-50 copies in some plants. Kar *et al.* (1996) noted multiple gene inserts in transgenic chickpea while, Krishnamurthy *et al.* (2000) and Tewari *et al.* (2004) found 50 per cent single insert and 50 per cent multiple (4-6) gene inserts. However, single copy

insertion of the target gene based on strong signal generated by hybridization of GUS and *npt* II specific homologous probes was reported by Sarmah *et al.* (2004) and Sanyal *et al.* (2003). Several other techniques have been used for detection of expression of inserted gene such as northern hybridization, immunoblotting and western blotting (Sambrook *et al*., 1989).

Northern hybridization detects transcription of DNA sequence that is used as a probe. This technique has been used to know the expression of cowpea protease inhibitor at mRNA level in transgenic pigeon pea (Lawrence and Koundal, 2001). Bhattacharya *et al.* (2002), used southern confirmed plants for northern blotting of *Bt* (*cry* IA(b)) transformants in cabbage and reported differences in the level of transgene expression.

Western blotting is used to detect proteins of a particular specificity. Particularly when a transferred gene expresses in transformed cells, the translated product in the from of protein can be identified by this technique. Sarmah *et al.* (2004) analysed transgenic lines, which were PCR positive for α-bean amylase inhibitor using western blotting. The chickpea α-AII polypeptide detected by western blotting were similar in size to those found in bean seeds which indicated the primary translation product proteolytically processed in bean seeds was similarly processed in chickpea seeds. This assay was also used to estimate the level of α-A II in the T_1 seeds and found that the level of α-AII was 2.1 per cent of seed protein and thus similar to the 2.5 per cent level in bran.

The technique, immunoblotting is based on antigen-antibody reaction. In this method total protein from plants is fractioned on SDS-PAGE (10% polyacrylamide) and transferred to PVDF and detected by antiserum of specific antigen. Bhattacharya *et al.* (2002) detected Bt *cry* I(A)(b) proteins using the rabbit anti *cry* IA(b) serum and a goat antirabbit IgG coupled to alkaline phosphtase as secondary antibody. They could detect 81.3 kDA cry protein through this method which was further tested to know the toxicity on second instar larvae of *Plutella xylostella*. They observed high reduction in growth rate and mortality.

Sarmah *et al.* (2004) used this technique to confirm the expression of the transformed gene bean α-amylase inhibitor against *Challesobruchus maculatus* in putative transgenic chickpea.

III. MATERIAL AND METHODS

The details of the material used and the techniques adopted during the course of investigation are described under this chapter. The current investigation was carried out at the tissue culture laboratory, Department of Genetics and Plant Breeding and Institute of Agri-Biotechnology, College of Agriculture, Dharwad.

3.1 Materials

3.1.1 Genotype

The popular chickpea cultivar of the area, Annigeri (A-1) was chosen for the present investigation. The characteristic features of the same are given below.

Annigeri-1

This is a popular cultivar of the region with semi spreading habit. It belongs to *desi* group and matures in about 95-100 days. The leaves are dark green with smaller leaflet size. The seed size is medium brown coloured with prominent beak. It is moderately susceptible to wilt and highly susceptible to pod borer.

3.1.2 *Agrobacterium* strain and binary vectors:

The disarmed and hyper virulent *Agrobacterium tumefaciens* strain LBA4404 harbouring pKKM2810 obtained from BARC, Mumbai was used as a vector system for *in planta* transformation. Plasmid pKKM2810 (Fig. 1) contains the *cryIAc* gene linked to the cauliflower mosaic virus (CaMV) 35S promoter and gene encoding neomycin phosphotransferase (*npt II*) under the control of nopaline synthase (nos) promoter and terminator. *npt II* was used as a selectable marker.

The disarmed and hyper virulent *Agrobacterium tumefaciens* strain LBA4404 harboring pKKK207B obtained from Dr. P. U Krishna Raj, IABT, Dharwad was used as a vector system for *in planta* transformation. Plasmid pKKK207B (Fig. 2) contains the *rafp2* gene linked to the cauliflower mosaic virus (CaMV) 35S promoter and gene encoding neomycin phosphotransferase (*npt II*) under the control of nopaline synthase (nos) promoter and terminator. *npt II* was used as a selectable marker.

3.2 Methods

3.2.1 *Agrobacterium* Transformation

pHS-100 vectors containing *cry1AC* and *rafp2* genes were transferred to *Agrobacterium tumefaciens* strain LBA4404 by separate trip rental mating. The vector pHS-100 is capable of replicating in both *E. coli* and *Agrobacterium* and carries unique cloning sites and a plant selectable marker between its T-DNA borders. The chromosomal selection is rifampicin (25µg/ml) and it contains disarmed Ti-plasmid, which has streptomycin (100µg/ml) selectable marker.

The recombinant plasmid with respective genes were propagated in *E. coli* DH5α cells and grown overnight in Luria broth containing (50µg/ml) kanamycin at 37^0C. The *A. tumefaciens* strain LBA4404 was grown for 16 to 22 hours at 28^0 C in Yeast Extract Manitol Agar (YEMA) containing rifampicin (25 µg/ml) and streptomycin (100µg/ml). The *E. coli* helper strain containing pRK-2013 vector was grown overnight in LB containing kanamycin (50 µg/ml).

The overnight grown cultures were centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet was washed with 0.01 M $MgSO_4$ for thrice to remove traces of antibiotics. It was again centrifuged at 13,000 rpm for 1 min and the pellet was dispensed in 50µl of 0.01 M $MgSO_4$ *A. tumefaciens* LBA4404 *E. coli* DH5α (pRK-2013)

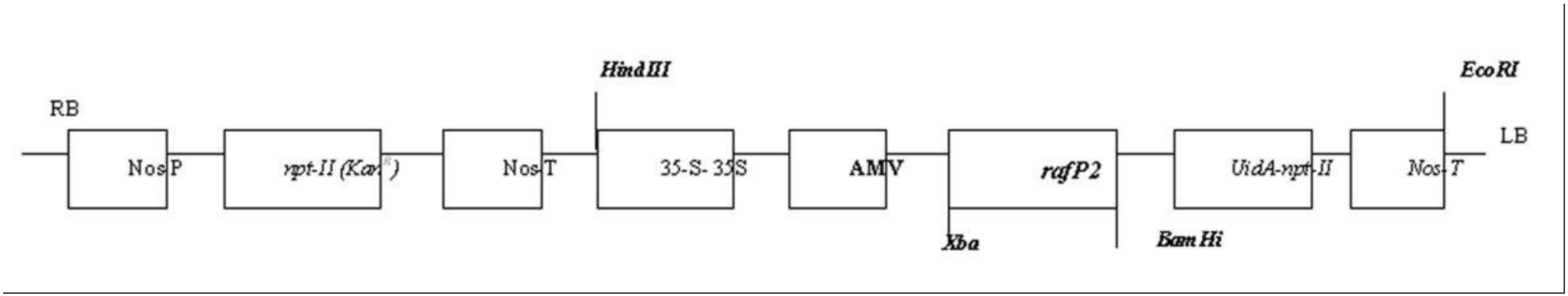

Fig 1. T-DNA construct containing rafp2 in pKKK 207B transformation vector

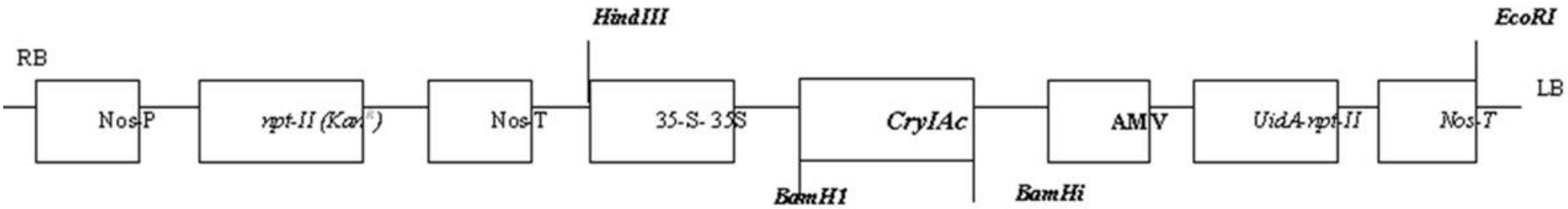

Fig 2. T-DNA construct containing *CryIAc* in pKKM 2810 plant transformation vecto

and *E. coli* containing pHS-100 with respective genes were mixed in 1:1:1 ratio separately. The mixture was spotted on plain LA medium and incubated overnight at 28^0 C. The spotted mixture was scraped and dissolved in 200 µl of 0.01 M $MgSO_4$ and spotted on YEMA medium containing kanamycin (50 µg/ml), rifampicin (25 µg/ml) and streptomycin (100 µg/ml) along with *A. tumefaciens* LBA4404 *E. coli* DH5α (pRK-2013) and *E. coli* containing recombinant strains as negative controls.

The presence of recombinant plasmid in the *Agrobacterium* was confirmed by PCR amplification.

3.2.2 Methods of *in planta* transformation

In planta transformation was attempted as described below with minor manipulations to allow the bacterial suspension to reach target cell/tissues in *ex-vitro* conditions.

3.2.2.1 Shoot tip infection

Bold and cleaned seeds of chickpea were surface sterilized with 0.1 per cent mercuric chloride and kept for germination in germination paper (Plate 1) to know the appropriate concentration of Triton X-100 to be used based on mean number of germinated plants, 30 shoot tips each in five replications were treated with each concentration of 0.01%, 0.05%, 0.10% and 0.20% of Triton X-100 and sown in pots. Germination count was taken 10 days after sowing.

As 0.01% concentration of Triton-X100 gave maximum germination percentage emerging plumules were treated with Agro-inoculum for 10, 20 and 30 min at 0.01 per cent Triton-X100, blot dried and directly sown @ 2 per pot. For the control plumules were dipped in 5 per cent sucrose solution. The established plants were counted after 10 days. Plants were screened 25 days after sowing through kanamycin leaf paint assay method. Plants showing yellowing/ chloratic symptoms as in control were discarded. The seeds from remaining plants were forwarded to next generation for confirmation through kanamycin leaf paint assay method and PCR.

3.2.2.2 Seed imbibition method

Bold and cleaned seeds of chickpea were surface sterilized with 0.1 per cent mercuric chloride and soaked in Agro-inoculum. For this method different incubation periods followed were 1.30, 2.00, 2.30 and 3.00 h. Different concentrations of Triton-X100 followed in 2.00 h incubation treatment were 0.01%. 0.05%, 0.1% and 0.2% Agro-inoculum was decanted and seeds were sown in greenhouse. Seeds soaked in 5 per cent sucrose solution without bacteria were used as control for each treatment. Ten days after sowing, the number of seeds germinated was counted treatment wise. Plants were screened 25 days after sowing through kanamycin leaf paint assay method. Plants showing yellowing/chloratic symptoms as in control were discarded. The seeds from remaining plants were forwarded to next generation for confirmation through kanamycin leaf paint assay method and PCR.

3.2.2.3 Vacuum Infiltration

To know appropriate duration of vacuum infiltration based on mean number of germinated seeds, 30 embryos with one cotyledon each in five replications were vacuum infiltrated at 10 min, 20 min, 30 min and 40 min duration and sown in pots. Germination count was taken 10 days after sowing.

As 10 min duration of vacuum infiltration resulted in maximum seed germination for all the experiments involving excised embryos with one cotyledon and germinating seeds (Plate 1) were vacuum infiltrated with *Agrobacterium* for 10 minutes and were sown in pots in greenhouse. For each treatment vacuum infiltrated seeds without Agro-inoculum were used as control. Ten days after sowing, the number of seeds germinated was counted treatment wise. Plants were screened 25 days after sowing through kanamycin leaf paint assay method. Plants showing yellowing/chloratic symptoms as in control were discarded. The seeds from

Plate 1. Germination seeds with plumule used for shoot tip infection and vacuum infiltration

remaining plants were forwarded to next generation for confirmation through kanamycin leaf paint assay method and PCR.

3.2.2.4 Infection of embryo with one cotyledon

To find out appropriate Agro-inoculum infection period based on mean number of germinated embryos, embryos were treated with Agro-inoculum for 5 min, 10 min, 15 min and 20 min duration. For each treatment, 30 embryos in five replications were treated and sown in pots. Germination count was taken 10 days after sowing.

Bold and healthy seeds were sterilized with 0.01 per cent (W/V) mercuric chloride for 4 minutes, washed 3-4 times with sterile distilled water and soaked over night. Then next day, embryos with one cotyledon intact were excised and grown for 2 days on Murashige and Skoog's medium in petriplates in culture room (Plate 2). Pre-cultured embryos with one cotyledon were injured at cotyledenary nodes, apical meristamatic regions and treated with *Agrobacterium* culture in three ways

i. Pricking the embryos with needle dipped in *Agrobacterium* culture.

ii. Dipping the pricked embryos in *Agrobacterium* culture for 10 min with intermittent shaking.

iii. Dipping the embryos in *Agrobacterium* culture and kept on shaker for overnight.

To find out appropriate co-cultivation period for treated embryos, another small experiment was conducted, in which Agro-inoculated embryos were co-cultivated for 24 hrs, 48 hrs, 72 hrs and 96 hrs. For each duration 30 embryos in five replications were kept and sown in pots. Germination count was taken 10 days after sowing. As 48 hrs of co-cultivation period was found appropriate, embryos with one cotyledon were co-cultured in dark for 48

Plate 2. Embryos with single cotyledon used for *in planta* transformation by infecting embryos

hours at 28^0C temperature Thereafter, germinating embryos were washed once in cefotaxime (400 mg L^{-1}) and three to four times with sterile water and then transferred to small cups containing autoclaved vermiculate and soil in 1:1 ratio. After 10 days, seedlings were shifted to greenhouse. Plants were screened 25 days after sowing through kanamycin leaf paint assay method. Plants showing yellowing/chloratic symptoms as in control were discarded. The seeds from remaining plants were forwarded to next generation for confirmation through kanamycin leaf paint assay method and PCR.

3.2.3 Culture conditions

All the laboratory experiments were conducted under defined conditions of the culture room, maintained at 25±2℃ under a 16 hour/day photoperiod. Uniform light intensity (Ca 1000/Lux) was provided by fluorescent tubes.

The culture work was carried out aseptically in a laminar airflow chamber. For all the experiments, borosilicate glassware and analytical grade chemicals were used. Sixteen-hour day photoperiod, 20℃ night and 25-30℃ day temperature was maintained in transgenic green house. Regular watering was done to the seedlings.

3.2.4 Nutrient medium

Based on the earlier study (Suma, 2006) Murashige and Skoog's (MS) (1962) medium identified as ideal, was used in the present investigation for growing embryos with one cotyledon. The composition of the medium used is furnished below.

3.2.4.1 Preparation of stock solution

Separate stock solutions of macrosalts, microsalts-I, microsalts-II, iron, $CaCl_2.2H_2O$ and organics (except inoisitol) were prepared and stored in coloured bottles in refrigerator. They were as follows.

1. Macrosalts (20x)	
KNO_3	38.00 g
NH_4NO_3	33.00 g
$MgSO_4.7H_2O$	7.40 g
KH_2PO_4	3.40 g

Taken one at a time and dissolved in small quantity of water, one after the other. Volume made upto 500 ml which gives 25 ml L^{-1}.

2. Microsalts-I (50 x)	
$MnSO_4.4H_2O$	1115.00 mg
$ZnSO_4.7H_2O$	430.00 mg
H_3BO_3	315.00 mg
KI	41.50 mg
Na_2MoO_4	12.50 mg

Volume made upto 500 ml which will become 10 ml L^{-1}

3. Microsalts II	
$CuSO_4.5H_2O$	25 mg
$CoCl_4.6H_2O$	25 mg

Volume made upto 100 ml. 10 m1 is taken from this solution and final volume is made upto 500 m1 which will become 5 ml L^{-1}.

4. Iron	50 x
Na_2EDTA	1865.00 mg
$FeSO_4.7H_2O$	1390.00 mg

The volume was made upto 500 ml, which will become 10 ml L^{-1} and stored in amber bottles.

5. $CaCl_2.2H_2O$ (50 ×)	22.00 g

Volume made upto 200 ml, which will become 10 ml L^{-1}

6. Organics	50 x
Thiamine HCl	500.00 mg
Nicotinic acid	50.00 mg
Pyridoxine HCl	50.00 mg
Biotin	25.00 mg
Glycine	500.00 mg

Volume made upto 200 ml, which will become 10 ml L^{-1}

All stocks were kept in refrigerator (at 4°C)

3.2.5 Preparation of antibiotics

Kanamycin, rifampcilin, streptomycin and cefotaxime (Hi-media) stocks of 200 mg mL^{-1} prepared in double distilled sterile water, filter sterilized and stored at 4°C. However, for leaf paint assay, kanamycin stock of 10 mg mL^{-1} was prepared separately.

3.2.6 Preparation of culture media

Media were prepared by adding required amount of stock solutions. Inositol, sucrose and agar were weighed and added in required amounts at the time of media preparation. After adding all the ingredients except agar, making up the volume little short of final volume, the pH was adjusted to 5.7 to 5.8 with 0.1 N NaOH and 0.1 N HCl using a digital pH meter. The final volume was made up and the required amount of agar added and autoclaved at 121°C for 15 minutes and dispensed into petriplates.

3.2.7 Maintenance and culture of *Agrobacterium*

The *Agrobacterium* LBA4404 (pHS100) culture was maintained on solid YEMA (Appendix IV) medium containing 100 mg L^{-1} of streptomycin, 50 mg L^{-1} of kanamycin and 25 mg L^{-1} of rifampicin. Sub culturing was done every fortnight in fresh media with selection pressure. Single *Agrobacterium* colony was taken from the Yeast Extract Mannitol Agar (YEMA) plate and inoculated to 100 ml yeast extract mannitol (YEM) broth containing 100 mg L^{-1} of streptomycin, 50 mg L^{-1} kanamycin and 25 mg L^{-1} of rifampicin and incubated on shaker for 36 to 48 h and fresh culture used for transformation work.

3.2.8 Method of co-cultivation

A loopful of *Agrobacterium* was taken from overnight grown culture on YEMA containing 100 mg L^{-1} of streptomycin, 50 mg L^{-1} kanamycin 25 mg L^{-1} rifampcin and inoculated into YEM broth along with selection agent. After 36-48 hours, the culture was centrifuged to harvest the cells and then suspended in 5 per cent sucrose solution along with Triton-X100 and called as

Agro-inoculum was used treatments with seed imbibition, germinating seeds and for vacuum infiltration.

The *Agrobacterium* strain LBA4404 (pHS100) was grown at 28°C in YEM broth containing 100 mg L^{-1} of streptomycin, 50 mg L^{-1} kanamycin and 25 mg L^{-1} rifampicin. After 36-48 hours, the culture was centrifuged to harvest the cells and *Agrobacterium* inoculum suspended in ½ MS liquid medium. Embryo with half cotyledon which were grown for two days on MS-medium, were pricked at apical and hypocotyledonary regions with sharp scalpel dipped in inoculum and co-cultivated in dark for two days on MS-medium. Then seedlings were planted in pots and kept in greenhouse.

3.2.9 Progeny analysis

In the present study, initially the non-transgenic plants were eliminated by kanamycin leaf paint assay in T_o generation Plants showing yellowing/chloratic symptoms as in control were discarded. The seeds from remaining plants were forwarded to next generation. All the T_1 plants so raised were subjected to kanamycin screening. DNA was isolated from kanamycin resistant plants by rapid method. To test the integration of target gene PCR analysis was done for the resistant plants.

3.2.9.1 Screening for putative transgenics

Kanamycin resistance is conferred by transgenic expression of neomycin phosphotransferase, the product of *nptII* gene from the bacterial transposon Tn5. The enzyme neomycin phosphotransferase transfers a phosphate group from ATP to the aminoglycoside and thereby inactivates it. Hence kanamycin resistance was used as a criterion for screening the transformants.

To standardize dose of kanamycin used to screen the putative transgenics embryos were cultured on MS medium supplemented with kanamycin ranging from 50 to 1200 mg L^{-1}. In another experiment, leaf paint assay was carried out. The control plants were tested by painting the first three leaves with 5 mg mL^{-1}, 10 mg mL^{-1}, 15 mg mL^{-1} and 20 mg mL^{-1} kanamycin solution during evening hours. After 4-5 days observations were made for mottled green sectors on leaves, burnt symptoms and leaf survival. The test was carried out following the protocol of Schroeder *et al*. (1993). The entire experiment was carried out in the controlled conditions in the greenhouse.

3.2.9.2 Confirmation for gene integration

Isolation of plasmid DNA was done from *Agrobacterium* by alkali lysis method (Sambrook and Russel, 2001)

- A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation needle and grown overnight in 100 ml YEM medium containing respective selective agents in a flask by keeping on shaker at 150 rpm at 27 to 30°C for 1 to 2 days.
- Overnight grown 1.5 ml culture was taken in 1.5 ml eppendorf tubes and centrifuged at 12,000 rpm for 15 min.
- Removing the supernatant the cell pellet was resuspended in 0.1 ml of solution I (Appendix I) per tube. The mixture was vortexted and kept on ice immediately.
- After incubation, 0.2 ml of solution II (Appendix I) was added to each tube and inverted gently 4-5 times and then transferred on ice for 5 minutes.
- For the above tubes 0.15 ml of solution III (Appendix I) was added, mixed well and kept at -80°C for 20 minutes. This was melted for 3-5 minutes before centrifugation for 10-15 minutes at 12,000 rpm and supernatant transferred to new tube.
- 0.5 ml of the supernatant was mixed with phenol to form an emulsion, vortexted and centrifuged for 5 min at 2,000 rpm. This supernatant was collected, poured to fresh 1.5 ml tubes.

- To the supernatant, 0.6 ml of CIA (chloroform: isoamylacohol = 24:1) was added,. centrifuged at 12,000 rpm after mixing gently. The upper aqueous layer was carefully removed with 1 ml pipette as 0.2 ml aliquots.

- The supernatant was treated with 0.2 ml sodium acetate and 0.6 ml isopropanol and stored at -80°C for 20 minutes (or overnight at room temperature). The solution was centrifuged at 12000 rpm for 10 minutes and supernatant was discarded leaving behind the DNA pellet.

- DNA pellet was washed with 70 per cent ethanol @ 500 µL/tube kept for 5 minute at room temperature and centrifuged for 5 minutes at 12000 rpm. Later, 70 per cent ethanol was decanted and tubes were placed in sterile hood to dry.

- The pellet was suspended in TE buffer and dissolved in water bath (37°C) for 20 min and stored at 4°C.

Extraction of genomic DNA for PCR analysis (Edwards *et al.*, 1991)

- Leaf tissue from putative transgenic and control plants was collected using 1.5 ml eppendorf tube lid to ensure uniform size.

- The tissue was macerated with pestle and mortar at room temperature without buffer for 15 sec.

- Later, extraction buffer (0.4 ml) (Appendix II) was added and sample vortexted for 5 sec.

- The solution was centrifuged at 13000 rpm for 1 minute and 300 µl supernatant transferred to fresh eppendorf tube.

- The supernatant was mixed with 300 µl isopropanol and incubated at RT for 2 minutes.

- The solution was centrifuged at 13000 rpm for 1 minute and supernatant discarded.

- The pellet was dried and suspended in 100 µl 1x TE.

- 100 µl RNase was added (1 mg mL^{-1}) to the DNA and incubated at 37°C in water bath for half an hour.

- The DNA was precipitated using $1/10^{th}$ volume of 3M sodium acetate and ethanol and incubated overnight at 4°C.

- The solution was centrifuged at 13000 rpm for 2 min and pellet dried again.

- The pellet was suspended in 50 µl 1x TE.

DNA quantity and quality estimation

The concentration of DNA was assessed spectrophotometrically and also by gel electrophoresis using 0.8 per cent agarose with known concentration of uncut DNA.

In spectrophotometric analysis, 5 µl of DNA sample diluted with TE buffer and volume made up to 3000 µl was subjected to spectrophotometer readings at absorbance of 230 nm, 260 nm and 280 nm. A good DNA preparation generally exhibits the following spectral properties.

$A_{230} < 0.10$, $A_{230}/A_{260} < 0.45$, $A_{280}/A_{260} < 0.55$, or $A_{260}/A_{280} > 1.80$

DNA concentration was calculated using O.D. at 260 nm with following formula.

Concentration of DNA (µg/ml) = O.D. at 260 x 50

To test the quality of DNA, samples were run on 0.80 per cent agarose gel in 1x TAE (Appendix III) buffer and stained with ethidium bromide and checked for contamination by RNA.

PCR amplification and analysis of amplified DNA fragments

Requirements

Template DNA: Genomic DNA extracted from young unopened leaves of putative transformants was used as template DNA.

Taq DNA polymerase: Taq DNA polymerase and 10x Taq dilution buffer were obtained from M/S Bangalore Genei Pvt. Ltd., Bangalore.

dNTPs: Individual dNTPs *viz*., dATP, dGTP, dCTP and dTTP were obtained from M/S Bangalore Genei Pvt. Ltd., Bangalore.

Thermocycler: Eppendorf was used for cyclic amplification of DNA.

Primers

The primers used were as follows.

nptII

5' GAG GCT ATT CGG CTA TGA CTG 3' (F)

5' ATC GGG AGA GGC GAT ACC GAT 3' (R)

cryIAc

5' ATG GAT AAC AAT CCG AAC ATC AAA GA 3' (F)

5' TTA TTA GCC CRA GTT GGT TTG TAC A 3' (R)

rafp2

5'GCG GAT CCA GTA GTG ATC ATG GCT AA 3' (F)

5'GCG AAT TCA ATT AAC AAG GGA AAT AAC A 3' (R)

Reaction mixture

The PCR reaction mixture comprised of 50 ng template DNA, 5 pmoles primers (forward and reverse each), 2.5 mM dNTPs, 0.33 µL (1 unit) of Taq DNA polymerase, 1x Taq assay buffer in a final volume of 20 µl.

Amplification conditions

The standardized amplification conditions were as follows.

For *nptII* specific primer.

Stage	Temperature (°C)	Duration	Cycles
Initial denaturation	94	5	1
Denaturation	94	1	39
Annealing	53	2	
Extension	72	2	
Final extension	72	10	
Hold	4	-	-

For *cry I Ac* specific primer.

Stage	Temperature (℃)	Duration	Cycles
Initial denaturation	94	5	1
Denaturation	94	1	39
Annealing	54.5	2	
Extension	72	2	
Final extension	72	10	
Hold	4	-	-

For *rafp2* specific primer.

Stage	Temperature (℃)	Duration	Cycles
Initial denaturation	94	5	1
Denaturation	94	1	39
Annealing	58.1	1	
Extension	72	1	
Final extensions	72	20	
Hold	4	-	-

After the completion of required cycles of amplification the samples were stored at 4℃ in a refrigerator and the contents loaded on to agarose gels for electrophoresis.

Separation of PCR amplified products by agarose gel electrophoresis

Procedure

- The frame of the gel-casting unit was cleaned, dried and sealed with a tape to form a mold. The frame was placed on a flat platform to ensure a flat and level base. The comb was then positioned parallel to the open edge of the frame about 2 mm above the surface.
- Sufficient (1x) electrophoresis buffer was prepared from 50x stock (Appendix III).
- Agarose powder was added (1%) to TAE buffer (1x) and dissolved by melting at 100℃. The solution was cooled to 50℃ and ethidium bromide added (0.5 µg/ml) and the comb positioned 0.5-1.0 mM above the base of the plate. Then agarose solution was poured into the gel frame and allowed to set. After setting, the gel was transferred to the gel tank such that the wells were towards the negative pole. The gel tank was filled with TAE buffer (1x) just enough to cover the surface of the gel.
- The DNA sample was mixed with gel loading buffer (Appendix II) and slowly loaded into the wells of the submerged gel using a disposable micropipette tip. λDNA *EcoRI* + *HindIII* double digest and 100bp ladder were used as a marker.

- The electrodes were connected to the power supply and electrophoresis was carried out at 100 volts for 1 hr or till the dye migrated to the end of the gel.
- The DNA was visualized on a UV transilluminator and documented in a gel documentation system (Uvi Tech, UK).

3.3 Statistical analysis

As all the studies were done in the laboratory under well defined conditions of temperature and light, completely randomized design (CRD) was employed for the experiments.

The analysis and interpretation of data were done using the Fisher's method of analysis of variance (ANOVA) as given by Panse and Sukhatme (1967). The level of significance used in 'F' test was P = 0.01.

3.3.1 Calculation of standard error of means and critical differences

$$\text{Standard error of means } (SEm\pm) = \sqrt{\frac{EMSS}{\text{No. of replications}}}$$

Critical difference (CD) values were calculated for P = 0.01, 0.05 probability levels.

$$CD = \sqrt{\frac{2 \times EMSS}{\text{No. of replications}}} \times \text{'t' error degrees of freedom}$$

4. EXPERIMENTAL RESULTS

The results of the experiments conducted on *Agrobacterium* mediated *in planta* transformation studies in chickpea are presented here.

4.1 Transformation of *Agrobacterium tumefaciens* strain LBA 4404

Different *E. coli* clones containing pHS-100 with *cry1Ac* and *rafp2* genes were transferred to *A. tumefaciens* LBA 4404 strain in separate tri-parental matings using *E. coli* (pRK-2013) as helper strain. Patch mating in the ratios 1:2:1 and 1:2:2 of donor : helper : recipient were done and all were found successful. The transconjugants were picked on YME medium containing kanamycin (50 μg/ml), streptomycin (100 μg/ml) and rifampicin (25 μg/ml). The recombinant *A. tumefaciens* LBA 4404 (pHS-100) strains were confirmed through PCR amplification of *npt II* (Plate 3) and *cry1Ac* (Plate 4) genes for *A. tumefaciens* with *cry1Ac* gene and in case of *A. tumefaciens* with *rafp2* gene, strains were confirmed through PCR amplification of *rafp2* gene (Plate 5).

4.2 Standardization of germination medium for embryos with one cotyledon

Experiments were conducted to standardize medium for germination of injured embryos with one cotyledon as germination and establishment of seedlings was very poor when directly sown in pots. For this, three treatments were done i.e., sowing injured embryos in cups containing 1) soil 2) vermiculate and 3) soil : vermiculate in 1:1 ratio. Along with these treatments injured embryos were also sown in pots directly as control. In each case, 30 injured embryos were sown. Though, good root growth was observed in embryos sown in vermiculate, establishment of seedlings after transplanting in pots was less (62.50%) when compared with seedlings grown in soil : vermiculate in 1:1 ratio (Table 4.1). Number of germinated embryos and number of seedlings established after transplanting was more in case of embryos sown in vermiculate and soil in 1:1 ratio. Hence, for better establishment of *Agrobacterium* treated, injured embryo with one cotyledon, vermiculate and soil 1:1 ratio was used as germination medium.

4.3 Screening of putative transgenics with kanamycin

To standardize killing dose of kanamycin used to screen the putative transgenics embryos were cultured on MS medium supplemented with kanamycin ranging from 50 to 1200 mg L^{-1}. Green shoot development with no proper root development was observed at 50 and 100 mg L^{-1} concentrations of kanamycin. Albino shoots with no proper root development was observed in 150 to 400 mg L^{-1} concentrations of kanamycin. In other concentrations, germination was not observed (Table 4.2).

In another experiment, response of chickpea to kanamycin was detected with the help of cotton swab saturated with kanamycin on 2^{nd} and 3^{rd} leaf from tip of the plant. Observations were taken on 5^{th} day of the treatment. The response was noted by observing the symptoms manifested in terms of chlorotic patches. Twenty plants with two replications were treated with 5, 10, 15 and 20 mg mL^{-1} kanamycin. The kanamycin level of 5 mg mL^{-1} did not develop any symptoms on treated plants. At concentration of 10 mg mL^{-1} onwards development of chlorotic patches on leaflets was observed. High concentration (20 mg mL^{-1}) turned leaflets completely chlorotic and plants died after few days. Hence, for screening of putative transgenics 10 mg L^{-1} kanamycin was employed.

4.4 *In planta* transformation by shoot tip infection

To know appropriate concentration of Triton-X100 to be used based on mean number of germinated plants, 30 shoot tips each in five replications were treated with each concentration of 0.01%, 0.05%, 0.10% and 0.20% of Triton-X100 and sown in pots. It was

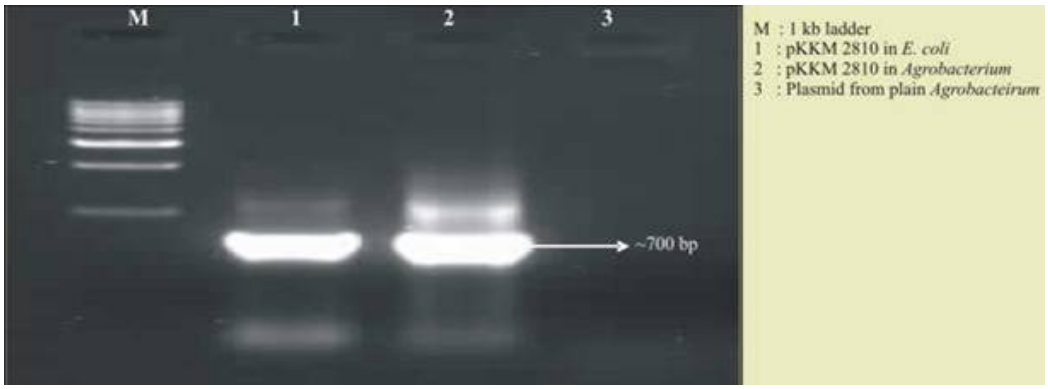

Plate 3. Amplification of *npt-II* gene in *Agrobacterium tumefaciens* in LBA 4404 (pKKM 2810)

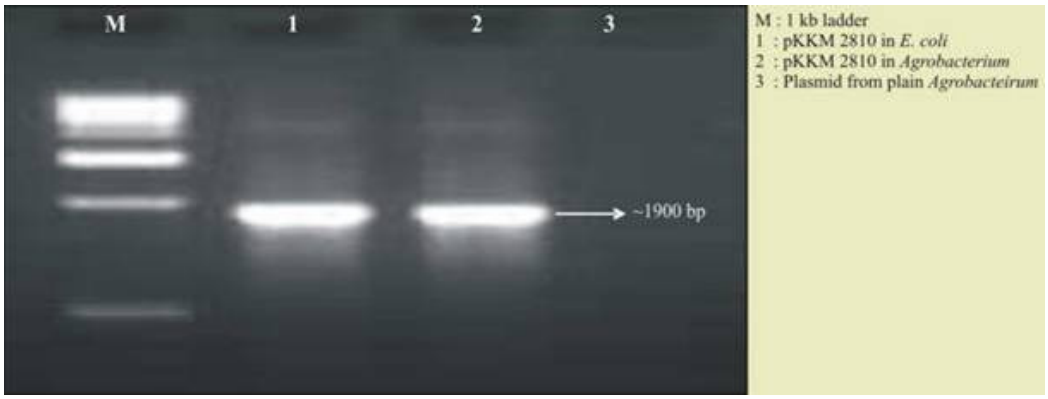

Plate 4. Amplification of *cry1Ac* gene in *Agrobacterium tumefaciens* in LBA 4404 (pKKM 2810)

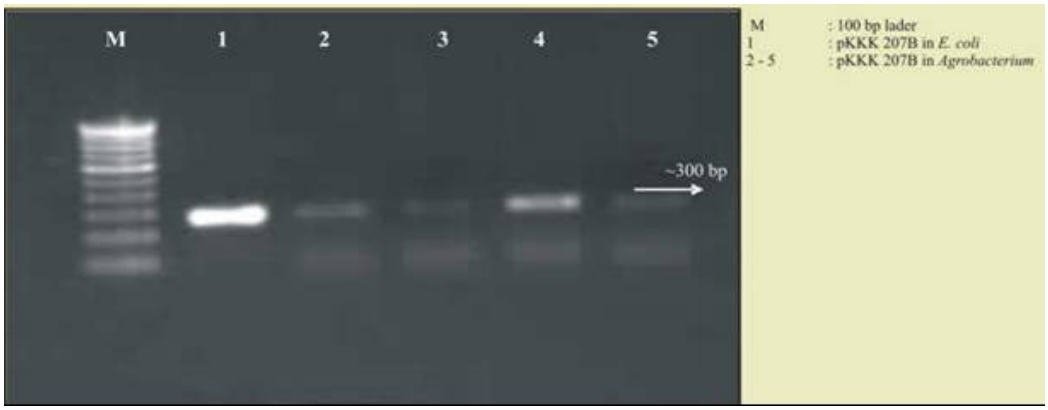

Plate 5. Amplification of *rafp2* gene in *Agrobacterium tumefaciens* in LBA 4404 (pKKK 207B)

clear from the results that Triton-X100 affects the germination. All the treatments were critically different at both 5 and 1 per cent levels of significance (Table 4.3). However, mean number of germinated seeds was more at 0.01 per cent of Triton-X100 (77.2).

In this method, germinating seeds with just emerging plumule were subjected to *Agrobacterium* treatment for 10, 20 and 30 min durations at 0.01 per cent of Triton-X100. For control, germinating seeds dipped in 5 per cent sucrose solution were used. Hundred per cent germination was not obtained even in control seeds. Seeds treated with *Agrobacterium* showed reduction in germination (Table 4.4 and 4.5).

In *cry1Ac* gene transfer, in T_0 generation the lowest incubation period (10 min) resulted in higher kanamycin resistant plants (4 out of 32), followed by 20 min incubation period (3 out of 30). The least number of kanamycin resistant plants were obtained in incubation period of 30 min (1 out of 25). In T_1 generation plants obtained from 30 min

Table 4.1: Standardization of germination medium for embryo with single cotyledon

Medium (Treatments)	Number of embryos sown	Number of germinated embryos	Per cent germination	Number of seedlings established	Per cent seedling establishment
Soil (in cups)	30	18	60	10	55.56
Vermiculate	30	24	80	15	62.50
Soil:Vermiculate (1:1)	30	26	86	22	84.61
Soil (in pots)	30	9	30	5	55.56
SEm±		0.122		0.141	
CD at 5%		0.367		0.424	
CD at 1%		0.505		0.584	

Table 4.2: Kanamycin sensitivity of embryos with one cotyledon

Sl. No.	Concentration of kanamycin (mg L^{Nil1})	Germination of embryos with one cotyledon
1.	Control	NG
2.	50	G
3.	100	G
4.	150	A
5.	200	A
6.	250	A
7.	300	A
8.	400	A
9.	800	Nil
10.	1200	Nil

NG – Normal germination
G – Green shoot development with no proper root development
A – Albino shoot with no proper root development

Table 4.3: Standardization of Triton X100 concentration for shoot tip infection

Sl. No.	Concentration of Triton X100	Mean number of germinated seeds
1.	0.01	77.2
2.	0.05	71.8
3.	0.10	68.7
4.	0.20	64.9
	SEm$\pm$	0.117
	CD at 5%	0.351
	CD at 1%	0.484

Table 4.4: Effect of incubation period on *in planta* transfer of *crylAc* gene by shoot tip infection

Incubation period (min)	Number of shoot tips treated	Number of plants established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
10	50	32	4	28	1	24
20	50	30	3	27	1	10
30	50	25	1	24	Nil	7
Control	50	36	Nil	36	Nil	Nil

Table 4.5: Effect of incubation period on *in planta* transfer of *rafp2* gene by shoot tip infection

Incubation period (min)	Number of shoot tips treated	Number of plants established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
10	200	138	6	132	6	24
20	200	123	12	111	7	63
30	200	117	1	116	Nil	7
Control	100	78	Nil	78	Nil	Nil

incubation period did not show resistance to kanamycin. One plant in each incubation periods of 10 min and 20 min showed resistance to kanamycin (Table 4.4).

In *rafp2* gene transfer, incubation period of 20 min produced high number of resistant plants in T_0 generations (12 out of 123) followed by 10 min incubation period (6 out of 136). Very few resistant plants were observed in 30 min incubation period (1 out of 117). In T1 generation, 6 out of 30 plants from 10 min incubation period, 7 out of 70 plants from 20 min incubation period and none from 30 min incubation period showed resistance to kanamycin (Table 4.5).

4.5 *In planta* transformation by seed imbibition method

Sterilized seeds were directly soaked in *Agrobacterium* suspension with 0.01 per cent of Triton-X100 for 1.30, 2.00, 2.30 and 3.00 h durations. Treatments with 0.05, 0.10, 0.20 per cent of Triton-X100 were also done for 2 hours incubation period. Hundred per cent germination was not obtained in treated as well as control. The least germination was noted at highest Triton-X100 concentration i.e., 0.2 per cent (Table 4.6 and 4.7).

In case of *cry1Ac* gene transfer 2.00 h incubation period with 0.01 per cent Triton-X100 gave kanamycin resistant plants (2 out of 30) in T_0 generation. However, in T_1 generation none of the plants showed resistance to kanamycin (Table 4.6).

In *rafp2* gene transfer, in T_0 generation 2.00 h incubation period with 0.01 per cent Triton-X100 gave maximum number of kanamycin resistant plants (4 out of 60) followed by 0.10 per cent Triton-X100, which gave 2 resistant plants out of 40. The remaining treatments with 2.00 h incubation period gave one resistant plant each (Table 4.7). For 1, 2.30 and 3.00 hr incubation period, no resistant plants were not observed in T_0 generation. Plants that showed resistance to kanamycin at 2.00 h incubation period with 0.01 and 0.20 per cent of Triton-X100 in T_0 generation alone gave resistant plants in T_1 generation (Table 4.7).

4.6 *In planta* transformation by vacuum infiltration

To know appropriate duration of vacuum infiltration based on mean number of germinated seeds, 30 embryos with one cotyledon each in five replications were vacuum infiltrated at 10 min, 20 min, 30 min and 40 min durations and sown in pots. It was clear from the experiment that with the increase in duration of vacuum infiltration, mean number of germinated seeds decreased. All the treatments differed significantly both at 5 and 1 per cent levels of probability. The mean number of germinated seeds was more at 10 min duration of infiltration (Table 4.8).

Excised embryos with one cotyledon and germinating seeds were vacuum infiltrated with Agro-inoculum for 10 min and sown in pots along with control seeds for each treatment. The establishment of seedlings from vacuum infiltrated embryos was less than control. Further, slow growth was observed in case of vacuum infiltrated embryos. In the T_0 generation in *cry1Ac* gene transformation 4 out of 37 plants obtained from vacuum infiltrated germinating seeds showed resistance to kanamycin. However, in T_1 generation only 2 out of 14 plants exhibited resistance (Table 4.9).

In T_0 generation of *rafp2* gene transfer, 2 out of 22 plants and 9 out of 42 plants obtained from vacuum infiltrated embryos and germinating seeds respectively showed resistance to kanamycin. In T_1 generation 2 out of 13 plants obtained from embryos and 15 out of 60 plants from geminating seeds showed resistance to kanamycin (Table 4.10).

4.7 *In planta* transformation by infecting embryos

To find out appropriate Agro-inoculum infection period based on mean number of germinated embryos, embryos were treated with Agro-inoculum for 5 min, 10 min, 15 min and 20 min duration. The mean number of well established plants decreased with the increase in duration of treatment. Mean number of well established plants for 5 and 10 min treatment did not differed at 1 per cent level of significance (Table 4.11).

Table 4.6: Effect of incubation period and concentration of Triton-X100 on *in planta* transfer of *crylAc* gene by seed imbibition method

Incubation period (h)	Triton-X100 concentration (%)	Number of seeds treated	Number of seeds germination	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
				Resistant	Susceptible	Resistant	Susceptible
1.30	0.01	50	41	Nil	41	Nil	Nil
2.00	0.01	50	32	2	30	Nil	13
	0.05	50	26	Nil	26	Nil	Nil
	0.10	50	21	Nil	21	Nil	Nil
	0.20	50	16	Nil	16	Nil	Nil
2.30	0.01	50	22	Nil	22	Nil	Nil
3.00	0.01	50	25	Nil	25	Nil	Nil
Control	0.01	50	40	Nil	40	Nil	Nil

Table 4.7: Effect of incubation period and concentration of Triton-X100 on *in planta* transfer of *rafp2* gene by seed imbibition method

Incubation period (h)	Triton-X100 concentration (%)	Number of seeds treated	Number of seeds germination	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
				Resistant	Susceptible	Resistant	Susceptible
1.30	0.01	100	56	Nil	55	Nil	4
2.00	0.01	100	60	4	56	3	13
	0.05	100	61	1	61	Nil	Nil
	0.10	100	40	2	38	Nil	7
	0.20	100	36	1	35	1	4
2.30	0.01	100	65	Nil	65	Nil	Nil
3.00	0.01	100	69	Nil	69	Nil	Nil
Control	0.01	100	82	Nil	82	Nil	Nil

Table 4.8: Standardization of vacuum infiltration duration with germinating seeds

Sl. No.	Duration of infiltration (min)	Mean number of well established plants
1.	10	75.2
2.	20	62.2
3.	30	56.1
4.	40	45.4
	SEm±	0.136
	CD at 5%	0.410
	CD at 1%	0.565

Embryos with one cotyledon were excised from overnight soaked seeds and grown for two days on Murashige-Skoogs medium. They have shown good glumule and radicle growth. These pre-cultured embryos with one cotyledon were treated with Agro-inoculum in 3 different ways.

1. Cotyledonary nodes and apical meristem were pricked with needle dipped in inoculum.
2. Injured embryos were dipped in inoculum for 10 min with intermitant shaking.
3. Injured embryos were dipped in inoculum and kept on shaker for 8 h.

To find out appropriate co-cultivation period for treated embryos, another small experiment was conducted, in which agroinoculated embryos were co-cultivated for 24 hrs, 48 hrs, 72 hrs and 96 hrs. Mean number of germinated seeds was low at 72 hrs (57.12) and 96 hrs (45.8) co-cultivation periods. Means of 24 and 48 hrs co-cultivation periods were not critically different at 5 and 1 per cent level of significance (Table 4.12).

After treatment embryos were cultured on MS-medium and kept in dark for 48 h at 28^0C temperature. Embryos became pale in appearance after co-cultivation. Embryos were washed with cefatoxime after 2 days and sown in small cups with autoclaved vermiculated and soil in 1:1 ratio and shifted to greenhouse after 10 days.

In T_0 generation of *cry1Ac* gene transfer very poor seedling establishment (3 out of 50) was observed from embryo dipped in inoculum for overnight (8 h) and did not yield any plants showing resistance to kanamycin. On the contrary, seedlings from injured embryos, dipped in inoculum for 10 min established well (34 out of 50) followed by seedlings obtained from pricked embryos with needle dipped in inoculum (29 out of 50).

In T_0 generation, 4 out of 29 plants from first method and 3 out of 34 plants from second method showed resistance to kanamycin. In T_1 generation, 4 out of 36 plants from

Table 4.9: *In planta* transfer of *crylAc* gene by vacuum infiltration

Treatment	Number of seeds treated	Number of plants established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
Embryoswith one cotyledon	50	13	Nil	13	Nil	Nil
Control	50	28	Nil	28	Nil	Nil
Germinating seeds	50	37	4	33	2	12
Control	50	40	Nil	40	Nil	Nil

Table 4.10: *In planta* transformation of *rafp2* gene by vacuum infiltration

Treatment	Number of seeds treated	Number of plants established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
Embryoswith one cotyledon	50	22	2	20	2	11
Control	50	33	Nil	33	Nil	Nil
Germinating seeds	50	42	9	33	15	45
Control	50	39	Nil	39	Nil	Nil

Table 4.11: Standardization of Agro-inoculum treatment period to injured embryos with one cotyledon

Sl. No.	Treatment period (min)	Mean number of germinated seeds
1.	5	68.0
2.	10	67.68
3.	15	63.4
4.	20	58.0
	SEm±	0.101
	CD at 5%	0.305
	CD at 1%	0.420

Table 4.12: Standardization of co-cultivation period for embryos with one cotyledon

Sl. No.	Co-cultivation period (hr)	Mean number of germinated seeds
1.	24	63.82
2.	48	63.60
3.	72	57.12
4.	96	45.80
	SEm±	0.130
	CD at 5%	0.039
	CD at 1%	0.540

Table 4.13: Effect of pricking embryos with one cotyledon on *in planta* transfer of *crylAc* gene

Treatment	Number of embryos treated	Number of seedlings established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
Pricking with needle dipped in inoculum	50	29	4	25	4	32
Dipping injured embryo in inoculum	50	34	3	31	2	14
Dipping in inoculation for overnight shaker	50	3	Nil	3	Nil	Nil

Table 4.14: Effect of pricking embryos with one cotyledon on *in planta* transfer of *rafp2* gene

Treatment	Number of embryos treated	Number of seedlings established	Reaction of T_0 generation plants to kanamycin		Reaction of T_1 generation plants to kanamycin	
			Resistant	Susceptible	Resistant	Susceptible
Pricking with needle dipped in inoculum	100	68	7	61	5	37
Dipping injured embryo in inoculum	100	61	19	42	9	80
Dipping in inoculation for overnight shaker	100	5	Nil	5	Nil	Nil

first method and 2 out of 16 plants from second method of treatments showed resistance to kanamycin (Table 4.13).

In *rafp2* gene transfer too very poor seedling establishment was observed for embryos dipped in inoculum and kept on shaker for 8 hours (5 out of 100) and plants did not show resistance to kanamycin. Maximum number of seedlings (68 out of 100) obtained from first method of treatment established well followed by seedlings from second method of treatment (61 out of 100) (Table 4.14).

In T_0 generation, more number of plants (19 out of 61) from second method of treatment showed resistance to kanamycin followed by first method of treatment (7 out of 68). In T_1 generation, 9 out of 89 plants from second method of treatment, 5 out of 42 from first method of treatment showed resistance to kanamycin.

4.8 Confirmation of gene integration in T_1 generation plants

In T_0 generation kanamycin screening was done to eliminate non-transformants. Plants showing chlorotic symptoms as in control were discarded and remaining plants (Plate 6) were advanced to next generation to confirm gene integration by kanamycin screening and PCR.

In *cry1Ac* gene transfer, DNA was isolated from all T_1 generation kanamycin resistant plants. None of them showed amplification for *npt II* and *cry1Ac* primers, indicating all are false positive for kanamycin resistance and no integration of *npt II* and *cry1Ac* genes was confirmed (Table 4.15).

In case of *rafp2* gene transfer DNA was isolated from T_1 generation plants showing resistance to kanamycin and screened through PCR with *rafp2* gene primers. In shoot tip infection for 10 min incubation period, one out of 6 kanamycin resistant plants showed amplification. In vacuum infiltration of germinating seeds for 10 min, one out of 15 kanamycin resistant plants showed amplification. Infecting embryo with one cotyledon gave maximum result. In second method (dipping injured embryo in inoculation for 10 min with intermittent shaking) three plants out of 9 kanamycin resistant plants showed amplification. Two out of 5 kanamycin resistant plants from first method (pricking embryos with needle dipped in inoculum) gave amplification (Plate 7, Table 4.16).

Table 4.15: PCR analysis of putative T_1 generation transgenics developed by different methods of *Agrobacterium in planta* mediated transfer for *crylAc* gene

Method	Incubation period	Triton-X100 concentration (1n%)	Kanamycin resistant plants in T_0 plants	Reaction of T_1 generation plants to kanamycin		Number of PCR positive plants
				Resistant	Susceptible	
Shoot tip infeciton	10 min	0.0	4	1	24	Nil
	20 min	0.01	3	1	10	Nil
	30 min	0.01	1	Nil	7	Nil
Seed imbibition	1.30 h	0.01	Nil	Nil	Nil	Nil
	2.00 h	0.01	2	Nil	13	Nil
		0.05	Nil	Nil	Nil	Nil
		0.10	Nil	Nil	Nil	Nil
		0.20	Nil	Nil	Nil	Nil
	2.30 h	0.01	Nil	Nil	Nil	Nil
	3.00 h	0.01	Nil	Nil	Nil	Nil
Vacuume infiltration						
1) Embryo with one cotyledon	10 min	0.01	Nil	Nil	Nil	Nil
2) Germinating seeds	10 min	0.01	7	2	12	Nil
Infecting embryo with one cotyledon						
1) Pricking with needle dipped in inoculum	Nil	Nil	4	4	32	Nil
2) Dipping injured embryo inoculum	10 min	Nil	3	2	14	Nil
3) Dipping in inoculum for overnight on shaker	Overnight (8 h)	Nil	Nil	Nil	Nil	Nil

Table 4.16: PCR analysis of putative T_1 generation transgenics developed by different methods of *Agrobacterium in planta* mediated transformation for *rafp2* gene

Method	Incubation period	Triton-X100 concentration (1n%)	Kanamycin resistant plants in T_0 plants	Reaction of T_1 generation plants to kanamycin		Number of PCR positive plants
				Resistant	Susceptible	
Shoot tip infeciton	10 min	0.0	6	6	24	1
	20 min	0.01	12	7	63	Nil
	30 min	0.01	1	Nil	7	Nil
Seed imbibition	1.30 h	0.01	1	Nil	4	Nil
	2.00 h	0.01	1	3	13	Nil
		0.05	4	Nil	Nil	Nil
		0.10	Nil	Nil	7	Nil
		0.20	2	1	4	Nil
	2.30 h	0.01	1	Nil	Nil	Nil
	3.00 h	0.01	Nil	Nil	Nil	Nil
Vacuume infiltration						
1) Embryo with one cotyledon	10 min	0.01	2	2	11	Nil
2) Germinating seeds	10 min	0.01	9	15	45	1
Infecting embryo with one cotyledon						
1) Pricking with needle dipped in inoculum	Nil	Nil	7	5	37	2
2) Dipping injured embryo inoculum	10 min	Nil	19	Nil	80	3
3) Dipping in inoculum for overnight on shaker	Overnight (8 h)	Nil	Nil	Nil	Nil	Nil

Screening and confirmation of transgenics

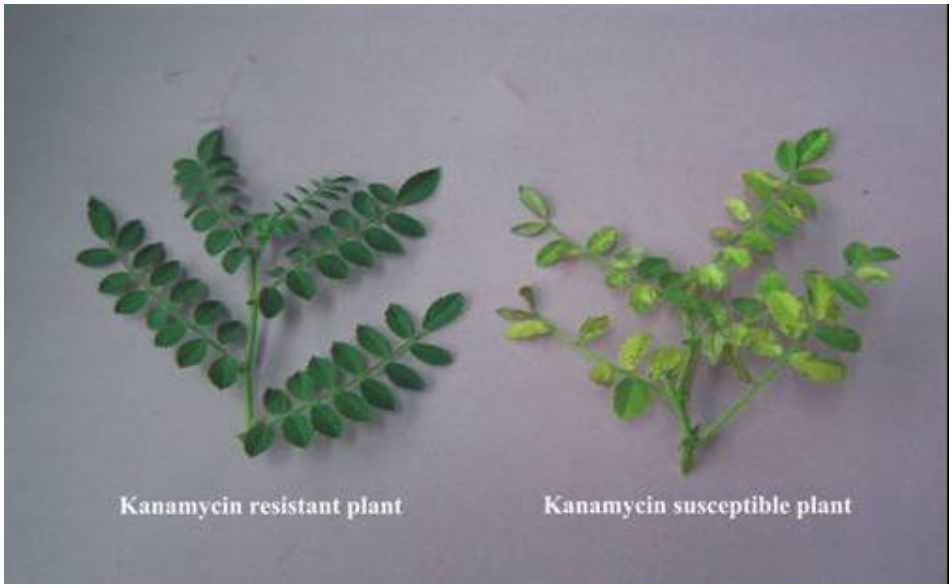

Plate 6. Leaf paint assay to screen for Kanamycin resistance

Plate 7. Amplification of *rafp2* gene in transgenic plants

5. DISCUSSION

Transformation of crop plants with desired genes is the focus of many plant genetic engineering programs. The stable introduction of foreign genes into plants is one of the significant advances in crop improvement programme, which would help in transforming desirable genes into otherwise well adopted varieties, thus supplement conventional breeding programme.

Among the several methods used for transformation of plants, *Agrobacterium tumefaciens* mediated transformation is preferred in many cases because of several distinct advantages over other methods. These include single copy integration, greater precision with excellent stability.

The development of *in planta* transformation system for *Arabidopsis* (Clough and Bent, 1998) radically accelerated research in basic plant molecular biology. After that a number of laboratories have pursued plant transformation methods that avoid tissue culture and/or regeneration. The benefits are clear : transformation without tissue culture can provide a high throughput method that requires minimal labour, expense and expertise.

The rate of transformation is influenced by various factors like plant genotype, *Agrobacterium* strains, plasmid vectors, temperature etc. Along with these factors, others factors like Triton-X100 concentration, vacuum infiltration duration, Agro-inoculum treatment duration and co-cultivation period also influences transformation rate. To know appropriate concentration, the effect of above factors on explant germination were conducted.

The mean number of germinated seeds observed was highest (77.20) at 0.01 per cent of Triton-X100 concentration and 10 min duration of vacuum infiltration compared to other treatments. Higher concentration of Triton-X100 and more duration of vacuum infiltration might have caused injury of embryos/tissues leading to low germination.

Among different infection periods tried, 5 and 10 min gave maximum number of germinated seeds compared to other treatments. The mean number of seeds germinated after 24 h and 48 h of co-cultivation did not differed significantly. Since, 10 min infection period and 48 h co-cultivation periods provide relatively more time for interaction *Agrobacterium* and injured tissues, same was followed for further transformation experiments.

The main difficulty associated with *in planta* transformation protocol is screening large number of plants in T_0 and T_1 generations. A suitable technique needs to be developed for easy screening of seeds in any crop species. In *Arabidopsis*, germination medium with kanamycin is routinely used for screening seeds obtained from T_0 plants (Bechtold *et al*., 1993 and Reddy *et al*., 2002). In chickpea same procedure was followed but resistant plants obtained by this screening method exhibited very poor establishment in greenhouse. Same observations were reported by Suma (2006). Hence, *ex vitro* method i.e., leaf paint assay was adopted to screen for transformants both in T_0 and T_1 generations. Similar method was followed by Sarma *et al.* (2004), Tewari *et al.* (2004) and Suma (2006) to screen transformants.

Wide gap was found between kanamycin resistant plants and PCR plants. The discrepancy between values obtained for selection by kanamycin and PCR detection was related to some escapes from the selection procedure. However, this procedure was effective in eliminating the non-transformants and in reducing the number of plants for PCR screening.

In planta transformation methods have targeted meristem or other tissues that ultimately give rise to gametes (Chee and Slighton, 1992 and Birch, 1997). Successful transformation has been reported in *Glycine max* (Chee *et al*., 1989), *Brassica* species (Liu *et al*., 1998), *Arabidopsis* (Desfeux *et al*. 2000), *Medicago truncatula* (Trieu *et al*., 2000), peanut (Rohini and Rao, 2000), pigeonpea (Sandyarani, 2002) and *Hibiscus sabduffa* (Yaye *et al*., 2004). Cultivated genotypes or germplasm do not to inherent resistance to pod borer and *Fusarium* wilt (Vander Have, 1970, Sharma and Ortiz, 2000). Interspeicific hybridization in chickpea has been largely unsuccessful (Ahmad *et al*., 1988) and further the wild species

have not responded well to introgression through conventional breeding techniques (Van Rheenen *et al*., 1993). Hence, developing transgenics in chickpea is need of hour. Since, chickpea is recalcitrant to *in vitro* regeneration and genetic transformation, development of transgenes in chickpea through tissue culture independent methods are required. In the present investigation, the possibilities of *in planta* transformation in chickpea was explored.

Traditional mutagenesis studies showed that imbibition of dry seeds followed by chemical mutagenesis provide a high rate of mutations in progeny resulting from such treated seeds. In similar fashion, we also tried to explore the susceptibility of chickpea at different growth stages of germinating seed. For the first time, Feldmann and Marks (1987) applied *Agrobacterium* to germinating seeds of *A. thatiana* and obtained very few transformants. In the present study, whole chickpea seeds were infected by *Agrobacterium* by soaking in Agro-inoculum (seed imbibition method) and in another experiment, germinating seeds were infected.

Suma (2006) reported 26 per cent transformation through seed imbibition method at 2 hr incubation period and 0.01 per cent Triton-X100. To refine the incubation period and concentration of Triton-X100, different incubation periods (1.30 h, 2.00 h, 2.30 h and 3.00 h) and different concentrations of Triton-X100 (0.01%, 0.05%, 0.10% and 0.2%) were tried in the present study. However, seed imbibition method did not result in any transgenics. Feldmann and Marks (1987) reported that *Arabidopsis* seed imbibition procedure was difficult to reproduce transgenics consistently.

In *rafp2* gene transfer through infection of shoot tips, only one transformant was found in T_1 generation, out of 100 shoot tips, treated at 10 min incubation period in T_0 generation. However, infection of shoot tips resulted in no transformation events in *cry1Ac* gene transformation.

The gap between seed imbibition and infection of shoot tips, for frequency of transgenics recovered can be attributed to physical or chemical nature of germination process. Germination may involve wounding process in which cells of the embryo become susceptible to infection only after the testa has broken open. However, the cells may be susceptible to *Agrobacterium* for only limited time due to healing of the tissue or other processes (Stachel *et al*., 1985) occurring during germination process.

The poor response in the above two methods was attributed to inefficiency of co-cultivation environment or due to actual low efficiency of methods used. Even if an integration had occurred it might have been occurred in somatic cells and thus will not be inherited to seed progeny (Parrot *et al*., 1989).

Occurrence of chimeric plants is common when meristem explants are used as targets for transformation (Tarek *et al.,* 2002). And there is a chance that the seed forwarded to T_1 generation may be collected from untransformed branch of T_0 plant or transformed sectors of T_0 plants do not produced the seeds.

Bechtold *et al.* (1993) proposed vacuum infiltration method where, applied pressure produce the micro-wounds which helps to increase the efficiency of transformation. Anthony *et al.* (2000) developed transformation procedure involving infiltration of seedlings with *Agrobacterium*. In the same way in the present study, embryo with one cotyledon and germinating seeds (shoot tips) were infiltered with *Agrobacterium.* Infiltration of shoot tips resulted in one transformation event in *rafp2* gene transfer, which might be due to the susceptibility of meristamatic cells during germination process to *Agrobacterium*.

There is a possibility to increase the frequency of transformation by altering the relevant factors that include duration of co-cultivation, vacuum pressure and the duration of vacuum treatment.

In principle, vacuum infiltration, applying vacuum to plant organs in the presence of *Agrobacterium* removes intercellular fluids and air which bacteria replace when the vacuum is released. The bacteria remain alive within the intercellular spaces of the plant tissue and later gametophyte, gametophytes or fertilized embryo are transformed (Bechtold *et al*., 1993 and

Feldmann and Marks, 1987). In the seedling infiltration procedure, the *Agrobacterium* is in contact with the plant from the very early stages of development. Hence, it is more likely that transformation of meristem cells could occur, giving rise to multiple sibling transformation. In the seedling infiltration procedure, transformation of germ line cells occur more frequently, resulting in a greater proportion of independent transformants (Bechtold *et al*., 1993).

Embryo axes with one of the cotyledon wounded by pricking with a needle were subjected to *Agrobacterium* infection to produce transgenics in *Arabidospsis* (Fieldmann and Marks, 1987), soybean (Chee *et al.,* 1989), in peanut, safflower and sunflower (Rohini and Rao, 2000) and rice and wheat (Supartana *et al.,* 2006) have earlier demonstrated *in planta* methods of similar nature.

In present study embryo axes with one cotyledon were treated in three different ways given different results.

1) Pricking embryos at apical meristem and cotyledonary node with needle dipped in inoculum : Ping *et al.* (2003) and Supartana (2006) earlier demonstrated *in planta* methods of similar nature. Hundred embryos treated in this way grew (T_0 generation) to maturity and advanced to next generation. In T_1 generation two transformation events were confirmed out of 42 plants in mulberry and wheat, respectively.

2) Pricked embryos were dipped in inoculum for 10 min with intermittent shaking. Rohini and Rao (2000) followed *in planta* method in the similar way in peanut also. Hundred embryos treated in this way grew (T_0 generation) to maturity and advanced to next generation. In T_1 generation, three transformation events were confirmed out of 89 plants.

3) Pricked embryos were dipped in inoculum and kept on shaker overnight (8 h). Yaye (2004) have demonstrated similar procedure in *Hibiscus sabdarifa*. This method gave few viable seedlings which were kanamycin susceptible.

In all the above methods, time of infection appeared to effect transformation efficiency. Dipping the pricked embryos in inoculum might have provided sufficient inoculum as embryos were dipped for 10 min in inoculum when compared to pricking the embryos with needle dipped in inoculum. In another method where embryos dipped in inoculum and kept on shaker for 8 h, long durations of treatment might have led to decreased survival of embryos.

A. tumefaciens is a phytopathogenic bacterium in nature, which induces a crown gall on plants by introducing its T-DNA on to the plant genome. Strain A208 of *A. tumefaciens* was inoculated on to the leaves or stems of *Kalanchoe daigremontiana* in pots by piersing with the tooth pick previously smeared with *A. tumefaciens*, galls were formed on all (100%) inoculated sites after six weeks (Majumder *et al*., 2001). Thus, *in planta* transformation through injuring embryo mimicked the process of *A. tumefaciens* of plants in nature.

However, in *cry1Ac* gene transfer experiments, none of the plant showed *npt II* and *cry1Ac* gene amplification indicating no gene integration. This might be due to less number of seeds treated and not as such failure of gene delivery. There is need to enhance the quantum of initial experimental material to raise the probability of getting transgenics. Since the probability of getting transgenics through *in planta* method is less (Birch, 1997).

However, quantity of phenolics from the wounded embryo axes would be much less as compared to the amount excluding from seedlings or from a well developed (matured) plant. To overcome this, prolonged infection period, adding wounded tobacco leaf extracts to co-cultivation medium (Rohini and Rao, 2000) can be followed.

Designing new strategies to introduce foreign genes requires a understanding of the specific requirements of the crop under study. To transform genotype or species that has not been manipulated for *in vitro* regeneration previously, one must either adapt a standardized protocol or evolve a new one keeping the efficiency of transformation in mind. Embryo transformation strategy presents certain advantages. It does not require *in vitro* plant regeneration, allows rapid screening of large number of transformants is genotype and variety independent and precludes culture induced variations.

The procedure therefore can be applied to all those genotypes and cultivars of crops which are susceptible to *A. tumefaciens* infection, although the efficiency might vary between them. The degree of susceptibility of these crops to *Agrobacterium* infection could be the reason for this difference.

Further, tissue culture independent transformation such as embryo transformation is relatively simple, highly reproducible and less labour intensive. Incorporation of such methods of gene transfer will accelerate crop improvement considerably.

Future line of work

The effect of different factors on *in planta* transformation need to be investigated in chickpea.

- Durations of co-cultivation period, Triton-X100 concentrations on pricked embryos with one cotyledon.
- Vernalizaiton, dehydration of seeds, embryos before co-cultivation period.
- Effect of wounded leaf extracts and acetosyrezone in co-cultivation media.
- Vacuum infiltration of well established seedlings and flowers.

6. SUMMARY AND CONCLUSIONS

In chickpea, gram pod borer causes 80 to 90 per cent of total pest damage (Srivastava *et al.*, 1996) and *Fusarium* wilt causes losses upto 60 to 70 per cent. Chemical control for both the biotic stresses became impractical (Willium, 1982). As the screening of cultivated genotypes or germplasm has not identified inherent resistance (Sharma and Ortiz, 2000; Van Der Have, 1970), breeders are turning towards annual *Cicer* species as a possible source of desired traits. Unfortunately, interspecific hybridization in chickpea has been largely unsuccessful (Ahmad *et al.*, 1988) and the wild species have not responded well to introgression through conventional breeding techniques for yield improvement (Van Rheenen *et al.*, 1993).

Developing transgenics in chickpea is need of hour. The current investigation was aimed at developing pod borer and *Fusarium* wilt resistant transgenic chickpea. The lack of reproducibility of regeneration protocols and highly problematic rooting and subsequent transplantation of the *in vitro* regenerated shoots was a major limiting factor for obtaining complete transgenic plants. Hence, in the present investigation attempts were made to develop *in planta* transformation procedures.

In the present investigation, different *in planta* transformation methods with different time duration of treatment, Triton-X100, co-cultivation periods were done to investigate most effective method for gene delivery.

Putative transgenics were screened by employing leaf paint assay with kanamycin at 10 mg L^{-1}. Leaf paint assay though not efficient in selecting positive transgenics, it helped to eliminate non-tarnsgenics. The method also decreased the time and effort involved in the screening process.

Seed imbibition method did not result in any transgenics. In *rafp2* gene transfer through infection of shoot tips (germinating seeds) one transformant was found in T_1 generation out of 100 shoot tips treated in T_0 generation.

In case of vacuum infiltration of embryos with one cotyledon and shoot tips, later one resulted in single transformation event in *rafp2* gene transfer.

In another study embryo axes with one cotyledon were treated in three different ways;

i. Pricking embryos at apical meristems and cotyledonary nodes with needle dipped in inoculum resulted in two transformation events in T_1 generation out of 100 embryos treated in T_0 generation.

ii. Pricked embryos dipped in inoculum for 10 min with intermitant shaking resulted in three transformation events in T_1 generation out of 100 embryos treated in T_0 generation.

iii. Pricked embryos dipped in inoculum and kept on shaker for overnight (8 h) gave few viable seedling, which were kanamycin susceptible.

In *cryIAc* gene transfer experiments, none of the methods resulted in transformants. Among all the methods followed, treatments given to injured embryos with one cotyledon gave more transformation events.

The main observations made from this study were;

- Kanamyin leaf paint assay method was effective in eliminating non-tarnsgenics.
- Transformation through seed imbibition did not give any transformants.
- Transformation procedures targeting meristamatic tissues, after initiation of germination process gave results.
- Of different methods used, transformation through injured embryos with one cotyledon was more effective in producing transgenics.

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APPENDIX I

Extraction solutions for plasmid extraction

Solution I stocks : 1) 1 M glucose (stored at 4℃)

2) 0.5 M EDTA

3) 1 M Tris-HCl (pH 8.0)

Working solutions : 5 mL of 1 M glucose + 2 mL of 0.5 M EDTA + 2.5 mL of 1 M Tris HCl were combined and 5 mg/mL lysozyme was added to solution I before use.

Solution II : Stock solutions : 1) 10 N NaOH

2) 10% SDS

Working solution : 0.8 mL 10 N NaOH + 4 mL 10% SDS + 35.2 mL of sterile distilled water.

Solution III: Stock solution : 1.5 M potassium acetate (stored at 4℃)

Working solutions : 60 mL of 5 M potassium acetate was mixed with 28.5 mL of glacial acetic acid and 11.5 mL of sterile distilled water. pH of the final solution was adjusted to 4.8-5.3 using glacial acetic acid.

Solution IV: 3 M sodium acetate

200 µL sodium acetate and 600 µL Isopropanol were added in sequence.

APPENDIX II

DNA extraction buffer (Edwards *et al.*, 1991)

Tris Hcl (pH 7.5)	- 200 mM
NaCl	- 250 mM
EDTA	- 25 mM
SDS (w/v)	- 0.5%

APPENDIX III

TAE tris acetate (Sambrook *et al.*, 1989) 50x

Tris Base	- 242 g
Glacial acetic acid	- 57.1 mL
0.5 M EDTA (pH 8.0)	- 100 mL
Distilled water	- 1000 mL

Loading dye (50x)

0.25% Bromophenol blue

40% (w/v) sucrose in water

Stored at 4℃

APPENDIX IV

YEMA (Yeast Extract Mannitol Agar Medium)

Mannitol	- 10 g
Yeast extract	- 1 g
KH_2PO_4 (2%)	- 10 mL
K_2HPO_4 (2%)	- 10 mL
$MgSO_4 . 7H_2O$ (1 m)	- 0.8 mL
$CaCl_2 . 2H_2O$ (1 m)	- 0.4 mL
Agar	- 16 g
Distilled water	- 1000 mL

IN PLANTA TRANSFORMATION STUDIES IN CHICK PEA (*Cicer arietinum* L.)

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CHAIRMAN

ABSTRACT

An investigation was carried out to develop transgenic events in chickpea for *Fusarium* wilt and pod borer resistance through *in planta Agrobacterium* mediated transformation. Different *in planta* transformation methods with different time durations of treatment, Triton-X 100, co-cultivation periods were attempted to investigate most effective method for gene delivery. Putative transgenics were screened by employing leaf paint assay with kanamycin at 10 mg L^{-1}. Leaf paint assay though not efficient in selecting transgenics, helped to reduce time, season and eliminate most of the non transgenics.

In *rafp2* gene transfer against *Fusarium* wilt, infection of shoot tips (germinating seeds), one transformant was found in T_1 generation out of 100 shoot tips treated in T_0 generation. In case of vacuum infiltration of embryos with one cotyledon and shoot tips, the latter resulted in a single transformation event.

In another study, embryo axes with one cotyledon were treated in three different ways in which pricked embryos were dipped in inoculam and kept on shaker for over night. It gave few viable seedlings, which were kanamycin susceptible. In another method in which pricking embryos at apical meristems and cotyledonary nodes with needle dipped in inoculam, resulted in two transformation events in T_1 generation out of 100 embryos treated in T_0 generation. In the third method pricked embryos dipped in inoculum for 10 minutes with intermittent shaking resulted in three transformation events in T_1 generation out of 100 embryos treated in T_0 generation.

In *cry 1 Ac* gene transfer experiments none of the methods resulted in transformants. Thus, some transformation events was achieved in recalcitrant species like chick pea through *in planta* method.