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AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF DIANTHUS (CARNATION)

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CERTIFICATE

This is to certify that the work entitled, "Agrobacterium mediated genetic transformation of dianthus (carnation)" submitted by SWATI GOSWAMI and VIKASH SACHAN in partial fulfillment for the award of degree of Bachelor of Technology in 2007 of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Anil Kant

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We take this privilege to thank Dr Anil Kant for providing us an opportunity to carry out this project and for his active involvement and guidance throughout this project. We also thank to rest of the faculty members who have been instrumental in the completion of our project. This project has increased our knowledge immensely in various aspects of plant genetic transformation

LIST OF ABBREVIATIONS

BAP	Benzyl Adenine Purine
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
2, 4-D	2, 4-Dichlorophenoxyacetic acid

LIST OF FIGURES

Fig.1 Pre-Culturing of leaf explants on the regeneration media

Fig. 2 Leaf explants of carnation transferred to the selective media

TABLE OF CONTENTS

S. No.		Pages
1.	ABSTRACT	1
2.	INTRODUCTION	2-4
3.	REVIEW OF LITERATURE	5-8
4.	MATERIALS AND METHODS	9-12
5.	RESULTS AND DISCUSSIONS	13-14
6.	BIBLIOGRAPHY	15

ABSTRACT

In this investigation an attempt was made to standardize the protocol for *Agrobacterium mediated transformation* of carnation using nptII as the selectable marker gene and GUS as the scorable marker gene. The method comprises preparing leaf explants from carnation rooted cuttings, pre-culturing on regeneration media, cocultivating the explants with Agrobacterium and then selection of transformed cells and tissue.

CHAPTER 1

INTRODUCTION

Biotechnological applications in crop improvement have led to the gene revolution which is often compared with the green revolution of the 1960s and 1970s. The pace of gene revolution is enhanced by the new recombinant DNA technology or genetic engineering, which leads to the manipulation of the genes across species boundaries. Thus many desirable traits from entirely different organisms can be combined in one background, which is not feasible in conventional modes of breeding. Since 1970 rapid progress is being made in developing tools for manipulation of the genetic information of plants by recombinant DNA methods and genetic engineering. The overall process of genetic transformation involves introduction, integration and expression of foreign gene in the receipt host plants. Plants which carry additional stably integrated and expressed foreign genes are referred to as transgenic plants. The development of transgenic plants is the result of integrated application of recombinant DNA technology, gene transfer methods and tissue culture techniques. The first generation application of genetic engineering to crop plants has been targeted toward the generation of transgenic plants expressing foreign gene that confer resistance to insect pests, diseases, viruses, herbicides and some quality traits.

Agrobacterium system was historically the first successful plant transformation system, making the breakthrough in the plant genetic engineering. This breakthrough came by characterizing and exploiting the plasmids carried by *Agrobacterium* species. These provide natural gene transfer, gene expression and selection system. *Agrobacterium* are gram negative rods that belong to the bacterial family of rhizobiacae. *Agrobacterium tumiefaciens* is the causative organism of the crown gall disease of plants and another species known as *A. rhizogenes* causes hairy root disease. An understanding of the molecular basis of these diseases led to utilization of these bacteria for development of gene transfer system for plants.

Smith and Townsend showed in 1907 that this bacterium is the causative agent of the crown gall tumors. Experiments of Braun and his coworkers showed that the bacteria

were not necessarily present in the crown gall tumors. Attention then focused on the identification of the tumor inducing principle. Zaenen et al 1974 first noted that virulent strain of Agrobacterium harbor a large plasmid and virulence trait is plasmid borne. Virulence is lost when bacteria is cured of the plasmid. Plasmid of *A. tumiefaciens* is called tumor inducing plasmid (Ti Plasmid) and that of *A. rhizogene*s as Root inducing plasmid (Ri Plasmid). It was later revealed that only small portion of Ti or Ri plasmid is transferred and integrated to the host plant nuclear genome (Chilton et al 1972). The discovery of the mechanism of transfer of T-DNA to plant genome formed the basis of the development of gene transfer vectors based on Ti plasmid. *Agro bacterium tumiefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is stably integrated into the host genome and transcribed. There are several significant advantages of transferring DNA via *Agrobacterium*, including a reduction in transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA with defined ends.

To be sure that the gene you are trying to insert is actually present, the added segment of DNA usually includes a "marker gene" which is most often a gene for antibiotic resistance. The organism is then grown in a culture containing the antibiotic. Only those individuals with the added segment of DNA will survive, since they are the only organisms that are resistant to the antibiotic. At least this is how desired genes are usually identified, with a marker for antibiotic resistance. Once the desired genes are inserted into the selected organism, the new genetically engineered organism is reproduced to obtain a generation of individuals that possess the desired trait.

The carnation, *Dianthus caryophyllus*, is a popular ornamental plant and highly valued for its cut flowers. As with many ornamental plant species, breeders have long sought to improve existing varieties and to create new cultivars using conventional techniques, such as cross-breeding and somatic clonal variation. Phenotypic variations of particular interest include colour, fragrance, morphology, herbicide resistance, pesticide resistance, environmental tolerance, vase life of the cut flower, and the like. While improvements and variations in many or all of these characteristics have been achieved, progress is slow because of the inherently random nature of such breeding approaches.

Indeed, the introduction of any particular characteristic requires a substantial effort if it can be achieved at all. Carnation being an economically important crop, the application of plant tissue culture and plant genetic engineering is of special value to obtain improved or desirable traits. Before embarking on any crop improvement programme using a specific trait of interest through gene transfer, a high frequency gene transfer and plant regeneration method should have been standardized for that particular crop. Although there are few reports describing the successful transformation of carnation using Agrobacterium method but routine genetic transformation in this way is currently restricted partly due to lack of clearly-written, complete, publicly-available protocols. Besides this the response of crop plants also varies with the genotypes. This has been a driving force in the development of methods using *Agrobacterium tumiefaciens* to deliver DNA. In this investigation genetic transformation studies were carried out using Agrobacterium strain LBA4404 carrying a recombinant Ti plasmid having nptII gene which impart resistance against kanamycin and β-glucuronidase gene as reporter gene.

OBJECTIVE

To standardize the method of genetic transformation of carnation tissues/cells using Agrobacterium-mediated gene transfer.

CHAPTER 2

REVIEW OF LITERATURE

The stable transformation of foreign gene(s) into plants represents one of most significant development in the field of plant genetic engineering. Genetic engineering method complements plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into crops and by shortening the time required for production of new varieties and hybrids. It is most suitable if a well established crop variety has to be improved with respect to one or few characters without disturbing the general genetic background. Carnation is one of the most important ornamental crop which stands second after rose in term of economy as well as preference. Some of the works done in this crop with regard to genetic transformation have been reviewed here.

Chin et al (1991) of Calgene recovered transgenic carnation plants on kanamycin-containing medium after co-cultivation of stems with disarmed or armed strains of *Agrobacterium tumefaciens*. Both strains contained a vector carrying the neomycin phosphotransferase II (nptII) and β-glucuronidase (GUS) genes.

Tsugawa et al (2002) used three different strains of *A. tumefaciens* for genetic transformation of Carnation, *Lilium formosanum*, *Agapanthus praecox* ssp. orientalis and *Muscari armeniacum* all of which harbored the binary vector carrying the nptll, hpt and gus-intron genes in the T-DNA region. For *L. formosanum*, neither transgenic tissues nor plants were obtained after co-cultivation of organogenic calli with *A. tumefaciens*, although transient expression of the gus gene could be detected in the calli during co-cultivation. On the other hand, several hygromycin-resistant cell clusters were obtained for both *A. praecox ssp.* orientalis and *M. armeniacum* following the transfer of co-cultivated embryogenic calli onto hygromycin containing media. Calli developed into complete plants via somatic embryogenesis, and most of them were confirmed to be transgenic plants based on GUS histochemical assay and PCR analysis. Southern blot

analysis revealed the integration of 1 to 5 copies of the transgene into the genome of the transgenic plants of both 2 species, but most of them had 1 or 2 copies.

Since carnation is an ornamental plant much work is directed at improving its ornamental value. Scientists of Florigene (Calgene Pacific) have already marketed two types of transgenic carnation (*Dianthus caryophyllus*). The white carnation lacks delphinidin synthesis, after it accepts two of the anthocyanin biosynthesis genes ("flavonoid 3'5' hydroxylase" and "dihtdroflavonol reductase"), the transgenic carnation produces delphinidin and shows a unique violet/mauve colour. Another transgenic carnation has long vase - life. After being harvested, flower death is triggered by production of a gaseous hormone, ethylene. By transferring a second copy of ACC synthase gene, the production of ACC synthase in the transgenic carnation was suppressed. And the plants do not synthesize ethylene. Hence this new type of long vase-life carnation flowers will stay fresh in your home for much a longer time.

H. Salehi (2006) conducted investigations to find the best shoot proliferation and rooting media for 13 virus free cultivars of carnation (Dianthus caryophyllus L.) recently imported from The Netherlands to Iran. The best shoot proliferation media were Murashige and Skoog (MS) containing 3 mgL-1 (13.95 μM) kinetin and 0.5 mgL-1 (2.69 μM) NAA or 1 mgL-1 (4.44 μM) BA and 1 mgL-1 (5.37 μM) NAA. Average of shoot numbers produced in the establishment media was 2-3, which increased to 30 or higher with some cultivars in subsequent subcultures. For rooting, the in vitro shoots were cultured on the best MS rooting media which is discussed. Transferring to a mixture of sand, leaf-mold and vermiculite (1:1:1, v/v/v) successfully acclimatized the plantlets. It can be concluded that using a general medium for shoot proliferation of used carnation cultivars is possible, but for rooting it is impossible.

Miller et al.(1991) of the Horticultural Research Institute, Knoxfield, established that for axillary bud explants, a suitable optimum adventitious regeneration medium contained Murashige and Skoog basal medium solidified with Gelrite and supplemented with 15 μm benzyladenine and 0.5 μM a-napthaleneacetic acid was used. Adventitious primordia arose from the cut basal end of bud explants erupting as individual shoots after

2–3 weeks incubation. The axillary bud size and the time between subcultures of source material influenced the production of adventitious shoots. Transfer of regenerated shoots onto a medium solidified with agar minimized visible signs of vitrification. Regenerated shoots could be easily rooted, transferred to glasshouse conditions and grown to flowering.

Conditions for efficient direct somatic embryogenesis and plant regeneration of leaf explants from carnation cultivars Lena (SIM group) and Bulgarian spray cultivars Nasslada, Yanita, Regina and Line 84 were established by Yantcheva et al (2004). Murashige and Skoog (MS) liquid medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.2 mg/l 6-benzylaminopurine was used for direct induction of embryoids without an additional callus phase. The first globular structures were observed after 20 days of cultivation. Their further development to the torpedo stage was correlated with the presence of polyethylene glycol (PEG 6000). Somatic embryo maturation was promoted by casein hydrolysate (1000 mg/l) in MS liquid media. The percentage conversion of embryos and polyembryos to whole plants varied between 10 and 75% among studied cultivars. Plantlets regenerated by this procedure were morphologically identical to the donor material and developed normally in a greenhouse.

Jones (1997) transformed carnation by *Agrobacterium tumefaciens*. The first experiment was based on antibiotic selection using either hpt (aph4-Ib) or nptII (aph3'II) which encode phosphotransferase enzymes that confer tolerance to the aminoglycoside antibiotics such as kanamycin, neomycin, paromomycin, G418 and hygromycin. A second system utilized the bar gene which confers tolerance to glufosinate ammonium-based herbicides such as PPT, Basta, Bialaphos etc. The use of 0.02 mM glyphosate on regenerating meristems has been reported to reduce the number of plants escaping selection to zero. A third system was based on the aroA: CP4 genes conferring tolerance to glyphosate-based herbicides such as Roundup.

CHAPTER 3

MATERIALS AND METHODS

PLANT MATERIAL

The rooted cuttings of carnation were obtained from Dr. Y.S.Parmar University of Horticulture and Forestry and were maintained in pots in the lab area. Leaves were used as explants from these plants in all the genetic transformation experiments.

NUTRIENT MEDIA

MURASHIGE AND SKOOG'S MEDIA

The basal MS media (Murashige and skoog 1962) with 3% sucrose solidified with 0.8% agar-agar was used to culture plant cells/tissues in vitro. The pH of media was adjusted to 5.8 before autoclaving. The media was supplemented with various growth regulators depending upon the experiment before autoclaving at 15lb/inch2. The basal MS media was supplemented with following growth regulators.

MS + Kinetin (2.0mg/l) + NAA (0.5mg/l)

MS + BA (2.0mg/l) + IAA (0.5mg/l)

YEAST EXTRACT MEDIA

The YMB media was used for the maintenance and preparation of fresh culture of Agrobacterium as given below.

Yeast Extract-0.4g/l

Mannitol-10g/l

NaCl-0.1g/l

$MgSO_4$-0.2g/l

K_2HPO_4-0.5g/l

Kanamycin-50mg/l

The pH of the medium was adjusted to 7.5 using 1N HCl and 1N KOH. Kanamycin was added into sterilized molten or liquid YMB medium by filter sterilization using membrane filter of 0.22um because of the heat sensitive nature of the antibiotic.

SURFACE STERLISATION OF EXPLANTS

The explants used for the initiation of in vitro culture need to be surface sterilized because they contain inoculums of various organisms. Overzealous treatment with chemical sterilants removes the organisms but is also lethal to plant tissue. So it is important to determine the optimal conditions for each plant, species and tissue used. The explants were rinsed thoroughly under running tap water and then washed in a mild detergent before treatment with chemical sterilant. The explants were then rinsed with autoclaved distilled water to remove the traces of chemicals.

GENETIC TRANSFORMATION EXPERIMENTS

AGROBACTERIUM STRAIN AND PLASMID

Disarmed *Agrobacterium tumiefaciens* LBA 4404 strain containing a reporter/marker β-glucuronidase (GUS) gene in binary vector pBI121 system along with a kanamycin resistant gene for selection in both bacteria and plant was used for co-cultivation experiment to transfer Gus gene in carnation cells.

MAINTAINENCE OF AGROBACTERIUM STRAIN

Agrobacterium strain was maintained by sub culturing the bacterial colonies on the YMB medium containing 50 mg/l kanamycin. After the proper growth of the Agrobacterium, the plates were kept at low temperature for storage.

PREPARATION OF FRESH AGROBACTERIUM CULTURE

Overnight fresh cultures of Agrobacterium strain were prepared by inoculating a small colony into the 10ml liquid YMB medium containing 50 mg/l kanamycin. These culture were kept overnight at 28^0C in orbital shaking incubator for growth.

PRE-CULTURE OF LEAF EXPLANTS

Pre-culture involves culturing the explants in suitable media for some time so as to rejuvenate the cells. Surface sterilized explants , cut into small pieces of about 0.5 – 1 cm in size were cultured in MS media with growth regulators for varying amounts of time like 48, 72, 96 hrs etc to standardize the pre-culture time before co-cultivating with the Agrobacterium.

COCULTIVATION

Co-cultivation is the procedure of growing explants along with Agrobacterium cells containing appropriate gene construct during which the acetosyringose released from plant cells induces the vir genes which bring about transformation. The fresh culture of Agrobacterium was centrifuged for 10 minutes and supernatant was discarded to get a pallet. This pallet was resuspended in MS liquid medium. This bacterial suspension and pre-cultured leaf explants were used for co-cultivation experiment. The leaf explants were immersed into bacterial suspension for 10-20 seconds then these were blotted dry on sterile filter paper and inoculated on growth medium for co-cultivation. After inoculation these were in culture room for varying interval of time viz. 48, 76, 96 hour to standardize the time of co-cultivation.

SELECTION OF TRANSFORMED CELLS/TISSUE

Selective regeneration medium was prepared by adding kanamycin (50 mg/l) and cefotaxime (500mg/l) through filter sterilization to the molten regeneration medium. The antibiotic kanamycin acts as selection agent and untransformed plant cell will not grow on this medium. The transformed plant cells contain the nptII gene which confers resistance against kanamycin hence they will be able to grow on this medium.

Cefotaxime is a broad spectrum antibiotic which is used to check the further growth of Agrobacterium which is not desirable at this stage.

After co-cultivation of leaf explants for varying interval of time periods leaf explants were transferred to selective regeneration medium. All the cultures were kept in culture room at 26^0C for further growth and differentiation. Explants were subsequently sub cultured on fresh selective medium in order to check the excessive bacterial growth. Selection involves growing co cultivated explants into media having the antibiotic whose resistance gene has been transferred via the Agrobacterium into the explants.

CHAPTER 4

RESULTS AND DISCUSSION

In this investigation the leaf explants from plants grown in pots were used for the genetic transformation experiments hence they had to be surfaced sterilized. In the beginning of the experiments the attempt was made to establish the in vitro cultures by culturing the leaf explants on the regeneration media after surface sterilizing the explants with 0.1% mercuric chloride for one minute. However all the explants cultured turned brown and ultimately became dead after few days. These result forced us to reduce the exposure time of the treatment to 20-25 seconds. Thus the genotype of the carnation plant taken for the study was extra sensitive to sterilization treatment. We could not obtain the callus growth from any of the explants cultured on the selective media after the co-cultivation experiments. It is a general fact that due to the surface sterilization treatment the cultured explants taken from the field grown material take extra time for the culture establishment. The genetic transformation is generally done by using leaf or any explants taken form the in vitro growing plant material to avoid the pressure of surface sterilization treatment. Since we had used explants from the pot grown plants which needed to be surface sterilized due to non availability of axenic cultures and paucity of the time to establish them. This could be one of the reasons for not getting the transformed callus. The other possible reason could be the sensitivity of this carnation genotype to the kanamycin. In the selection media 50mg/l kanamycin was used. Thus in our opinion sensitivity of carnations explants to kanamycin needs to optimized which was beyond the scope of this small project , inflexibility of experiment and shortage of time as well.

Miller et al (1991) reported that the use of leaf and stem explants of carnation was not successful, largely due to explants senescence in the presence of BAP, kinetin and, to a lesser extent, zeatin. However they could obtain adventitious shoots from axillary bud explants of 15 carnation cultivars. Since our media also had the same two hormones this can be one of the reasons for no callus growth.

The transformation frequency of Agrobacterium mediated methods can be increased by different approaches. Acetosyrngone is the phenolic compound released from wounded explants which trigger the *vir* gene functions of the Ti plasmid thus playing key role in the T-DNA transfer. This compound is nowadays being used in media to increase the T-DNA transfer efficiency. Alexander et al (2006) used 100 micro molar acetosyringose in co- cultivation media for the transformation of the carnation with agrobacterim mediated method.

Walbot et al. (1987) subjected the cultured shoots to a heat shock treatment of about 40 degrees for a time period of about 4 hours so as to improve transformation efficiency.

Fig.1 Pre-Culturing of leaf explants on the regeneration media

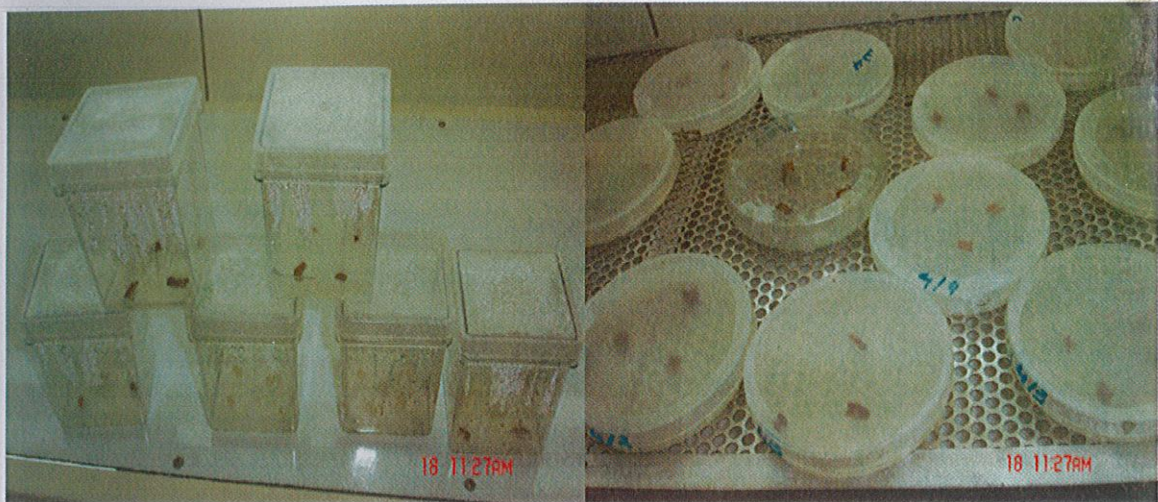

Fig. 2 Leaf explants of carnation transferred to the selective media

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