STUDIES ON TRANSFER OF FOREIGN DNA IN PLANT CELLS

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To My Parents

PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Scineces, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma at any other Univesity.

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A.S. LAKSHMI

CONTENTS

ABBREVIATIONS

BAP	benzylaminopurine
°C	Celsius (degree)
cm	centimeter
2,4-D	2,4-dichlorophenoxy acetic acid.
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
hr	hour
Kan	kanamycin
LB	Luriabroth
LS	Linsmaier and Skoog's medium
ug	microgram
mg	milligram
ml	millilitre
mm	millimeter
M	molar
MS	Murashige and Skoog's medium
NAA	∝-Naphthalene acetic acid
NADH	nicotinamide adenine dinucleotide (reduced)

NED	N-1-Naphthylethylene diamine dichloride
nia	Apoenzyme defective tobacco NR^- mutant.
nos	nopaline synthase
p.s.i.	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane
UV	ultra-violet

INTRODUCTION

Genetic modification of plants by man has long been central to his activities in continually generating an efficient agriculture for food and fiber. Of late, it is realized that the boundaries set by natural genetic barriers have placed limits on extending the gene pool and further governing new genetic variability to enhance agricultural efficiency. Endeavours to circumvent classical genetic technologies that are limited by natural genetic barriers are the approaches being examined recently for genetic improvement of crops. Technologies for efficient insertion of foreign genes into crop plants is one of these endeavours (Kado and Kleinhofs, 1980). Somatic cell genetics and recombinant DNA technology have become handy to achieve the set targets.

The fact that various eukaryotic genes are expressed into biologically functional products in bacteria, generated much euthusiasm among scientists. Furthermore, various alien genes of prokaryotic and eukaryotic origin were shown to be transcribed and translated in the easily amenable animal system of Xenopus cells.

Although success with prokaryotic systems is encouraging, it is indeed a great deal to understand the expression characteristics of genes from higher systems in an altogether foreign environment. The physical (Mechanical), biochemical (molecular) and genetic barriers of the cells of the higher systems are found to be exceedingly difficult to obviate. Though the mechanical barrier of the cell wall is eliminated by the use of plant protoplasts, the regeneration of protoplasts of certain species is of a challeng-

ing nature (Evans and de Brovo, 1983). The success of plant cell transformation was also found to be a function of the degree of amenability of the plant species to tissue culture techniques (Vasil, 1982; Cocking, 1981; Lurquin, 1982). Some plant species may prove refractory to all efforts at transformation (Chilton, 1980). Most cereals, which are of very high agronomic importance are known to be recalcitrant in culture.

Several methods have been developed to introduce foreign DNA into plant cells. Amongst these, DNA transfer through the Ti plasmid of Agrobacterium tumefaciens, is considered to be the simplest and the most successful one (Hooykoas and Schilperoort, 1984). This gram-negative, soil bacterium causes the crown-gall disease in many dicotyledonous plants, by introducing a part of its Ti plasmid DNA (T-region) into the wounded plant cells. The much cherished advantage of this system is that the genetic engineer can exploit the pathogen's highly evolved and efficient route of penetration into the plant cell (Chilton, 1980).

A large number of successful attempts of transformation have been reported by using Agrobacterium tumefaciens in dicotyledonous plants (Decleene and De Ley, 1976; Chilton et al., 1977). Recent reports suggested that the monocotyledonous plants could also be transformed by A. tumefaciens (Hernalsteens et al, 1984).

Regenerating mesophyll protoplasts have been used in many of the transformation studies. However, protoplasts of many plant species were found to be refractive towards regeneration (Evans and Brovo, 1983). Alternative procedures for transformation were developed e.g. co-cultivation of

leaf tissue slices with A. tumefaciens (Horsch et al., 1985). Promising results have been reported with callus cells (Muller et al.,1984) and with suspension cultured plant cells (An, 1985). The scarcity of biochemically defined plant auxotrophs was found to be the difficulty in DNA transformation studies (Cocking, 1981; Lurquin and Sheehy, 1982). The development of drug resistance markers such as that for kanamycin has allowed the direct selection of transformants without the induction of tumerigenic growth. In the wishful sense, incorporation of the desired trait together with the knocking down of the tumorigenic growth of transformed tissue is set to be the goal in genetic engineering of plants. (Cocking, 1981; Barton and Chilton, 1983).

The present investigation was undertaken to study the characteristics of crown gall tumor system in order to develop a good transformation system for its efficiency and desirable expression of the incorporated traits. The crown gall system was investigated by studying some of the characteristics of A. tumefaciens such as the formation of crown gall tumors and establishment of callus cultures from tumor tissue under selection pressure. One of the nitrate reductase deficient cell lines of Nicotiana tabacum (nia) has been used as the transformation system. Levels of kanamycin sensitivity of this cell line have been studied. Attempts have also been made to transform the callus cells of this cell line by cocultivating with Agrobacterium tumefaciens.

REVIEW OF LITERATURE

Apart from its potentials in applied aspects, transformation of plant cells provides suitable system for understanding certain aspects of genome organization and regulation of gene expression in higher plants. A number of approaches have been suggested for such transformation of plants which could not be achieved by conventional plant breeding techniques. These approaches have been reviewed extensively in recent years (Cocking et al., 1981, Kado and Kleinhofs, 1980).

Of all the methods of transformation, the method which makes use of the natural vector, Agrobacterium seems to be the simplest and the most successful one. Recent interest in A. tumefaciens (Crown-gall bacteria) and A. rhizogenes (root-inducing bacteria) stems primarily from the finding that these bacteria have systems for the horizontal transfer of genetic elements from a prokaryote to eukaryote (Hooykaas and Schilperoort, 1984). In fact, the Ti (Tumor-inducing) plasmid has already been used for the transfer of foreign DNA into plant cells. It has been shown that the transposons and other DNA fragments from alien sources when introduced into the T-region of the Ti-plasmid were integrated into the nuclear DNA of the plant alongwith the T-region (Hernalsteens et al., 1980; Garfinkel et al., 1981; Zambryski et al., 1984). There are good prospects that Ti and Ri plasmids can be exploited in the near future as the first vectors for the genetic manipulation of plants by man (Hooykaas and Schilperoort, 1984).

In the following few pages an attempt has been made to briefly review the different modes of delivery of exogenous DNA to plant cells

with emphasis on Ti plasmid-mediated DNA transfer, Further, methods of detecting DNA transfer and selection procedures will be dealt with.

Methods For The Introduction Of Foreign DNA To Plant Cells

As mentioned earlier, there are a number of approaches to introduce exogenous DNA to plant cells.

Direct DNA transfer - Studies on direct DNA uptake have been performed both in in situ and in vitro systems of plant species. Direct DNA uptake by intact seedlings has been reported in barley (Ledoux and Huart, 1969) pea (Bendich and Filner, 1971), Petunia (Hess, 1972), Arabidopsis thaliana (Hemleben et al., 1975; Gradmann-Rebel and Hemleben, 1976). Zhou and Weng (1982) reported the injection of DNA into the axile placenta of cotton. Phage-mediated changes were studied (Doy et al., 1973) in callus of tomato and Arabidopsis thaliana and in suspension cultures of Sycamore (Johnson and Grierson, 1974).

Isolated plant protoplasts have proved to be a promising system for DNA uptake studies, where the cell wall which acts as a mechanical barrier is removed enzymatically.

Liposome-mediated DNA transfer - The use of phospholipid vesicles (liposomes) to introduce nucleic acids into plant cells has gained considerable importance (Rollo, 1982). Possible advantages in using liposomes for delivery of nucleic acids include ,

(i). Low toxicity and applicability with many plant species.

(ii) Protection of entrapped nucleic acids from degradation by nucleases present in the culture medium and,

(iii) More efficient delivery of nucleic acids to plant protoplasts (Rollo et al., 1981; Lurquin and Sheehy, 1982).

Liposomes have been used for delivering infective poliovirus particles (Wilson et al., 1977), and SV 40 DNA (Fraley et al., 1980) into mammalian cells.

Liposome-mediated nucleic acid delivery has been reported for Vinca rosea (Fukunaga et al., 1981), tobacco (Nagata et al., 1981; Fraley et al., 1982), cowpea (Fraley and Papahadjopoulos, 1981) and turnip (Rollo and Hill, 1982). Nucleic acids for the above experiments were derived from TMV (Tobacco mosaic virus), CPMV (Cowpea mosaic virus), CaMV (Cauliflower mosaic virus) TROSV (Turnip rosette virus). It has been found that the involvement of a fusogenic agent such as polyethyleneglycol, polyvinyl alcohol and calcium was necessary for infectivity of protoplasts suggesting that the process of DNA transfer involved the fusion of the liposome membrane with the plasmamembrane of protoplasts.

Recent reports (Caboche and Deshayes, 1984; Deshayes et al., 1985) provided encouraging results with liposome-mediated DNA delivery into plant cells. The average frequency of the transformed colonies was found to be 4×10^{-5} and the transferred DNA was also found to be inherited in the daughter generations in a typical mendelian fashion.

Delivery of DNA through viruses - Success in cloning with bacteria, yeasts and mammalian cell systems has resulted from the development of plasmid and viral vectors and it is towards such vectors that a number of groups are concentrating their efforts to develop methods for the cloning and expression of foreign genes in plant cells. The DNA viruses of plants were found to be the ideal choice to serve as vectors (Glover, 1984). Those of the DNA viruses known so far are presently classified in two broad groups viz., (i) Caulimoviruses and (ii) Gemini viruses. The characteristic features of the different group of viruses are as follows:

(i) The Caulimoviruses - These are large icosahedral particles containing dsDNA; Cauliflower mosaic virus (CaMV), Dahlia mosaic virus (DaMV), Figwort mosaic virus (FMV), and Mirabilis mosaic virus (MMV) are some examples.

(ii) The Gemini Viruses - They have geminate particles with ssDNA. Bean Golden yellow mosaic virus Cassava latent virus, Euphorbia mosaic virus and Potato leaf roll virus are some examples of this group.

Of these groups, the genome of CaMV has been best characterized. It has a double stranded DNA with the capability to infect crucifers directly (Shepherd et al., 1979). Therefore, the mechanical barrier imposed by the cell wall was not found to be limiting for the DNA transfer of this virus. However, due to certain other characteristics of this system it is found to be less appealing than other transducers. They are as follows:

(i) The CaMV have a narrow host range mainly restricted to the crucifers (Shepherd, 1979).

(ii) The process of obtaining virions is time consuming and purification of the viral DNA is especially tedious, and provides less than desirable amounts for use in gene cloning (Kado and Kleinhofs, 1980).

(iii) The observed loss of infectivity by a single cleavage of the genome still remains unexplained (Szeto et al., 1977).

(iv) They are frequently found to be replicated in the cytoplasm of the host cells instead of their desired occurrance in the nucleus (Fujisawa et al., 1967).

Microinjection as a method to introduce exogenous DNA into plant cells - Microinjection is perceivably the most direct way of introducing foreign DNA into plant cells. This technique has been extensively used for the delivery of a number of macromolecules and metabolities into animal cells and found to be highly efficient in the transformation of mammalian cells (Capecchi, 1980).

Microinjection of DNA has been suggested as one of the ways for introducing foreign DNA into the cells of monocotyledonous plants, especially cereals, as they are found to be highly recalcitrant to culture conditions and the DNA transfer to them through natural infection processes is difficult (Flavell and Mathias, 1984). However, application of this technique to plant cells is known to have certain inherent limitaitons as follows:

(a) Plant protoplasts do not anchor to the substratum like cultured mammalian cells, and therefore, are more difficult to impale with a microneedle.

(b) the injected DNA might get introduced into the vacuole rather than into the cytoplasm or nucleus (Howell, 1982).

Recently, some refinements have been proposed to obviate the above problems and it has been shown that the DNA could be injected into the nucleus (Steinbiss, 1983; Hiromichi and Yamada, 1985).

Somatic hydridization - The application of this procedure has been handy when the transfer of more or less complete genomes was desired (Cocking, 1981). It was also found to be useful in the transfer of polygenic traits that would affect characters such as yield in agricultural production. Successful integration of the parental genomes through somatic hybridization has been achieved by several groups (Cocking, 1982), in both intergeneric and intrageneric protoplast fusions. Subsequent to protoplast fusion, incompatibility of the fusing protoplasts and elimination of chromosomes of one parental species has been reported (Harms, 1983). Irradiated protoplasts have also been used as a fusion partner with actively dividing protoplasts in the transfer of nuclear markers and of cytoplasmic male sterility (Dudits et al., 1980; Gupta et al., 1983). Selection procedures for isolating heterokaryons resulting from protoplast fusion were mainly based on fluorescence techniques (Cocking, 1982). Recently, electric field induced fusion was proposed to be an ideal method in obtaining synchronous protoplast aggregation

and fusion and also to trace the events during the fusion process (Zimmermann and Scheurich, 1981; Kohn et al., 1985).

The fusion of bacterial protoplasts (Schaffner, 1980; Rassoulzadegan et al. 1982) with mammalian cells has been used to transfer cloned DNA directly from bacterial cells to mammalian cells, in the presence of a fusogen, viz., PEG. A similar approach has been used for the uptake of E. coil cells or Sphaeroplasts (Matsui et al., 1983) and A. tumefaciens sphaeroplasts (Hasezawa et al. 1981; Kazuya Okada et al., 1985) by Vinca rosea protoplasts. The co-cultivation method wherein protoplasts regenerating cell wall are incubated with A. tumefaciens cells has been extensively used to transform plant cells (Marton et. al., 1979).

The Crown-Gall Tumor System

Recently, the focus on Agrobacterium tumefaciens has gained considerable significance. In fact, of all the methods, the one which employs A. tumefaciens is the simplest and the most successful one in genetic engineering of plants.

Many species of higher plants, mostly from dictyledonous group are susceptible to crown-gall disease. (Decleene and De Ley, 1976). As a result of infection, tumors could be induced at the wounded site by A. tumefaciens, often at the crown separating the stem from roots. Wounding appears to be an essential requirement to develop "conditioned cells" that are susceptible for tumor induction. During infection, the bacteria were found to get attached to the cell wall and inject a part of their DNA into

plant cells. It has been suggested that no tumor formation occurred in monocots either due to the lack of attachment of agrobacteria to the cell walls (Rao et al., 1982) or because of an abnormal phytohormonal balance in such cells (Krens et al., 1983), until recent reports suggested that transformation of monocots is a possible one (Hernalsteens et al., 1984). These gram-negative soil bacteria are found associated with crown gall harbouring plants in nature. It was also shown that the presence of these bacteria was not necessary for tumor maintenance and growth (Braun, 1958).

Tumor induction was found to be accompanied by transfer and integration of a specific part of the tumor-inducing plasmid into the nuclei of the host cells. (Chilton et al., 1980). This part of the bacterial DNA has been designated as the T-region and that in tumor cells as T-DNA. Thus, the crown gall cells are persistently altered cells and are characterized by,

(a) hormone-independent growth, and

(b) synthesis of a variety of compounds termed as opines, that can be metabolized by the tumor inciting bacteria.

Ti-plasmid organization and properties - Ti-plasmids are known to be quite large, in the range of 150-200 kb. Significant homology was found to exist between different Ti-plasmids. The factors required for subsequent attachment of Agrobacterium to the plant cell wall, T-DNA transfer, processing and conditioning of sensitive plant cells are known to be the virulence functions and are encoded by vir genes. The functions related to the transformation of normal plant cells into tumor cells are called

oncogenic factors, which are encoded by onc genes (Hooykaas et al., 1984).

Ti-plasmids are generally divided into octopine type, nopaline type or agropine type wherein they direct the synthesis of octopine, nopaline and agropine, respectively (Guyon et al., 1980). Octopine tumors are usually rough with adventitious roots, whereas nopaline tumors are smooth and often result in teratomas with leaf-like structures.

In general, Ti plasmids are responsible for the following properties of A. tumerfaciens :

(i) Crown gall induction, tumor morphology and host range.

(ii) Specificity of opine synthesis in tumor cells.

(iii) Catabolic utilization of opines.

(iv) Conjugative transfer of Ti-plasmids.

(v) Agrocin sensitivity.

(vi) Exclusion of the virulence phage APel.

The relevant genes containing the above mentioned characteristics of the bacterium have been mapped by transposon - insertion mutagenesis and deletion mapping techniques.

Four distinct and separate homologous regions have been recognised from octopine (pTiACH5) and nopaline (pTiC58) Ti plasmids (Engler et al., 1981). They are,

(a) the T-region

(b) the vir-region

(c) the replication control region, and

(d) region coding for conjugative functions.

Transfer of Ti-plasmid sequences from A. tumefaciens to plant cells - In established plant tumor lines only the T-region of the Ti-plasmid has been known to be present. The presence of Ti-plasmid sequences in tumor cells has been shown by genetic analysis of Ti-plasmid functions (Bomhoff et al., 1976), renaturation kinetics analysis (Chilton et al., 1977) and by Southern gel blotting techniques. It has been characterised that a processing step involving the separation of T-region from the rest of the Ti-plasmid was operational during the tumor induction process. However, controversy exists as to whether the excision of T-region occurs prior to its transfer to the plant cell or the entire Ti-plasmid is tranferred to the plant cell and subsequently the Ti plasmid is lost leaving only the T-region with the plant genome. But recent evidences support the former view (Hoekema et al., 1983; De Framond et al., 1983; Hoekema et al., 1984; Hooykaas et al., 1985) Lately, it has been suggested that the T-DNA segment is circularized in the bacterial cells after its excision from the Ti plasmid. These circular molecules are believed to be the intermediates in DNA tranfer from the A. tumefaciens genome to the plant cells (Zdena et al., 1985).

Studies have revealed that T-DNA is present only in the nucleus (Willimitzer et al., 1980; Chilton et al., 1980). There it is covalently joined to the plant DNA and inherited as a mendelian trait (Otten et al., 1981). The T-DNA in most nopaline tumor lines is found to be a 23 kb DNA segment, co-linear with the corresponding T-region in the Ti plasmid (Lemmers et

al., 1980). In octopine type tumors, a 13.5kb TL-DNA was found to be always present and an additional 6-7 kb TR-DNA was also sometimes detected (Thomashow et al., 1980; De Beuckeleer et al., 1981). Deletion studies have shown that only TL-DNA is required for tumor development. A direct repeat of 25 bp has been found flanking the T-region of nopaline Ti plasmids and also in TL-region of octopine Ti-plasmids (Zambryski et al., 1984; Yadav et al., 1982; Simpson et al., 1982). It has been observed that the deletions which remove the right border from the T-region of the Ti-plasmids resulted in strains having diminished virulence (Ooms et al., 1982). Therefore, it has been concluded that the right border of the T-region contributed to the transfer of T-DNA to plant cells (Hooykaas, 1985).

The genes located in the T-region of the Ti plasmid were shown to be faithfully expressed in plant cells, as they carried eukaryotic 5' and 3' signals for transcription. T-DNA was found to be transcribed in crown-gall tumors into discrete polydenylated transcripts by an ∝-amanitin-sensitive RNA polymerase (Willimitzer et al., 1982; Bevan and Chilton, 1982). Besides genes for opine synthesis, T-DNA was characterised to contain onc genes which have not yet been assigned any function. Further, there were more sequences coding for the enzymes involved in auxin biosynthesis and cytokinin biosynthesis (Ooms et al., 1981; Schroder et al., 1984; [illegible]. This observation provided an explanation for the hormone independent growth of plant tumors. The processes of transfer and insertion of T-DNA into plant DNA was found to be due to the border sequences of the T-DNA and also the vir genes located on the Ti-plasmid outside the T-region (Caplan et al., 1983).

Ti-plasmid as a vehicle to introduce foreign DNA into plants - It is well established that Ti-plasmid is a potential vector to transfer genes into plants. The first such attempts were carried out with bacterial transposons Tn 7 (Hernalsteens et al., 1980) and Tn 5 (Garfinkel et al., 1981) which were inserted in vivo into the T-region of Ti-plasmids and were found to be cotransferred with the T-DNA. However, these genes could not be expressed owing to their prokaryotic regulatory sequences. Further, heterologous eukaryotic genes, such as that of yeast alcohol dehydrogenase (Barton et al., 1983), mammalian β -globin (Shaw et al., 1983), interferon, and genes under the control of SV40 early promoter, also failed to get expressed in plant cells. Therefore, it has been concluded that the expression of such foreign genes would require use of transcription signals from a gene that is known to be functional in plant cells. The opine synthase genes which are known to carry eukaryotic transcription signals have been employed to insert the desired genes and a number of vectors have thus been constructed via a method devised by Ruvkin and Ausbel, (1981)(Herrera-Estrella et al., 1983; Fraley et al., 1983; Bevan et al., 1983). Dominant selectable markers such as APH II from Tn5 and APHI from Tn 903, dihydrofolate reductase from R67 have been constructed based on nopaline synthase regulatory sequences which conferred resistance to kanamycin, G418 or methotrexate to host plant cells respectively. Maize alcohol dehydrogenase (ADH) gene was inserted in the coding sequences of nos gene and transferred into tabocco (Llewellyn et al., 1985).

Recently, a sophisticated binary plant vector system was developed based on the finding that when the T-region and vir-region of the Ti-plasmid

were physically separated, this did not lead to a diminished virulence (Hoekema et al., 1983). These new plant vectors contained a number of unique restriction sites in which desired genes could be cloned directly alongwith a marker gene surrounded by border sequences for the vir-system. Such a construction could then be used by Agrobacterium to transfer the cloned DNA into plant cells. Therefore, the introduction of such plant vectors in a strain of A. tumefaciens carrying a Ti-plasmid vir-region will affect their transfer to plant cells. It is therefore, now possible to obtain normal, fertile plants with phytohormone dependence for growth and regeneration by the use of vectors from which the undesired onc genes have been deleted (Zambryski et al., 1984).

The ultimate aim of all the above described transformation studies has been to improve the quantity and quality of many of the agricultural products. Cereals, which form the most important agronomic group, were till now thought to be recalcitrant to regenerate in culture conditions and all the efforts to induce tumors on monocots with A. tumefaciens turned blank. But, recent reports indicated a degree of success in this line. Hooykaas et al.,(1984) showed opine synthesis in the infected tissues of Narcissus cv. paperwhite and Chlorophytum capense. Hernalsteens et al.,(1984) reported tumor formation, opine synthesis and integration of T-DNA sequences in the monocot , Asparagus officinalis. Therefore, A. tumerfaciens Ti plasmid would undoubtedly prove to be a very important tool for genetic engineering of plants including monocots.

Assays for DNA Uptake and Selection of Transformants

To detect the uptake and integration of foreign DNA, DNA-DNA hybridization and autoradiography, and routinely used. Other methods include detection of radioactively labelled donor DNA in treated plants, and density differences in DNA from treated plants. However, only a few selection methods have been proposed for the detection and recovery of transformants.

Role of mutants in selection of transformants - Selection of transformants was found easy where mutants were used for transformation studies in such groups as bacteria, fungi, yeasts and some mammalian systems.

Himmen et al., (1978) reported the transformation of a stable leu$^-$ yeast strain to leu$^+$ by employing a chimaeric Col.El plasmid carrying the yeast leu 2 gene. Transformation of human (Bacchetti and Graham, 1977) or mouse (Wigler et al., 1977) cells from the thymidine kinase-negative (tk$^-$) to the thymidine kinase-positive (tk$^+$) phenotype was achieved using purified DNA or DNA fragments from Herpes Simplex Virus (HSV)

Lack of such stable, biochemical mutant systems in higher plants is an nevitable disguise for transformation studies. The most extensively studied plant mutant system is that of Arbidopsis thaliana, where mutants lacking in the metabolic steps of the thiamine pathway are available. It was reported that homozygous mutant seeds treated with DNA from a large number of bacterial sources lead to the production of tranformed, viable and fertile plants with varied frequency (Ledoux et al., 1971, 1972, 1974). Jean Peirre-Bourgin (1982) reported the isolation of a few aminoacid resistant mutants recently by UV - mutagenesis of haploid tobacco

protoplasts. Some auxotrophs have also been isolated from Hyoscyamus muticus (Potrykus et al., 1982).

Nitrate reductase deficient mutants as biochemical markers - The major factors delaying the establishment of a reliable plant cell transformation system were realised to be the lack of suitable selectable markers and the corresponding gene (s) in appropriate vectors. One of the candidates that is projected for achieving a suitable system for transformation studies is nitrate reductase (Lurquin and Kleinhofs, 1982). Nitrate reductase dificient (NR^-) mutants in higher plants were first reported in A. thaliana (Braaksma, 1973). Later, such mutants have been described in Hordeum vulgare (Warner et al., 1977; Kleinhofs et al., 1980), Nicotiana tabacum (Mendel and Muller, 1980), Pisum sativum (Kleinhofs et al., 1978; Warner et al., 1982; Feenstra, 1980), Datura innoxia (King and Khanna, 1980), N. plumbaginifolia (Marton et al., 1982), Hyoscyamus muticus (Strauss et al., 1981).

Nitrate reductase catalyzes the initial step in NO_3^- assimilation reducing NO_3^- -----> NO_2^-. The nia mutants are characterised to be defective in the apoenzyme whereas cnx mutants possess a defective molybdenum-cofactor (Mo-co); These mutants which can be selected on a chlorate medium, cannot grow on a medium with nitrate as the sole N_2 source. Somatic hybridization between nia and cnx was found to restore NR activity in the hybrids (Gupta and Schieder 1982,; Marton et al., 1982; Kohn et al., 1985). Successful attempts have been made to restore NR activity in cnx mutants by using ch1M gene from E. coli (Kleinhofs et al., 1982). The major advantage of NR system is the availability of conditional lethal mutants. Thus, the NR system offers to develop an effcient plant cell transformation model.

Dominant selectable markers for selection of transformants - The dominant selectable markers have the great advantage of allowing the selection of transformed cells of any species. Mulligan and Berg (1980) have used a bacterial gene for xanthine guanine phosphoribosyl transferase (XGPRT) as a selectable marker in animal cells. O'Hare et al., (1981) have cloned a bacterial dihydrofolate reductase gene derived from R67 on an SV40 plasmid which conferred resistance to methotrexate. The cloning of chloramphenicol-acetyltransferase (CAT) gene derived from E. coli transposon Tn9 was also shown to confer resistance to chloramphenicol.

Aminoglycoside phosphotransferase (APH 3' II) encoded by Tn5 and Tn 903 allowed resistance to the aminoglycosides kanamycin and G-418, which otherwise, were toxic and lethal to the cells (Garapin et al., 1982).

A further source of selectable genes was found to be that of the T-DNA from Ti plasmid; e.g., the area coding for hormone independence and opine synthase genes. At present, APH (3') II is the most widely used gene in the construction of chimaeric genes encoding for antibiotic resistance as well as for plant genes of heterologous origin. (Herrera-Estrella et al., 1983a,b, 1984; Bevan et al., 1984). These dominant selectable markers are used to recover non-selectable transferred genes.

MATERIALS AND METHODS

Bacterial Plasmids

Agrobacterium tumefaciens plasmids pLGVTi23 neo, pLGVTi23 DHFR and E. coli plasmids pLGV23 neo, pLGV23 DHFR were obtained through the courtesy of Dr. M. Van Montagu, Belgium.

Plant Material

Tobacco (Nicotiana tabacum L. var. Xanthi) seeds were obtained from Division of Genetics, IARI, NEW DELHI, Pea (Pisum sativum L. var. Boneville) seeds were obtained from National Seeds Corporation and nia mutants (Nicotiana tabacum nia - 120) through the courtesy of Dr. Otto Schieder, West Germany.

Antibiotics

Carbenicillin, kanamycin, rifampicin were obtained from Sigma Chemical Co., U.S.A.

Chemicals

L-glutamine, kinetin, lactose, myo-inositol, naphthalene acetic acid, N-l-naphthylethylene-diamine dihydrochloride, nicotinamide adenine dinucleotide (reduced), nicotinic acid and pyridoxine were obtained from Sigma Chemical Co., U.S.A., Bactoagar, bactotryptone and yeast extract were obtained from DIFCO laboratories, U.S.A.

Common laboratory chemicals and reagents, preferably of analar grade, were obtained from Glaxo laboratries, India Ltd., E. Merck, Sarabhai M. Chemicals or Bengal Chemicals and Pharmacuticals Ltd., India.

Other Materials

Millipore 0.45 μm HAWP membrane filters and filter assembly were obtained from Millipore Corp., U.S.A.

Preparation of Benedict's Reagent - Tri-sodium citrate 173g and sodium carbonate anhydrous 100g, were dissolved in 800 ml of water while heating. The solution was allowed to cool and 17.3 g of copper sulphate, dissolved separately in 100ml of water was added to it slowly while stirring.

Preparation of Media - All media were prepared in double distilled water. Ingredients were mixed and autoclaved at 15 p.s.i for 15 min or separate stock solutions were mixed after autoclaving as desired. Heat labile components were filter sterilized by passing through autoclaved 0.45 μm membrane filters and then added to the cooled medium.

Bacterial Media - Following bacterial media were prepared for growing A. tumefaciens and E. coli strains.

1) LB (Miller, 1971)

	g/litre
Bactotryptone	10.0
Yeast extract	5.0
NaCl	10.0
pH 7.2	
Agar	15

2) Lactose medium (Bernaerts and Deley, 1963)

Lactose	10.0
Yeast extract	1.0
Agar	20
pH 7.2	

Plant Tissue Culture Media

1) LS medium

LS medium is essentially MS medium (Murashige and Skoog, 1962) except for the following two modifications.

i) Thiamine is used in higher concentrations (0.4 mg/l instead of 0.1 mg/l)

ii) Glycine, pyridoxine and nicotinic acid are deleted.

Composition of Linsmaier and Skoog medium (LS, 1965).

Ingredients	Concentration in the medium mg/l
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	22.3

Ingredients	Concentration in the medium mg/l
$ZnSO_4.7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2.EDTA	37.2
$FeSO_4.7H_2O$	27.8
Organic	
Sucrose	30,000
Myo-inositol	100
Thiamine-HCl	0.4
pH	5.7
Agar	6-8 g/l
Hormones - For nontransformed tissue.	
NAA	2.0
Kinetin	0.3

2. NH4S medium - (Evola, S.V. 1983). This was employed for culturing nia callus.

Macronctrients	Murashige and skoog(1962)
Micronutrients	"
Fe-EDTA	"
Succinic acid	2.36 g/l
Thiamine-HCl	1 mg/l
Hormones	
2,4-D	1 mg/l
BAP	0.2 mg/l
pH	5.8
Agar	0.8%

This medium is essentially MS except for the following modifications

i) KCl (1446 mg/l) is added

ii) KNO_3 (1900 mg/l) is deleted

iii) Succinic acid is added

iv) Thiamine-HCl is added in higher concentrations (1 mg/l instead of 0.1 mg/l).

v) Glycine, pyridoxine and nicotinic acid are deleted.

3. MS413AA medium - (Muller and Grafe, 1978). This was employed for culturing nia callus.

Macroelements	Murashinge and Skoog (1962)
Microelements	"
Fe-EDTA	"
Vitamins	mg/l
Thiamine - HCl	1

Nicotinic - acid	1
Pyridoxine - HCl	1
Organic	Murashige and Skoog
Amino acids	
	mg/l
L-glutamine	877
L-Aspartic acid	266
L-Arginine	174
pH	5.8
Agar	0.8%
Hormones employed for callus growth were	
	mg/l
2,4-D	1
BAP	0.2
For differentiation	
BAP	1

Preparation of Antibiotics - Antibiotic stock solutions were freshly prepared in distilled water (kanamycin and carbenicillin) or DMSO (rifampicin) and added to the media before use. Stock solutions, prepared in water, were filter sterilized, while those prepared in DMSO were added as such.

Antibiotics alongwith their concentrations used are given in the following table

Antibiotics	Stock (mg/ml)	Solvent	Final concentration (μg/ml) *E. coli*	*A. tumefaciens*	tumor tissue
Kanamycin	12.5	Water	25	25	100
Carbenicillin	50	Water	100	100	1000
Rifampicin	50	DMSO	100	100	-----

Growth and Culture of Bacterial Strains - *Agrobacterium tumefaciens* strains were grown in LB medium at 28°C and *E. coli* at 37°C. Strains containing plasmid with gene(s) for antibiotic resistance, were always grown in the presence of the respective antibiotic(s).

Agrobacterium tumefaciens (pLGVTi23 neo) cells were inoculated from solid medium into a small volume(20ml) of liquid LB medium with kanamycin and shaken overnight at 28° C.

Cells were stored in LB-agar slants at 0.4°C and subcultured every month.

Growth and Culture of Plant Material - Pea seeds were surface sterilized by treatment with 70% ethanol for 2-3 min followed by treatment with 0.1% $HgCl_2$ solution for 5 min. They were throughly rinsed 3-4 times with sterile water and soaked overnight in sterile water. The soaked seeds were kept for germination on 0.8% agar in water, in culture tubes at 27° C in dark.

Tobacco seeds were surface sterilized by the procedure used for pea seeds, rinsed 3-4 times with sterile water and placed on Murashige

and Skoog mineral medium with 0.7% agar in culture tubes or flasks. The seeds were kept for germination in dark at 27°C for 14 days following which they were transferred to light.

Nicotiana tabacum nia callus was maintained on NH4S or MS413AA medium containing 1 mg/l 2, 4-D and 0.2 mg/l BAP and solidified with 0.8% agar. The callus was subcultured at regular intervals on the same medium.

For differentiation, nia callus grown on the above media was transferred to MS413AA or NH4S medium without hormones and kept in light at 27°C. After 2 weeks the callus turned green. This greening callus was then transferred to MS413AA medium with 1 mg/l BAP and kept in light. This led to shoot bud regeneration.

Oncogenicity Assay - Three to five day old pea seedlings were either decapitated or wounded using a sterile needle. A. tumefaciens (pLGVTi23 neo) cells grown in LB medium were applied to the cut or wound surface using a platinum wire loop. The culture tubes were then kept either in light or dark at 27° C.

Tumor Formation in Tobacco with A. tumefaciens (pLGVTi23 neo)

Tobacco seedlings cultured on MS medium were transferred to the fresh medium in 250 ml Ehrlenmyer flasks and kept in light. When the plants were 6-8 week old, stems were wounded with a sterile needle. After 24 hr the wound was inoculated with a loopful of an overnight culture of A. tumefaciens (pLGVTi23 neo). Visible tumors could be observed

after 10 days of inoculation. When the tumors had grown sizeably, they were excised from the stem, dipped in alcohol and briefly flammed. They were then cut into pieces and the inner regions were removed for culture.

Culture of Excised Tumor Tissue - Inner regions of the excised tumor tissue were placed on 0.8% agar solidified LS medium containing 1 mg/ml carbenicillin but no hormones and kept in dark for callus growth. After 14 days, when no bacterial growth was observed on the medium, the tumor callus was transferred to fresh medium with carbenicillin.

Culture of tobacco tumor tissue on selective medium- The tumor callus growing on hormone-free LS medium containing carbenicillin was transferred to the same medium with 100 μg/ml kanamycin and kept in dark. This was subcultured at regular intervals on the same medium.

Detection of Nitrate Reductase Activity in N. tabacum Wild Type and nia Tissue

Preparation of enzyme extract - About 100 mg of callus tissue from wild type and nia was transferred separately to the petriplates containing 0.06 M KNO_3 and kept in light for the induction of nitrate reductase activity. After 24 hr of the incubation, the tissue was washed, dried on blotting paper and ice chilled before homogenization in 0.1 M Tris-HCl buffer (pH 8.5) containing 3 mM EDTA and 3 mM cysteine. The homogenate was centrifuged at 15,000 rpm for 30 min. All operations were carried out at 4°C. The supernatant was used for assaying the enzyme.

NADH-nitrate reductase assay - Assay of the enzyme was performed esstntially by the procedure of Hageman and Hucklesby (1971). The reaction mixture contained 1 ml of 0.1 M phosphate buffer, (pH 7.5), 0.2 ml of 0.1 M KNO_3, and 0.2 ml of 2 mM freshly prepared NADH. In the control set, NADH was not added. Reaction was initiated by adding 0.2 ml of crude enzyme extract and was carried out for 30 min at 30°C in a water bath. The reaction was terminated by the addition of 0.1 ml of 1 M Zinc acetate. The rection mixture centrifuged for 20 min. To 1 ml of the supernatant, 1 ml each of 0.1% sulfanilamide (W/V), prepared in 1.5 M HCl and 0.02 % (W/V) NED were added. After 20 min, absorbance was measured at 540 mm with a spectrophotometer (Shimadzu, model UV-260). Amount of nitite was calculated by means of a standard curve. The enzyme activity has been expressed as nano moles of nitrite formed/gm fresh weight/hr.

Determination of Kanamycin Sensitivity Levels of nia Callus

For studying kanamycin sensitity level of N. tabacum nia - 120, 2 week old, actively growing callus was employed. All the operations were done in a laminar air flow bench; and sterile glassware were used throughout.

MS413AA medium solidified with 0.8% agar and contining 1 mg/l 2,4-D and 0.2 mg/l BAP was prepared. Equal volumes of the medium were poured into five 250 ml flasks. Filter sterilized concentrated kanamycin solution was added to the media separately to obtain the final concentrations of 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml. Kanamycin was not added in one of the flasks which served as control. The contents of the flask were shaken in order to mix them well; The medium

with or without kanamycin was poured into sterile plastic petriplates (35 mm diameter; about 5 ml in each) and was allowed to solidify. Plates were sealed with parafilm strips of equal size. Individual weights of the plates with medium were noted down. nia callus of about 1 cm diameter was transferred to each of the plates which were sealed with parafilm and weighed again. From these two readings the initial weight of the callus was calculated. Petridishes with callus were kept in dark at 27°C and the growth pattern was followed. The callus was removed from the plates at regular intervals and weighed. The increase in fresh weight and percentage growth were calculated for each concentration of kanamycin. After weighing the callus was transferred onto a preweighed aluminium foil and kept in an incubator at 60°C for 12 hr. During this period, the callus got completely dried. The dry weight of the callus was taken. Percentage increase in dry weight at all concentrations of kanamycin was calculated and results were plotted on a graph.

$$\% \text{ growth} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100$$

Co-Cultivation of nia Callus Cells with A. tumefaciens (pLGVTi23 neo)

The method followed for co-cultivation was that of Muller et al (1984), with slight modifications. About 0.5 g of nia callus pregrown on MS413AA medium was taken after 2 weeks of its transfer. This callus was transferred tc a 15 ml capped glass centrifuge tubes and squashed gently with a sterile stainless steel spatula. To this, about 6 ml of MS medium without hormones or antibiotics was added and centrifuged. Supernatant was discarded with a sterile pasteur pipette. Another 6 ml of the same medium was added and the contents of the suspension were poured in a sterile plastic pertidish (90 mm diameter). To this 0.2 ml culture

of A. tumefaciens (pLGVTi23 neo) was added; the plates were sealed with parafilm and incubated in dark at 28°C. Control set in which agrobacteria were not added was also incubated as above. After incubation for 24 hr the suspension was centrifuged and the supernatant was discarded. Washing of the cells was done with 6 ml of medium without amino acids or hormone. Another 6 ml of the same medium was added and poured in petridish. At this step, filter sterilized carbenicillin was added to the washed cells at a concentration of 1 mg/ml to kill agrobacteria. The petridishes were sealed and again incubated at 28°C in dark. Amino acids were excluded from the medium to inhibit the growth of Agrobacterium. After two days, the cells were washed and 6 ml of MS medium with aminoacids but without hormones was added; carbenicillin was also added. The procedure was repeated and antibiotic was added twice during the next 14 days. After 14 days, the suspension of growing cells was poured as overlay onto 4 petriplates, containing solidified MS medium with amino acids and with or without kanamycin but without carbenicillin and hormones. The plates were incubated in light at 28°C. Control set was also processed and plated as above.

RESULTS

Characterization of A. tumefaciens Strains

The properties of A. tumefaciens and E. coli which were employed in the present study are given in table I.

Some of the known characteristics of the Agrobacterium tumefaciens which distinguish it from other bacterial species were checked.

3-ketolactose test - Based on the production of 3-ketoglycosides from the corresponding disaccharides and acids by A. tumefaciens and A. radiobacter, Bernaerts and DeLey (1963) devised a biochemical test that is limited to members of the genus Agrobacterium and used for their classification. Bacterial cultures were grown at 28°C for 1 to 2 days on medium containing 1% Yeast extract, 2% glucose, 2% $CaCO_3$ and 2% agar. A loopful of bacteria were then deposited on solid lactose medium in a petridish as a small heap of about 0.5 cm or less in diameter. The plates were incubated at 28°C for 1 to 2 days. They were flooded with a shallow layer of Benedict's reagent, and left at room temperature for 1-2 hr. A. tumefaciens strains with pLGVTi23neo and pLGVTi23DHFR showed formation of a yellow ring around the cell mass suggesting that they belong to the predominantly 3-ketolactose positive group. E. coli strains with pLGV23neo and pLGV23DHFR did not show such coloration.

Virulent Characteristics of A. tumefaciens (pLGVTi23neo)

The formation of tumors at the wound site following inoculation indicates the virulence of the inciting A. tumefaciens strain. In the present study virulence was tested following infection of pea seedlings and tobacco

Table 1: *Plasmids and their properties*

Plasmids	Properties
A. tumefaciens	
pLGVTi23neo	Present in strain C58 C1 Rif^R. It contains kan^R marker from Tn 5 between nos regulatory sequences. Virulent; resistant to kanamycin and rifampicin.
pLGVTi23DHFR	Present in strain C58 C1 Rif^R. co-integrate of PTiC58: PLGV23DHFR; virulent; resistant to rifampicin and methotrexate.
E. coli	
pLGV23neo	Present in strain HB101. Resistant to kanamycin and rifampicin.
pLGV23DHFR	In strain HB101. Resistant to carbenicillin, kanamycin and methotrexate.

plants with A. tumefaciens (p LGVTi23neo).

Tumor formation in pea seedlings - Virulence of A. tumefaciens (pLGVTi23neo) was tested by infecting pea seedlings. Bacterial cells were applied either by a lateral infection or after cutting the stem. Tumor formation was observed 10 days after the infection.

Induction and in vitro Culture of Tumor Tissue from Tobacco Plants

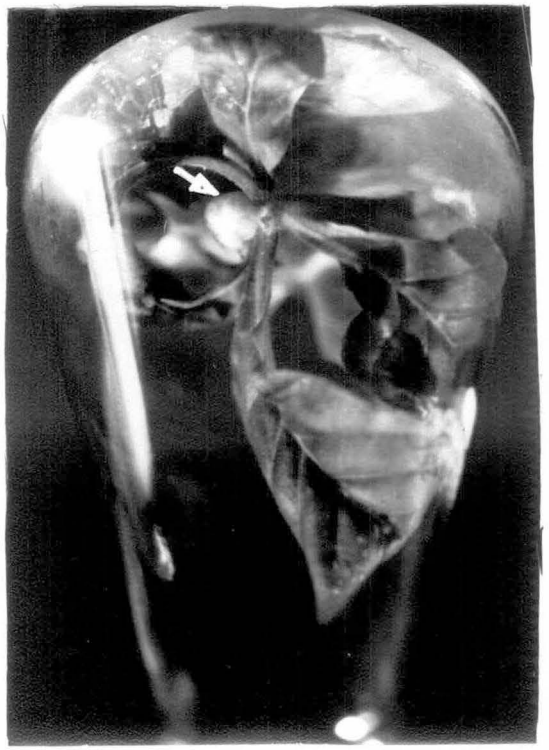
The transformation of plant cells by DNA transfer from A. tumefaciens was studied in crown gall tumors induced on tobacco plants. Nicotiana tabacum plants grown under sterile conditions on MS mineral medium were wounded on the stem and inoculated after 24 hr, with overnight culture of A. tumefaciens (pLGVTi23neo). As control, wounds in some plants were left uninoculated. The plants were kept in light at 27°C and tumor formation was observed 10 days after bacterial inoculation. Only a small wound callus was seen in plants where bacterial inoculation was not made. When the tumors had grown sizeably (Fig.1) they were excised from the stem and employed for culture.

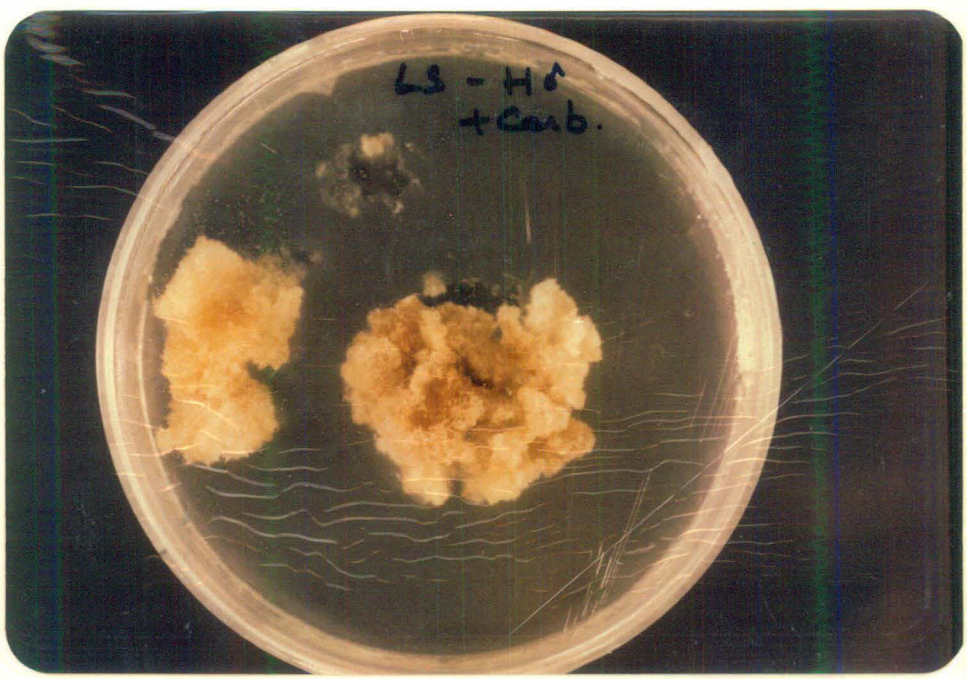
After initial surface sterilization, the tumor was cut into pieces and placed on hormone-free LS medium with 1mg/ml carbenicillin to eliminate the inciting bacterium and kept in dark. Tumor tissue continued to grow on this medium and no bacterial growth was observed surrounding the tumor tissue. After 10 days, the tumor callus was transferred to fresh medium with carbenicillin and finally to medium without carbenicillin. Wound

Fig. 1: *Tumor induced by A. tumefaciens (pLGVTi 23 neo) on Nicotiana tabacum plant.*

Fig.2a: *Tumor callus growing on hormone-free LS medium containing 1mg/ml carnencillin.*

Fig.2b: *Callus obtained from uninoculated tobacco plant and growing on hormone-free LS medium containing 1mg/ml carbenicillin.*




LS - H$
+ Carb.

callus excised from tobacco stem and cultured on hormone-free LS medium failed to grow. Some parts of the tumor callus turned brown and ceased to grow when subcultured on hormone-free LS medium. The rest of the tumor callus was bright, friable and grew well. (Fig. 2a & 2b).

Growth of Tobacco Tumor Tissue on Medium Containing Kanamycin

As the plasmid pLGVTi23neo which was employed for tumor induction on tobacco, has a marker gene encoding for kanamycin resistance in its T-region, the transformed tumor tissue was tested for kanamycin resistance. For this, tumor callus growing on hormone-free Ls medium with carbenicillin was transferred to the same medium with 100 g/ml kanamycin. The tumor callus continued to grow on this medium whereas control callus turned brown and failed to grow (Fig. 3a & 3b).

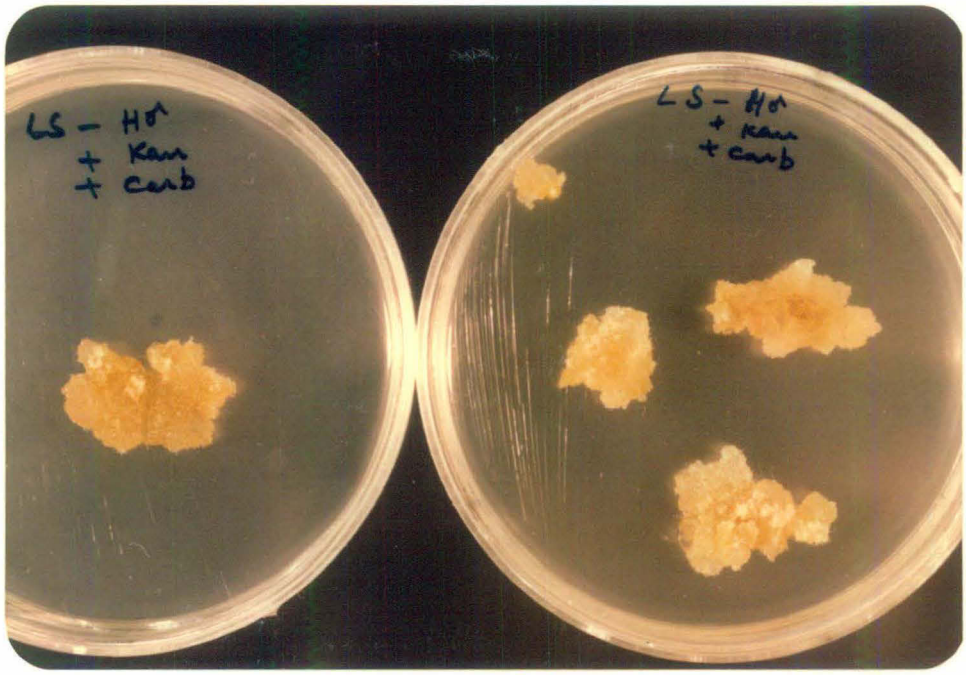
Characterization of nia The assimilation of nitrate is achieved through the eight -electron reduction of oxidized inorganic anion (NO_3^-), resulting in the formation of ammonium (NH_4^-). This conversion requires two enzymatic steps, the first of which is catalyzed by the enzyme nitrate reductase (EC 1.6.6.1-3).

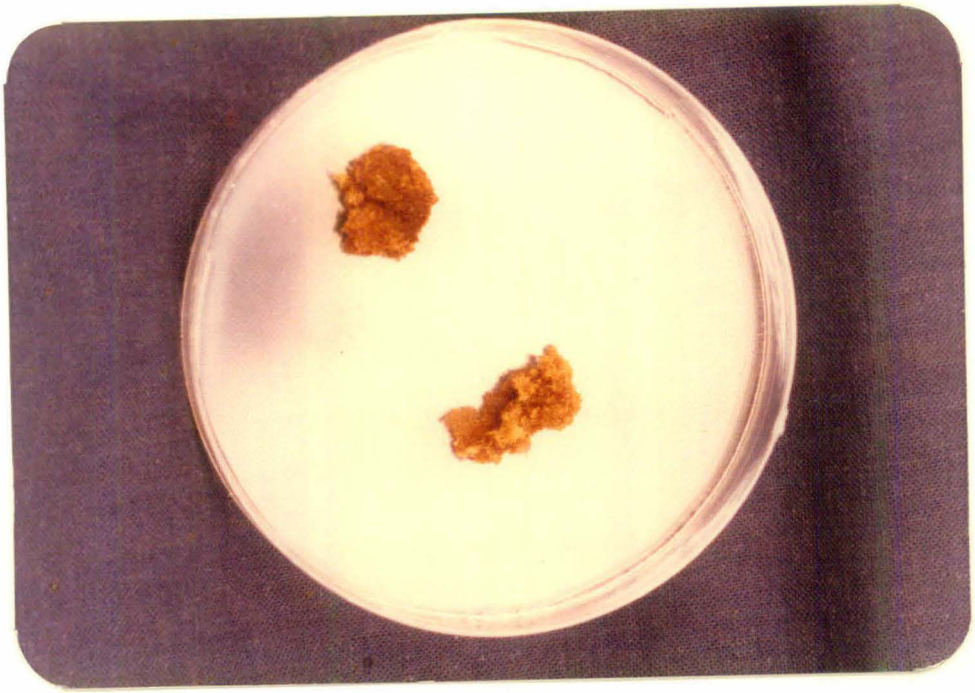
$$NO_3^- \xrightarrow[\text{nitrate reductase}]{2e^-} NO_2^- \xrightarrow[\text{nitrite reductase}]{6e^-} NH_4^+$$

The nitrate reductase is soluble, electrontransferring protein, 200,000-300,000 in molecular weight. Electron transfer is generally regarded

Fig. 3a: *Tumor callus 2 weeks after its transfer onto hormone-free medium containing 1mg/ml carbenicillin and 100 µg/ml kanamycin.*

Fig. 3b: *Control callus from uninoculated tobacco plant 2 weeks after its transfer onto hormone-free LS medium containing antibiotics as above.*


LS – Hδ
+ Kan
+ carb
LS – Hδ
+ Kan
+ carb



to be mediated by enzyme bound heme iron, flavin adenine dinucleotide (FAD) and molybdenum cofactor. The physiological activity involves the reduction of nitrate to nitrite occurring at the molybdenum site. Mutants impaired in nitrate reductase activity have been obtained by many groups. Studies on these mutants revealed that they belong to two categories. Tobacco mutants which have a defective apoenzyme of nitrate reductase are named as nia mutants, and those defective in molybdenum cofactor (Mo-co) are called cnx mutants. The NR activity of these mutants is either very low or zero. (Muller and Grafe, 1978). When callus tissue of nia - 120 was tested for nitrate reductase activity, it was zero whereas wild type tissue showed normal activity. Nitrate reductase activity was expressed as nano moles of NO_2^- formed/g fresh weight /hr (Table II).

Culture and Differentiation of nia Callus

As nia mutants are unable to utilize NO_3^-, reduced N_2 was supplied in the media. For this, either succinate medium (NH4S) or aminoacid medium (MS413AA) was used for growth and differentiation. On medium containing 2,4-D (1mg/l) and BAP (0.2 mg/l), nia callus grew well and was bright and friable (Fig.4).

To obtain differentiation, 2-3 week old callus pregrown on MS413AA medium with 2,4-D (1 mg/l) and BAP (0.2 mg/l) was transferred to the same medium without hormones. After two weeks the callus started greening (Fig. 5a). This callus was transferred to the medium containing BAP (1mg/l). After 3 weeks shoot buds appeared all over the callus (Fig.5b)

Table II: *Enzyme activity of Tobacco wild type and nia*

Cell line	Enzyme activity[a]
N. tabacum (wild type)	382
N. tabacum nia-120	0

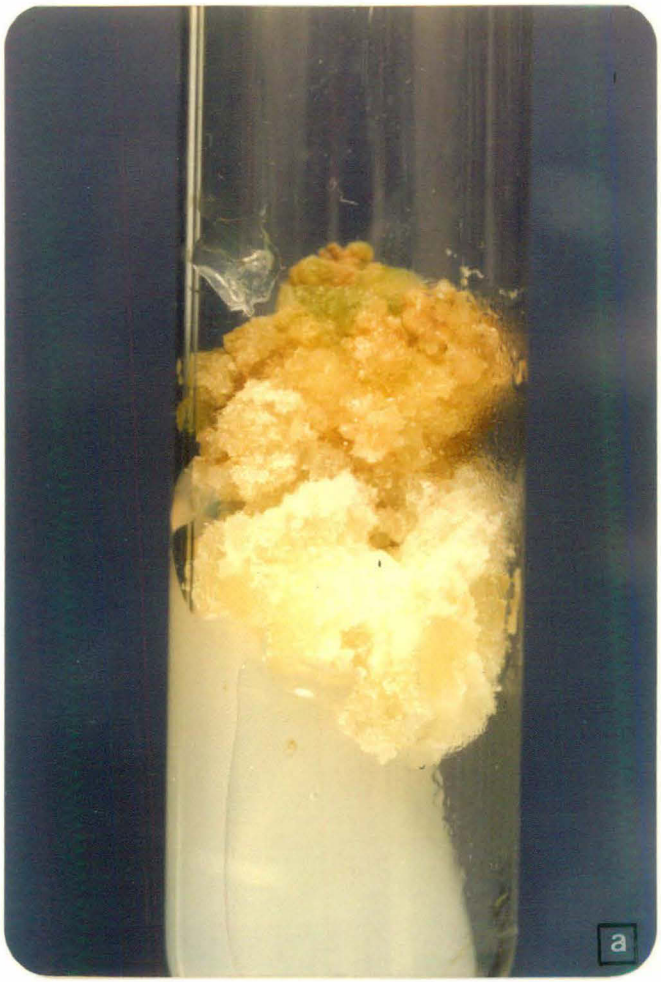
a. Enzyme activity was expressed as nano moles of NO_2^- formed / g fresh weight/hr.

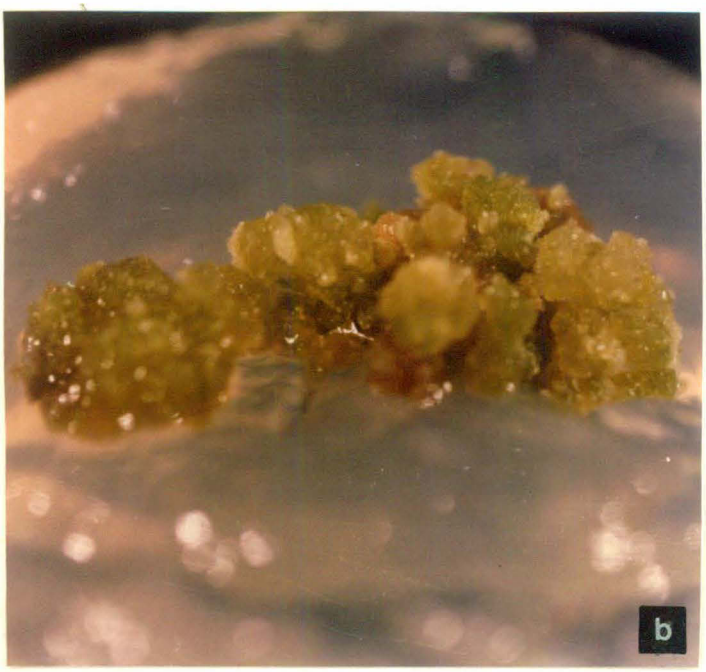
Fig. 4: Three week old nongreen callus cultures of nia maintained on MS413AA medium containing 1mg/l 2,4-D and 0.2 mg/l BAP.

Fig. 5a: *Two week old greening nia callus on MS413AA medium without hormones.*

Fig. 5b: *3 week old differentiating nia callus on MS413AA medium containing 1mg/l BAP. Note shoot bud initiation.*

Fig. 5c: *5 week old nia culture, developing leaves on medium containing 1mg/l BAP.*


a


b


c

and thick leaves started developing (Fig.5c).

When the undifferentiated callus was transferred from MS413AA with 2,4-D (1mg/l) and BAP (0.2mg/l) to a medium either with BAP (1mg/l) or with IAA (0.5mg/l) and BAP (0.1mg/l) to obtain differentiation, the callus turned brown (Fig.6) and eventually died. Hence, the callus was first transferred to hormone-free medium prior to shooting medium.

Growth Pattern of nia Callus

nia callus grown on MS413AA medium containing 2,4-D (1mg/l) and BAP (0.2 mg/l) for two weeks was transferred to the same medium in petriplates as described in Materials and Methods. The growth of the callus was followed upto 26 days after which it was found to decline. Growth was studied in terms of increase in fresh weight as well as dry weight. The callus growth was found to reach log phase by seventh day of the culture (lag phase was not studied). Maximum increase was observed on 22nd day of the culture. Twentysix day onwards there was no change in fresh weight, however decrease in dry weight was observed. Thus, nia callus showed maximum growth on twenty second day (Fig. 7).

Determination of Kanamycin Sensitivity Level of nia Callus

nia callus was transferred to plates containing medium with or without kanamycin of different concentrations as described in Materials and Methods. The effect of kanamycin on the callus growth was studied in terms of fresh weight and dry weight, on 22nd day after the transfer.

The callus growth was expressed as the percentage of 'O' kan control.

Kanamycin inhibited the callus growth at all the concentrations tested (25 µg/ml, 50µg/ml,75µg and 100µg/ml)(Fig.8). But maximum inhibition was observed at 100 µg/ml kanamycin concentration. At this concentration callus growth was inhibited by 90% in terms of dry weight and 92% inhibition was observed in terms of fresh weight (Fig.9).

Co-cultivation of N. tabacum nia callus Cells with A. tumefaciens (pLGVT-i23neo)

Co-cultivation of nia callus cells was done according to the procedure of Muller et al., (1984) with the following modifications:

1) 1mg/ml carbenicillin was used to kill the agrobacteria instead of cefoxitin and cloxacillin

2) Succinate was replaced by aminoacids as reduced nitrogen source for the growth of nia callus cells.

3) As the plasmid pLGVTi23neo, which was used for co-cultivation has a gene encoding for kanamycin resistance, 100 µg/ml of kanamycin was added to the solid medium on which plating was done in order to select transformed colonies. At this concentration, kanamycin was found to inhibit normal nia callus growth.

Callus cells of nia were co-incubated with Agrobacterium cells for 24 hr in dark. After this period, they were washed and allowed to grow

Fig. 6: *Three week old browning nia Callus on MS413AA + BAP (1mg/ml), when transferred directly from medium containing 2,4-D (1mg/l) and BAP (0.2 mg/l).*

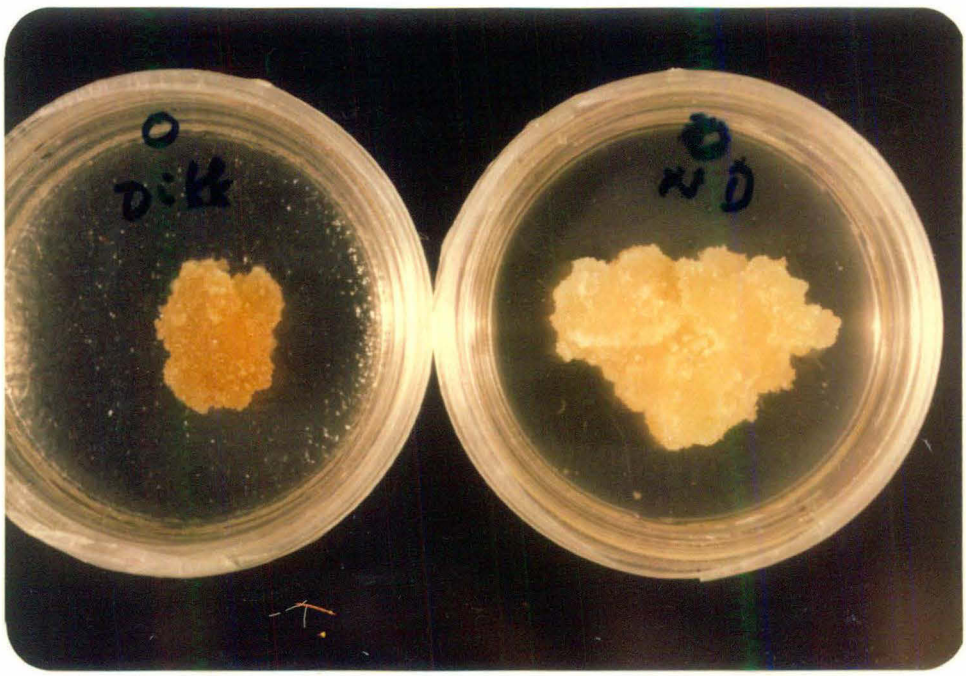

O
Dikk
O
ND

Fig. 7: *Growth pattern of nia Callus on MS413AA medium containing 1mg/l 2,4-D and 0.2 mg/l BAP.*

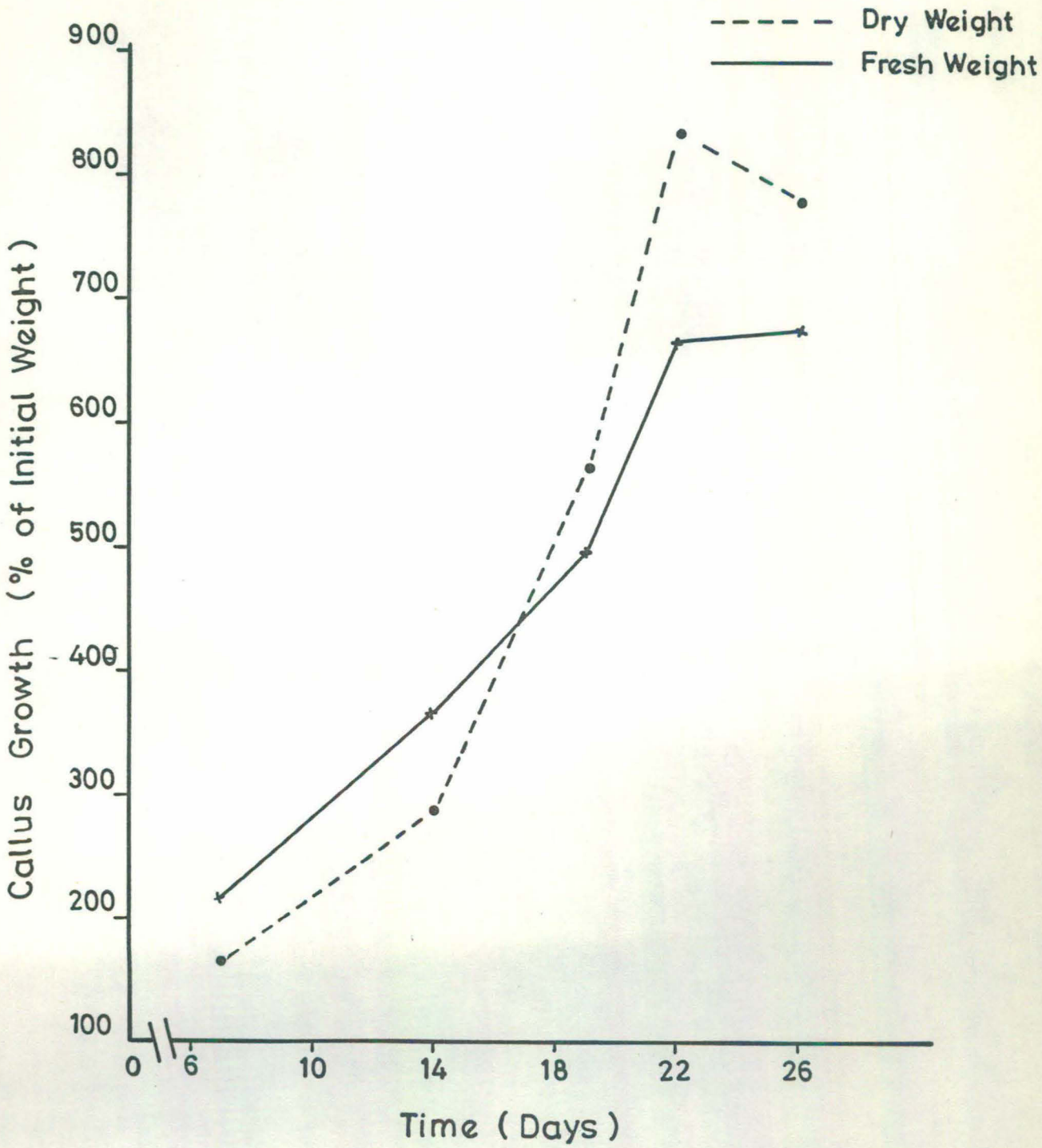

Dry Weight
Fresh Weight
900
800
700
600
500
400
300
200
100
Callus Growth (% of Initial Weight)
0
6
10
14
18
22
26
Time (Days)

Fig. 8: *nia callus growth on 22nd day on medium containing different concentrations of Kanamycin.*

a) '0' Kan.
b) 25 µg/ml.
c) 50 µg/ml.
d) 75 µg/ml.
e) 100 µg/ml.

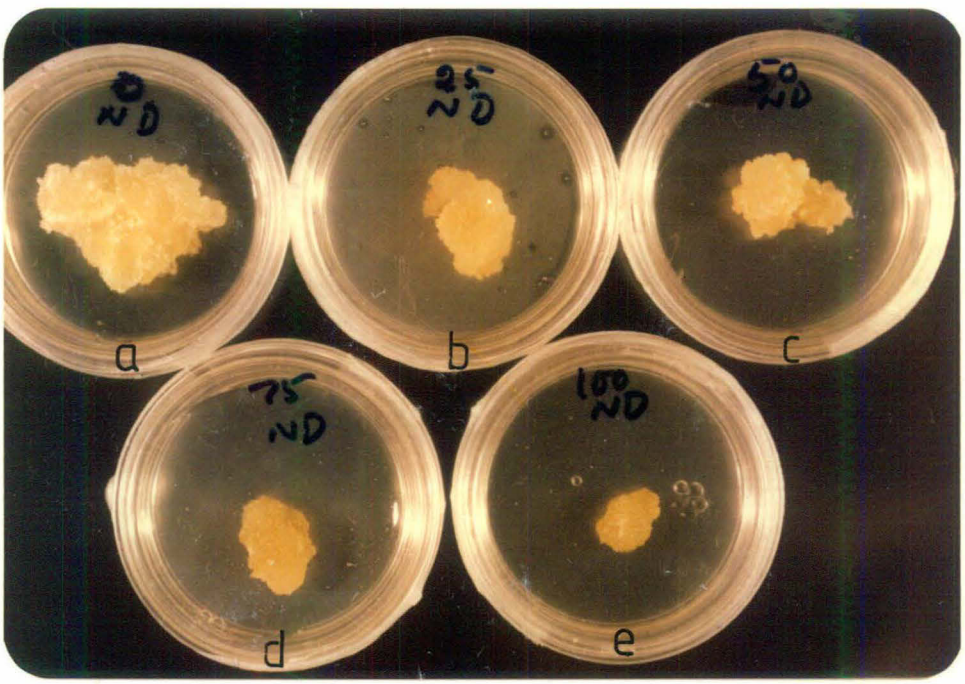
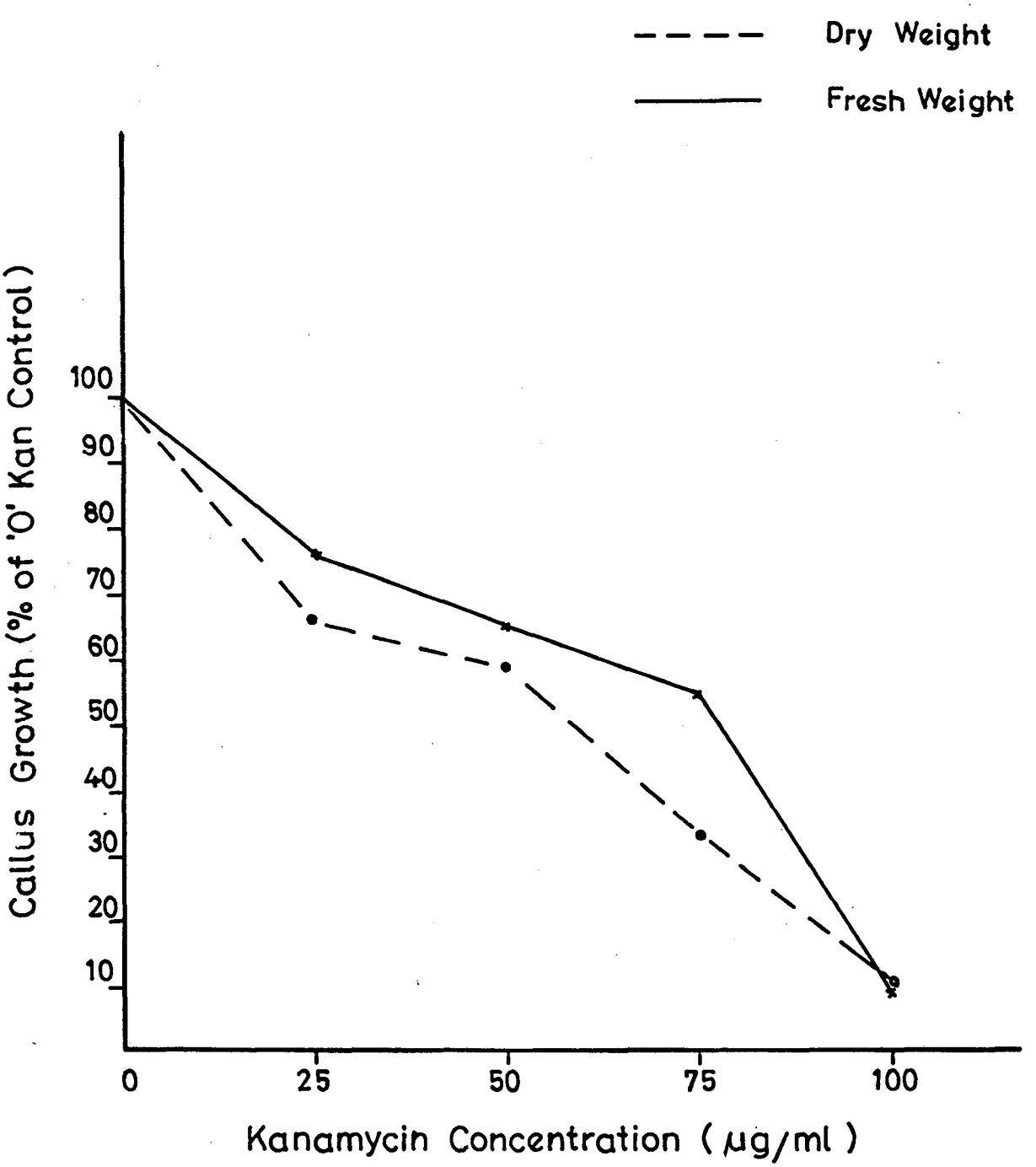

0 ND
a
25 ND
b
50 ND
c
75 ND
d
100 ND
e

Fig. 9: *Effect of kanamycin concentration on nia callus growth on twenty second day of the culture on MS413AA medium containing different concentrations of kanamycin.*


Dry Weight
Fresh Weight
Callus Growth (% of '0' Kan Control)
100
90
80
70
60
50
40
30
20
10
0
25
50
75
100
Kanamycin Concentration (µg/ml)

on hormone-free medium containing 1mg/ml carbenicillin. Antibiotic solution was added twice during the next 14 days. Later, the growing suspension was plated on hormone-free solid MS413AA medium with or without kanamycin (100 g/ml). The co-cultivated Callus Cells showed some growth initially during the 14 days incubation period, but when plated on hormone-free solid medium, no significant growth was observed in plates with treated or untreated cells.

DISCUSSION

The present study was aimed at the investigation of foreign DNA transfer into plant cells via A. tumefaciens and also developing a genetic transformation system in a nitrate reductase deficient cell line of Nicotiana tabacum. For this study A. tumefaciens (pLGVTi23neo) and N. tabacum nia-120 were employed.

The Crown-Gall Tumor System

The transfer of DNA from A. tumefaciens to plant cells during tumor formation has been actively pursued since Chilton et al., (1977) have demonstrated stable incorporation of Ti-plasmid sequences in tumor cells of tobacco.

The biochemical characteristics of Agrobacterium were studied by employing A. tumefaciens (pLGVTi23neo) and A. tumefaciens (pLGVTi-23DHFR). When tested for 3-ketoglycoside production by the procedure of Bernaerts and DeLey (1963), both strains showed positive result (yellow coloration with Benedict's reagent), whereas E. coli strains with plasmids pLGV23neo and pLGV23DHFR did not show the reaction. Thus, these strains were confirmed to be those of A. tumefaciens.

Virulent strains induce tumors on plants when inoculated. The tumor morphology varies depending on the bacterial strain. Nopaline strains induce smooth tumors, which often develop leaf-like structures (teratoma), if tumor induction has occurred on the upper half of the stem or on the leaves. This position effect on teratoma development is ascribed to differences in the local levels of auxins and cytokinins in the plant (Hooykaas, 1982;

Stonier, 1982). We tested A. tumefaciens (p LGVTi23neo), a nopaline strain for virulence on pea seedlings and tobacco plants. Smooth tumors appeared when tobacco plants were infected on the lower half of the stem. No teratomas developed.

It is well known that crown gall tumors produce auxins and cytokinins (Braun, 1978). it is assumed that this is the reason for the growth of the tumor cells in the absence of added phytohormones. In the present study tumors induced on tobacco plants by pLGVTi23neo were tested for hormone autotrophy. When grown on hormone-free LS medium, tumor tissue grew well. The callus formed from this tumor tissue was bright and friable. Parts of untransformed tumor tissue were also observed, which eventually turned brown and ceased to grow on subsequent culturing on hormone-free medium. It appeared that tumors induced by A. tumefaciens were a mixture of normal and transformed cells as were also reported earlier (Sacristan and Melchers, 1977).

It has been reported that the plasmid p LGVTi23neo carries a chimaeric gene encoding for the aminoglycoside 3' -phosphotransferase-II [APH (3') II] from Tn 5 under the control of the regulatory sequences of the nopaline synthase gene (Herrera-Estrella et al., 1983). Hence we tested the tumor tissue induced by this plasmid for kanamycin resistance. When tobacco tumor callus growing on hormone-free LS medium was transferred to the medium containing 100μg/ml kanamycin, it continued to grow whereas control callus from uninoculated tobacco plant turned brown and ceased to grow on kanamycin medium. Plants, such as tobacco, are very

sensitive to antibiotics G418, kanamycin, neomycin and gentamycin (Leemans et al., 1982). Hence, the resistance of tumor callus to kanamycin in our study was attributed to T-DNA transfer from pLGVTi23neo to plant cells.

Studies with N. tabacum nia-120

Even though a number of reports suggested nitrate reductase deficient mutants as promising system for transformation studies, very few attempts have been made to ascertain this fact. The work with NR^- mutants was mainly concerned with protoplast fusion and restoration of nitrate reductase activity through complementation (Marton et al., 1982; Kohn et al., 1985). Only Muller et al., (1984) reported successful transformation of tobacco NR^- cell line, Cnx A-68 using A. tumefaciens.

In the present study, attempts were made to establish a good transformation system in one of the NR^- cell lines of tobacco, viz., nia-120. These attempts started with the study of cultural conditions and obtaining differentiation of nia-120. Growth pattern and kanamycin sensitivity level of nia were studied. Finally, co-cultivation of nia callus cells with A. tumefaciens (pLGVTi23neo) was also attempted.

nia-120 which is defective in the apoenzyme of nitrate reductase was tested for the enzyme activity. When in vitro assay was done with nia and tobacco wild type callus tissue, nia showed no activity whereas wild type tissue exhibited normal NR activity. This is in accordance with the results of Muller and Grafe (1978).

Nitrate reductase deficient mutants (NR^-) cannot utilise NO_3^- as the sole N_2 source (Muller and Grafe, 1978; King and Khanna, 1980; Kleinhofs et al 1978). Thus they were maintained on MS medium supplemented with succinate or aminoacids. On MS413AA medium with 1mg/l 2,4-D and 0.2mg/l BAP, the callus was bright, friable and grew well. For obtaining differentiation, the callus pregrown on the above medium was first transferred to hormone-free medium and after 2 weeks it was transferred to BAP (1mg/l) medium. Callus started greening on hormone-free medium and when transferred to BAP medium, shoot buds appeared all over the green callus. Later, small thick leaves developed.

Growth characteristics of nia callus on MS413AA medium containing 1mg/l 2,4-D and 0.2mg/l BAP was studied. Callus growth was in exponential stage upto 3 weeks. The maximum growth was observed on twenty second day.

Unless plants carry genes encoding for antibiotic resistance, they are killed by higher concentrations of antibiotics. Based on this phenomenon, several plasmids with antibiotic resistance markers have been constructed and upon their transfer to sensitive strains, the transformants are selected on a selective medium containing respective antibiotics. Aminoglycosides are one of the most widely used group of antibiotics in transformation studies. This group of antibiotics (gentamycin, neomycin, kanamycin, G418 etc.) cause misreading of mRNA by binding to the ribosomes. Davies and Davis (1968), studied in in vitro, the effect of variation in concentration

of antibiotic on the degree of misreading of mRNA. Kanamycin showed 3-5 fold increase in the level of misreading as the drug concentration was raised from 10^{-6} to 10^{-4}M. N. tabacum wild type plants never grew on a medium with kanamycin at a concentration of 100µg/ml (Deshayes et al., 1985). In the present study, we observed that kanamycin inhibits callus growth of nia-120. Though inhibition occurred at all concentrations tested, maximum inhibition was observed at 100µg/ml of kanamycin. At this concentration inhibition upto 90% was observed.

Transformation of nia callus cells in this study was attempted by the method of Muller et al., (1984). For this A. tumefaciens (p LGVTi23neo) which has a gene encoding for kanamycin resistance was employed. The procedure with minor modifications has been described in Materials and Methods. The co-cultivated cells were plated on hormone-free medium with or without kanamycin. The growth was followed upto 8 weeks. Though the cells showed some growth initially, it was not sustained. Transformation frequency was found to be much lower when callus cells are co-cultivated with Agrobacterium, than that obtained in co-cultivation of protoplasts (Muller et al, 1984). This may be due to many reasons. Transformation is dependant on the physiological state of the cells, nature of Agrobacterium strain, and less so on the expression of the 'Vir' genes of the Ti-plasmid (An, 1985). Rapidly dividing suspension cultured cells were found to be transformed with high efficiency (An, 1985). It is not yet clear whether transfer mechanism of Ti-plasmids is dependant on DNA replication in the host plant cells, or recognition and interaction are produced only when the plant

cells are dividing rapidly. Moreover, protoplasts are found to be killed during treatment with A. tumefaciens (An, 1985). This may be true for callus cells also. Refined techniques are being developed to co-cultivate callus cells with the bacteria.

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SUMMARY

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In the present study, the process of transformation of plants through A. tumefaciens and possibilities of establishing a good transformation system in one of the nitrate reductase deficient (NR^-) cell lines of tobacco, viz., Nicotiana tabacum nia - 120 were investigated.

Characteristics of A. tumefaciens were studied by 3-ketolactose test and tumor induction.

Tumors induced on tobacco plants by A. tumefaciens (pLGVTi23neo) were tested for hormone autotrophy by growing on LS medium containing no hormones.

Actively growing tumor tissue was further tested for kanamycin resistance. When grown on medium containing 100µg/ml kanamycin, tumor tissue continued to grow and thus confirmed to be transformed.

Biochemical and cultural characteristics of nia - 120 were studied. Growth and differentiation of nia were obtained on media containing reduced N_2 source (succinate or amino acids).

Growth pattern of nia callus was studied and it was found to attain the maximum growth on 22^{nd} day.

Kanamycin sensitivity of nia callus was studied and about 90% inhibition was observed in nia callus growth on medium containing 100µg/ml kanamycin.

Transformation of nia callus cells by Co-cultivation with A. tumefaciens was attempted.

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* Original not seen.