PRODUCTION OF EDIBLE VACCINE AGAINST RABIES IN TRANSGENIC MUSKMELON (*Cucumis melo* L.) BY ELECTROPORATION

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BANGALORE

1998

PRODUCTION OF EDIBLE VACCINE AGAINST RABIES IN TRANSGENIC MUSKMELON (*Cucumis melo* L.) BY ELECTROPORATION

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Thesis submitted to the
University of Agricultural Sciences, Bangalore
in partial fulfilment of the requirements
for the award of the Degree of

Master of Science

in

BIOTECHNOLOGY

BANGALORE AUGUST 1998

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DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled ***"PRODUCTION OF EDIBLE VACCINE AGAINST RABIES IN TRANSGENIC MUSKMELON (Cucumis melo L) BY ELECTROPORATION"*** submitted by ***Miss. BINDU RAGHAVAN*** for the award of degree of ***MASTER OF SCIENCE*** in ***BIOTECHNOLOGY*** to the University of Agricultural Sciences, Bangalore is a record of *bonafide* research work done by her during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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Dedicated to
My Parents
and
My Sister

ACKNOWLEDGEMENT

I would first like to express my gratitude to my guide ***Dr. Akella Vani,*** *Senior Scientist, Division of Biotechnology, IIHR, Bangalore for her able guidance throughout my research work and preparation of my thesis. I am also indebted to* ***Dr. Ramanjini Gowda****, Assistant Professor, Department of Biotechnology, my major advisor for all the help he has extended to me during my M.Sc. programme. I express my gratitude to* ***Dr. T.K.S. Gowda****, Professor and Head, Department of Biotechnology,* ***Dr. Uday Kumar****, Professor and Head, Department of Crop-physiology and* ***Dr. Krishnamurthy,*** *Associate Professor, Department of Veterinary Microbiology, members of my advisory council for all their helpful suggestions during my research work.*

I take this opportunity to acknowledge my parents and my sister for all their support.

I would like to thank all my class mates for the assistance they have given me at different points of time during my M.Sc. course.

I am thankful to my friends ***Aparna, Deepaja, Jayanthi, Anindita*** *and* ***Chitra*** *for their moral support. I express my special thanks to my friends* ***Anil*** *and* ***Asish*** *who helped me in getting my thesis ready.*

I thank, ***Mr. Siddaraju****, technician of the Molecular Diagnostic laboratory, IIHR, for looking after my plants in the glass house. I am grateful to* ***Mr. Desai,*** *and* ***Mrs. Sowmya*** *of the Molecular Diagnostic Laboratory, IIHR for their helpful hints during my research work.*

Finally, I express my gratitude to ***Chandrashekar*** *for helping me produce a neat thesis in a short time.*

Bindu

(Bindu Raghavan)

CONTENTS

LIST OF TABLES

LIST OF PLATES

INTRODUCTION

I. INTRODUCTION

Rabies is an important disease which was recognised as far back as 23rd century B.C, where there were references to 'Mad dog' in the legal documents of Mesopotamia, Greek and Roman mythology and writings of Homer Vigil and Aristotle (Steel , 1975). The word Rabies originates from Sanskrit 'Rabbahs' meaning 'to do violence'. (Hand, 1989). It is termed hydrophobia in human beings. Even today rabies is endemic in many areas of the world. This is because there are huge global reservoirs of the virus in both domestic and wild animals which includes dogs, fox, racoons, skunks, coyotes, mongoose and bats.

Rabies is caused by a virus belonging to the family Rhabdo-Viridae under the genus Lyssa. Virtually all warm-blooded animals are susceptible to rabies with the degree of susceptibility varying from species to species. Humans serve only as accidental hosts and become infected with the rabies virus either by a bite from a rabid animal or by mucosal exposure. After a bite the virus enters unmyelinated nerve fibers or muscle end plates. It then travels by retrograde axonal transport to the nearest sensory neuron in the dorsal root ganglion or anterior horn cells of the spinal cord, where it replicates. The virus may then travel to the brain where it infects neurons in almost all brain regions. After replication in the brain the virus travels to other parts of the body via neuronal pathways and can be found in the salivary glands, tears, skin and adrenals. Incubation period may last a few weeks beginning with fever, restlessness, hydrophobia and finally death.(Wiktar, 1985).

Piere Victor Galton was the first to suggest vaccination against this deadly disease (Wiktar,1985). It was Louis Pasteur in 1885 who developed the first rabies vaccine using rabies infected brain and spinal cord of rabbit. The virus was inactivated through heat and drying of the infected materials. Vaccination involves daily injections for 14 days with severe local reactions in many cases. The adverse effect of such neural tissue vaccines was overcome by the diploid cell vaccines developed by Koprowski. These included human DCV, purified chick embryo cell vaccines, purified vero cell rabies vaccines and purified duck embryo cell vaccine. Current research is directed at developing safer and cheaper vaccines for both human and animals. Lately, researchers have developed recombinant vaccines where cloned genes coding for glycoprotein and nucleoprotein of the rabies virus are tagged to a virus with a large genome like vaccinia or adenovirus. The most recent effort is to produce vaccines in transgenic plants. These transgenic plants can be used as edible vaccines. Such a system overcomes the need for fermentation technology, stringent purification protocols, refrigeration costs and maintenance of cold chains during shipment and delivery. Also it does away with the risk and pain associated with parentral delivery. In a developing country like India, such a cost-effective system would be most appropriate to bring rabies under control.

For the production of transgenic plants expressing the rabies glycoprotein a number of plant transformation protocols may be used. One of the techniques being used widely at present is the Agrobacterium mediated gene transfer wherein

a disarmed Agrobacterium Ti plasmid harbouring the gene of interest is used. Agrobacterium however has a limited host range consisting mainly of dicots. There are also direct gene transfer methods like microinjection (Neahaus and Spangenberg, 1990) biolistic method (Sanford, 1988) and electroporation of protoplasts (Fromm *et al.,* 1987) All these methods require tissue culture and regeneration of the plant following gene transfer. In case of electroporation protoplasts were used since it was believed that plant cell walls act as an impermeable barrier for DNA uptake (Potrykus *et al.*, 1991). However Topfer *et al.*. (1989) demonstrated that direct gene transfer in intact seed derived embryos is possible in cereals and legumes. Several groups (Dekeyser *et al.,* 1990 ; Senaratna *et al.*, 1993 and Songstad *et al.,* 1993) have also demonstrated naked DNA uptake and expression in leaf sections, somatic and zygotic embryos as well as intact suspension cells of several plant species after electroporation mediated transfer. The inference drawn is that plant cell walls are not impervious to DNA. Chowrira *et al.* (1995) have shown that meristematic tissue located on the apical dome of nodal buds can take up and express foreign DNA by electroporation. The branches arising from the electroporated tissue will bear fruits which will contain transgenic seeds. This technique thus allows the production of transgenic plants without the need for *in vitro* tissue culture and regeneration. Electroporation is the application of high voltage electric pulse to cells to induce transient membrane pores allowing entry of macromolecules including DNA. Tissue electroporation could have a general application as a facile gene transfer method (Luong *et al.*,1995)

For the purpose of expression of the rabies glycoprotein in a transgenic plant, certain characters are desirable in the plant. The plant must have an edible portion which can be eaten raw, because cooking may denature the glycoprotein. Also the fruit should be acceptable to the consumers. The plant should be easy to cultivate and should bear a large number of fruits in a short duration and should be amenable for transformation. Thus muskmelon is a crop of choice for production of edible vaccine. Muskmelon (*Cucumis melo*) is grown almost all over India particularly in Bihar, UttarPradesh, Punjab, Rajasthan, AndhraPradesh, Tamil Nadu and Karnataka. It is a popular fruit not only in India but also throughout out the tropical and subtropical regions of the world. It is grown in an area of 803 ha with a production of 13894 x 10^3 MT in the world. Muskmelon contains 92.8% water, 0.6g protein, 0.1g fat, and 5.4g carbohydrates and has a calorific value of 2.5 per 100g. It is also a rich source of Ca, Mg, K, P and Vitamin A (Choudhury 1990). It is consumed raw as a summer fruit all over the world.

Muskmelon 2n=20 belongs to the family cucurbitaceae. It has duration of 80-120 days and a yield of 17 tons per hectare and there are many varieties of muskmelon being cultivated in India .

For the present study Arka Jeet was used. This is an improvement over a local collection IIHR-103 from Lucknow. The fruits are small round with flat ends and smooth skin. Each fruit weights 300-500 g and has white flesh with medium soft texture, excellent flavour and 15-17% TSS. Arka Jeet yields 15 tons per hectare in 90 days. The present investigation was undertaken in the Molecular

Diagnostic Laboratory, Division of Biotechnology, IIHR, Hessarghatta, Bangalore with the following objectives:

To standardise the electroporation parameters for muskmelon.

Transformation of muskmelon plants with the rabies glycoprotein gene.

Testing of the putative transformants by dot blot analysis.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Using plants as vaccine production systems is a new concept. Work has been done at a few centres in the world for the production of vaccines in plants. Also work is going on to develop improved vaccines against rabies (Celis *et al.*, 1990).

Many plant transformation techniques are available for the introduction of foreign genes into plants. Use of electroporation as a method of inserting exotic genes into plants has been experimented with. This chapter deals with earlier work done in the following areas.

2.1 Vaccines for Rabies

2.2 Vaccine production in plants

2.3 Electroporation

2.4 Muskmelon transformation.

2.1 Vaccines for Rabies

The first ever vaccine for rabies was developed by Louis Pasteur in 1885. He along with his assistant Emil Roux made a preparation of progressively desiccated rabies infected rabbit spinal cord and brain. Even today in many countries this neural tissue vaccine is being used. Here, phenol is used as the inactivating agent instead of heat. This nervous tissue vaccine however causes adverse reactions due to myelin content of the nervous tissue. Subsequently,

Hillary Koprowski developed the diploid cell vaccines. These tissue culture vaccines were safer than Pasteur semple vaccine and more effective. The human diploid cell vaccine is found to develop high neutralising antibody titres as early as 10 days after inoculation compared to the semple vaccine which requires atleast 30 days to induce protective levels of antibodies (Anonymous, 1997).

Now other tissue culture vaccines have been developed which are easier to prepare and less expensive like the purified chick embryo cell vaccine. This is very safe to use and can even be administered during pregnancy with no damage to the mother or child. Purified duck embryo cell vaccine has also been produced.Purified verocell vaccine makes use of continuous cell line of heteroploid nature, which have tumourigenic propensity. Thus this vaccine is not safe for humans.

Oral live attenuated vaccines in baits have been used to bring rabies in wild animals under control in Europe and North America (Winker and Bogel, 1992). At present in the U.S.A, the rabies vaccine being used are inactivated virus are being used for dogs, cats, sheep, cattle, horses, ferrets. Combination (inactivated rabies) is used in cats and horses. Oral rabies vaccine called Roboral has been licensed for use in federal rabies control programmes to control rabies in racoons. This is rabies glycoprotein in live vaccine sector (JAMVA, 1998).

In India the semple vaccine which causes neuroparalytic accidents is still manufactured and used for post bite treatment.(Ramalingaswamy. 1997). Rabies is the only communicable disease where post exposure vaccination is strongly

recommended after exposure to infection. This involves the use of anti rabies immunoglobulin of equine or human origin, along with vaccines. Since the virus takes on an average two to four months to produce disease symptoms, post exposure vaccination is resorted to evoke immunity in time. Mouse monoclonal antibodies used for typing rabies strains from various regions and different animal species may replace immunoglobulin of human or equine in serum therapy. (Koprowski, 1989).

2.1.1 Recombinant Rabies Vaccine

Dietzchold *et al.* (1987) reported the introduction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. The rabies nucleocapsid was found to be an oral immunological enhancer by Hooper *et al.* (1994). Dietzschold *et al.* (1990) reported the expression of rabies vaccine nucleoprotein in insect cells and this protein can be purified from the insect cells and used as a vaccine. The cDNA of the RNA, gene that codes the rabies virus nucleoprotein was cloned into baculovirus. The recombinant baculovirus expressed the N protein in *Spodoptella frugipeda*. From the insect cells the N protein was purified and inoculated into mice, which induced specific T-cells and virus neutralising antibody & protected against a lethal challenge of rabies. Rupprecht *et al.* (1986) reported the oral immunization and protection of racoons with a vaccinia rabies glycoprotien recombinant virus vaccine.

Wiktor *et al.* (1984) reported protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Blancou and co-workers (1986) obtained oral vaccination for fox against rabies using a live recombinant vaccinia virus. Brochier *et al.* (1991) reported large scale eradication of rabies using recombinant vaccinia rabies vaccine. Structural and immunological characterisation of a linear virus neutralising epitopes of the rabies virus glycoprotein and their possible use in a synthetic vaccine was suggested by Dietzschold *et al.* (1990).

The glycoprotein of rabies virus ERA strain was abundantly expressed in a baculovirus expression system. Oral vaccination of racoons with the baculovirus expressed glycoprotein resulted in the production of rabies virus neutralising antibodies and protection against lethal challenge with a street rabies virus. Zhen Fang Fu *et al.* (1993). Moens *et al.* (1993) reported that Roboral, a vaccinia rabies recombinant vaccine was tested in Belgium, France and USA during 1987-93. It was assessed under confined use on the basis of residual pathogenicity, horizontal transmission, genetic stability, efficacy, etc. Deliberate release into the environment was monitored with respect to design of bait and biosafety. Rabies was declared eradicated in Belgium as a result of these efforts. Although rabies was declared eradicated by 1993, in 1994 it was confirmed in thirteen foxes collected in the areas close to the French border, indicating the entry form across the border. This demonstrated the need for extensive vaccination campaigns using the recombinant rabies vaccine (Pastoret *et al.*, 1995).

Pastoret and Bochier (1996) used a recombinant virus expressing the rabies glycoprotein for oral vaccination in foxes. To study the stability of the recombinant vaccine baits containing the vaccine were placed in the field. Despite considerable variations of environmental temperature the vaccine remained stable for atleast one month. The recombinant is presently used to control wildlife rabies in fields in several European countries and USA. Chantal *et al.,* 1997 administered vaccinia rabies recombinant vaccine (Raboral VRG) as direct buccal drop or by feeding with impregnated granules. The vaccinia virus recombinant is assessed to be a suitable vector for oral vaccination of rabbits using baits.

McGarvey *et al.* (1995) engineered tomato plants to express a gene for rabies virus glycoprotein, which coats the outer surface of the rabies virus. The plants were transformed by Agrobacterium mediated transformation. The protein product was expressed in leaf and fruit tissue. Low levels of rabies glycoprotein per gram of tomato tissue were obtained. However, the levels were sufficient to occasionally engender a glycoprotein specific immune response when freeze dried material was administered in complete Freunds adjuvent to mice.

2.2 Vaccine Production in Plants

Plants have traditionally supplied a wide range of products for use by man. In addition to feed and foodstuffs, plant tissues also act as a source of components used in the production of medicines, oils, fuels, fiber's, fats, detergents and dyes. However, the concept of vaccine production in plants was introduced by Mason

and Arntzen in 1992. This effort was stimulated by interest in evaluating the capacity of plants to produce different classes of proteins of pharmaceutical value, and because of the practical need for new technology for the production and delivery of inexpensive vaccines, especially in the developing world.

According to Arntzen (1997) there are two major strategies for the production of subunit vaccines in plants, one of them being the genetic transformation of the nuclear genome of plants using gene vectors. The other is the manipulation of the genome of plant pathogenic viruses. The first approach gives rise to plants that have the permanent capacity to produce the desired vaccine. The viral engineering approach gives a transient expression system.

In a pioneering work done by Dr. Hugh Mason and associates in 1992 tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a constitutive promoter using Agrobacterium medicated transfer. By 1995 it was proved that a crude extract from these transgenic tobacco plants could induce an immune response against Hepatitis upon parenteral immunization of mice. They also showed that B and T cells epitopes of HBsAg are preserved when the antigen is expressed in transgenic plants.

Haq *et al.* in1995 reported the transformation of tobacco and potato plants with genes encoding the heat labile enterotoxin subunit, LT-B, of enterotoxigenic *E.coli*. When the recombinant LT-B was given equally to mice the plant derived antigen stimulated humoral and mucosal immune response with titres comparable to bacterial LT-B. Feeding of raw transgenic potato tubers to mice also caused the

production of mucosal and serum antibodies. The production of rabies virus glycoprotein has been reported in transgenic tomatoes by McGarvey *et al.* (1955). Preliminary animal studies have shown the immunogenicity of the plant derived glycoproteins but improved plant expression many be necessary to elicit stronger responses.

Arntzen and associates in 1996 reported the expression of Norwalk virus capsid protein in transgenic tobacco and potato. The plant expressed recombinant Norwalk virus like particle which was orally immunogenic in mice. This virus causes epidemics of acute gastro-enteritis in humans. Therefore these transgenics may in future be used as vaccines for humans. Apart from using the plants to express antigenic subunits of pathogens for active immunity they may also be used for providing passive immunity. Ma and Hein reported the generation and assembly of secretary antibodies in plants in 1995. Four transgenic Tobacco plants were generated that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A.G heavy chain, a murine joining chain and a rabbit secretary component, respectively. A series of crosses were carried out to obtain a plant expressing all four proteins assembled into a functional immunoglobulin. This plant-derived immunoglobulin could recognise the streptococcus mutans cell surface adhesion molecule against tooth decay, in the future.

Vaccine production in plants has also been done by the modification of plants pathogenic viruses. Material epitope fusion with TMV capsid protein was done by Trupen *et al.* (1995). The Epitopes from material sporozoites were fused

into the coat protein of TMV the recombinant virus was then used to infect tobacco plants. Western blot analysis of the recombinant capsid protein expressed in plants showed recognition by monoclonal anti-malarial antibodies. Epitopes for foot and mouth disease of cattle, human rhino virus-24 (HRV-14) and human immuno deficiency virus-1 (HRV-1) have been fused into the capsid of CPMV. The chimeric CPMV could induce expression of the epitope in the host plant and could be used as plant derived vaccine (Porta *et al.,* 1994).

Transformation of the CPMV by inserting an epitope of the mink enteritis virus (MEV) and infection of cowpea plants for expression of MEV antigens was done by Dalsgaard and associates (1993). This plant-derived vaccine could be used to prevent enteritis in minks, dogs and cats. For the production of edible vaccines in plants it is necessary to express the desired protein in a food that is consumed raw, to avoid denaturation of the candidate vaccine protein. Blake and Arntzen (1995) have developed a transformation system for banana, which is an ideal crop for edible vaccine production.

Plants are increasingly being considered as a new, unconventional source of vaccine. 'Edible vaccines' hold an enormous potential to make plants once again a dominant source of therapeutic drugs (Moffat, 1996). According to Whitelam (1995) the development of edible transgenic plant materials for use as oral vaccines can be seen to exploit the unique properties of plants for the storage, distribution and administration of the vaccine or the therapeutic agent. Thus seeds can provide a convenient means of storing and distributing vaccine encoding

genes, whilst expression of the encoded protein in, say, the fruit of the plant derived from the seed could be envisaged as the simplest means of administering the vaccine.

2.3 Electroporation

The dielectric break down of the membrane was explained by Zimmermann (1974,1982). According to him, cells suspended in liquid medium can be compared to a structure consisting of a non-conducting membrane with aqueous solutions on both sides exposed to an electric field leading to separation of charges in the membrane, similar to the charge separation in the dielectric layers of an electrical capacitor. This builds up a potential difference across the membrane. Opposite electric charges on the membrane attract each other creating pressure because of which membrane thinning is induced. At a critical potential difference, localised breakdown of membrane occurs and pores are formed.

In 1986, Stanger and Hui reported that a large number of adjacent pores in the membrane might coalesce resulting in large cracks in the membrane. This was demonstrated using freeze fracture electron microscopy. Upon removal of the electric field the pores get resealed provided that the field strength and pulse width are not excessive. The term electroporation was coined by Newman in 1962 to refer to the formation of pores under the influence of electrical field. Joersbo *et al.* (1990) studied the size of pores generated under different electroporation conditions. They found that field strength at short pulses exerts a relatively higher

electroporatic effect. When pulse duration is decreased the critical voltage required for pore formation decreases.

Kinoshita *et al.* (1988) have shown that pore formation takes less than 1 μ sec in high salt medium and pores recovered in less than 2sec. With intense and long pulses some leakage continued resulting in osmotic imbalance, leading to bursting. Zimmermann and Veinken (1982) found that membrane disruption at lipid-protein junctions recover more slowly. It may take a few minutes at 25^0C to 30 minutes at 30^0C.

2.3.1 Electroporation in plants

Fromm *et al.* (1986) developed the electroporation mediated gene transfer technique. Electroporation has been used in generating transgenic plants (Fraley, 1989). Using electroporation, foreign genes can be introduced into intact protoplasts of dicots and monocots, which can be regenerated into plants (Zang *et al.,* 1989 ; Fromm *et al.,* 1985).

Saunders *et al.* (1989) compared square and exponential decay pulses. They obtained a higher percentage of transformants using exponential decay pulses. The activity of CAT gene introduced through electroporation into protoplasts of tobacco was studied by Taylor and Larkin (1988). They found increased activity of CAT gene with increased pulse length upto 5ms and then it plateaued. Voltage was optimum at 1500-1750 V/cm. Highest activity was seen with a pulse number of 6. In 1987, Lindsey and Jones found that by increasing delay between pulses from

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0.5 to 99.9 m sec, pulse number and duration could be increased without adversely effecting viability of sugarbeet suspension cultures. Thus, more expression of CAT gene was observed.

It was Fromm *et al.* (1985) who first developed a method to introduce DNA into plant protoplast by applying a high voltage current to carrot protoplasts in solution containing DNA. They reported the expression of CAT gene in maize and tobacco cells by electroporation medicated transfer. Fromm *et al.* (1986) demonstrated the stable transformation of maize with the Npt-II gene following electroporation.

The stable transformation of soybean callus with Npt-II gene was reported by Christou *et al.* (1987). This was confirmed by southern blot analysis. Bower and Birch 1990 found transient expression of GUS gene in carrot protoplasts by electroporation. GUS gene expression increased with increase in DNA concentration from 50 μg/ml to 100 mg/ml. Dekeyser *et al.* (1990) demonstrated that intact and organised rice leaf tissues of 2 sq cm size could take up foreign DNA by electroporation and express it. This method of gene delivery involved overcoming host nucleases, prolonging DNA-plant tissue incubation time and other parameters of electroporation.

In 1991, Senaratna *et al.* transformed somatic embryos of alfalfa with plasmid DNA containing the GUS gene. Akella and Lurquin (1993) electroporated seed derived embryos of cowpea and obtained seedlings expressing chimeric transgenes. Gene transfer into intact scutellum cells of wheat embryos was

reported by Kloti *et al.* (1993). The transient expression of GUS gene and anthocyanin constructs in intact maize immature embryos by electroporation was demonstrated by Songstad *et al.* (1993). Chaudhury *et al.* (1995) obtained transient expression of GUS gene in intact seed derived embryos of rice.

Obermeyer and Weisenseel (1995) used electroporation to introduce plasma membrane impermeable molecules into the cytoplasm of pollen grains of *Lilium longiflorum*. They found that the size of the pores produced in the plasma membrane by electroporation allowed uptake of 40-KDa dextran but not 70-KDa dextran. They suggested that this method could be used to loan genetic material into pollen grains for the production of transgenic plants. Direct electroporation into intact cells of wheat embryogenic callus was demonstrated by Zaghmout (1993). Greatest activity of the introduced GUS gene was found in cells electroporated at 6000 V/cm in the presence of 6 % glycerol and 20-25% (v/v) polyethylene glycol. Tissue culture medium was used as an elctroporation buffer

Zhou *et al.* (1993) transformed protoplasts of wheat using pBARGUS by electroporation. The integration of BAR gene in wheat was confirmed by southern hybridization. The integrated DNA sequence may be tandemly repeated atleast once and partially methylated by this method of transformation. Mukhopadhyay *et al.* (1994) reported gene transfer to protoplasts of *Asparagus officinalis* by electroporation. The transient expression level of the introduced GUS gene was enhanced by increasing plasmid DNA concentration and the presence of PEG in the electroporation medium Songstad *et al.* (1995) advocated the exploration of

alternative DNA delivery techniques such as electroporation of intact tissues including meristems especially under *in vivo* conditions. Introduction of foreign genes and stable integration was reported by Akella *et al.* (1992-93) where they generated stable transformed pea plants in the R1 generation of plants electroporated with GUS gene.

Gangamma *et al.* (1996) demonstrated the uptake and stable integration of chimeric GUS gene by electroporation in intact nodal meristems in plants. The stability of the introduced gene was studied in R1 and R2 generations of electroporated pea, cowpea, lentil and soybean. The stable integration untill the R2 generation indicated that this simple and reproducible method of electroporation could be used in future for transformation of plants with desired genes. Jarel and Rietveld (1996) compared the transformation efficiency of tobacco protoplasts by EG mediated uptake and electroporation. They concluded that electroporation gave higher and more reproducible results.

Hailing *et al.* (1996) introduced two defective mutants of TMV (Tobacco Mosaic Virus), one without coat protein gene and the other without replicase, into tobacco protoplasts by electroporation. In the transgenic tobacco plants, normal TMV coat protein and minus strand of the viral RNA were detected. Hong Chao *et al.* (1996) reported enhanced GUS activity in protoplasts of wheat electroporated with calf DNA as a carrier material. Bommineni and Jauhar (1997) conducted an evaluation of target cells and tissues used in genetic transformation of cereals. Cereals were considered recalcitrant for transformation mainly because tissue

culture was difficult and agrobacterium mediated transfer was not successful in cereals. However, introduction of DNA into cereals has become a routine procedure where electroporation mediated transformation is of great importance. Jing Bo *et al.* (1997) has reported the expression of goat growth hormone in popular protoplasts by electroporation. Expression of cDNA encoding goat growth hormone was confirmed at the mRNA level, 24 hours after electroporation mediated DNA transfer.

These recent reports indicate that electroporation can be used for the expression of foreign genes of varied origin like viral, animal and bacterial, in plants. This can be done both under *in vitro* and *in vivo* conditions in both monocots and dicots.

2.4 Muskmelon transformation

Most of the transformation work in muskmelon has been done using *Agrobacterium tumefactions*. Tahar *et al.* (1989) reported the transformation of muskmelon using the particle gun. Fang and Grumert (1990) have reported *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Muskmelon cotyledon pieces were dipped in cultures of disarmed *Agrobacterium tumefaciens* containing a binary transformation vector bearing a selectable marker gene Npt-II for Kanamycin resistance. Regenerated plants were found to be transgenic by southern blot assay. There was stable integration of Npt-II gene in the R1 generation.

Gonsalves *et al.* (1994) reported the transfer of cucumber mosaic virus, white leaf strain coat protein gene into *Cucumis melo*. They found nearly the same percentage of transgenic plants using Agrobacterium and microprojectile bombardment method. The level of R1 plant resistance to cucumber mosaic virus varied with different transformed genotypes. Pech *et al.* (1996) demonstrated the expression of ACC oxidase antisense gene in cantaloupe melon fruits by Agrobacterium mediated transformation. Due to the expression of this gene, ethylene reduction of the transgenic fruit was less than 1% of control fruit. The transgenic fruits displayed extended storage life and improved quality.

In the present study transformation of muskmelon was attempted through electroporation.

MATERIAL AND METHODS

III. MATERIALS AND METHODS

For the production of edible vaccine against rabies in transgenic muskmelon, one has to first transform muskmelon plant with the rabies glycoprotein gene. For this purpose, the rabies glycoprotein gene must be introduced into a suitable vector which can be used to transform muskmelon plants and the gene must be driven by a promoter which is compatible with the plant system.

The transformation vector used was the plasmid name pRGRgp (Fig) A binary A tumefaciens vector RG-2 was taken which is a derivative of Bin 19.Rg-2 was modified by first removing the GUS gene by digestion with Sma I and Sst I and treated with T4 DNA polymerase to remove the overhangs. Then the 2 ends were relegated to form RG-G containing an unique Bam HI site between CaMV35S promoter and Nos. terminated A complete C DNA of the glycoprotein of rabies virus ERA strain was removed from the plasmid PT g 155 by digestion with Bgl II, agarose gel purified and inserted into the complete Bam HI site of RG-G thus obtaining the plasmid pRGRgp the site of the plasmid is about 12.5 Kb while the insert franked by the CaMV35S promoter and Nos terminator has a total size of 2.0 Kb. This vector also has a bacterial and plant selectable marker namely Kanamycin resistance. This plasmid pRGRgp was obtained from Dr. C.S. Prakash of Tuskegee University, USA.

Plate 1 : pRGRgp construct

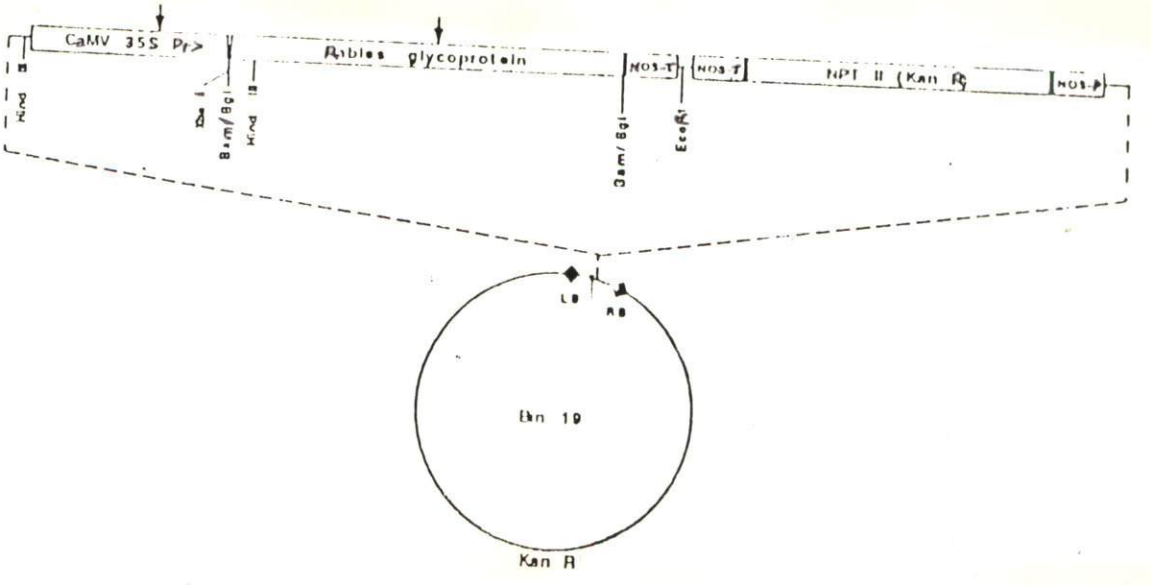
CaMV 35S Pr
Rabies glycoprotein
NOS-T
NOS-T
NPT II (Kan R)
NOS-P
Hind III
Bam/Bgl
Hind III
Bam/Bgl
EcoRI
LB
RB
Ban 19
Kan R

Plate 1

The present study was done using the pRGRgp vector to transform muskmelon variety Arka Jeet. The work was carried out at the Molecular Diagnostics Laboratory, Division of Biotechnology, IIHR, and Hessarghatta, Bangalore during 1997-98. The materials and methods used for conducting experiments during the course of investigation are presented in this chapter.

Preparation and Transformation of Competent Cells of *E. coli* strain DH5α

This is a procedure for transforming bacterial cells with circular DNA plasmids containing an origin of replication and a selectable marker (antibiotic resistance). Bacterial cells that have successfully taken up the plasmid DNA will form discrete bacterial colonies, which are subsequently selected on a plate containing the required antibiotic.

The bacterial cell used for the transformation in this experiment is the strain DH5α which is recombination deficient. The bacteria were transformed with pRGRgp. All the reagents and chemicals used were of analytical grade and procured from standard companies.

MATERIALS

1. 500ml screw-cap conical flasks.
2. 500ml screw-cap polypropylene tubes.
3. Micropipeter and autoclaved microtips.

4. Sterile microfuge tubes.
5. LB-liquid medium-sterile.
6. LB-agar plates (1.5% agar)
7. LB-agar plates with Kanamycin (50mg / ml)
8. Stock plate of bacterial colonies of DH5(
9. Vector DNA-pRGRgp.
10. Sterile and cold calcium chloride solution (0.1M)
11. Ice.

EQUIPMENT

1. Ultracentrifuge.
2. Microfuge.
3. Laminar flow hood.
4. Rotary shaker.
5. Incubator.

Cleaning and Sterilisation of Materials and Reagents

All the steps in the experiment were conducted under aseptic conditions in the laminar flow cabinet. So before using, the laminar flow chamber was exposed to UV-light for 10-15 min. Before working under the hood, the working surface was sterilised by swabbing with 70% ethanol. The walls of the chamber were also sprayed with 70% ethanol to ensure total sterility, Any material coming from

outside were also sprayed with alcohol. In case of glassware, the mouth of the bottles, etc. were flamed before and after use. Also before starting the experiments the hands were sprayed well with ethanol.

Experimental Details :

Preparation of LB-medium

For liquid medium Bactotryptone	10g/L
Yeast extract	5g/L
NaCl	10g/L

All the three chemicals were taken in 800ml double distilled water and mixed well on a magnetic stirrer. The pH of the medium was adjusted to 7.5 using NaOH (1N) and the volume was made up to 1L using double distilled water and then autoclaved.

For preparing LB-agar medium, to the above liquid medium agar powder was added @15g/L after adjusting the pH, autoclaved and poured into sterile petri plates under the laminar hood.

For preparing LB-agar medium with kanamycin, the above medium was cooled after autoclaving. Filter sterilized kanamycin was added at a final concentration of 50μg / ml mixed well and poured into sterile petriplates under the hood. (The required amount of Kanamycin was added from a stock solution of 50mg/ml)

Preparation of Competent Cells of DH5α

PROCEDURE

1. One day in advance; 1ml culture of *E coli* strain DH5α was grown overnight in LB-liquid medium at 37^0C
2. The following day; using this culture; a 20ml culture was grown in LB-medium for 2-3 hours on a rotary shaker. During this time, the bacterial cells would have multiplied and reached the log phase where the cells would be nascent with an OD of 0.5 at 600nmand their concentration approximately equal to 2-5x10 cells/ml.
3. The culture was then transferred aseptically into sterile 50ml screw-cap polypropylene tubes.
4. Cells were spun in an high speed centrifuge at 7000 rpm for 7minutes at 4^0C.
5. The cell pellet was resuspended in 1/2 volume (10ml) sterile and cold 0.1 M calcium chloride solution by gentle shaking and iced for 20 minutes. This treatment caused the cells to swell and the Ca^{2+} ions bind to the surface of the cells.
6. Cells were harvested by centrifuging at 7000 rpm for 7 minutes at 4 °C.
7. The pellet was resuspended in 1/10 volume (2ml) sterile and cold Calcium Chloride (0.1 M) solution and iced for 20 minutes. The cells were then ready to take up DNA and were said to be competent.
8. To test the viability of the competent cells, they were streaked on a LB-agar plate, sealed with parafilm, labelled and incubated overnight at 37^0C.

9. The cells at the point were dispensed into 100ul aliquots in sterile microfuge tubes and stored at 4^0C until further use.

Transformation of fresh Competent cells

100 µl of the fresh competent cells were taken in a sterile microfuge tube and 1µl of plasmid DNA pRGRgp (2µg/µl concentration) was added to the tube and iced for 30 minutes.

After 30min, the cells were heat shocked at 42°C for 2 minutes in a water-bath and immediately quenched on ice. This treatment facilitates the entry of DNA into the cells. 900 µl of sterile LB-broth was added to this and incubated at 37^0C for 60-90 min. with gentle shaking for multiplication of the cells and expression of the Kanamycin R gene.

It was then microfuged at 5000 rpm for 2 min. and the resulting pellet was spread on a LB-agar plate containing Kanamycin 50 µg/ml final concentration) and sealed with parafilm, labelled and incubated overnight at 37°C.

The control tube was treated in an identical manner except that, instead of DNA, 1 µl T.E. buffer was added. From this a 10^{-6} dilution was made (10µl in 990 µl of sterile water, done thrice: 10^{-2},10^{-4}, 10^{-6}) and 10µl of the final dilution was spread on a LB-agar plate, sealed with parafilm, labelled and incubated overnight at 37 ^{0}C. This was used to estimate the total number of cells in the reaction mixture.

The following day the colonies were counted using a colony counter. The frequency of transformation in terms of number transformants (that is Kanamycin resistant colonies) to the total number of cells in the reaction mixture (that is the colonies growing on the control plate without Kanamyacin) was calculated.

RAPID ISOLATION OF PLASMID DNA (QUICK Mini-prep)

This procedure involves a quick method of preparing partially purified plasmid DNA in a small quantity from a number of transformants in order to verify the presence of the plasmid in the transformed cells and also to obtain an approximate estimation of the size of the plasmid in the transformed bacterial cells. It involves the lysing of the bacterial cells in Tris-EDTA solution and subsequent precipitation of proteins with phenol: chloroform (1:1). In most instances, the contaminating chemicals, proteins and chromosomal DNA can be reduced to a level that allows the plasmid DNA to be easily visualised by gel electrophoresis.

MATERIALS

1. Sterile microfuge tubes.
2. Micropipeter and autoclaved microtips.
3. Vortex mixer.
4. Microfuge.

5. Laminar flow chamber.
6. Inoculation needle or sterile toothpick.
7. Bacterial culture plate harbouring the desired plasmid DNA.
8. STE-buffer NaC1(100mM) ; Tris. HC1(pH 8.0; 10mM); EDTA (pH 8.0; 1mM).
9. Phenol : Chloroform (1:1) mix. (Chloroform was prepared by mixing chloroform : isoamyl alcohol: 24:1).

PROCEDURE

A single transformed colony was picked up aseptically using a sterile inoculation needle and was grown overnight in 1ml LB-broth containing Kanamycin 50 μg/μl in a sterile microfuge tube. The culture was microfuged at 5000 rpm for 2 minutes. The supernatant was poured off and the cell pellet was resuspended in 40 μl STE buffer and vortexed. In the absence of an overnight grown culture, a single colony from the original transformation plate was taken into a sterile microfuge tube and resuspended in 40 μl STE-buffer using a vortex mixer. This helps in lysing the cell. 40 μl of phenol: chloroform (1:1) mix was added and the contents were mixed well using a vortex mixer to precipitate the proteins. The contents were then microfuged at 5000 rpm for 2 minutes. The top aqueous phase was collected and electrophoresed for confirming the presence of the plasmid by gel electrophoresis with a molecular weight marker.

AGAROSE GEL ELECTROPHORESIS OF PLASMID DNA

Electrophoresis involves the movement of charged molecules in an electric field. DNA molecules carry a net negative charge and therefore when placed in an electrical field they will migrate towards the positive pole. Electrophoresis of DNA is usually carried out in a gel made of agarose or polyacrylamide or a mixture of the two. In a gel, the migration rate of a macromolecule is influenced by its shape and size. Depending on the size of the DNA fragment to be separated, the concentration of the agarose varies. Smaller the DNA fragment, higher is the concentration of the agarose used in the gel.

Agarose gel electrophoresis is not only used to resolve DNA fragments of different lengths but also to separate different forms of DNA such as the supercoiled or covalently closed circular (ccc) DNA (form I), the nicked or relaxed DNA (form II), and the linear DNA (form III) molecules. The compact CCC DNA will migrate faster than the open circular forms. For a particular form of DNA, the migration rate in the gel is inversely proportional to the logarithm of the molecular weight of the DNA molecules. The size of the fragment is determined by electrophoresing simultaneously DNA fragments of known size such as Hind III digest lambda-DNA. However, the rate of migration differs depending on the amount of nicking and supercoiling. Hence, it is only the linear form of DNA, which can be compared with the molecular weight marker. To confirm the correct size of the plasmid, the DNA should be linearised by restriction enzyme digestion.

The DNA is visualised by using an intercalating dye, such as ethidium bromide, which fluoresces orange when irradiated with UV-light at 230-280nm.

MATERIALS

1. TBE buffer (5x) stock: Tris base (54g/L); Boric acid (27.5g/L); EDTA (0.5M, pH 8.0) 20ml/L. The pH of the buffer was adjusted to 8.0.
2. Loading buffer 0.25% Bromophenol blue + 30% glycerol.
3. 0.7 % Agarose gel (horizontal).
4. Gel frames and comb (with a teeth size of 5mm and spaced at a distance of 3mm)
5. Ethidium bromide (0.5 μg/ml final concentration).
6. UV-transilluminator (230-280nm).

PROCEDURE

The frame of the gel casting unit was cleaned, dried and sealed with a tape to form a mould. 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 2mm above the surface.

Agarose powder was added to TBE buffer (1x) and was dissolved by melting at 100 ^{0}C. The solution was cooled to 50 ^{0}C, 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 TBE buffer just enough to cover the surface of the gel.

DNA samples were mixed with 5 µl of loading dye, bromophenol blue and loaded in the wells of the submerged gel using a micropipeter. 10 µl of DNA marker was also mixed with bromphenol blue and loaded onto the of the wells.

About 100 µl of ethidium bromide (0.5 µg/ml final concentration) was added to the gel tank.

The electrophoresis apparatus was connected to the power supply and electrophoresis was carried out at 80 V for 4 hours or when the deep blue dye (bromophenol blue) had migrated to end of the gel. It was then visualized on an UV-transilluminator.

Calculation of RF-values.

The size of the plasmid construct was estimated by calculation the RF-values. This was done by measuring the distance travelled by the DNA fragments of the molecular weight marker, from the wells. The distance travelled (mm) by the DNA fragments was plotted on a linear scale on the X-axis and the known molecular weight of the -DNA fragments (kb) were plotted on the Y-axis on the log scale. The point, at which the plasmid DNA fragments cut the curve of the -DNA, gives the size of the plasmid of interest.

LARGE-SCALE ISOLATION OF PLASMID DNA (by alkali lysis method)

After the initial confirmation of the plasmid construct, a single bacterial colony that contained the desired construct was selected, for inoculation in large quantities.

This method involves treating the cells with glucose- Tris. HCl-EDTA mixture that serves as an osmoticum and helps to keep the cells in suspension. Lysis of the cell is completed by the addition of SDS, a detergent. The high salt concentration also results in the precipitation of SDS-protein complexes. Most of the proteins along with much of the SDS added can therefore be removed by subsequent centrifugation. On neutralisation with potassium acetate, the acetate ions precipitate the proteins which are removed by centrifugation. The intact plasmids which are circular molecules (ccc) remain in the solution. On centrifugation at a high speed, the chromosomal DNA and most of the proteins are pelleted. The supernatant containing the plasmid DNA is precipitated in isopropanol

MATERIALS

1. Fresh culture of *E. coli* strain DH5α harbouring pRGRgp in LB-liquid medium containing kanamycin (50 μg/ml final concentration).
2. Sterile 50 ml polypropylene tubes with caps
3. Micropipeter and autoclaved microtips.

4. Vortex.

5. High speed cold centrifuge.

6. Incubator oven.

7. Autoclaved microfuge tubes.

8. Vacuum drying apparatus.

9. Solution-I , 25mM Tris-HCL; pH 8.0 ; 50 mM Glucose ; 10 mM EDTA

10.Solution-II 0.2M NaOH SDS 1 %

11.Solution-III 5M potassium acetate + 3M Acetic acid

(5 M with respect to potassium and 3M with respect to acetate) pH 8.0.

12. Isopropanol.

13. 70 % Ethanol.

14. Ice.

PROCEDURE

Bacteria containing the plasmid was grown overnight in a 500ml conical flask containing LB-medium and appropriate antibiotic on a rotary shaker at 210 rpm at 37^0C. The culture was taken in 50ml sterile centrifuge tubes and the tubes were balanced. Before harvesting the cells, 1ml culture from this was also taken in a microfuge tube and quick mini isolation was done to check whether the bacteria grown contain the right plasmid. The cells were then centrifuged at 4000g for 15minutes at 4^0C. The supernatant was removed and the cell pellet was thoroughly resuspended in 4 ml solution-I by vortexing and was incubated at 37^0C

for 10-15minutes in an incubator. 8 ml of solution-II were added and the contents were mixed gently and iced for 10-15 minutes or more till all the cells had completely lysed. 4 ml of solution-III was added, mixed gently and were iced for 10-15minutes for neutralisation as well as precipitation of protein and high molecular weight chromosomal DNA. The contents were centrifuged at 20,000 rpm for 20 minutes at 4^0C. The supernatant was taken into a fresh tube and 0.6 volumes of isopropanol were added, mixed well and as incubated overnight at 4^0C. The contents were then centrifuged at 12,000g for 30 minutes at room temperature (27^0C). The pellet was recovered and washed with 70 % ethanol. The ethanol was recovered and the pellet was dried in a vacuum desiccator. The dried pellet was then dissolved in T.E. buffer (minimum volume) and stored at 4^0 C until further use.

PURIFICATION OF PLASMID DNA

The plasmid DNA obtained from large-scale preparation still contains some contaminating proteins and RNA and is not sufficiently clean for restriction digestion, southern blot analysis, etc. RNA was first removed by treating the plasmid with RNase and then further purification to remove the proteins was carried out by one of the two methods.

Cleaning with phenol : Chloroform.

or

Cleaning with polyethylene glycol (PEG)

Removal of RNA from plasmid DNA preparations

1. Pancreatic RNaseA (RNaseA) at a concentration of 10mg/ml was dissolved in 10 mM Tris.Cl (pH 7.5) and 15 mM NaCl.
2. The contents were heated to 100 ^{0}C for 15minutes in a water-bath to denature the DNAase and were then allowed to cool slowly to room temperature
3. 1 µl of this was added to the plasmid preparation and was incubated at 37 ^{0}C for 1-2 hours.

The digested RNA gets subsequently removed during the removal of proteins.

3.5.2 Phenol : Chloroform Cleaning of Plasmid DNA

In this method, phenol precipitates the proteins while the plasmid DNA remains in the aqueous phase. Chloroform helps in denaturing the proteins and also removes traces of phenol. While the plasmid DNA remains in the aqueous phase. Chloroform is prepared by mixing chloroform and isomyl alcohol in 24:1 proportions (v/v). Isoamyl alcohol helps in reducing foaming during extraction and facilitates separation of the aqueous phase and the organic phase. The DNA is subsequently precipitated by ethanol.

MATERIAL

1. Buffered phenol,
2. Chloroform mix (chloroform: isoamyl :: 24:1)

3. 3M Na.acetate (pH5.2)
4. ethanol.
5. IX T.E buffer (pH 8.0)
6. Microfuge.
7. Sterile Microfuge tubes.
8. Vortex.
9. Vacuum desicator.
10. Plasmid DNA preparation to be purified.

PROCEDURE

To the Plasmid preparation, an equal volume of buffered phenol was added and vortexed well for 2 mins. This was centrifuged at 1600g for 3minuted at room temperature. Using a 1ml micropipeter, the upper aqueous phase was taken into another fresh tube and to this an equal volume of chloroform mix was added and mixed well by vortexing for 2 mins. The contents were microfuged at 1600g for 3minutes at room temperature. The upper aqueous phase was taken into a fresh tube and to this 1/10th volume of 3M Na. Acetate and two volumes of 100 % ethanol were added, mixed well and incubated at 4 ^{0}C for 4-5hours. It was then centrifuged at 10,000 g for 20 minutes at 4 ^{0}C.The ethanol were poured off and the last traces of ethanol were removed by vacuum desication. The dried plasmid pellet was resuspended in T.E buffer (minimum volume) and stored at 4^{0}C.

Polythylene Glycol (PEG) Method of Plasmid Purification

PEG preferentially dissolves proteins and precipitates the Plasmid DNA. The DNA is subsequently isolated by centrifuging and extracted by ethanol precipitation.

MATERIAL

1. PEG solution autoclaved and stored at 4^0C (30 % PEG mol.wt.6000 ; 13 % NaCl)
2. IX T.E buffer (pH 8.0)
3. 3 M Na. Acetate (pH 5.2).
4. 100 % ethanol.
5. Microfuge.
6. Plasmid DNA from large-scale preparation.
7. Vortex.
8. Sterile microfuge tubes.

PROCEDURE

0.8 ml PEG solution was added to the plasmid preparation (in approximately 2 ml T.E buffer) and mixed well. The contents were incubated at 0 ^{0}C for 1-15hours. Centrifuged at 10,000g for 20minutes at 4 ^{0}C. The pellet was taken and resuspended in 1ml T.E buffer. To this, 1/10 th volume Sodium Acetate (3M) and two volumes 100 % ethanol was added, mixed well by vortexing and

was incubated at 4 ^{0}C for 2-15 hours. The contents were centrifuged at 10,000g for 20minutes at 4 ^{0}C. Ethanol was drained off and the trace of ethanol was removed by vacuum desiccation. The dried pellet was dissolved in T.E buffer minimum volume and stored a microfuge tube at 4 ^{0}C.

Spectrophotometric Assay of plasmid DNA Concentration

It is crucial to know exactly how much DNA is present in the solution before carrying out electroporation experiments. DNA concentration can be accurately measure by ultraviolet absorbance by a solution of DNA. It is directly proportional to the amount of DNA present in the sample. Usually the absorbance is measured at wavelengths 260 and 28mm. The reading at 260 nm allows the calculation of DNA concentration in the sample. An O.D of 1 at 260 nm corresponds to 50ug/ml of double-stranded DNA.

UV-absorbance can also be used, to check the purify of the DNA preparation. With a pure sample of DNA, the ratio of the absorbance at 260 nm and 280 nm OD/OD is 1.8. Ratios less than 1.8 indicate that the preparation is contaminated either with phenol or proteins. Values higher than this indicates the presence of RNA in the preparation.

MATERIAL

1. UV-Spectrophotometer (Shimadzu-UV 160A)

2. 1ml quartz curettes (1pair).

3. IX T.E buffer (pH 8.0)

4. Plasmid DNA samples to be tested.

5. Micropipeter and autoclaved microtips.

PROCEDURE

The spectrophotometer was first turned on for 20 minutes for the equipment to stabilise. 1ml T.E. buffer was taken the quartz cuvette and placed in the reference slot as the reference sample and the equipment was autozeroed. 10 μl of the plasmid sample was taken in 990 μl of T.E. buffer (1X) in the other cuvette and mixed well. This was placed in the main reading slot and the absorbance at 260 nm and 280 nm wavelength were measured.

Three readings were taken and the average was calculated. Using the relationship, 1 O.D. at A 260 = 50 μg/ul was calculated. The ratio A 260 / A280 was also calculated to check the purity of the sample.

Restriction Enzyme Digestion of Plasmid DNA

This experiment was conducted to verify the presence of the Plasmid construct by linearizing it and also to check the presence of an intact Rabies glycoprotein in the plasmid being used. The pRGRgp plasmid has the rabies glycoprotein gene with CaMV35S promoter and Nos terminator. There is a Hae II site just downstream of the promoter and a unique Eco RI site just downstream of

the terminator. By carrying out a double cut with Eco RI and Hae II the rabies glycoprotein gene with its promoter and terminator gets released. The size of released fragment is about 2 kb while the size of the whole plasmid is 12.5 kb.

The restriction enzymes used in the experiments were of prescribed grade and form and were procured from standard companies (Sigma / Bangalore Genei).

MATERIALS

1. Sterile double distilled waster.
2. Micropipeter and autoclaved microtips.
3. Plasmid DNA.
4. Restriction enzymes Hae II and Eco RI.with their respective assay buffers.
5. EDTA 0.5M(pH 7.5).
6. Ice.
7. Sterile microfuge tubes.
8. Vortex.
9. Microfuge.
10. Incubator 37^0C.

PROCEDURE

20 µl (tube 1) and 10µl (tube 2) of plasmid DNA (concentration approximately 2 µg/ml) was taken in two separate microfuge tubes. 15 µl and 7 µl of sterile double distilled water was added to give a final volume of 40 µl and 20

µl respectively. 4µl and 2µl of the appropriate 10X enzyme assay buffer (Buffer C ad Buffer B) was added to tube 1 and tube 2 respectively. Tube 1 was treated with 1 µl of Hae II and tube 2 with 1 µl of Eco RI enzymes respectively. The contents were mixed well by vortexing, spun in a microfuge and incubated at 37^0C for 2 hours.

After 2 hours, 20 µl of the Hae II digested sample from tube I was aliquoted into a fresh sterile microfuge tube for further cutting with the second enzyme Eco RI to make a double cut in order to release the rabies glycoprotein gene.

To the remaining 20 µl of the Hae II digested sample from tube 1 and 20 µl of Eco RI digested sample of tube 2 were treated with 0.5 M EDTA (pH 7.5) to get a final concentration of 10 mM, to stop the reaction.

To the fresh tube containing 20 µl of Hae II digested sample, 6 µl of sterile double distilled water, 3 µl of the appropriate enzyme assay buffer (Buffer B 10X concentration) and 1 µl of the enzyme Eco RI was added, mixed well by vortexing, microfuged and incubated at 37 ^{0}C for 2 hours. The reaction was stopped by the addition of EDTA 0.5 M to get a final concentration of 10 mM.

The uncut and cut plasmid DNA samples were analysed by agarose gel electrophoresis along with the molecular weight marker, DNA Hae II digest as described in the earlier experiment, and visualised in a UV-transilluminator.

Electroelution of the Rabies Glycoprotein Gene

This experiment was conducted to recover the Rabies glycoprotein gene from the gel for labelling and probe preparation.

Experimental Details

DNA was electro-eluted from the gel. For this purpose the dialysis bag had to be prepared.

Preparation of dialysis tubing

The dialysis tube was cut into a convenient length (10 cms) and boiled in large volumes of 2 % sodium bicarbonate and 1mM EDTA (pH 8), for 10min. The tube was rinsed thoroughly in double distilled water and boiled again in 1mM EDTA (pH 8) for 10min. It was then cooled and stored at 4 ^{0}C submerged in 1 mM EDTA. Before using, the tubing was washed in double distilled water. The tube was always handled with gloves.

MATERIAL

1. UV Trainsilluminator
2. Scalpel.
3. Treated Dialysis tube and Clips.
4. IX TBE buffers (pH 8.0).

5. Gel electrophoresis apparatus.

6. Sterile microfuge tubes.

7. Micropipeter and autoclaved microtips.

8. Agarose gel containing the DNA fragment.

PROCEDURE

The 2 Kb double digested rabies glycoprotein fragment was excised from the agarose gel using a clean sharp scalpel, under the UV-transilluminator. One end of the treated dialysis tube was folded and clipped, and the tube was filled with TBE buffer. The gel slice containing the DNA fragment was transferred into the dialysis tube and the gel piece was allowed to sink to the bottom of the tube. Most of the buffer was removed from the bag leaving only 1ml of the TBE buffer in the dialysis tube. The other end of the tube was also folded and clipped just above the gel. Care was taken to see that no air bubbles were present in the bag during clipping. The bag was immersed in TBE buffer in the electrophoresis tank such that the bag was submerged completely and was above the base of the gel tank. It was then electrophoresed at 40V for 2-3 hours. During this time, the DNA is electro-eluted out of the gel into the inner wall of the dialysis tube and into the buffer in the bag. The polarity was the reversed for 1 minute in order to release the DNA from the walls of the dialysis bag. The bag was opened and the buffer removed into a clean and sterile microfuge tube. The gel slice was stained with

ethidium bromide for 15 minutes and examined under the UV-light to check if all the DNA had been eluted.

Dig Labeling of DNA

Nucleic acid molecules are labelled by covalent attachment of digoxigenin-label (DIG) to dUTP. This is then incorporated into the complementary polynucleotide chain. The steroid haptan digoxigenin (DIG), is linked via a spacer arm, consisting of 11-16 carbon atoms to the dUTP. DNA fragments are labelled by random-primed incorporation of DIG-labelled deoxyuridine triphosphate (dUTP). DIG-labelled DNA probes are detected after hybridization to target nucleic acids, by enzyme linked immuno-assay using an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate Anti-DIG-AP) and then visualised with the chemiluminiscent substrates CSPD or CDP*. The DIG nucleic acid labelling and detection system allows the detection of 0.1 g of homologous DNA or RNA.

In this experiment, the labelled probe was the electro-eluted Rabies glycoprotein gene fragment. Labelling was done by random-priming, which allows efficient labelling of small (10 ng) to large (3 μg) amounts of DNA per reaction mixture.

MATERIALS

1. Sterile microfuge tubes
2. Micropipeter and autoclaved microtips
3. Ice
4. Microfuge.
5. 37 ^{0}C incubator.
6. Template DNA
7. Hexanuleotide mixture (10X).
8. dNTP (210X) labelling mixture (1mM dATP; 1mM ; dCTP;1mM TP;0.65mM ; dTTP; 0.35mM DIG-11-UTP)
9. Klenow enzyme.
10. Double distilled water.

PROCEDURE

20 µl of eluted DNA was taken in a sterile microfuge tubes and heat denatured by boiling in hot-water bath for 10 minutes and immediately quenched in ice for at least 30 seconds. The contents were then spun in a microfuge at 5000 rpm for 1 min. and again placed on ice.

The following reagents were added to the denatured template DNA (on ice) in the following order to a final volume of 30 µl.

Double distilled water	5 µl

Hexanucleotide mixture (10X)	2 µl
DNTP mixture (10X)	2 µl
Klenow enzyme (2 units/µl)	1 µl

The tubes were incubated at 37 ^{0}C for 18-20 hours for the labelling to take place and were later stored at 4 ^{0}C.

***In vivo* Electroporation of Nodal Buds of Muskmelon**

The present investigation was done to study the uptake and stable integration of the rabies glycoprotein gene in the plant genome to generate transgenic plants, which might be used as edible vaccine. The pRGRgp plasmid harbouring the rabies glycoprotein gene with spermine as the DNA protectant was used under different electroporation conditions.

The experiment was carried out by sowing muskmelon seed variety Arka Jeet in polythene bags filled with soil. The soil was treated with furadon prior to sowing. About 30 days after sowing the plants were electroporated. About 10-20 days after electroporation the plants with surviving growing vegetative shoots were transplanted into large crates in the glasshouse. The soil was pre-treated with furadon. The plants were irrigated once in 2 to 3 days. The crop was sprayed with neem seed extract to ward off leaf miners. Periodic sprays of redomil @ 2g/L and kerathane @ 2 g/L were given to bring under control downy mildew and powdery mildew diseases respectively

MATERIALS

1. Muskmelon plants variety Arka Jeet raised in polythene bags.
2. Hamilton syringe (25 μl)
3. Plasmid DNA (200 μg) complexes with either Spermine (2 mM)
4. Sterile MS-salt solution.
5. Electroporator with circular electrode with 1cm gap between the two electrodes.
6. DC power supply (0-500 V and 0-100 mA).
7. Labels and pencils.

PROCEDURE

One day prior to electroporation, the apical portion of the plant was removed, only a single nodal bud was retained and all the other buds on the plant were removed in order to force this single bud to grow. The following day, the electroporation buffer was prepared by complexing 200 μg/ml of plasmid DNA pRGRgp with a DNA protectant, Spermine (2mM final concentration) for 20 minutes in sterile microfuge tubes. The volume was then made upto 1ml using sterile MS-salt solution (without sugar and having a pH of 4.33). Using a Hamilton syringe, a drop of the DNA solution was deposited close to the meristematic dome of the bud. The buds were then dipped in 2ml-electroporation buffer taken in the circular electrode and electroporated at a specific voltage for duration of 99 msec. The buds were subjected to 5 pulses, given at a interval of 3 seconds.

Plate 2 : Electroporator with Musk melon plant being electroporated

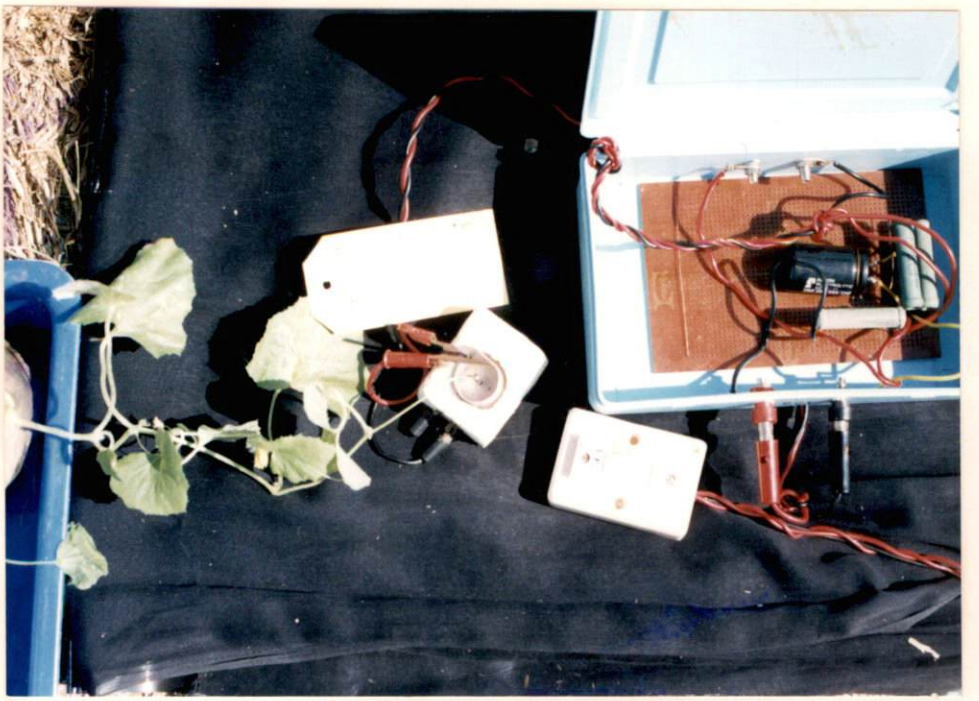

Plate 2

The treatments given were 5 pulses at 100 V, 200 V, 300 V and 400 V. The electroplated buds on the plant were allowed to grow, bear flowers and set fruits. Any new vegetative growth coming on the plant below the bud was removed periodically to allow only treated bud to grow. These plants will hence forth be referred to as R0 plants.

Control plants were not electroporated. Periodical observations were also recorded to see if there was any difference in the morphology and plant behaviour of the plant growing from the electroporated bud and the untreated plants. The fruits borne on the stem/branches growing from the electroporated bud were harvested and the seeds were collected. The fruit size, weight and the number of seeds obtained from each fruit were also recorded. The seeds from the R0 and control plants were sown in the playhouse to raise the R1 progeny. The crop was raised and maintained as was done for the R0 plants. Putative transformants were confirmed by doing a dot blot analysis.

CTAB Method of Isolation of Total Plant Genomic DNA of Muskmelon

This is an efficient method for isolating plant genomic DNA from leaf tissues. It provides high quality preparation of high molecular DNA. CTAB was used to liberate the nucleic acid from the cell, which is further purified by phenol: chloroform to remove proteins and other contaminating plant debris. The outline of the experiment is presented below.

MATERIALS

1. Extraction buffer 100mM Tris.C1 (pH 8.0) 2 % C-TAB, 1.4 M NaC1, 20mM EDTA, 0.1% Mercaptoethanol
2. Chloroform: Isoamyl mix (24:1 v/v)
3. T.E. buffer 10mM Tris, pH 8.0, 1mM EDTA
4. 70% Ethanol
5. Pestle and mortar
6. 50 ml screw-cap polypropylene tubes autoclaved
7. Vortex
8. Waterbath
9. Ultra-centrifuge and Microfuge
10. Microfuge tubes
11. Vacuum desicator
12. Micropipeter and autoclaved microtips
13. Leaf tissue from R1 plants
14. Balance
15. Rotary shaker

PROCEDURE

The leaf tissue was washed in water and the excess water was wiped with blotting paper and air-dried briefly. 5g of leaf tissue were weighed from each R1 generation plant and control plant. The leaves from individual plants were cut into

pieces and ground well using mortar and pestle in equal volume (15 ml) of hot (65 ^{0}C) extraction buffer. The extract was poured into 50 ml polypropylene tubes and was incubated at 65 ^{0}C in a hot water bath for 15-20 min. With gentle shaking. Equal volume (15 ml) of chloroform-Isoamyl mixture was added and was vortexed thoroughly for 15-20min. The contents were centrifuged at 11,000 rpm for 10 min. The supernatant was taken and to these 2.5 volumes of Absolute alcohol was added mixed and kept at 0 ^{0}C overnight. The contents were centrifuged at 5000 rpm for 10 minutes. The pellet was dried. The pellet was dissolved in high salt T.E. buffer, minimum volume. The DNA was purified by Phenol : chloroform cleaning as described in the earlier experiments and further recovered by ethanol precipitation. The resulting DNA pellet was dissolved in T.E. buffer (minimum volume) and stored at 4 ^{0}C.

Dot Blot Analysis

This method provides a rapid screen to identify plant samples carrying the desired DNA. A drop of DNA is spotted onto a nylon membrane, which is then hybridized with a labelled probe to test for the presence of the DNA of interest.

MATERIALS

1. Dot blot apparatus connected to a vacuum.
2. Positively charged nylon membrane of 0.45 µm mesh (Boehringer and Mannheim).

3. Micropipeter and autoclaved microtips.
4. Plant DNA
5. Bromophenol blue
6. Reagents 2M NaOH, 2M Ammonium acetate

PROCEDURE

The nylon membrane was cut to a size so as to fit the dot blot apparatus. The membrane was wetted first in double distilled water and then in 2X SSC briefly for a few minutes and air-dried. The dried membrane was placed in the dot blot unit, fastened tightly and the unit was connected to the vacuum. 8 µl of NaOH was taken in each of the wells in the 96-well plates. 32 µl of plant DNA sample was added and mixed well with Na0H and kept for denaturation for 15-20 minutes. 8 µl of 2M Ammonium acetate was added to each dwell mixed and incubated for 5 minutes for neutralization. 2 µl of Bromophenol blue were mixed with the sample and loaded on to the wells. Positive and negative controls were maintained by loading plasmid DNA pRGRgp and the total DNA from control plants respectively. After all the samples were loaded, the vacuum unit was turned-off. The spotted nylon membrane was air-dried and used for hybridization.

Dot Blot Hybridization

This method identifies homologous DNA fragments bound to a filter by hybridization with a labelled probe. The base pairing between the labelled probe

and DNA is governed by complementary hydrogen bonding of nucleic acids. The probe used in the hybridization procedure is the DIG-labelled rabies glycoprotein gene. The plant DNA spotted on a dot blot membrane

MATERIALS

1. Dot blot membranes.
2. Incubator oven 68 ^{0}C.
3. Plastic bags.
4. Sealer
5. Ho-.water bath.
6. Micropipeter and autoclaved microtips
7. Sterile microfuge tubes.
8. Heater, microfuge.
9. Ice.
10. Plastic containers and plastic forceps.
11. X-ray cassette and X-ray film, (Kodak Diagnostic films-X-Omat).

REAGENTS

1. 6X SSC.
2. Pre-hybridization and hybridization solution, 5X SSC, 0.1% N-lauryl sarcosine 0.02% SDS, 1% blocking reagent

3. DIG-labelled probe Rabies glycoprotein gene probe.

4. Wash solution-I 2X SSC, 0.1%SDS

5. Wash solution-II 0.1X SSC, 0.1% SDS

6. Buffer I 0.1M maleic acid, 0.15M NaCl (pH 7.0)

7. Buffer II Buffer 1, 0.3% Tween 20

8. Detection buffer 0.1M Tris, 0.1M NaCl, 50mM MgCl2 (pH 9.5)

9. CSPD (1: 100 dilution prepared in detection buffer) or CDP* (1: 6000 dilution).

10. DIG-Antibodies (1:10,000 dilution in buffer 1).

11. 1 % blocking reagent (prepared in buffer 1)

12. Developer, Fixer and 5% acetic acid.

13. Double distilled water.

PROCEDURE

The baked membrane were wetted and submerged in 6X SSC for 2 minutes. The membranes were then slipped into a heat-sealable plastic bag and pre-hybridization solution @ 20ml/100 sq.cm was added. The bag was sealed after squeezing as much air as possible. The bag was incubated at 68 ^{0}C for 2-7 hrs. It was then cut open at one corner and the pre-hybridization solution was squeezed out. Hybridization solution was added to the same bag @ 2.5 ml/100 Sq.cm of the membrane. The probe (1µl) dissolved in 9 µl T.E buffer was boiled for 10, min in hot water and quenched on ice for at least 30 sec. It was then spun down in a microfuge and added to the hybridization bag. The cut end of the bag was resealed

after removing all the air bubbles. The bag was incubated at 68 ^{0}C for 18-20 hrs (overnight) for hybridization. After hybridization, the bag was cut open and the hybridization solution was squeezed out and stored in a vial for future use. The membrane was then taken out quickly and submerged immediately in wash solution I (2x SSC + 0.1% SDS). The membrane was washed twice, by shaking gently for 10 min. each, at room temperature. The membrane was then washed twice, for 10 min. in wash solution-II in a 60 ^{0}C hot water bath where the temperature of the wash solution II was also maintained at 60 ^{0}C. Washing was done under slightly higher stringency, as there was a 1:1 homology between the probe and the plasmid DNA used in the Experiment. These two washing steps helps in the removal of unbound probe and prevents non-specific binding of the probe. The membrane was equilibrated in Buffer-2 for 5 min. Membrane was taken in heat sealable plastic bag with 20 ml of 1 % blocking solution and the bag was sealed. Blocking was done at room temperature for 1 hr with gentle shaking.

2 µl of the diluted DIG antibody was added to the same bag resealed and incubated at room temperature for 30 min. The membrane was washed in buffer II, twice for 20 min. at room temperature with gentle shaking. It was equilibrated in Detection buffer for 1-5 min. The membrane was then taken in the X-ray cassette between two sheets of acetate and about 100 µl (0.5 ml/100 Sq.cm) of diluted CSPD or CDP* was added on the membrane and spread evenly. It was incubated for 15-20 min at 37 ^{0}C or overnight at room temperature for the enzymatic reaction to stabilise. During this period the alkaline phosphatase

enzyme present on the antibody bound to the DIG-labelled probe reacted with substrate and resulted in the emission of light at a wavelength of 477 nm. So, at all those places where the probe has bound to the complementary DNA strand on the membrane, light is emitted which is recorded on X-ray film. A x-ray film was placed over the membrane in a dark room and the cassette was closed tightly. The film was exposed for till optimum intensity of the signal was obtained. The film was then developed in the dark room in the developer for 2 min., rinsed in 5 % acetic acid for 1-2 min. and then fixed in the fixer for 2-3 min. After fixing, it was washed thoroughly in doubly distilled water for 10-15 min. and air-dried. At regions where there is emission of light (from the membrane) the signals were recorded on the x-ray film as dark dots in case of dot blot analysis.

Deprobing or Removal of Probe from Nylon Membranes

This procedure involves the removal of the labelled probe bound to the nylon membrane, by using an alkaline solution. The DIG-label is denatured under alkaline condition. Further the bound probe is stripped off the DNA which is bound to the membrane. The stripped membranes can be to reprove with the same or a different probe.

MATERIALS

1. Sterile water.

2. Deprobing solution NaOH (0.2 M), SDS 0.1% solution,
3. 2X SSC
4. Plastic containers.

PROCEDURE

1. The nylon membranes (dot blot) were briefly rinsed in sterile water to remove the CSPD or CDP*.
2. It was then washed with several volumes of Deprobing solution, at room temperature for 5 Minutes. This step was repeated once more.
3. It was then washed with 2X SSC for 5 min., air dried and stored in Aluminium foils at room temperature. They were reprobed when necessary.

EXPERIMENTAL RESULTS

IV. RESULTS

Experiments were conducted to standardize the electroporation parameters for the transfer of the rabies glycoprotein gene to Muskmelon plants. The progeny of the electroporated plants known as the R1 generation were tested for the integration of the rabies glycoprotein gene by dot blot analysis. The results of these experiments are presented here.

Transformation of *E. coli* strain DH5α

Using rabies glycoprotein gene competent cells of *E. coli* strain DH5 -alpha was transformed using the plasmid pRGRgp. The transformed colonies were selected on Kanamycin media. These transformed Kanamycin resistant colonies were counted on a colony counter. 800 transformants were obtained from a 100μl transformation mixture. The total number of cells in 100 μl of the reaction mixture was found by counting the number of colonies obtained on a plain LB medium after serial dilutions (10^{-2} - 10^{-4} - 10^{-6})

The transformation frequency is the ratio of number of transformed cells to the total number of cells in the reaction mixture expressed as a percentage. The transformation frequency was found to be 2.85×10^{-5}(Table 1)

Table 1 : The number of transformants obtained after transformation of E coli strain DH5α with pRGRgp

Bacterial Colony	Number of Colonies obtained
Non-tranformed (Kan sensitive)	2800
Transformed (Kan resistant)	800

$$\text{Tranformation frequency} = \frac{\text{No. of transformed colonies}}{\text{Total no. of colonies x dilution factor}} \times 100$$

$$= 0.285 \times 10^{-6} \times 100$$

$$= 2.85 \times 10^{-5} \%$$

Plate 3 : Transformed colonies of *E.coli* DH5 α on Kanamycin containing media

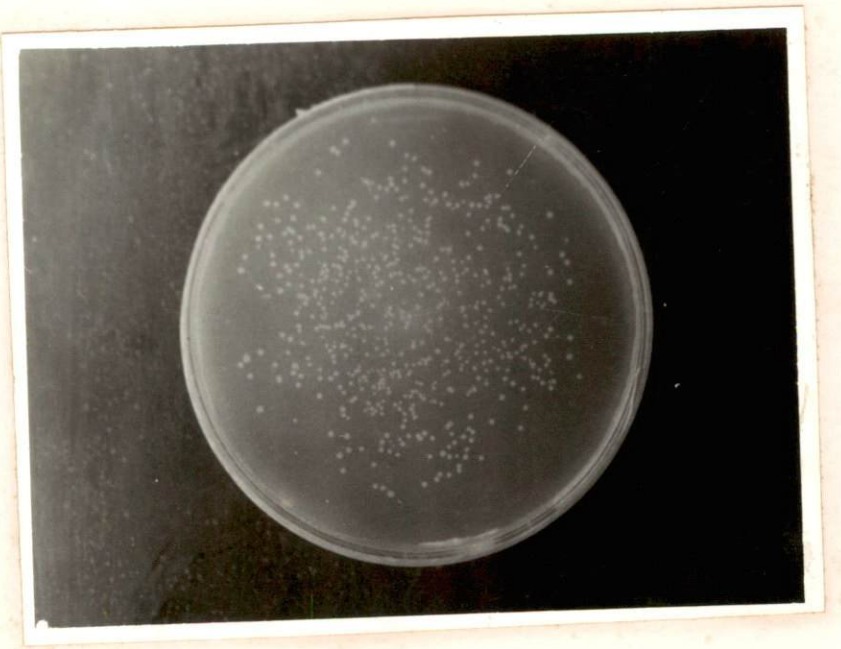

Plate 3

Calculation of RF-values

For the calculation of RF-values the molecular weight marker,λ DNA Hind III digest was used as the standard. The distance travelled by different fragments of λ DNA Hind III digest in the gel was plotted against their respective sizes on a semilog graph paper to obtain the standard curve. The distance travelled in the gel by the pRGRgp plasmid uncut and also the EcoRI Hae II double cut fragments were measured. These distances were plotted on the standard curve and the size of the pRGRgp plasmid and its cut fragments was estimated.

The complete uncut pRGRgp plasmid runs to a distance of 24 mm and had a size of 12.5 Kb. One of the cut fragments travelled to a distance of 34 mm and the other to a distance of 43 mm and their respective sizes were found to be 4 Kb and 2 Kb. (Table 2)

Electroporation of nodal buds of Muskmelon *in vivo*.

The survival of the electroporated nodal bud of each Ro plant was observed. Table 3 shows that in treatment 1 64.28% of the electroporated buds survived. In treatment 2 there was 100% survival. The survival of electroporated buds was 75% and 50% in treatments 3 and 4 respectively.

After the electroporated and control plants were transplanted into the glass house, some died due to transplantation shock. In treatment 1,2,3,4 and 5 (control) the percentage of plants which survived transplantation was 66.6%,70.58%,100% and 75% receptively.

Table 2 : RF values of restriction digested and undigested samples of pRGRgp

Fragment	DNA Hind III digest		Plasmid DNA (pRGRgp)	
	Distance travelled (mm)	Fragment size (Kb)	Distance travelled (mm)	Fragment size (Kb)
1	22	23.1	24	12.5
2	25	9.42	34	4.00
3	29	6.56	43	2.00
4	33	4.36		
5	41	2.32		
6	43	2.05		

Plate 4 : Restriction digested and undigested samples of pRGRgp

Lane 1 : λ DNA Hind III Digest

Lane 2 : pRGRgp uncut

Lane 3 : pRGRgp Eco RI Hind III Digest

Plate 4

Table 3 : Survival of electroporated buds of R0 and control plants

Treatments	R0				Control
	T1(%)	T2(%)	T3(%)	T4(%)	
No. of Plants electroporated	14	17	8	8	4*
No. of plants with surviving electroporated bud	9 (64.28)	17 (100)	6 (75)	4 (50)	4*

Table 4 : Survival of R0 and control plants after transplanting

Treatments	R0				Control
	T1(%)	T2(%)	T3(%)	T4(%)	
No. of Plants with surviving electroporated bud	9	17	6	4	4*
No. of plants with surviving electroporated bud	6 (66.6)	12 (70.58)	4 (66.6)	4 (100)	3* (75)

(* Not electroporated)
T1 = 5 pulses at 100 V
T2 = 5 pulses at 200 V
T3 = 5 pulses at 300 V
T4= 5 pulses at 400 V

Plate 5 : Glasshouse where Muskmelon plants were grown

Plate 6 : R0 plants in the glasshouse

Plate 5

Plate 6

Table 5 : Number of fruits harvested from R0 and control plants

Treatment	Number of surviving electroporated plants	Plant No.	Number of fruits harvested	Average
T1	6	3	8	
		5	15	
		10	7	7.5
		11	6	
		12	4	
		13	5	
T2	12	16	12	
		17	3	
		18	5	
		19	4	
		20	2	
		21	5	
		22	1	4.4
		23	1	
		24	10	
		25	1	
		29	7	
		30	2	
T3	4	40	1	
		41	0	
		42	0	0.3
		47	0	
T4	4	33	1	
		34	1	
		37	1	1
		39	1	
Control	3*	I	3	
		II	1	2
		III	2	

(* Not electroporated)
T1 = 5 pulses at 100 V
T2 = 5 pulses at 200 V
T3 = 5 pulses at 300 V
T4= 5 pulses at 400 V

Plate 7 : Fruits harvested from R0 and control plants

Plate 7

Table 6 : Weights and Diameter of fruits harvested from R0 and Control plants

Treatment	Fruit No.	Weight (g)	Average (g)	Diameter Equatorial (cms)	Average (cm)	Pole to pole (cms)	Average (cm)
T1	3A	140		24		23	
	3B	173		25		21	
	3C	235		27		27	
	3D	355		31		28	
	3E	169		25		22	
	3F	118		17		22	
	3G	88		26		17	
	3H	364		31		27	
	5A	143		26		23	
	5B	112		17		21	
	5C	202		26		22	
	5D	250		30		26	
	5E	126		23		20	
	5F	68		16		16	
	5G	55		21		18	
	5H	129		23		20	
	5I	175		25		21	
	5J	263		30		25	
	5K	202		21		23	
	5L	129		22		21	
	5M	221		29		25	
	5N	266		33		25	
	5O	188		28		23	
	10A	117		22		20	
	10B	169		24		21	
	10C	238		30		24	
	10D	192		26		23	
	10E	233		30		25	
	10F	198		28		23	
	10G	87		22		22	
	11A	225		29		24	
	11B	166		26		22	
	11C	110		22		19	
	11D	115		22		20	
	11E	203		28		24	
	11F	192		25		22	
	12A	88		15		20	
	12B	156		24		21	
	12C	80		20		17	
	12D	102		21		18	

	13A	176		27		22	
	13B	178		26		22	
	13C	186		27		22	
	13D	69		18		15	
	13E	307	172.4	30	24.61	27	21.97
T2	16A	168		25		21	
	16B	194		26		21	
	16C	169		26		21	
	16D	212		27		23	
	16E	54		12		15	
	16F	194		26		22	
	16G	500		35		32	
	16H	165		24		23	
	16I	150		23		21	
	16J	200		28		23	
	16K	108		17		21	
	16L	211		28		23	
	17A	142		25		21	
	17B	107		23		18	
	17C	195		29		23	
	18A	107		22		19	
	18B	195		27		22	
	18C	244		29		24	
	18D	134		21		20	
	18E	202		21		23	
	19A	125		23		22	
	19B	286		37		27	
	19C	102		23		19	
	19D	183		26		22	
	20A	131		20		19	
	20B	84		19		19	
	21A	109		22		19	
	21B	19		11		11	
	21C	178		25		22	
	21D	277		29		26	
	21E	175		27		22	
	22A	157		26		21	
	23A	150		24		21	
	24A	116		23		20	
	24B	163		26		21	
	24C	92		21		18	

	24D	335		31		26	
	24E	145		25		21	
	24F	252		30		25	
	24G	142		24		21	
	24H	157		25		21	
	24I	50		12		16	
	24J	173		27		22	
	25A	173		26		21	
	29A	181		26		22	
	29B	115		23		19	
	29C	253		29		25	
	29D	141		25		21	
	29E	246		30		25	
	29F	265		30		25	
	29G	150		25		22	
	30A	53		16		15	
	30B	201	164.6	27	26.66	23	21.81
T3	40A	6	6	10	10	8	10
T4	33A	8		9		8	
	34A	7		8		7	
	37A	10		9		8	
	39A	7	8	8	8.5	7	7.5
Control	IA	162		25		20	
	IB	182		25		22	
	IC	165		24		22	
	IIA	180		26		22	
	IIIA	215		27		24	
	IIIB	148	175.3	24	25.16	22	22

T1 = 5 pulses at 100 V
T2 = 5 pulses at 200 V
T3 = 5 pulses at 300 V
T4= 5 pulses at 400 V

Plate 8 : R1 plants in the glasshouse

Plate 8

Table 7 : Germination and survival of R1 plants

Treatment	Fruit No.	Number of seeds germinated (%)	Number of R1 plants survived (%)
T1	3A	10 (100)	1 (10)
	5A	5 (50)	2 (40)
	10A	8 (80)	7 (87.5)
	11A	10 (100)	8 (80)
	12A	9 (90)	7 (77)
	13A	9 (90)	6 (66)
T2	16A	7 (70)	7 (100)
	17A	9 (90)	6 (6)
	18A	8 (80)	5 (62.5)
	19A	8 (80)	8 (100)
	20A	8 (80)	7 (87.5)
	21A	7 (70)	4 (57.1)
	22A	5 (50)	2 (40)
	23A	5 (50)	2 (40)
	24A	9 (90)	4 (44.4)
	25A	5 (50)	1 (20)
	29A	7 (70)	3 (42.5)
	30A	---	---
T3	40A	---	---
T4	33A	5 (50)	3 (60)
	34A	6 (60)	2 (33.3)
	37A	9 (90)	4 (44.4)
	39A	7 (70)	3 (42.8)
Control	IA	8 (80)	3 (37.5)

T1 = 5 pulses at 100 V
T2 = 5 pulses at 200 V
T3 = 5 pulses at 300 V
T4= 5 pulses at 400 V

Plate 9 : Results of Dot blot analysis

1 : 12 A1
2 : 13 A1
3 : 29 AI
4 : 17 A3
5 : 3A1
6 : 11A1
7 : pRGRgp (positive control)
8 : 13A2
9 : 17A1
10 : 18A2
11 : 18A5
12 : 19A1
13 : 19A2
14 : 19A8
15 : 20A1
16 : 20A2
17 : 29A1
18 : Control plant (Negative control)

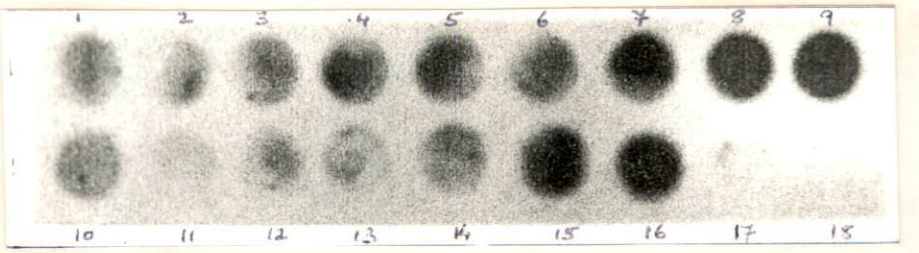

Plate 9

The R0 and control plants in the glass house flowered and gave rise to fruits. The number of fruits harvested from each R0 plant and control plant is shown in Table 5. Plant number 41, 42 and 47 did not produce any fruits. The average number of fruits produced per plant respectively in the treatments 1, 2, 3, 4 and 5 are 7.5, 4.4, 0.3, 1 and 2.

The fruit weights and diameters (pole to pole and equatorial) were recorded and are presented in Table 6. The average weights of fruits in T1, T2, T3, T4 and T5 were 172.4g, 164.6g, 6g, 8g and 175.3g respectively. The average equatorial diameter of the fruits was found to be 24.61cm, 24.66cm, 10cm, 8.5cm and 25.16 cm in T1, T2, T3, T4d T5 respectively. The average pole to pole diameter of the fruits in T1, T2, T3, T4 and T5 were 21.97 cm, 21.81 cm, 10 cm, 75 cm and 22 cm respectively.

10 seeds from the first fruit of each Ro plant and one control plant were sown to produce the R1 plants. Table 7 gives the germination percentage of the R1 seeds and their subsequent survival in the polyhouse. The germination percentage ranged from 50 to 100% in the R1 and control plants. The survival of the germinated R1 and control plants ranged from 20 to 100%.

Dot Blot Analysis of R1 plants

Genomic DNA extracted from all the surviving R1 plants was subjected to dot blot analysis. The results are tabulated in Plate 9. In all, 92 R1 plants were tested out of which 19 plants were consistently positive for hybridization with the

rabies glycoprotein gene probe when probed repeatedly. Six plants belonged to T1 and 13 plants to T2.

DISCUSSION

V. DISCUSSION

Rabies is a deadly disease, which is prevalent widely in feral dogs and also in domestic animals in our country. Rabies being a zoonotic disease it is very important to bring it under control. A cheap and effective vaccine against rabies is required for this purpose. Production of an edible vaccine against rabies in a plant like muskmelon would perfectly serve this purpose. Transgenic muskmelon plants expressing the rabies glycoprotein gene can synthesize the foreign protein. When the fruits of the transgenic plants are consumed it will be able to induce the production of rabies virus neutralizing antibodies in the host.

The present investigation aimed at the transfer of the rabies glycoprotein gene to muskmelon and stable integration in the plant genome.

The gene transfer method employed was electroporation of the nodal buds. This transformation technique is simple to carry out and doesn't require expensive and sophisticated equipment. Also this *in vivo* transformation of nodal buds does away with the tissue culture regeneration and hardening problems associated with other transformation methods.

This work also led to the standardization of the electroporation parameters for the transformation of muskmelon. The standardized protocol may be used for transfer of other genes to muskmelon.

The results of the present investigation are discussed here.

Transformation of *E. coli* strain DH5α

E. coli cells were transformed with the pRGRgp plasmid and were selected on kanamycin media. Since this plasmid has the neomycin phosphotransferase gene which detoxifies Kanamycin only the transformed cells show resistance to Kanamycin and can grow on Kanamycin containing media.

Eight hundred transformed colonies were obtained. The transformation frequency was 2.85×10^{-5} which is the expected level with the calcium chloride method of transformation. However, frequency of transformation perse was not important in this case as even a single cell would be enough for large-scale multiplication and plasmid isolation. The transformation frequency of 2.85×10^{-5} was sufficient for the purpose.

Calculation of RF - values

The distance travelled by the fragments of the molecular size markers (λ Hind III digest) in agarose gel was measured. This was used to construct a standard curve. The RF values of the pRGRgp plasmid cut and uncut fragments were determined using the standard curve. The uncut pRGRgp plasmid was found to have a size of 12.5 Kb. Upon cutting the plasmid with Hae II and EcoRI, the rabies glycoporten gene along with CaMV35S promoter and Nos terminators was realised. The size of the fragment was found to be 2 Kb which is in accordance with the calculated size of the fragment

***In vivo* electroporation of muskmelon nodal buds**

The rabies glycoprotein gene present in pRGRgp plasmid was introduced into intact nodal buds of muskmelon by electroporation using spermine as a DNA protectant.

Effect of electroporation voltage on transformation of muskmelon nodal buds

Four different voltage treatments were given to the muskmelon plants namely 100V, 200V, 300V and 400V. Of these treatments T4 of 400V caused death of 50% of the buds and is therefore too high a voltage for muskmelon plants. T3 of 300V increased the survival of the electrorated buds to 75%. However, T2 of 200V is optimum for muskmelon, as there was 100% survival of the electroporated buds. The lowest voltage treatment of 100 V gave only 64.28 % survival of electroporated buds. This was due to direct contact between the buds and the walls of the electroporation cuvette, which led to burning of the buds.

The fruit yield, weight, diameter of the T1, T2 and control plants was similar in R0 generation. In treatments T3 and T4 the fruit yield and size is comparatively low. The reason attributed to this is the severe attack of downy mildew when the T3 and T4 plants had just been transplanted. The T1, T2 and control plants were already well established and could regain their vigour after control measures were taken. The T3 and T4 plants failed to establish well due to disease and could not regain their vigour and gave low yields. Thus the

electroporation treatment itself may not have adversely effected the fruit yield weight, diameter and colour of the fruits.

Dot Blot and Hybridization Analysis

The nodal buds of R0 muskmelon plants were electroporated with the pRGRgp plasmid. The aim of this treatment is to make the plasmid enter the cells in the bud and integrate into the genomic DNA in the nucleus of the cells. The cells of the bud being meristematic would multiply rapidly and all the daughter cells arising from these transformed cells would have entire pRGRgp plasmid or some portion of it integrated in the nuclear genome. Some of these transformed cells would form flower primordial and eventually bare fruits containing some seeds, which might be transgenic. These transformed seeds gave rise to transgenic R1 plants.

To find out whether the electroporation treatment was successful in transforming the cells of the nodal bud, the R1 plant genomic DNA was isolated and dot blot analysis was done. The probe used was a gel eluted fragment of the plasmid containing the rabies glycoprotein gene, which was labelled with DIG. Since, we want transgenic plants expressing the rabies glycoprotein, which can be, used as edible vaccine, integration of just the rabies glycoprotein gene portion of the pRGRgp plasmid is considered as a success.

Out of 92 R1 plants tested 19 showed positive hybridization signals. Six of these belonged to treatment T1 and 13 to treatment T2. This indicates that both

these treatment allow the entry of foreign DNA into the muskmelon plant followed by stable integration. We have every reason to believe that this is a stable integration since the testing for the presence of the gene was done in the R1 generation. We do not believe that naked DNA can go through the gamete and be passed on to the next generation without first being integrated.

SUMMARY

VI. SUMMARY

Production of edible vaccines in plants is a novel concept. If actually put into application successfully it should be a cheap source of vaccination. Availability of an edible vaccine against rabies would prove to be a boon to bring under control this deadly disease which costs as many as 30,000 lives a year all over the world.

The present investigation is an initial step in the production of edible vaccine against rabies. It was carried out to introduce the rabies glycoprotein gene into muskmelon to get a stable integration of this gene. It also led to standardization of parameters for the electroporation of nodal buds of muskmelon. The work done and results obtained are summarised below :

1. Transformation of *E. coli* strain DH5-alpha with the pRGRgp plasmid and selection of transformants on Kanamycin media was done. These transformed colonies were used for large-scale isolation of the plasmid pRGRgp.
2. The pRGRgp plasmid DNA was complexed with spermine (DNA protectant) was used to electroporate muskmelon nodal buds. Treatment 5 pulses at 200 V over duration of 99-m sec gave 100 % survival compared to lower survival rates of nodal buds at 100 V, 300 V, and 400 V.
3. The yield of fruits and their morphological character (size, color and weight) in R0 plants were on par with the control plants.

4. R0 seeds were used to raise the R1 crop. The R1 plants were tested for the presence of rabies glycoprotein gene by dot blot analysis. R1 plants were found to be positive for transformation with the rabies glycoprotein gene.
5. The R1 plants belonging to treatments T1 and T2 were positive for the presence of the rabies glycoprotein gene, determined by dot blot analysis. A 5 pulse electroporation treatment at 100 V or 200 V is optimum for transformation of muskmelon.
6. The present investigation demonstrated that electroporation is a potent method for gene delivery into plants. It is a simple method, which doesn't require expensive equipment. It doesn't need to be carried out under sterile conditions and it does away with the need for tissue culture.
7. Also for the first time the gene for the antigenic subunit of an animal pathogenic virus was introduced into muskmelon plants. The fruits of the transgenic muskmelon plants carrying the rabies glycoprotein gene, if confirmed to express the protein, can be used in future as edible vaccine against rabies.

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VII. REFERENCES

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