EXPRESSION OF PROTECTIVE ANTIGEN GENE IN *Brassica juncea*

Thesis submitted to the Jawaharlal Nehru University in fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

G. Jyotsna

SCHOOL OF BIOTECHNOLOGY
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067
INDIA

2009

School of Biotechnology
Jawaharlal Nehru University
New Delhi-110067

CERTIFICATE

This is to certify that the work titled **'Expression of protective antigen gene in *Brassica juncea*'** submitted to the School of Biotechnology, Jawaharlal Nehru University, New Delhi in fulfillment of the requirements for the award of the degree of Doctor of Philosophy, embodies faithful record of original research work carried out by **G. JYOTSNA**. She has worked under my guidance and supervision. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

G. Jyotsna
(Ph.D. Candidate)
School of Biotechnology
JNU, New Delhi

Prof. Rakesh Bhatnagar
(Supervisor)
School of Biotechnology
Jawaharlal Nehru University
New Delhi-110067

Prof. Rajiv Bhat
(Dean)
School of Biotechnology
Jawaharlal Nehru University
New Delhi-110067

July 2009
School of Biotechnology
Jawaharlal Nehru University
New Delhi-110067

Dedicated to my parents

who have always instilled enormous optimism, reforming me in to a better person with each passing day!!

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Jyotsna

ABBREVIATIONS

$A_{280/350/595/600}$	Absorbance at 280/350/595/600 nm
Ab	Antibody
ADP rib.	Adenosine Diphosphate ribosyltransferase
Amp	Ampicillin
APC	Antigen Presenting Cells
APS	Ammonium persulfate
ATCC	American type culture collection
aad A	Aminoglycide-3′-adenyltransferase
ATP	Adenosine Triphosphate
bp	Base pair
BAP	Benzyl amino purine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
°C	Degree Celsius
cAMP	Cyclic adenosine monophosphate
CDC	Centre for Disease Control
CT	Cholera toxin
CT-B	Cholera toxin B subunit
C terminal	Carboxy terminal
DAB	Diamino Benzidine
DEB	DNA Extraction Buffer
D-IV	Domain-IV
ddH_2O	Double Distilled water
ddNTP	Di-Deoxyribose nucleotide
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
EF	Edema Factor
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
g	Gravitational force
GFP	Green Flouroscent Protein
Gms	Grams
GM	Genetically modified
HRP	Horseradish peroxidase
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2 ethanesulfonic acid)
hr./hrs.	Hour/hours
IBA	Indole Butyric acid
Ig	Immunoglobulin
I.P.	Intraperitoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
I.V.	Intravenous
I.M.	Intramuscular
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
LF	Lethal Factor
LT/LeTx	Lethal Toxin
LTR	Left Targeting Region
M	Molarity
MCS	Multiple Cloning Site
MIS	Mucosal Immune System
mg	Milligram
ml	Milliliter
min./mins	Minute/Minutes
mM	Milli Molar

MTT	3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide
N	Normailty
ng	Nanogram
nm	Nanometer
NAA	α- Naphthalene acetic acid
NBT	NitroBlue Tetrazolium
npt II	neomycin phosphotransferase
N terminal	Amino-Terminal
O/N	Over night
PA	Protective antigen
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PGA	Poly-D-glutamic acid
PMSF	Phenyl Methyl Sulfonyl Fluoride
prrn	Plastid Ribosomal RNA
RBS	Ribosome Binding Site
rpm	Revolutions per minute
RM	Regeneration Medium
RNase	Ribonuclease
RPMI	Roswell Park memorial Institute
RTR	Right Targeting Region
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophorsis
Sec.	Second
s.c.	Subcutaneous
SM	Selection Medium
TAE	Tris acetate EDTA
TCR	Translational Control Sequences
TEMED	N,N,N',N' tetramethyl ethylene diamine

TMB	3,3',5,5'-Tetramehylbenzidine
Tris.	Tris (hydroxymethyl) amino methane
TSP	Total Soluble protein
TTBS	Tris buffered Saline with Tween 20
U	Unit
UTR	Untranslated Region
UV	Ultra Violet
WHO	World Health Oraganization
x g	Times gravity (centrifugal force)
YEM	Yeast Extract Medium
β-ME	Beta mercaptoethanol
µg	Microgram
µl	Microliter
%	Percentage
~	Approximately

CONTENTS

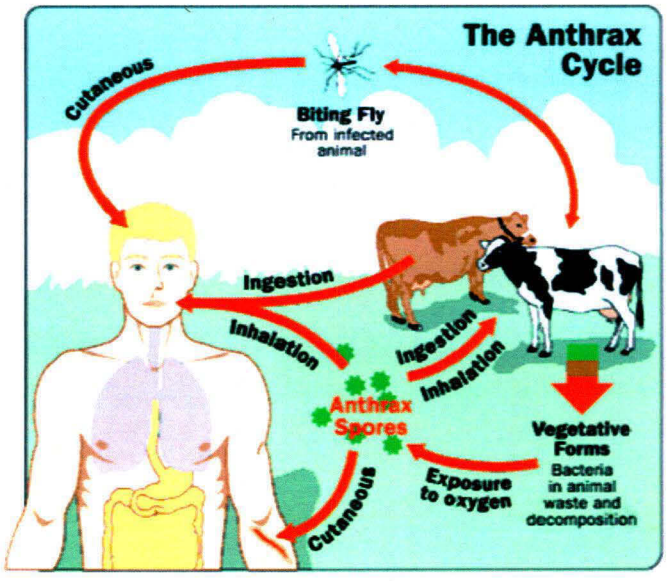
Chapter 1

Review of Literature

Anthrax: an old disease with a new face

Anthrax, a disease of antiquity has recently gained prominence in late 2001 because of its implication as biological weapon. The epidemic transmission of the disease under natural circumstances has not been greatly appreciated. The historical perspectives of the disease enrich us with wealth of information regarding the epidemic developments and also the disconcerting human efforts towards bioterrorism. Anthrax is a zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. The term anthrax is derived from the Greek word for coal, *anthrakis*, because of the black skin lesions characteristic of the disease. A disease that appears to have been anthrax was described in the biblical book of *Exodus* as the fifth plague in about 1490 BCE. The descriptions of anthrax affecting both animals and humans are found in early Indian and Greek writings. An epidemic of anthrax in 17th century Europe caused an estimated 60,000 human deaths. During the Middle Ages, anthrax then referred to as *"The Black Bane"*, killed large number of humans and animals in Europe. The organism served as a prototype for the famous Robert Koch's postulates which provided the framework for all the infectious diseases. The live attenuated strain of *Bacillus anthracis* used to protect livestock in 1881 by Louis Pasteur introduced the concept of vaccination. These milestones of the late 19^{th} century formed the roots of medical microbiology and laid the foundation for immunology. *Bacillus anthracis* exists as endospores in the soil in many areas. The spores are resistant to extreme conditions such as heat and radiations and remain infective and viable in the environment for many years even decades (Manchee, 1990), as illustrated by biological warfare experiments during World War II on the Scottish island of Gruinard (Manchee, 1990). During 1943 and 1944, an estimated 4×10^{14} anthrax spores were dispersed on the island through explosive means. Spores were still detectable more than 40 years later. This obstinate nature of the spores impels us to be prepared for any kind of epidemic outbreak springing out from engraved spores and unforeseen emergency attacks by bioterrorists. Spores are a direct source of infection for grazing animals and livestock, but they generally do not represent a direct infection risk for humans under natural conditions. Animals become infected when they ingest or inhale the spores while grazing. Humans can become infected with *B. anthracis* by skin contact, ingestion, or inhalation

of *B. anthracis* spores originating from products of infected animals or from inhalation of spores from the environment.

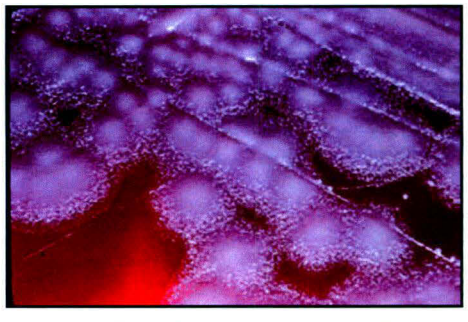


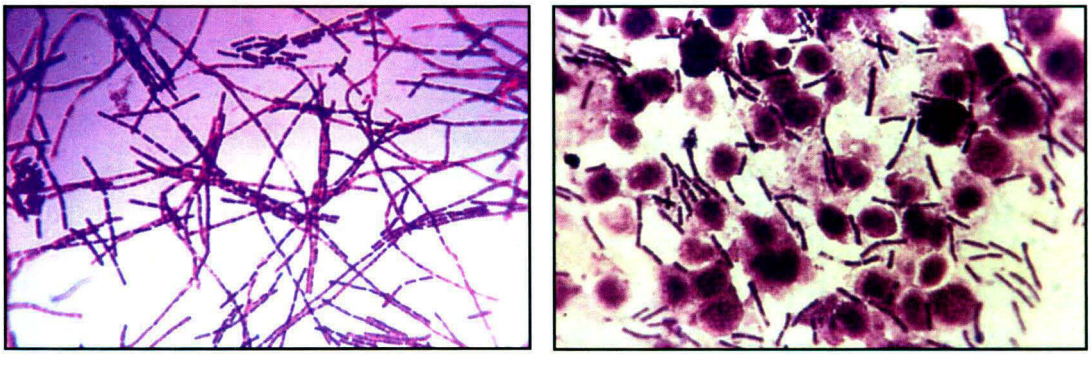
Anthrax spores germinate when they enter an environment rich in amino acids, nucleosides, and glucose, such as the blood or tissues of an animal. The replicating bacteria produce three important proteins namely protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins combine to form two toxins known as lethal toxin and edema toxin. PA and LF form lethal toxin, a protease that is believed to be responsible for tissue damage, shock and death, although the mechanism is not clear. PA and EF form edema toxin, an adenylate cyclase that perturbs the control of ion and water transport across cell membranes and causes extensive edema. PA gets proteolytically cleaved, binds to receptors on mammalian cells and then binds with LF or EF. The toxin complexes are internalized via endocytosis into the cell and then transported to the cytosol, where they exert their effect.

BACTERIOLOGY

Bacillus anthracis

The vegetative cell of the organism is a non-motile, aerobic, gram-positive bacillus, 1.2 to 10 µm in length and 0.5 to 2.5 µm in width having a joined bamboo rod like appearance and is capable of forming endospores. They are protected by a morphologically complex protein coat (Giorno, 2007). The Endospore is oval, and forms long chains of vegetative cells *in vitro*; single cells or short chains of 2 to 4 cells in direct clinical samples. They are characterized by being non-hemolytic on sheep blood agar and unsusceptible to lysis by gamma phage. They grow rapidly on sheep blood agar (SBA) and comma-shaped projections may give "Medusa head" appearance on SBA. Colonies have been described as having a "ground glass" or "curled hair" appearance and have the consistency of beaten egg whites. Colonies are 2 to 5 mm in diameter after 16 to 18 hours of incubation. It forms mucoid capsule when grown on agar with sodium bicarbonate and incubated in CO_2-enriched atmosphere. Capsule can be visualized with India ink preparation. Spores germinate and form vegetative cells in environment rich in nutrients (e.g., glucose, amino acids, and nucleosides). Vegetative bacteria generally survive poorly outside of mammalian hosts. Conversely, vegetative cells form spores when nutrients in the environment are exhausted.

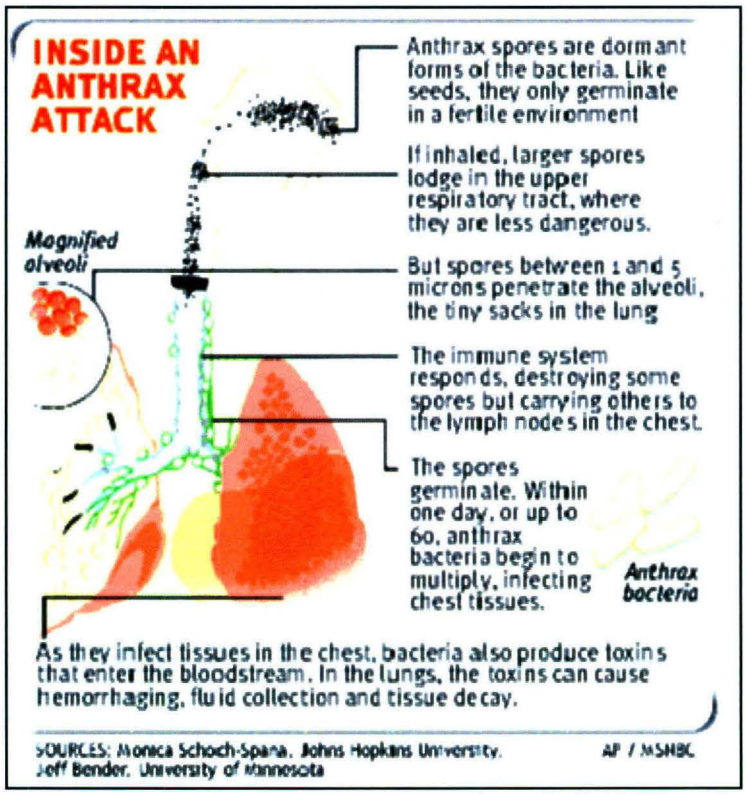




Gram stain of *Bacillus anthracis*: (a) from culture; (b) from infected tissue

PATHOGENESIS

The endospores enter through the skin abrasion or mucous membranes, are phagocytosed by macrophages and carried to regional lymph nodes. Endospores get transformed into vegetative bacteria by germination inside macrophages. The vegetative bacterium here produces a tough capsule that allows it to evade immune system by resisting phagocytosis and protects the organism from lysis by cationic proteins in the serum. The vegetative bacteria once released into the lymphatic system multiply and enter the blood stream until there are as many as 10^7-10^8 organisms per milliliter of blood. Bacteremia may ensue and lead to overwhelming septicemia, widespread tissue destruction, organ failure, shock like symptoms and death. The manifestations of advanced disease are believed to result from the action of exotoxins secreted by bacilli.



CLINICAL MANIFESTATIONS

There are three clinical forms of anthrax: cutaneous, gastrointestinal and inhalational. The symptoms and incubation period of human anthrax are determined by their route of transmission.

Cutaneous Anthrax

Cutaneous anthrax accounts for 95% of the anthrax infections and occur when the bacterium enters through a cut or abrasion on the skin (e.g., when handling *B. anthracis*–contaminated animals, animal products or other objects). The reported incubation period for cutaneous anthrax ranges from 1 to 12 days. Skin infection begins as a small papule that may be pruritic, progresses to a vesicle in 1–2 days and erodes leaving a necrotic ulcer (eschar) with a characteristic black center. Secondary vesicles may develop around the primary lesions. The lesion is usually painless. Other symptoms may include swelling of adjacent lymph nodes, fever, malaise and headache. The diagnosis of cutaneous anthrax is suggested by the presence of the eschar, the presence of edema out of proportion to the size of the lesion and the lack of pain during the initial phases of the infection. The case-fatality rate of cutaneous anthrax is 5–20% without antibiotic treatment and less than 1% with antibiotic treatment.

Gastrointestinal Anthrax

The intestinal form of anthrax usually occurs after eating contaminated meat. The incubation period for intestinal anthrax is believed to be 1–7 days. The involvement of pharynx is characterized by lesions at the base of the tongue or tonsils with sore throat, dysphagia, fever and regional lymphaenopathy. The involvement of lower intestine is characterized by acute inflammation of the bowel. Initial signs of nausea, loss of appetite, vomiting and fever are followed by abdominal pain, vomiting of blood and bloody diarrhea. The case-fatality rate of gastrointestinal anthrax is unknown but is estimated to be 25–60 %. Historically, illness has been thought to result from ingestion of *B. anthracis* spores; however, recently experts have postulated that illness predominantly results from

ingestion of large numbers of vegetative cells (such as those found in poorly cooked meat from infected animals) (Inglesby, *et al.,* 2002).

Inhalational anthrax

Originally known as woolsorter's disease, inhalational anthrax results from inhalation of 8,000–50,000 spores of *B. anthracis*. This form of anthrax would be expected to be the most common following an intentional release of *B. anthracis*. The incubation period for inhalational anthrax for humans appears to be 1–7 days, but may be as long as 43 days. Case fatality rate ranges from 75-97 % and even prolonged antibiotic therapy was found to be to be ineffective in acute cases. The initial symptoms most often reported are flu cough, myalgia and malaise. Later, during the course of infection after 2-3 days, the disease takes fulminant course with dyspnea, stringent cough and chills culminating in death (Penn C C, *et al.,* 1997). Most persons with inhalational anthrax have abnormalities on chest x-ray showing widened mediastenum.

EPIDEMIOLOGY

ANTHRAX AS A GLOBAL ZOONOSIS

Although anthrax is essentially under control in the industrialized world, it remains a serious zoonosis in the developing world and is enzootic in many natural parks. Anthrax has been one of the major causes of mortality in domestic livestock through recorded history. The development of an improved vaccine by Sterne in the 1930s and improved regulation of the animal trade (including quarantine) led to a drastic decline in the incidence of anthrax in most countries. National control programmes work very well against the disease. Indeed, anthrax is today considered an uncommon disease in Western Europe, Northern America and Australia, with exceptions in endemic foci in wild fauna in the national parks (Hugh-Jones, 1999). For example, in Canada, the situation is under control with the exception of a few epidemics in the bison population of the MacKenzie Bison Sanctuary and Wood Bison National Park (Nishi *et al.,* 2002, Hugh-Jones and de Vos, 2002), whereas in the USA, the disease is sporadic in some areas and is hyperendemic in south-west Texas (Hugh-Jones, 1999). In Australia, anthrax is sporadic, although a sudden and severe epidemic occurred in Northern Victoria in 1997 (Turner *et al.,* 1999). In Europe, the major endemic areas are Greece, Turkey, Albania and probably all of the Balkans (Hugh-Jones, 1999). France and Italy continue to record sporadic outbreaks (Fouet *et al.,* 2002).

The lack of suitable control programmes in Central Asia, South America and Africa makes the disease very common in these continents. Central and South America lack effective control programmes, with hyperendemic anthrax zones in Guatemala and El Salvador and endemic zones in Mexico, Honduras, Nicaragua, CostaRica, Brazil and Venezuela (Hugh-Jones, 1999). In contrast, vaccination programmes in Uruguay and Chile have resulted in good control. In Russia and countries of the former Soviet Union lack of effective control programmes is evidenced by the high percentage of human cases also reflecting the inadequacies of both the public health system and veterinary service (Hugh-Jones, 1999). In Asia, anthrax is widespread in the Philippines, South Korea, South East India and in western mountainous zones of China and Mongolia; in Papua New Guinea porcine anthrax is reported frequently (Hugh-Jones, 1999). Africa remains severely afflicted, with major epidemic areas in wildlife areas such as Queen Elizabeth

National Park (Uganda), Omo Mago National Park (Ethiopia), Selous National Reserve (Tanzania), Luangwa Valley (Zambia), Etosha National Park (Namibia), Kgalagadi Transfrontier Park (Botswana and South Africa) and Vaalbos & Kruger National Parks (South Africa) (Hugh-Jones and de Vos, 2002). Recently, anthrax has been reported as the cause of death among chimpanzees in Ivory Coast (Leendertz *et al.*2004) and chimpanzees and gorilla in Cameroon (Leendertz*et al.,* 2006). The isolates from the wild apes in both outbreaks, established a new "forest anthrax cluster", termed F, suggesting that *B. anthracis* is a far less homogeneous species than currently believed (Leendertz *et al.,* 2006).

The entire global scenario of anthrax enzoosis clearly emphasizes on the need to eradicate the disease in many parts of the affected areas of the world. Appropriate management of outbreaks should include the prompt and complete incineration of carcasses, rather than burial, together with prompt case recognition and regular vaccination of susceptible livestock in epidemic or endemic areas

ANTHRAX IN HUMANS—A GLOBAL PERSPECTIVE

Natural outbreaks in humans

Human cases generally follow disease occurrence in ruminants and are most prevalent in Africa, the Middle East and parts of South-east Asia. Outbreaks have been reported in industrial settings where animal products are processed and in agricultural settings following consumption of or exposure to ill animals. Notable examples include a major outbreak involving nearly 10,000 cases (most of them cutaneous infection) occurred in Zimbabwe during the late 1970s and early 1980s (Davies 1982). The estimations by Brachman in 1984 showed that 2,000 to 20,000 human cases of anthrax occur globally each year (Brachman 1984). The cases of naturally occurring anthrax have declined from 127 per year to only 1 per year in US alone. This achievement could be attributed to appropriate prophylactic and decontamination measures recruited routinely. In India, sporadic cases of anthrax have been reported from southern and central parts of the country (Ichhpujani RL *et al.,* 2004, Rao GR *et al.,* 2005).

ANTHRAX AS A BIOTERRORIST WEAPON

The potential for anthrax as bioterror agent has been witnessed by the chilling attacks of 2001. The magnitude of threat and mass destruction has been realized recently with the studies coming from various corners that project the weaponised form of the disease in true light. CDC lists anthrax as category 'A' bioterror agent. The studies done by Webb *et al.,* compare the destructive ability of anthrax spores to a nuclear bomb and categorized anthrax under weapons for mass destruction. The impact of a large aerosol release of weaponized anthrax remains unknown; however, a recent study by Wein *et al.,* presented a mathematical model that says, "An intentional release of only 1 kg of *Bacillus anthracis* spores could lead to the deaths of over 100,000 people in a city of 10 million". Modeling studies of viability of bio-weapon agents in the environment indicate that anthrax is among the most resilient agents, since spores are capable of surviving longer in the environment than are vegetative cells (Stuart, 2005).

Weaponization of *Bacillus anthracis* has been documented since historical times. During World War I, Germany sought to spread the bacteria to allied forces via infected feed and contaminated livestock. Russia (the former Soviet Union), United Kingdom, United States and Japan all instituted research on biological weapons before and during world war II .The dispersal of weaponised *Bacillus anthracis* spores on Gruinard Island under British biological warfare program lead to heavy contamination of the island with persistence of viable spores (Manchee *et al.,* 1988). Also Sverdlovsk Outbreak in 1979 mark the most alarming attacks in human history. The outbreak in the Union of the Soviet Socialist Republics resulted from accidental release of anthrax spores from a military microbiological facility where weaponized anthrax was being produced (Meselson, 1994).

DIAGNOSIS

The diagnosis of anthrax is made from a combination of microscopic, cultural and molecular-biological investigations. In case of specific clinical symptoms as described in clinical manifestations in previous section (e.g. oedema formation in and eschar), the microscopic detection of encapsulated, Gram-positive rods in the blood can aid in rapid diagnosis.

Clinical manifestations to look for are:

- *Ruminants:* Sudden death, bleeding from orifices, subcutaneous haemorrhage, without prior symptoms or following a brief period of fever and disorientation should lead to suspicion of anthrax.
- *Equines and some wild herbivores:* Some transient symptoms of fever, restlessness, dyspnoea and agitation may be apparent.
- *Pigs, carnivores, primates:* Local edemas and swelling of face and neck or of lymph nodes, particularly mandibular and pharyngeal and/or mesenteric.

Detection and confirmation

Characteristic colony morphology, non-hemolytic growth on blood agar, non-motile Gram-positive rods, sensitivity to the gamma phage and penicillin, the formation of a capsule under specific conditions *in vitro* and within the host body are indicative of *B. anthracis* (WHO, 1998). The original sample is always accompanied in parallel by an aliquot of the same material for the investigation of complex sample materials, artificially contaminated (spiked) with a defined number of spores of an avirulent strain of *B. anthracis*. Both the original and the spiked sample are incubated in a liquid enrichment broth and analysed on semi-selective solid media thereafter. The lysis of vegetative cells by the *B. anthracis* gamma phage has long been accepted as a highly specific diagnostic criterion, although rare exceptions exist. (Marston *et al.*, 2006).

Diagnostic infection by animal inoculation

The diagnostic animal trial may become necessary if samples yield a positive or suspicious PCR result, accompanied by a negative or non-interpretable culture test. The mice are inoculated with cultures isolated from the effected samples. If virulent *B.*

anthracis are present in the sample, animals die within 2–12 days (depending on the dose) after a phase of non-specific symptoms of systemic infection. The presence of *B.anthracis* as the cause of death must be verified by culture and/or molecular diagnosis.

Immunodiagnosis

Although attempts have been made for decades to develop immunological tests for the detection of cell derived and toxin antigens, there are still no test kits available commercially. Rapid test kits using monoclonal antibodies and microfluidics are in development and/or available only to the military sector. Specific ELISA based kits for major immunologic proteins *i.e.,* protective antigen and capsular components offers reliable method for diagnosis.

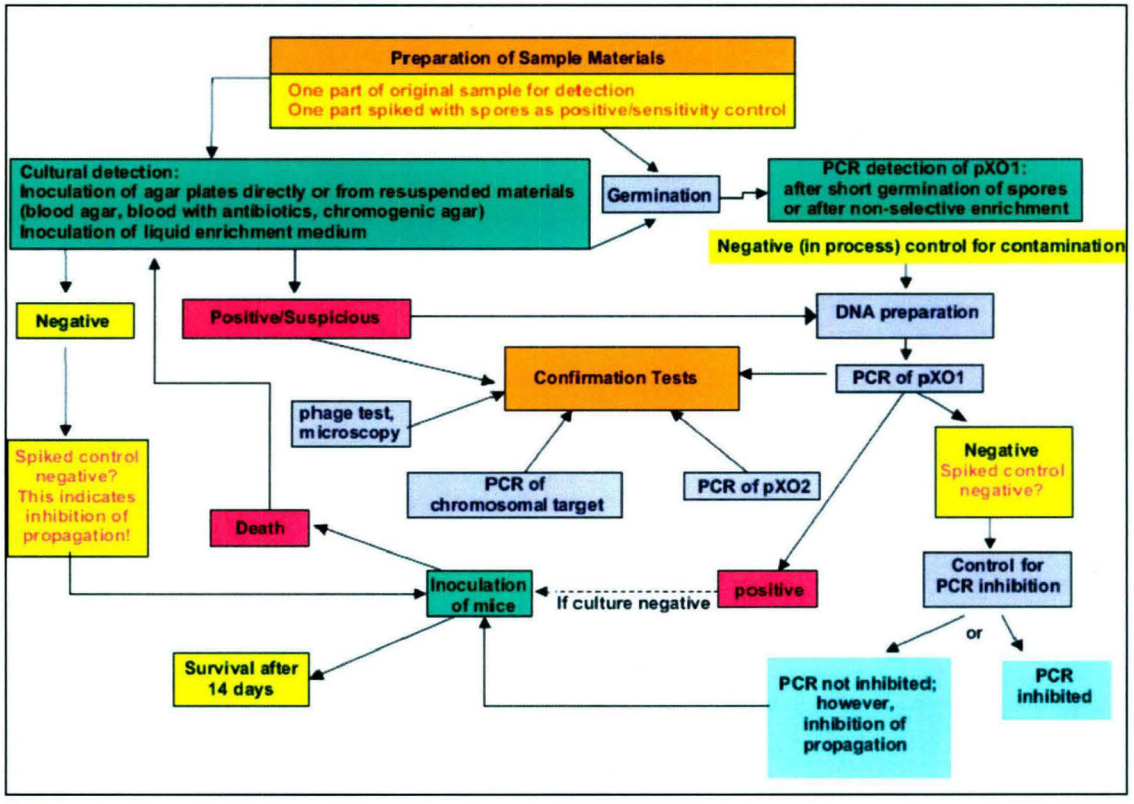
Polymerase chain reaction

The PCR is the method of choice as a parallel diagnostic test, whether performed directly on clinical samples after non-selective enrichment of mixed cultures or as a confirmation test for suspicious colonies. The presence of both the virulence plasmids pX01 (toxins) and pX02 (capsule formation) must be evaluated to identify virulent *B. anthracis* strains and for the differentiation of non-virulent strains. Because of the uncertainties connected with the presence of the virulence plasmids, a reliable chromosomal marker for *B. anthracis* is desirable. However, the published chromosomal targets of rpoB, S-layer protein genes and Ba813 very often lead to false-positive results from environmental samples (Papaparaskevas *et al.,* 2004). An alternative chromosomal target of the sasP gene was described by Beyer *et al.,* 2003.

Molecular characterization

Modern molecular fingerprinting techniques based on the analysis of multiple loci of variable numbers of tandem repeat sequences and canonical single-nucleotide polymorphisms have contributed to rapid advances in our understanding of the population genetics of the *B. cereus* group and *B. anthracis* in particular and now enable more precise molecular epidemiological investigation of outbreaks (Keim *et al.,* 2004; Lista *et al.,* 2006). Such methods are necessarily restricted to specialized laboratories at present. Although there are many diagnostic tools emerging, currently there is no single

diagnostic method considered sufficiently valid. Therefore a sample-based combination of diagnostic procedures is always necessary.



GENERAL SCHEME FOR THE DIAGNOSIS OF *BACILLUS ANTHRACIS* FROM COMPLEX SAMPLES.

THE TOXINS AND PGA CAPSULE

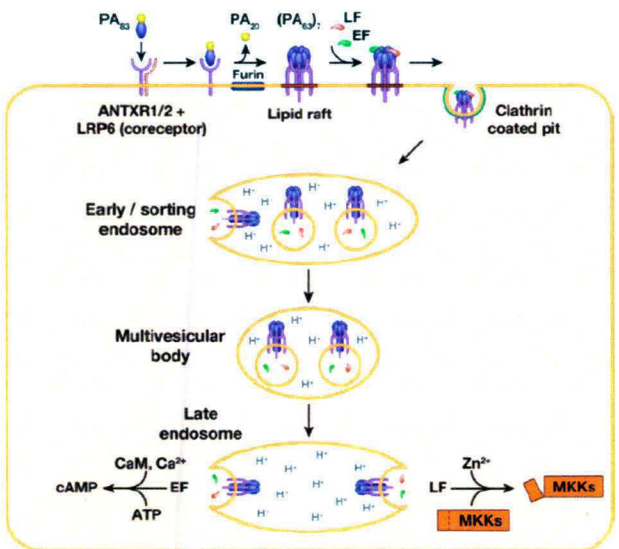
Bacillus anthracis owes its lethality to two major virulence factors: an antiphagocytic poly-D-glutamic acid capsule (PGA) (Green *et al.,* 1985) and a toxin (Smith 2000; Smith & Stoner, 1967). In addition to the cytoplasmic membrane and peptidoglycan layers found in all bacteria, *B. anthracis* bacilli have two other surface structures, namely a capsule and an S-layer. In the absence of the capsule, the cell wall of *B. anthracis* displays a highly patterned layered structure referred to as surface layer or S-layer. The capsule when present is exterior to and completely covers, the S-layer proteins, which form an array beneath it. The presence of S-layer is though not a prerequisite for the formation of the capsule.

The first description of *B. anthracis* capsule dates back to M'Fadyean's work in 1903. Capsules may be found on the bacilli in smears from an infected animal and capsule production *in vitro* is enhanced in the presence of elevated (>5 %) CO_2, bicarbonate and serum. The capsular material is a high molecular weight polypeptide composed exclusively of poly-D-glutamic acid (PGA) PGA is an anionic, poorly immunogenic polypeptide that disguises the bacteria from the host immune surveillance and, by virtue of its negative charges, inhibits bactericidal activity by the host. Thus, the PGA capsule allows virulent anthrax bacilli to grow virtually unimpeded in the infected host.

B. anthracis capsule synthesis is encoded by the plasmid pXO2 (Green BD *et al.,*1985). Initially 3 genes, *capB*, *capC* and *capA*, that encode membrane associated enzymes of 44.8, 16.5 and 46.4 kDa, respectively, were thought to be sufficient for PGA synthesis. Recent studies demonstrated that cap D and cap E genes were found within the same operon and proved to be essential for PGA synthesis. PGA biosynthesis involves two functions; the PGA synthesis, which primarily depends on Cap B- Cap C complex and PGA transport, which is effected by Cap A- Cap E complex. The interaction of Cap A and cap E proteins is essential for their activity (Candela, 2005). Cap D, a polyglutamate transpepidase, mediates the covalent anchoring of PGA filaments to peptidoglycan layer (Foeut *et al.,* 2005; Candela *et al.,* 2005). The understanding of capsule stucture and function provides new insights for developing potential drugs to block PGA synthesis or anchorage and decrease virulence and aid in host protection.

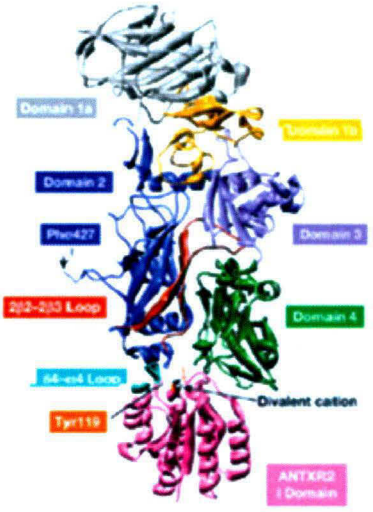
TOXINS

The toxins play a key role in pathogenesis of anthrax and are composed of three protein molecules acting in binary combinations (Stanley and Smith 1961), following an A-B toxin model. The toxin, discovered in the 1950s, consists of three proteins: protective antigen (PA; 83 kDa), lethal factor (LF; 90 kDa) and edema factor (EF; 89 kDa). Individually, none of the three is toxic, but a mixture of PA and LF (called lethal toxin; LeTx) can cause lethal shock in experimental animals and a mixture of PA and EF (called edema toxin; EdTx) induces edema at the site of injection. The genes for all three toxin components are located on pXO1, the larger of the two plasmids present in virulent *B. anthracis* isolates. The genes for PA (*pag*A, previously named (*pag*), LF (*lef*) and EF (*cya*) are not contiguous but are spread over a 25-kb region of the 184-kb pXO1 plasmid. This pathogenicity island may be a mobile genetic element as insinuated by the report of the presence of inverted regions (Uchida *et.al.,* 1993).



ENTRY OF ANTHRAX TOXIN INTO CELLS

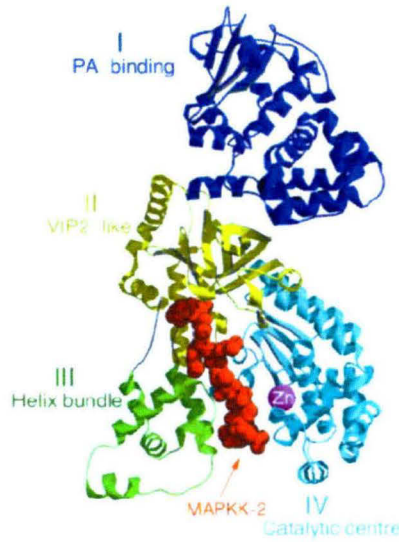
Protective antigen



This molecule plays a central role in the toxin action. The prophylactic use of PA in the form of subunit vaccine makes its discussion more germane in the present study. Its immunogenic potential will be discussed in a later section. The mature protein (PA 83) is 735 amino acids (MW 82,684) long. The crystal structure of monomeric PA83 has been determined at a resolution of 2.1Å (Petosa *et.al.,* 1997). The molecule is folded into four functional domains, each required for a particular step in the intoxication process. The functional moiety of PA (PA 63) is formed after the action of a furin like protease, which cleaves off N-terminal 20 kDa fragment and opens up the binding site for the LF or EF. PA consists of 4 domains:

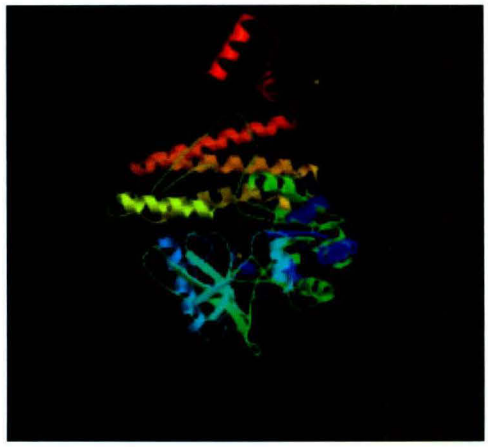
Domain 1 (residues 1–258) contains the cleavage site for furin (RKKR; residues164–167) in a surface loop. Thus, PA20 corresponds to residues 1–167, equivalent to about two thirds of the domain. The remainder of domain 1 (residues 168–258) forms the N terminus of PA63). Domain 2 (residues 259–487) consists of a β-barrel core with elaborate excursions and a modified Greek-key topology. The major function of this domain is to form a transmembrane pore to serve as the portal of entry of EF and LF into the cytosol. Recently, domain 2 has also been shown to participate in binding PA83 to cellular receptors (Lacy D B *et al.,* 2004, Santelli *et al.,* 2004). Domain 3 (residues 488–595) is believed to function primarily in self-association of PA63 (Mogridge *et al.,* 2001). Mutagenesis experiments have demonstrated its involvement in oligomerization (Mogridge *et al.,* 2001). Domain 4 (residues 596–735) consists primarily of a β-sandwich with an immunoglobulin-like fold and is primarily involved in receptor binding. This domain has relatively little contact with the rest of the protein (Rosovitz MJ *et al.,* 2003). Domain 4 is significantly reported to be the most immunogenic part, capable of conferring protection (Smith *et al.,* 2002).

Lethal factor



The crystal structure of LF was elucidated by (Pannifer *et al.,* 2001). Lethal factor is a 796 aa polypeptide and the functional toxin domain lies between aa 383 and 796. LF is highly specific metalloprotease (Duesbery *et al.,* 1998). The residues of LFn and EFn are crucial for recognizing the ligand site on oligomeric PA63 (Lacy *et al.,* 2002). LFn has been used as an intracellular delivery agent because of their unique capability to translocate antigen across the cell membrane without affecting cell viability and elicit immune response (K. McEvers *et al.,* 2005, Subhash Chandra *et al.,* 2006). It is still not very clear on how LeTx leads to macrophage destruction and death of host. A recent study using a mouse model demonstrated that both EF and LF could inhibit activation of T lymphocytes by T-cell receptor-mediated stimulation (Comer *et al.,* 2005). Experiments in mice show that lethal toxin suppresses cytokine secretion during infection (Drysdale *et al.,* 2007) and that cleavage of MKKs reduces production of NO and TNFα induced by LPS and IFNγ. These results suggest ways in which LeTx may impair the host immune system.

Edema Factor



Edema factor (EF) is a calmodulin-dependent adenylate cyclase. EF combines with PA to form edema toxin. Edema toxin converts adenosine triphosphate to cyclic adenosine monophosphate (cAMP). High intracellular levels of cAMP lead to impaired maintenance of water homeostasis and characteristic edema. The cAMP response generated by edema toxin requires an influx of calcium into the affected cells (Kumar *et al.,*

2002). Edema toxin also inhibits neutrophil function. It was found that edema toxin induces a dose dependent and time dependent increase in cAMP inside rabbit platelets (Alam S *et al.,* 2006). Recent studies in rabbit model show edema toxin acts indirectly or directly on an uncharacterized target stimulating the production or release of multiple inflammatory mediators, including neurokinins, prostanoids and histamine (Tessier *et al.,* 2007).

With the advent of the genomics providing the vital information regarding the sequence of plasmids pXO1 and pXO2, anthrax research has been much more diversified to gain new insights regarding anthrax pathogenesis. Virulence of *B. anthracis* appears to be related to plasmid quantities and clonality of the pX01 and PX02 plasmids within each bacterium (Coker *et al.,* 2003). The complete genomic sequence of *B. anthracis* (Ames strain) has been analyzed. Several chromosomally encoded potential virulence factors were identified, including hemolysin, phospholipases and iron acquisition proteins (Read *et al.,* 2003).

A newly described toxin, anthrolysin O (ALO), a cholesterol-dependent cytolysin, may represent a previously unidentified virulence factor of *B. anthracis*. When human neutrophils, monocytes and macrophages are treated with native or recombinant ALO, the protein provoked dose and time-dependent cytotoxicity (Mosser *et al.,* 2006). The experiments that paired ALO with three phospholipase C proteins (PLCs) in a murine mouse model revealed that ALO and PLCs might have overlapping roles in pathogenesis (Heffernan *et al.,* 2007).

PREVENTION BY VACCINATION

Be it the anthrax bioterror attacks or the emergence of the disease in the endemic areas, it has taught us adequate lessons reprimanding the need to encounter any such attacks more effectively. Considering the gruesome proportion of aftermath effects of anthrax, it is not very surprising that the current anthrax research pays undue attention to combat the disease in the most effectual ways. The most reliable means in this regard appears to be vaccination. From the earliest attempts of basic vaccination by Louis Pasteur to recent strategies of generating various subunit vaccines, the anthrax vaccines reveal a spectrum of developments and hold many more promises for the future.

Live spore vaccines

Anthrax has been one of the first bacterial diseases against which effective live vaccine was developed (Plotkin *et al.,* 1999). This veterinary vaccine developed by Louis Pasteur consisted of an attenuated strain of *B. anthracis* that resulted from continuous passaging of the culture for 8 days at elevated temperatures (42-43 °C). The culture used by Pasteur probably contained a mixture of bacilli with only the capsule-encoding plasmid pX02 and bacilli containing both the plasmids. The basis of the attenuation remained a mystery for about 100 years until Mikesell *et al.,* (1983) showed that increasing the culture temperature to 42 °C results in the partial loss of the toxin-encoding plasmid pX01.

Nowadays, live spores of the attenuated, non-encapsulated Sterne strain (34F2) are most commonly used to immunize animals. This strain, prepared by Max Sterne in South Africa in the late 1930s (Sterne, 1939), is a stable acapsulate mutant that produces all three toxin components of *B. anthracis* (PA, LF and EF), *i.e.,* possesses the pX01 plasmid, but lacks the pX02 plasmid responsible for capsule formation. Though this live-spore vaccine has proved to be safe and very effective, inducing a profound antibody response against the PA of *B. anthracis,* it wasn't precluded out of shortcomings. Problems with this vaccine include local inflammation at the site of inoculation, incomplete protection of vaccinated herd (Krishna PS *et al.,* 2007) and development of disease itself in some animals, e.g. llamas and mice (Cartwright ME *et al.,* 1987).

Protein vaccines

The demonstration of a ''protective antigen'' in cultures of *B. anthracis* (Gladstone, 1946) laid foundation for the development of two similar but distinct protein vaccines in the 1950s and 1960s, which are licensed and still used in USA and in UK. Both vaccines are produced from pX01/pX02$^-$ seed stocks. The UK vaccine in use since 1963 is prepared from cultures of the Sterne strain 34F2. The supernatant of a static culture grown in a protein hydrolysate medium containing charcoal is precipitated with potassium aluminium sulphate (alum) and preserved with thiomersal. All three toxin components are present in the vaccine although PA is the predominant antigen and the immune response is directed predominantly against PA and LF.

The US vaccine licensed by the Food and Drug Administration called 'AVA' (Anthrax Vaccine Adsorbed, known as BioThrax^{TM} since 2002), consists of an aluminium hydroxide (Alhydrogel) adsorbed culture filtrate of the avirulent strain V770-NP1-R. The main antigen of the vaccine is PA. Formaldehyde (0.02%) is added as a stabiliser and benzethonium chloride (0.0025%) as a preservative. The two licensed acellular vaccines have been shown to be protective in several animal models, including non-human primates. The protective properties of both BioThrax ^{TM} and the British vaccine appear to be very similar.

Problems associated with current vaccines

The above-described antiquated vaccines have been in use for more than a century but are accompanied by several side effects. The immunity generated by these vaccines is thought to be quite short-lived and annual boosters are recommended for both vaccines. As might be expected for rather old-fashioned vaccines, these complex acellular protein vaccines have a number of inherent problems, namely unknown duration of protective immunity as well as a number of clinically relevant side effects, such as local pain and inflammation at the site of injection and occasionally fever and general malaise. Since the inception of Vaccine Adverse Event Reporting System (VAERS) between 1990 and 2000, it received 1,544 reports of adverse events following anthrax vaccination. Recently, the association of lymphocytic vasculitis (Muniz AE, 2003) and an autoimmune disease – Oral Pemphigus vulgaris (Muellenhoff *et al.,* 2003) with the administration of AVA has

further instigated to find better substitutes to the existing vaccine. The Institute of Medicine (IOM) in 2002 also raised concerns over the safety of the anthrax vaccine, thus prompting for the venture of subunit vaccines and newer approaches for generation of better anthrax vaccines.

Novel strategies for better vaccine development

In the past years, there have been numerous research efforts to develop new and improved alternatives to currently used vaccines. One strategy to improve PA-based vaccines is to combine the antigen with adjuvants other than Alhydrogel. A CpG oligonucleotide motif compound added to the aluminum hydroxide adjuvant currently in AVA vaccine increased immune response kinetics, increased response rates, prolonged antibody decay rates and required less antigen per dose or fewer doses to achieve immunity in studies conducted in mice and guinea pigs (Gu *et al.,* 2007). Other approaches for developing a novel anthrax vaccine include the use of recombinant *B. subtilis* (Duc le H *et al.,* 2007), salmonellae (Strokes *et al.,* 2007), viral vectors (McConnell MJ *et al.,* 2007), vaccine expressed in plants (Aziz *et al.,* 2005) or plasmid DNA (Midha S and Bhatnagar R, 2008) as vehicles for PA. The recombinant organisms, as well as the bacterial DNA used in these studies, serve as natural adjutants, thus improving the immune response elicited. Different routes of administering anthrax vaccine, which is currently injected subcutaneously, have also been investigated. Novel approaches focused mainly on oral (Grienstein *et al.,* 2005), nasal (Beilinska *et al.,* 2007; Haung *et al.,* 2007) and also intradermal application (Mikszta *et al.,* 2007). The rationale behind this research is that these routes of delivery will elicit a strong mucosal immune response, which is thought to give better protection especially in the case of inhalational anthrax. Combinatorial vaccines of anthrax and plague to combat bioterrorism may also contribute to next generation vaccines (DuBois *et al.,* 2007).

Various other research efforts attempt to identify additional antigens of *B. anthracis* with protective properties. Some of these antigens like basic outer structures of the spore (*e.g.* BclA (Brahmbhatt TN *et al.,* 2007) or the poly-g-D-glutamic acid capsule (Rhie *et al.,* 2003), (Glomski *et al.,* 2007a, b) confirmed to be immunoprotective against anthrax. Apart from these, there are a number of genetically engineered protein molecules like

mutants of PA (Ahuja *et al.,* 2003), LF (Gupta *et al.,* 2006), EF (Kumar *et al.,* 2001), polyvalent inhibitors (Mourez *et al.,* 2001), peptide based LF inhibitors (Turk *et al.,* 2004) and recombinant antibody fragments (Maynard *et al.,* 2002, Cirino *et al.,* 1999) that can be used for therapeutic purpose. But therapy of anthrax remains mainly a damage control exercise and therefore a next generation vaccine becomes the need of the hour.

Protective antigen as the best vaccine candidate

Protective immunity against infection with *B. anthracis* is almost entirely based on a response to the protective antigen (Pezard et. al., 1995). The effectiveness of both animal and human vaccines depends on the induction of anti-PA antibodies. Antibodies to PA can also reduce the ability of *B. anthracis* spores to germinate and to survive phagocytosis by macrophages (Welkos *et al.,* 2001). Thus, the induction of PA specific antibodies is believed to be a key requirement for an improved anthrax vaccine. A third generation anthrax vaccine employing the concept of safe subunit vaccine has been accepted and recombinant PA based vaccine clinical trials are underway.

Initially, two slightly different types of rPA vaccines produced by VaxGen Inc. in the USA and Avecia in the UK were undergoing accelerated development under the US Bioshield Program. Although clinical trials of the VaxGen rPA vaccine showed it to be safe and immunogenic (Gorse *et al.,* 2006), the US Government contract to supply stockpiles of this vaccine was withdrawn in December 2006. These vaccines failed on the grounds of stability of the antigen, which is a prerequisite when considering the aspect of long term storage and stockpiling. Thus, a vaccine that is "safe, efficacious, easy to administer, having long shelf life and of course devoid of any side effects" is the need of the hour.

PLANT BASED VACCINES-A BETTER ALTERNATIVE?

Over the last decade, plants have emerged as a convenient, safe and economical alternative to mainstream expression systems, which are based on the large-scale culture of microbes or animal cells. Plants have the potential to provide virtually unlimited quantities of recombinant antibodies, vaccines, blood substitutes, growth factors, cytokines, chemokines and enzymes for use as diagnostic and therapeutic tools in healthcare, the life sciences and the chemical industry. After the introduction of the concept of transgenic plants as edible vaccines in 1992 by Arntzen and colleagues, the field of plant-based vaccines has increasingly attracted the attention of scientists.

Transgenic plants have been successfully utilized for the expression of various antigens. The primary goal of plant based vaccines is that the plant expressed antigen purified and delivered parenterally or with simple food processing techniques, when expressed in palatable parts of the system and administered orally, is to able generate sufficient immune response to protect against the disease in question. Plant produced vaccines can be grown locally where needed, avoiding storage and transportation costs. Relevant antigens are naturally stored in plant tissue and oral vaccines can be effectively administered directly in the food product in which they are grown, eliminating purification costs. In many instances, it appears that refrigeration will not be needed to preserve vaccine efficacy, removing a major impediment to international vaccination efforts of the past (Fischer R 2004). Plant vaccines can express entire selected proteins, but the use of DNA encoding only desired antigenic sequences from pathogenic viruses, bacteria and parasites has received considerable attention (Mahon BR *et al.,* 1998). The key immunogenic proteins or antigenic sequences can be synthesized in plant tissues and subsequently ingested as edible subunit vaccines (Ma JK *et al.,* 2005). Oral delivery is immunologically more advantageous in generating effective mucosal and systemic immune response. This dual immune response becomes pivotal especially in case of infectious diseases where the pathogen makes its entry in to the system crossing the first line of defense.

The first demonstration of expression of a vaccine antigen within plants occurred in 1990 when Curtiss and Cardineau expressed the *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco (Curtiss *et al.,* 1990). This demonstration was closely followed by

plant expression of the hepatitis B surface antigen (HbsAg), the *E. coli* heat–labile enterotoxin responsible for diarrhea, the Norwalk virus capsid protein (Wamsley *et al* ., 2000) and the rabies virus glycoprotein (Mc Garvey *et al.,* 1990). Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans. Indeed, today many of the plant based vaccines have progressed towards the road of commercialization. A plant produced poultry vaccine against Newcastle disease virus has already been commercialized (www.dowagrosciences.com). Several other plant based vaccines against human and animal pathogens in edible or non-edible crops are in pipeline. Traditionally, plant derived vaccines were confined to target infectious diseases, but recently the production of auto-antigens in plants for oral tolerance therapy of autoimmune diseases (Carter *et al.,* 2002) and allergic diseases (Yang *et al.,* 2007, 2008) has also been shown to be feasible In addition, attention is being directed to the production of epitopes in plants that target cytotoxic activity against tumours (Firat H *et al.,* 1998). The expansion of plant based vaccines to non infectious diseases is demonstrated by the recent success of vaccine against Non Hodgkins lymphoma (Artzen *et. al.,* 2008).

The quest for the development of anthrax plant based vaccine was explored for the first time with the expression of protective antigen of the anthrax toxin in tobacco by Agro bacterium-mediated transformation (Aziz *et al.,* 2003). This was followed by expression of protective antigen in tobacco chloroplasts and generation of protective immune response in mice when immunized parenterally (Watson *et al.,* 2004, Aziz *et al.,* 2005). Other documented reports include expression of domain 4 of PA in spinach (Sussman, 2003) and the generation of protective immune response with fusion protein Domain-4 of PA and N-terminal peptide of lethal factor via viral expression vectors (Chichester JA *et al.*, 2007). The concept of edible vaccine still needs to be materialized in case of anthrax vaccine.

BENEFITS OF ORAL VACCINES

Benifits	Characteristics
Oral delivery	The plant cell wall, consisting essentially of cellulose and sugars, provides protection in the stomach and gradual release of the antigen in the gut
Use as raw food or dry powder	The vaccinogenic plant tissue may be used as raw food, dried or, alternatively, proteins maybe partially or fully purified and administered in capsules as dry powder
No need for "cold chain"	The vaccinogenic plant parts or plant extracts can be stored and shipped at room temperature
Mucosal and serum immune response	Plant-derived vaccines are primarily designed to trigger the mucosal immune system (IgA),thus preventing pathogen entry at mucosal surfaces; they also elicit serum and, possibly,cytotoxic responses
Cost efficiency	Production cost will be reduced 100–1000 times as compared with that of traditional vaccines
Optimized expression system	Plants may be engineered to accumulate the antigen in convenient intracellular compartments(endoplasmic reticulum, chloroplast)
Ease of genetic manipulation	Procedures essentially rely on established molecular and genetic manipulation protocols; these are already available in developing countries
Ease of production and scale-up	GM-plants can be stored as seeds. Unlimited vaccine quantity can be produced from these unlimited time; production and management is suitable for developing countries
Safer than conventional vaccines	Lack of contamination with mammalian pathogens
Ideal to face bio-weapons	Safety and cost efficiency propose plants plant-derived vaccines as an ideal tool to face bio-terrorism
Ideal for veterinary use	Cost affordable Ready for use as food additive

THE GENERATION OF PLANT BASED VACCINES

For the generation of effective plant based vaccine we need to contemplate on the following aspects:

1. THE CHOICE OF CROP SPECIES

The plant species for vaccine production is chosen according to the desired mode of antigen delivery. Edible food crops are opted when the antigen has to be delivered orally whereas non food crops can be chosen in cases where antigen requires purification prior to use. The most common species selected to serve as an expression host for vaccine antigens is tobacco (Mason *et al.,* 1992, Sojikul *et al.,* 2004, Molina A *et al.,* 2004, Aziz *et al.,* 2003, 2005), for which well established transformation and regeneration techniques are available. Tobacco is also easy to propagate and grow on a laboratory scale, rapidly producing a large biomass, and it has no requirement for special growth conditions. One major drawback of the tobacco plant is the production of toxic secondary metabolites, particularly nicotine alkaloids, which limits its use for oral delivery but nevertheless can serve as plant biofactories. Fruit and vegetable, crops, Edible leafy crops, and seed crops are routinely used for edible vaccine production.

Fruit and vegetable crops have been utilized in antigen production for oral vaccines since they can be consumed raw or processed to various palatable forms without cooking. Reported examples include bananas (Kumar *et al.,* 2005), tomatoes (McGarvey *et al.,* 1995, Soria-Guerra RE *et al.,* 2007, Zhou F *et al.,* 2008), cherry tomatillos (Gao *et al.,* 2003), potatoes (Haq *et al.,* 1995) and carrots (Bouche *et al.,* 2003, Rosales-Mendoza S *et al.,* 2008). The tomato is a major vegetable plant grown worldwide that possesses a high fruit biomass yield per hectare. Another widely cultivated high-yield Solanaceae-family crop plant is potato. Well-established and efficient techniques for potato transformation are available, and more over, potato has served as a host for several vaccine antigens (Gomez E *et al.,* 2008, Choi N W *et al.*, 2005).

Edible leafy crops selected for vaccine antigen production include alfalfa (Dong *et al.,* 2005 Joensuu JJ *et a.,l* 2006), lettuce (Kapusta *et al.,* 1999, Kim TG *et al.,* 2007), spinach (Modelska *et al.,* 1998) and white clover (Lee *et al.,* 2001). Lettuce and spinach are regularly consumed by humans and thus, are suitable as delivery vehicles for human oral

vaccines. Several seed crops are being investigated as potential production systems including cereals, (rice, wheat, barley and maize) legumes (peas and soybean) and oilseeds (safflower, oilseed rape) (Stoger *et al.,* 2005). The expression of antigens in seeds enables long-term storage even at room temperature, because seeds have the appropriate natural biochemical processes to enable stable protein accumulation, dehydration and preservation.

Among cereals maize is the most common crop used for antigen production (Taket C O *et al.,* 2004). Recently, Rice has also been used to express vaccine antigens (Matsumoto Y *et al.,* 2008, Oszvald M *et al.,* 2008) The leguminous plants pigeon pea (Satyavathi *et al.,* 2003), soybean (Piller *et al.,* 2005, Moravec T *et al.,* 2007) and peanut (Khandelwal *et al.,* 2005) have been reported to express vaccine antigens. They are capable of Rhizobium related nitrogen fixation and possess a high protein content in terms of reduced fertilization costs.

2. METHODS OF TRANSFORMATION

The amenability of the crop to transformation, the expression yields, mode of antigen delivery decide as to which method has to be applied for transformation. Routinely three different methods of transformation are followed as per the requirement: The stable agro bacterium-mediated transformation, the chloroplast transformation and the transient viral expression method.

2.1 Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation has been the method of choice for plant biotechnologists for many decades. Briefly, in nature, the transformation process involves transfer of a defined segment of DNA (the T-DNA possessing a set of oncogenes and opine catabolism genes) that is bordered by 25 bp left and right border repeats to the plant cell genome. The entire process of transportation and integration into the genome is governed by a repertoire of bacterial and host cell factors. (Tzfira T *et al.,* 2002, Gelvin SB *et al.,* 2003). This natural genetic transformation process is exploited to produce recombinant agrobacterium strains in which the native T-DNA is replaced with genes of interest. These recombinant strains are used to transform plants and most of the first

generation plant vaccines have applied this method of transformation (Kong Q *et al.,* 2001, Chikwamba R *et al.,* 2002, Rigano M M *et al.,* 2004).

Despite widespread implications of this method, many crop species including monocotyledonous crops which could now be transformed, the question of inadvertent dissemination of transgenes into the environment remained unsolved. Production of male sterile lines could offer one possibility, but it is not the ultimate solution. Besides the safety concerns regarding the use of 'agrobacterium' itself, created real obstacles for plant biotechnologists to proclaim patents. In pursuit of safe methods of transformation scientists have explored the genetic transformation in non Agrobacterium species - *Rhizobium sp. NGR234, Sinorhizobium melilot* and *Mesorhizobium loti* (Broothaerts W *et al.,* 2005). Thus far, no vaccine antigen has been expressed in plants by using these bacteria.

2.2 Chloroplast Transformation

Chloroplast transformation technology has gained prominence over other current methods of transformation because it provides readily obtainable high protein levels (up to 45 % TSP has been reported), the feasibility of expressing multiple proteins from polycistronic mRNAs and gene containment through the lack of pollen transmission (Pal Maliga, 2002). Plastid transformation in higher plants is based on DNA delivery by the biolistic process (Svab Z *et al.,* 1990) or polyethylene (PEG) treatment (Golds T *et al.,* 1993). The transformation occurs via homologous recombination between the plastid-targeting sequences of the transformation vector and the targeted region of the plastid genome. In order to obtain a genetically stable plant, all genome copies have to be uniformly transformed (Bock R 2001, 2007). Immunologically active peptides in amounts of up to 33.1 % of total soluble have been obtained by chloroplast transforamtion (Molina *et al.,* 2004). Plastid transformation currently works efficiently only in solanaceous plants, such as tobacco (Daniel H *et al.,* 2001, Tregoning J *et al.,* 2004, Aziz M A *et al.,* 2005, Svab and Maliga, 1993), tomato (Ruf *et al.,* 2001) and potato (Sidorov *et al.,* 1999), but it has been introduced into other crop species, including carrot (Kumar *et al.,* 2004a), cotton (Kumar *et al.,* 2004b), soybean (Dufourmantel *et al.,* 2004), lettuce (Kanamoto H *et al.,* 2006) and oilseed rape (Hou *et al.,* 2003).

2.3 Transient Expression

Although stable transgenic plants offer several advantages, they often suffer from relatively low protein expression levels and long periods of time required for generation, screening, selection and characterization of plant lines. Several plant viruses have been engineered to accommodate a foreign gene, which provides replication *in planta* to boost mRNA levels and thus enhance translation and protein accumulation significantly. Transient expression with plant virus vectors can generate very high levels of recombinant protein within days after delivery of the vector into plant leaves (Fischer R *et al.,* 2004). High expression levels in short time span have made viral expression a very lucrative option especially with production of therapeutic proteins and vaccines in plants. Transient expression in plants by viruses has offered better alternative strategies in cases where antigen needs to be purified in large amounts and transgene integration is not an obligation.

Two processes are being routinely used today:

2.3.1 Viral vector-based transient amplification in plants

Transient expression with plant virus vectors can generate very high levels of recombinant protein within days after delivery of the vector into plant leaves. RNA viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) are engineered to contain a foreign gene under the control of a strong sub genomic promoter. Two strategies of viral expression in plants are generally followed:

The first one was to design a vector that is as capable of infecting a plant as a wild-type virus, but is in addition engineered to carry and express a heterologous sequence coding for a gene of interest (a 'full virus' vector strategy). Examples in which epitopes of vaccine antigens have been successfully displayed as a part of chimeric plant viruses include *Cowpea mosaic virus* (CPMV) (Dalsgaard *et al.,* 1997), *Tobacco mosaic virus* (TMV) (Koo *et al.,* 1999), *Alfalfa mosaic virus* (AlMV) (Yusibov *et al.,* 2005), *Plum pox potyvirus* (PPV) (Fernandez-Fernandez *et al.,* 1998) and *Potato virus X* (PVX) (Marusic *et al.,*2001). Successful co-expression of antigens has been employed with TMV

(Wigdorovitz *et al.,* 1999), PVX (Franconi *et al.,* 2002), and PPV (Fernandez-Fernandez *et al.,* 2001).

The other, later trend attempts to 'deconstruct' the virus by eliminating the functions that are limiting. In "deconstructed virus strategy" the TMV genome is delivered as two separate DNA constructs that undergo recombination to generate a full viral genome. The studies at Icon Genetics (Halle, Germany) showed that the efficiency of this system could be greatly enhanced by introduction of introns into the DNA copy of the viral genome in order to prevent aberrant mRNA splicing. Icon showed recombinant protein production at levels up to 5 mg per g leaf mass in *Nicotiana benthamiana*, only 10-14 days after vector delivery (Gleba *et al.,* 2005, 2007).

2.3.2 Transient Expression with Agroinfiltration

In agroinfiltration, the Agro bacterium culture is forced into intact or harvested plant leaf tissues by pressure, the transgene is allowed to get expressed in plant cells for some hours to a couple of days and subsequently harvested for the foreign protein (Kapila *et al.,* 1997). Initially, this technique was very convenient for preliminary laboratory-scale testing for transformation vector capacity and for production of small amounts of purified recombinant proteins. But now, proteins can be scaled up to milligram amounts, enabling it to develop into an efficient production platform (Twyman *et al.,* 2005). Recently, inhibition of gene silencing in agroinfiltrated leaves was shown to increase recombinant protein yields up to 50-fold (Voinnet *et al.,* 2003). Large scale agroinfiltration is currently being utilized by the Canadian biotechnology company, Medicaco (www.medicaco.com) that is processing alfalfa material (D'Aoust *et al.,* 2005). No large-scale production of candidate vaccine antigens with this technique has thus far been reported, but agroinfiltration has been utilized for preliminary testing purposes (Lee *et al.,* 2001). Recently, the German company Icon Genetics (www. icongenetics.com) has established an agroinfiltration-based application which combines the transfection efficiency of *Agrobacterium* and the high expression yield of viral vectors (Marillonnet *et al.,* 2005).

3. OPTIMIZATION OF GENE EXPRESSION IN PLANTS

A mass immunization program with vaccines requires that the vaccine be produced in large quantities. All the first generation plant based vaccines are limited by their production levels. Antigen expression in plants for large-scale production of vaccines demands high yields in *planta*. The overall biomass yield of the crop species and the intrinsic protein content of the plant tissue define the capacity of the chosen production system. However, multiple factors determine the final antigen content of the production platform. To achieve high yields, the expression construct design must include all stages of gene expression, from transcription to protein stability. Approaches that have been utilized to give high levels of expression of foreign proteins in transgenic plants are discussed below:

3.1. Transcription and mRNA stability

The choice of the promoter in the expression construct plays an important role in controlling the transcription. The cauliflower mosaic virus 35S promoter (Odell *et al.,* 1985) was the mainstay of early phase work focused on expressing recombinant proteins in dicot leaves. Using this promoter, fairly high levels of accumulation of recombinant proteins have been achieved (Fiedler *et al.,* 1997; Gutierrez-Ortega *et al.,* 2005). Duplication of the enhancer of this promoter amplifies the gene expression further (Smith *et al.,* Warzecha *et al.,* 2003, Dong *et al.,*2005). Other plant viral promoters include a cassava vein mosaic virus promoter (Verdaguer *et al.,* 1996), the C1 promoter of cotton leaf curl Multan virus (Xie *et al.,* 2003) and the promoter of component 8 of Milk vetch dwarf virus (Shirasawa-Seo *et al.,* 2005). Most of the viral promoters tested so far show activity in monocots (Shirasawa-Seo *et al.,* 2005). Inducible promoters and tissue specific promoters have also been used to enhance the expression levels. The auxin-inducible mannopine synthase (mas) P1, P2 dual promoter system (Arakawa *et al.,* 1997, Arakawa *et al.,* 2001, Yu and Langridge, 2001) have been widely used. The tuber-specific patatin promoters (Mason *et al.,* 1996, Lauterslager *et al.,* 2002) and maize seed-specific globulin-1 and γ-zein promoters (Chikwamba *et al.,* 2002b, Streatfield and Howard, 2003a) have also been used by various groups to increase expression levels of the antigens.

3.2 Introduction of scaffold regions

Introduction of global regulatory sequences next to promoters can boost transcription to a great extent. These scaffold regions interact with the plant scaffolds *in vitro* and aid in recruitment of transcription factors to the promoters (Li e *at al.,* 2002).When certain transcription factors are limiting to the host plant, the introduction of transcription factors by coexpressing with transgenes can also boost expression by 2-4 folds (Yang *et al.,* 2001). A transcription factor can also be provided transiently by the inoculation of a transgenic line with plant viral sequences encoding the factor (Hull *et al.,* 2005). In addition to stable nuclear transgenic systems, the co-expression of transacting factors has been applied to transplastomic plants. For example, T7 RNA polymerase has been expressed from the nuclear genome and then targeted to the plastid, where it can transcribe sequences integrated into the plastid genome (McBride *et al.,* 1994). So far, such modifications have not been applied in generating a plant-based vaccine.

3.3 Stabilization of transcript

After transcription, the mature transcript has to be protected from premature degradation and transported efficiently to the cytoplasmic translation machinery. The related posttranscriptional eukaryotic processing events (capping, splicing, polyadenylation) can have a major effect on the levels of protein produced in the plant cell (Gutierrez *et al.,* 1999). Polyadenylation signals strongly influence the stability of mRNA and the level of gene expression in plant cells (Ingelbrecht *et al.,* 1989, Hunt, 1994). Widely used polyadenylation signals in vaccine applications include those from the CaMV 35S transcript (Huang *et al.,* 2001, Piller *et al.,* 2005), the soybean vegetative storage protein (vsp) (Richter *et al.,* 2000, Viera da Silva *et al.,* 2002), and the Agrobacterium nopaline synthase (nos) and octopine synthase (ocs) genes (Mason *et al.,* 1992, Aziz M A *et al.,* 2005) Comparision of vsp, potato proteinase inhibitor 2 (pin2) polyadenylation signals with nos-terminator in potato revealed accumulation of Hepatitis B virus surface antigen (HBsAg) at several-fold higher level in constructs with vsp and pin2 polyadenylation signals indicating that posttranscriptional effects contribute strongly to the enhanced expression of HBsAg (Ritcher *et al.,* 2000).

3.4 Translation

Translation initiation is the rate limiting step in polypeptide synthesis of plants (Kawaguchi and Bailey-Serres, 2002). The initiation can be optimized by assuring that the translational start-site matches the Kozak consensus of the plants (Joshi *et al.,* 1997). After initiation, the rate of translation may become limited by a lack of suitable tRNAs. The codon composition of foreign gene may not be compatible for plant translational machinery. Modification of coding sequences of bacterial origin (Horvath *et al.,* 2000) or codon optimization of foreign genes with plant codon usage led to enhanced expression levels for various antigens such as *E. coli* heat-labile enterotoxin subunit B (LT-B) (Mason *et al.,* 1998), HIV 1 tat protein (Karasev A V *et al.,* 2005), cholera toxin subunit B (CT-B) (Kang *et al.,* 2004a) and TGEV spike glycoprotein (Tuboly *et al.,* 2000, Streatfield *et al.,* 2001). 5'non translated regions of plant viral origin have been identified to boost antigen expression in plants (Dowson Day *et al.,* 1993). Most widely used 5′ untranslated regions used in antigen applications include 5′ leader sequences from *Tobacco etch virus* (TEV) (Thanavala *et al.,* 1995, Mason *et al.,* 1996, Richter *et al.,* 2000, Dong *et al.,* 2005) and TMV Ω element (Richter *et al.,* 2000, Matsumura *et al.,* 2002,Biemelt *et al.,* 2003).

3.5 Subcellular targeting of foreign proteins

Once the recombinant protein is made it is subject to subcellular environment where various factors such as pH, presence of chaperons or proteases can influence the folding, assembly and post translational modification. Thus, the stability and the overall protein yield may be affected. To save the protein from the above said factors recombinant proteins can be directed to the secretory pathway by N-terminal signal peptide. Such proteins are co-translationally synthesized in the endoplasmic reticulum (ER) and transported by default through the golgi network to the apoplast, or in the presence of a suitable signal directed to the vacuole (Matsuoka and Nakamura, 1991). HBsAg when targeted to the apoplast or vacuoles increased the expression levels by 2- to 7-fold accumulation levels in comparision with cytoplasm-targeted potato plants (Ritcheret *et al.,* 2000). Similarly when LT-B in maize was targeted to vacuole and apoplast the expression levels increased by 3080-fold and 20,000-fold, respectively (Streatfield *et al.,*

2003). Enhanced expression levels have been noticed when protein is retrieved to ER lumen itself using H/KDEL/SEKDEL C- terminal peptide tag (Haq *et al.,* 1995). However, studies by Streitfield *et al.* show conflicting results where ten-fold less LT-B was produced when retained in the ER than targeted to the secretion pathway.

For some organelles, including plastids and mitochondria, alternative signal sequences offer suborganellar options for targeting recombinant protein. For recombinant proteins synthesized in the plastid from sequences integrated into the plastid genome, suborganellar targeting to particular locations within this organelle can be considered. Given their endosymbiotic origin, plastids may be particularly well suited to the production of bacterial proteins. Recombinant proteins are not glycosylated, but correct disulphide bonding can be accomplished (Staub *et al.,* 2000). In addition, other post-translational modifications such as lipidation, can be achieved, as for the outer surface lipoprotein A of *Borrelia burgdorferi* expressed from the tobacco plastid genome (Glenz *et al.,* 2006).

3.6 Protein fusions

Fusing peptides of whole proteins of poor stability to other known stable proteins can improve the stability of the selected antigen in the plant tissues and in the subsequent vaccine delivery. This approach is commonly used for peptides produced with the plant virus expression system (Canizares *et al.,* 2005) and has also been used to optimize the antigen expression in stably transformed plants. Such vaccine antigen fusion partners include green fluorescence protein (GFP) (Molina *et al.,* 2004), CT-B (Arakawa *et al.,* 2001, Yu and Langridge, 2001, Kim *et al.,* 2004b, Lee *et al.,* 2004, Molina *et al.,* 2004, Molina *et al.,* 2005), CT-A (Yu and Langridge, 2001), and β- glucuronidase (GUS) (Gil *et al.,* 2001). Antigen fusion with marker genes also allows antigen production to be screened conveniently. Fusion of a foreign protein or peptide to a second recombinant protein that has been shown to be stably expressed in plants can act to stabilize the target protein or peptide. The fusion of a tuberculosis antigen to the receptor binding subunit of the heat-labile toxin of *E. coli* followed this strategy (Rigano *et al.,* 2004). An added advantage in this case is that the heat-labile toxin subunit can direct the fused antigen to

ganglioside receptors on the surface of the gut, facilitating delivery of the product to the target tissue.

3.7 Genetic approaches to boost expression

Several genetic approaches have been applied to boost expression, including increasing the transgene copy number through selfing or crossing and introducing foreign genes into germplasm suited to their over-expression. Increasing transgene copy number with stable transgenics, taking a single transgene to homozygosity through selfing doubles transgene representation and typically boosts expression (Zhong *et al.,* 1999). Moreover, crossing high-expressing transgenic lines arising from independent transformation events can boost transgene copy number and expression levels. This approach to achieve high gene copy numbers appears to have a reduced risk of gene silencing in comparison with making multiple gene insertions during transformation.

Certain germplasm is well suited to the expression of proteins at high levels. For example, maize lines have been developed over many generations with elevated protein or oil levels in seeds (Bhattramakki and Kriz, 1996). High-protein lines have the potential for the production of more recombinant protein in the seed on a weight basis. In high-oil lines, the increased proportion of oil is primarily a result of increased embryo size. The use of high-oil lines has been shown to boost recombinant protein expression approximately four-fold in maize (Hood *et al.,* 2003).

4. USE OF SELECTION MARKERS

The success of genetic modification of plants is determined by the ability to deliver foreign DNA into the host plant and the efficiency with which shoots or somatic embryos can be regenerated from transformed cells. Marker genes aid in selectively choosing the transformed plants and saves time prior authenticating transgenics by molecular analysis. These conditional dominant genes confer resistance to an antibiotic that kills non-transformed cells or confer a metabolic advantage on transformed cells in the presence of a nontoxic selective agent. Genes encoding hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat or bar*) and neomycin phosphotransferase (*nptII*) are widely used to select transformed plants. Aminoglycosides like spectinomycin, and

streptomycin are used for selection of transformed lines for chloroplast transformation. Mannose-6-phosphate isomerase (MPI) is a recently developed selectable marker. The enzyme is encoded by *manA* from *E. coli*, which converts the unusable carbon source mannose-6P to fructose-6P (Hansen *et. al.*,2005) The positive selectable marker phosphomannose isomerase was first used for agrobacterium-mediated transformation of sugar beet and was recently used to enhance transformation of sorghum (Lucca *et al.*, 2001; Gao *et al.*, 2005).

The inclusion of marker genes in plant-based vaccines raises serious public concerns about the medical implications especially when the vaccine is consumed in edible form and its detrimental effects on the environment. Therefore, strategies have been developed for the removal of marker genes. The most recent development is the use of inducible site-specific recombinase system (Hare and Chua 2002).

5. MOLECULAR ANALYSIS OF TRANSGENE EXPRESSION

PCR amplification of the marker gene or transgene is often taken as an indication of transgenic status of transgenic plants. However, Southern analysis is essential to prove the integration of the foreign gene into host genome. Also, Southern hybridization is useful to assess the number of independent insertions of trans-gene. Since single, unaltered transgene insertions are ideal for analysis, it is also necessary to check the presence of multicopy tandem insertions and other rearrangements at the given locus. RT-PCR, northern and western hybridization techniques are employed to assess the expression of the introduced gene. Enzyme Linked Immunosorbent Assay (ELISA) is usually done to determine the expression level of biomolecules. Bioassays, if available, can also provide the proof of functionality of the transgene product.

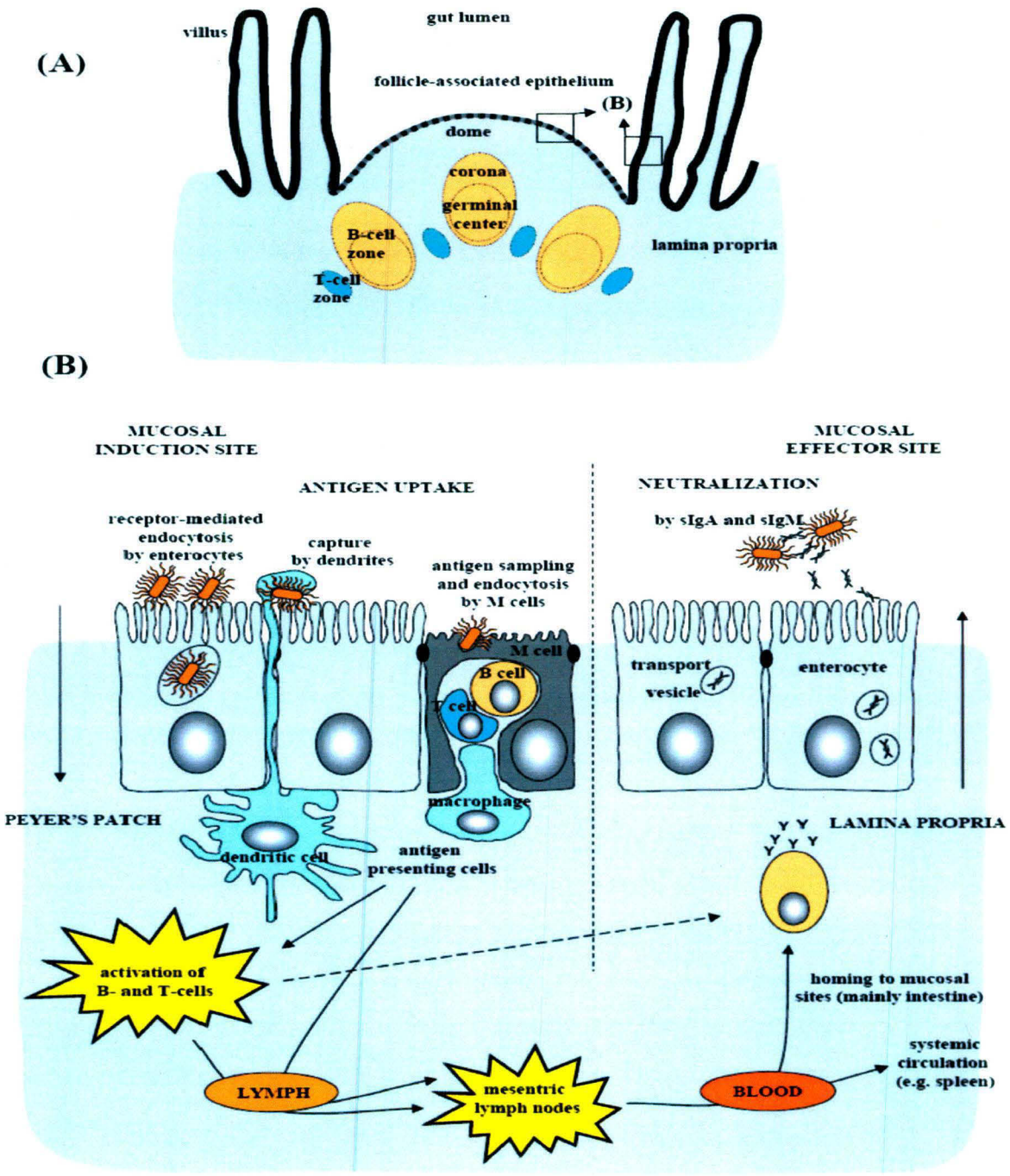
MUCOSAL IMMUNITY

The success of an edible vaccine requires induction of the mucosal immune system (MIS). The MIS is the primary defense of the surfaces where most human and animal pathogens initiate infection, that is, the mucosal surfaces found lining the digestive tract, respiratory tract and urino-reproductive tract. Induction of a mucosal immune response starts with the recognition of an antigen by specialized cells called M-cells. These cells are localized in the mucosal membranes of lymphoid tissues such as Peyer's patches within the small intestines. The M-cells channel the antigen to underlying tissues where antigen-presenting cells internalize and process the antigen. The resulting antigenic epitopes are presented on the APC surface and with the assistance of helper T cells activate B cells. The activated B cells migrate to the mesenteric lymph nodes where they mature into plasma cells and migrate to mucosal membranes to secrete immunoglobulin (IgA). Upon passing through the mucosal epithelial layer towards the lumen, the IgA molecules complex with membrane-bound secretary components and form secretary IgA (sIgA). Once transported into the lumen, the sIgA interacts with specific antigenic epitopes and neutralize the invading pathogen. The mucosal immunization of the gastrointestinal tract is an effective way to stimulate local and systemic responses. Oral vaccines must be formulated in such a way that antigens are protected as they pass through the adverse environments of the stomach and are delivered to the mucosal inductive sites. Vaccine antigens expressed in edible transgenic plants are promising new delivery systems for oral vaccine. Such vaccines could be safe, inexpensive and multi-component.

The studies completed so far in animals and humans have provided a proof of principle; they indicate that the strategy of Edible Vaccines is feasible. Yet, many issues must still be addressed. For one, the amount of vaccine made by a plant is low. As researchers solve that challenge, they will also have to ensure that any given amount of a vaccine food provides a predictable dose of antigen. Additionally, workers could try to enhance the odds that antigens will activate the immune system instead of passing out of the body unused. General stimulators (adjuvants) and better targeting to the immune system might compensate in part for low antigen production. One targeting strategy involves linking antigens to molecules that bind well to immune system components known as M cells in

the intestinal lining. M cells take in molecules that have entered the small intestine (including pathogens) and pass them to other cells of the immune system, such as antigen-presenting cells. Macrophages and other antigen-presenting cells chop up their acquisitions and display the resulting protein fragments on the cell surface. If white blood cells called helper T lymphocytes recognize the fragments as foreign, they may induce B lymphocytes (B cells) to secrete neutralizing antibodies and may also help initiate a broader attack on the perceived enemy.

It turns out that an innocuous segment of the *V. cholerae* toxin--the B subunit--binds readily to a molecule on M cells that ushers foreign material into those cells. By fusing antigens from other pathogens to this subunit, it should be possible to improve the uptake of antigens by M cells and to enhance immune responses to the added antigens. These features raise the prospect of producing a vaccine that brings several different antigens to M cells at once thus potentially fulfilling an urgent need for a single vaccine that can protect against multiple diseases simultaneously. There is a need to be sure that vaccines meant to enhance immune responses do not backfire and suppress immunity instead. Research into a phenomenon called oral tolerance has shown that ingesting certain proteins can at times cause the body to shut down its responses to those proteins. To determine safe, effective doses and feeding schedules for edible vaccines, manufacturers will need to gain a better handle on the manipulations that influence whether an orally delivered antigen will stimulate or depress immunity.



Induction of a mucosal immune response at the intestine. (A) Structure of Peyer's patches. (B) Antigens are transported across the mucosal barrier by M-cells, enterocytes, or dendritic cells, and antigen-presenting cells induce activation of antigen-specific mucosal and systemic immune responses. The main protection at mucosal surfaces is provided by the secretory immunoglobulins transported to the lumen. Adapted from Neutra and Kozlowski, 2006.

CHALLENGES POSED BY PLANT BASED VACCINES

Despite many advantages of plant based vaccines, a myriad of challenges are yet to be overcome before the promise of this technology will be fully realized. The challenges facing plant-based-vaccine development include technical, regulatory, economic aspects and public perception.

- Increasing the yield of expression so that crop provides adequate biomass for antigen extraction or storage.
- Protocols needed to ensure transcription, translation, intracellular localization, tissue specificity, adequate gene copy number, metabolism and accumulation of protein of interest (Buetow and Korban, 2000; Streatfield and Howard, 2003).
- Determining the level of expression of the transgene and stability of expression over generations of the transgenic line will be essential for determining the economic feasibility of a proposed plant-based vaccine.
- Standardizing of appropriate formulation of vaccine for edible crops to ensure stability of the vaccine and standardization of dosage and route of delivery.
- Prevention of environmental dissemination and opting for more environmental friendly approaches for plant generation e.g. by chloroplast transformation, use of male-sterile lines for transformation or self pollinating crops.
- Avoidance of commingling of vaccine crop with the food crop when the vaccine is produced in edible crop by complete physical separation of the transgenic crop.
- Obtaining regulatory approval by concerned authorities during clinical trials and authentication of efficacy, safety and reliability of the vaccines.

Chapter 2

Aims and objectives

INTRODUCTION

Anthrax is a zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*, normally associated with domestic livestock such as sheep, goats, and cattle, but humans also get infected due to exposure or consumption of infected animals. The recent bioterrorism attacks and the resurgence of the disease in endemic areas have once again cautioned the mankind of its devastating effects. This imposes an urgent need for effective prophylactic measures and therapeutic formulations to combat the disease. Vaccination has been the most humane means to prevent the disease. The currently used anthrax vaccine is limited by being incompletely characterized, associated with several side effects due to its potential reactogenecity and requires protracted and complicated dosage schedule. A vaccine which is safe, affordable, well characterized and induces long lasting protective immunity would be suitable for mass vaccination.

Recently, the successful use of plants as expression systems for a number of pathogenic antigens has opened new vistas for vaccine production. Plant based vaccines are especially attractive as plants are free of human or animal diseases, reducing screening costs for viruses and bacterial toxins. They offer several advantages in being economical, easily adaptable to large scale operations, may not require purification when produced in an edible crop and offer a palatable oral delivery system that would preclude the costly purification process required by traditional injectable vaccines. Besides, plant based oral vaccines stimulate both systemic and mucosal immune response, thereby combating the infectious disease more efficaciously.

Plant based vaccines can be a boon to the impoverished countries of the developing world, especially in rural areas which often cannot afford to buy current vaccines. Wildlife and live stock outbreaks in endemic areas can be most effectively prevented by including the vaccine in feed or fodder meal. Besides, vaccine in edible form and also plant vaccine germplasm can be stored safe for years, thus can aid to attend any emergency situation.

Immunization studies against anthrax confirm the role of protective antigen (PA) as the major immunogenic molecule in humoral response generated by all the current anthrax vaccines. The present vaccines contain protective antigen as the main component. Though full length Protective antigen has been shown to provide protective immunity,

concerns have been raised over introducing a potential toxin subunit to individuals at risk of exposure to *Bacillus anthracis*. It has been shown that receptor binding component of PA i.e., Domain-IV (D-IV) alone is sufficient to induce protective immunity. Therefore, expression of Domain-IV (D-IV) as a vaccine in plants against anthrax can be explored. The principal objective of this research was aimed towards developing a new generation vaccine against anthrax. In an endeavor to develop improved vaccine against anthrax; we first aimed to express the protective antigen gene in economically important crop *Brassica juncea* (Indian mustard) by Agrobacterium-mediated transformation. Further, we intended to develop chloroplast transformation vectors to augment the expression levels in tobacco plastids. The latter part of the study also focuses on exploring 'Domain-IV (D-IV)' the most immunogenic portion of protective antigen as a vaccine candidate in *E. coli* as well as plants. Finally, the plant expressed immunogens would be tested in mice animal model to investigate for the immunogenicity and protection acquired with these molecules.

The objectives of the study are listed as under:

1. **Expression of protective antigen (PA) in economically important fodder crop '*Brassica juncea*' by Agrobacterium-mediated transformation.**

2. **Molecular analysis of putative transgenic plants to confirm the presence of transgene and its expression.**

3. **Functional activity of plant expressed PA with cytotoxicity assay on J774A.1 mice macrophage cell-line.**

4. **Cloning of Domain-IV (D-IV), 'the most immunological portion of protective antigen' in pCAMBIA vector-1303.**

5. **Agrobacterium-mediated transformation of tobacco with pCAM-D-IV construct.**

6. **Molecular analysis of putative transgenic plants for transgene presence and expression.**

7. **Construction of chloroplast transformation vector for expression in tobacco plastids.**

8. **Cloning of D-IV in chloroplast transformation vector.**

9. **Expression of PA and D-IV in tobacco plastids to enhance the expression levels.**

10. **Cloning, expression and purification of D-IV in *E. coli* expression vectors.**

11. **Characterization and evaluation of biological activity of PA from chloroplast transformed plants *in vitro* using J774.A1 cell line.**

12. **To test the efficacy of plant expressed antigens (PA and Domain–IV) in mice animal model.**

Chapter 3

Expression of Protective antigen gene in *Brassica juncea*

INTRODUCTION

Vaccination has been the most reliable approach to effectively prevent the occurrence of anthrax. The present human and the veterinary anthrax vaccines rely on the antiquated methods. The veterinary anthrax vaccine developed in 1930,'s by Sterne is essentially an attenuated, nonencapsulated, toxigenic strain of *Bacillus anthracis*, while the licensed human vaccine predominantly contains 'protective antigen', the main immunogenic component of the tripartite anthrax toxin. Both these vaccines have potential side effects. The ancient veterinary anthrax vaccine suffers from declining potency and troublesome variations in virulence that led to the death of animals occasionally and in both of these vaccines, the presence of residual virulence can effect protection of the subjects. Thus, a safe and effective vaccine devoid of any side effects is required to combat the disease more effectively.

Plants have offered immense potential to serve as hosts for candidate antigen expression. They are advantageous in being a low cost, providing easy scale up, devoid of other contaminants and the ease of delivery evading the requirement of medical professionals when delivered orally. Oral delivery becomes important especially to combat diseases that make their entry via mucosal lining. Oral vaccines are capable of efficiently generating both mucosal and systemic immune response. The bioencapsulation of the antigen in plant cell environment prevents from antigen degradation as it passes through the adverse environment of the gut. There are instances where an oral vaccine expressed in plants has been very effectively used to prevent the diseases whose primary infection sites are the mucosal linings. Successful reports of generating an immune response against enterotoxigenic strains of *E. coli* (Haq *et al.,* 1995, Streatfield *et al.,* 2001) was emulated by scientists to apply this concept in combating other diseases. With this idea, the generation of transgenic plants expressing protective antigen was conceptualized.

The present study focuses on the expression of protective antigen gene in *Brassica juncea*. *Brassica juncea* is commonly known as 'oriental mustard'. Apart from being used as a spice, mustard is widely used as leaf and stem-vegetables and as a salad crop in the Far East and Southeast Asia, and for green manuring or as a fodder crop mainly in Western Asia. It is also an important oilseed crop in the Indian subcontinent, China and the South-western areas of the former Soviet Union, where the crop is grown in a similar

manner to spring rapeseed. The versatility of the crop for both human and animal feed implications makes this crop very lucrative for expressing antigen. The predominant production area in the American zone comprises the Canadian prairie provinces of Manitoba, Saskatchewan and Alberta, and the adjacent states of North Dakota and Montana in the United States. Incidentally, this area of America comprises the endemic zone for anthrax. Therefore, the cultivation in these regions would make even easier for transport and delivery of the vaccine. Furthermore, the availability of standard transformation protocols, efficient regeneration, large biomass, prolonged stability and safe storage of antigen in oilseeds promoting the increase in shelf life, make mustard an ideal choice for edible vaccine. The self pollinating nature of this crop effectively prevents from unintended environmental escapes.

As discussed earlier, PA is the most immunogenic molecule for conferring immunity against anthrax. The protective antigen gene contains a 2319-bp-long open reading frame (ORF) of which 2205 bp. encodes for an AT rich (69 %) cysteine free 735 amino acid (82.7 k Da) secreted protein. This chapter deals with the expression of protective antigen (PA) gene in economically important fodder crop 'mustard'. The native PA gene was cloned in a binary vector and transformed into Agrobacterium strains. The conventional and most widely used method of gene transfer "The Agrobacterium -mediated transformation method" was employed to transform hypocotyl explants of mustard. From these transformed hypocotyls, a set of mustard plants were regenerated and selected on a high level of kanamycin to ensure minimum number of false positives. The hardening of plants was carried out to acclimatize them to the external environmental conditions. The expression of a fully functional protective antigen was ascertained by carrying out biological assays on a macrophage like cell line preceded by the molecular analysis of putative transgenic plants. The present study paves the way for the generation of a novel anthrax vaccine especially for animals thus, promoting to tackle anthrax in endemic areas more efficiently.

MATERIALS AND METHODS

Reagents and Supplies

Mustard seeds (*Brassica juncea*) were purchased from the Indian Agricultural Research Institute (IARI), Pusa, New Delhi. The enzymes and chemicals used for DNA manipulation were purchased from MBI Fermentas (USA) and NEB (USA). The oligonucleotides were obtained from Microsynth (Switzerland). DNA purification kit was obtained from Qiagen (Germany). Growth media and its components were from Hi-Media Laboratories (India). Plant hormones were procured from Sigma Chemical Co. (USA). Cell Culture plasticware was obtained from Corning (USA). Fetal Calf Serum (FCS) was from Biological Industries (Israel). RPMI 1640, 3-(4,5-dimethylthiazol-3-yl)-5-diphenyltetrazolium bromide (MTT), Phenyl methylsulfonyl fluoride (PMSF), HEPES, NaCl, EGTA, EDTA and other chemicals were purchased from Sigma Chemical Co. (USA). *E. coli* strain DH5α and J774A.1, a macrophage like cell line was obtained from ATCC (American Type Culture Collection; USA).

Bacterial Strains and Culture Medium

E. coli DH5α was used as the host for cloning and propagation of plasmids and was cultured in Luria-Bertani broth supplemented with kanamycin (50 μg/ml). *Agrobacterium tumefaciens* strain GV2260 was routinely grown in YEM (Refer to appendix for composition) supplemented with kanamycin (50 μg/ml) and rifampicin (10 μg/ml) when required.

Plant expression vector- pBINPAG

A Binary vector pBINAR- a pBin19 derivative, which employs constitutive CaMV35S promoter for gene expression and also carries bacterial *nptII* gene, (which codes for neomycin phosphotransferase that confers kanamycin selection) has PA gene cloned in it (Previous studies of the lab (Aziz *et. al.,* 2003). This plasmid was mobilized to *Agrobacterium tumifaciens* (GV2260) using freeze thaw procedure.

Transformation of *Brassica juncea*

1. *Agrobacterium tumefaciens* strain GV2260 containing a binary plasmid was grown overnight in 5 ml of liquid YEM supplemented with appropriate antibiotics.
2. Around 1 ml of the overnight grown culture (OD_{600} 1.2) was inoculated in 30 ml of YEM medium without antibiotics and the culture was grown to reach an OD_{600} of 0.5–0.6.
3. The cells were harvested by centrifugation and suspended in N1B1 medium [MS medium containing NAA (1 mg/ml) and BAP (1 mg/ml), kanamycin (50mg/l), and rifmpicin (10 mg/l)] to obtain an OD_{600} of 0.3. These cells were then used for infection of hypocotyl explants as described below.
4. Seeds were germinated aseptically in glass tubes (4–5 seeds per tube) on MS medium in dark for 1 day, then transferred to light (200 lux, 16 h light, 8 h dark) and maintained at 23 ± 1 °C for 5 days.
5. Approximately, 100 hypocotyl explants (0.5–1.0 cm) from the 5-day-old hypocotyls explants were kept in a 500 ml Erlenmeyer flask containing ~ 80 ml liquid N1B1 medium on a shaker (100 rpm) in diffused light for 12 h. (Overnight).
6. N1B1 medium was decanted and the explants were cocultutred with harvested agrobacterium cells for about half an hour on a rotary shaker.
7. Explants were rinsed twice for 1 min with 25 ml of N1B1 medium containing 200 mg/l augmentin and twice for 30 min with 80 to 100 ml of same medium on a rotary shaker (at 100 rpm).
8. The medium with all the explants was carefully poured in a petri-dish and the medium was removed with a pipette.
9. Excess bacterial suspension was removed by placing the explants on a filter paper and the explants were plated on selection media (MS + N1B1 medium containing augmentin 200 mg/l, AgNO3 20 mM).
10. Green plantlets that differentiated on the selection medium were transferred to the MS medium with IBA (2 mg/l), augmentin (200 mg/l) for rooting. Only one shoot from each explant was transferred in this manner.

11. Following rooting, which took place within 15–20 days; the shoots were subcultured at monthly intervals on the same medium till they were transferred to the soil. Control plants (untransformed) were also grown under similar conditions.

DNA isolation from plant tissues

DNA from mustard leaves was isolated with "DNeasy Plant Mini Kit" (Qiagen,). DNA isolated this way was subjected to PCR analysis. 100-200 mg of leaf material was used for DNA extraction. To increase final concentration, the amount of elution buffer was reduced by a factor of two.

PCR Analysis

Presence of the PA gene in the plantlets was confirmed by PCR using primers of PA gene.

A. Forward Primer: 5' GGA TTG GAT TTC AAG TTG TAC TGG ACC 3'

Reverse Primer: 5' CTT AAC TAC TGA CTC ATC CGC CCC AAC 3'

PCR reaction with primers for full length PA:

Step		**Recommended conditions**
Denaturation	**5 min**	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	94 °C
Annealing	0.5 min	60 °C
Extension	1 min/kbp (2 min)	72 °C (2.2 min)
Final extension	**7 min**	**72 °C**

Primers specific to CamV 35S (forward) and PA at internal site (reverse) {B}; *npt II* gene specific primers{C} were also used for PCR analysis to ensure the incorporation of the whole cassette. PCR reaction was carried out in Biorad iCycler under the following conditions:

B. Forward Primer: 5'ACC ACG TCT TCA AAG CAA GTG 3'

Reverse Primer: 5' CTT AAC TAC TGA CTC ATC CGC CCC AAC 3'

Step		Recommended conditions
Denaturation	**5 min**	**95 °C**
3-step cycling (30 cycles)		
Denaturation	1 min	94 °C
Annealing	1 min	58 °C
Extension	1 min/kbp (2 min)	72 °C (1.45 min)
Final extension	**7 min**	**72 °C**

C. Forward Primer: 5' ATG GGG ATT GAA CAA GAT GGA TTG CA 3'

Reverse Primer: 5' GCC CAT TCG CCG CCA AGC TCT TCA GC 3'

Step		Recommended conditions
Denaturation	**5 min**	**95 °C**
3-step cycling (30 cycles)		
Denaturation	1 min	94 °C
Annealing	0.5 min	56 °C
Extension	1 min/kbp (2 min)	72 °C (0.45 min)
Final extension	**7 min**	**72 °C**

Selected transgenic plantlets were potted in vermiculite mixture and maintained in the green house. Gradual hardening of these potted plants was done during winter season as the crop easily adapts to cold climate.

Extraction of total soluble protein and Western blot analysis

Total soluble protein was extracted from leaves of transformed plants by grinding them in liquid nitrogen in a pre-cooled mortar and pestle. 1 ml of extraction buffer (20 mM HEPES, 5 mM EGTA, 2 mM EDTA, 2 mM Phenylmethyl sulfonyl fluoride, DTT and protease inhibitor cocktail (Cat # P9599, Sigma chemicals) per gram of leaves was added and the homogenate was centrifuged at 13000 g for 20 minutes at 4 °C. The supernatant containing total soluble protein was used for further analysis. Extracted protein was resolved on a 12 % SDS-PAGE along with prestained molecular weight marker (MBI

Fermentas), and transferred to nitrocellulose membrane at a constant voltage of 20 V for 12 h. in the BioRad mini transblot cell. Blocking was carried out by using 3 % BSA for 1h. For probing the blot with antibody, the membrane was incubated with 1:1000 dilution of anti-PA antibody for one hour. Blot was probed with antimouse Ig G alkaline phosphatase conjugated secondary antibody raised in goat (1:5,000) for 45 minutes and subsequently developed by addition of substrate NBT and BCIP.

Estimation of PA in TSP from transgenic plants

Total soluble protein was estimated by ELISA taking a known amount of protective antigen protein as standard.

1. *Coating:* Protective antigen was plated in to the 96 well microtitre plate at the concentrations 1 μg/ml, 0.5 μg/ml, 0.25 μg/ml, 0.125 μg/ml, 0.63 μg/ml and 100 μl of TSP was added in each well. The plates were incubated at 37 °C for one hour and at 4 °C overnight in a moist environment.
2. *Washing:* The unbound protein was removed by washing the plates thrice with PBST (1X PBS + 0.1 % Tween-20).
3. *Blocking:* Bovine serum albumin (2 %) was used for blocking purpose. A volume of 100 μl was added to each well and incubated for 1 h. followed by washing.
4. *Addition of Primary antibody:* 100 μl antimouse PA antibody was added at a dilution of 1: 1000. Plates were incubated for 2 h. at 37 °C. Washing was done thrice to remove the unbound antibodies after incubation.
5. *Addition of secondary antibody:* Horse radish Peroxidase (HRP) conjugated with anti-mouse Ig G (raised in sheep) secondary antibody was used for detecting the primary antibody. 100 μl of diluted secondary antibody (1: 5000) was added to each well and incubated for 1 h. at 37 °C. Unbound antibodies were removed by washing thrice with PBST.
6. *Substrate addition:* 3, 3, 5, 5'-Tetramehylbenzidine (TMB) was used for color development by adding 100 μl of the commercially available substrate.
7. After incubation for 20 min, absorbance was recorded at 630 nm in an ELISA reader (Bio-Rad).

Functional assay

The biological activity of the plant expressed PA was determined by the cytotoxicity assay. Cytotoxicity was determined by percentage viability of J774A.1 cells after incubation with anthrax lethal toxin (PA+LF) using MTT dye. Macrophage like cell line J774A.1 was maintained in RPMI 1640 medium containing 10 % FCS. The cell suspension (3x 10^4 cells) was plated at 100 µl/well in 96-well flat-bottomed plates, and cells were allowed to adhere by incubation at 37 °C for 16 h (95 % humidity and 5 % CO_2). After 16 h the medium and detached cells were removed by gentle aspiration and replaced (100 µl/well) with RPMI containing 1.0 µg/ml lethal factor (LF) and total soluble protein extracted from transformed plants containing PA and incubated for 3h at 37 °C in a humidified CO_2 incubator. All experiments were done in triplicates. After 3 h MTT dye dissolved in RPMI was added to the cells to the final concentration of 0.5 mg/ml and the cells were incubated for 30 min at 37 °C to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 100 µl of 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl in 90% isopropyl alcohol, and vortexed to dissolve the precipitate. The absorption was read at 540 nm using a microplate reader (Bio-Rad 550) and percentage cytotoxicity was calculated.

RESULTS

Generation of transgenic mustard plants with PA by Agrobacterium Mediated transformation.

A 2.2 kb PA gene was cloned in a binary vector pBINAR – a pBin19 derivative. Hypocotyl transformation of 6 day old mustard plantlets was carried out using *Agrobacterium tumefaciens* harboring pBINPAG. The transformed tissue was selected at a high level of kanamycin (40 mg/litre). The reason for using a high level of kanamycin was to increase the selection pressure and thus, minimize the number of false positives. Untransformed hypocotyls were kept on the same regeneration/selection medium as controls. While transformed plants were able to grow at a normal pace (Fig.3.1), none of the control explants were able to survive at this selection level. All the control tissues were found bleached on the selection medium, while on regeneration medium have shown 100 % regeneration efficiency.

Fifteen plants were selected for further studies after several rounds of subculturing on selection media. Since mustard crop adapts easily to cold climates hardening was generally done during winter season (day temperature ranging from 12-22°C). The acclimatized plants were able to endure variable conditions of cold temperature and humidity. Control plants were grown (without antibiotic) along with the transformed ones under similar conditions to monitor and compare the morphology. Out of 15 selected plants, 4 plants were found to have abnormal growth pattern (rosettes and stunted growth) due to the infection of aphids. Rest of the plants were able to grow normally like control plants.

Fig. 3.1)
GENERATION OF TRANSGENIC *Brassica juncea* PLANTS EXPRESSING PA.
Pictures showing different stages towards gereration of transgenic mustard plants expressing PA
a) Initial stages of transformed mustard explants surviving on the selection media with phytohormones and kanamycin 40 mg/l (Left); b) Regeneration of explants on regeneration media without antibiotic selection showing 100 % regeneration efficiency (Right). c) Shoot regeneration from callus in selection media. d) Fully developed transformed plants in the rooting media e).Transgenic mustard plants expressing PA transferred to agropeat to get acclimatized to external conditions.

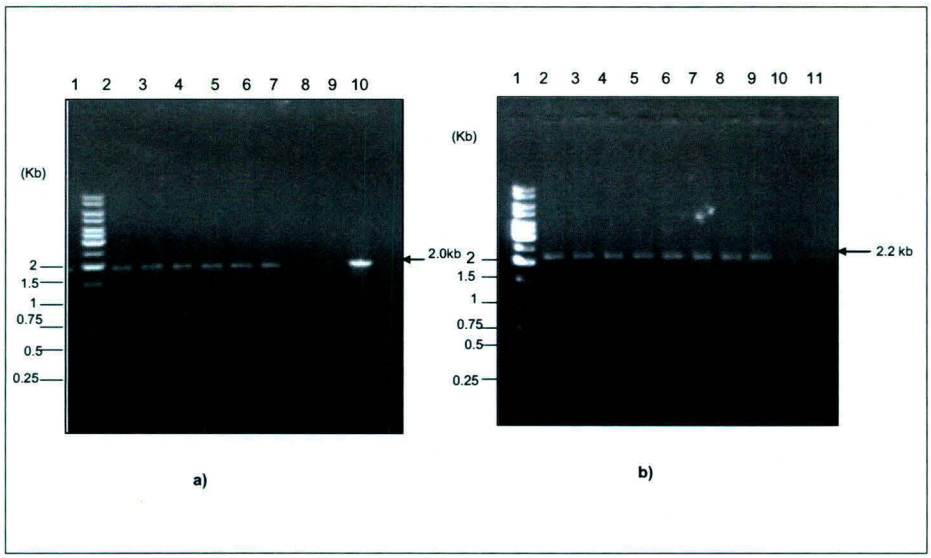


Fig.3.2)
PCR ANALYSIS OF TRANSGENIC MUSTARD PLANTS.
a) PCR amplification of internal region of *pag* A gene
PCR amplification of genomic DNA isolated from transgenic mustard plants using primers P1-CamV35S specific and P2- PA internal primers showing a 2 kb amplicon. Lane 1- 1kb ladder, Lane 2-8 PCR from putative transgenic plants. Lane 9-Wild type, Lane 10-PCR from *Bacillus anthracis* genomic DNA.
b) PCR amplification of genomic DNA from transgenic mustard plants with full length PA primers. Lane 1- 1 kb ladder, lanes 2-10; 2.2 kb amplicon PA, lane 11: Wild type DNA.

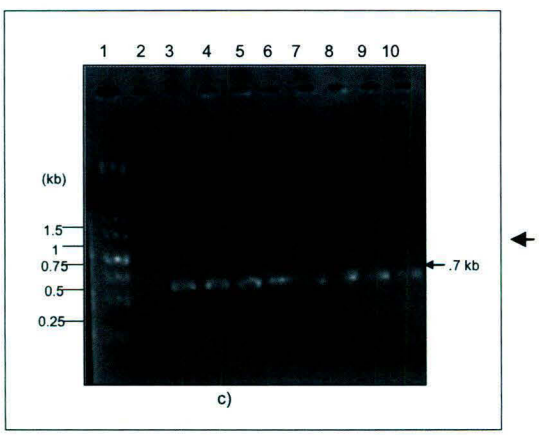


Fig. 3.2)
PCR ANALYSIS
c) PCR amplification of genomic DNA isolated from transgenic mustard plants using primers specific for *neomycin phosphotransferase II.*
Lane 1-1 kb ladder, Lane 3: Wild Type DNA, Lanes 2, 4-8: Brassica juncea plants transformed with pBINpag carrying npt II as a selection marker.

Confirmation of transgene (PA gene) integration by extensive PCR analysis

PA gene integration in nuclear genome of mustard plants was ascertained by PCR analysis carried on genomic DNA isolated from mustard leaves of transformed plants. A set of forward and reverse primers corresponding full length PA were used to check for the presence of PA gene. The expected amplification product of approximately 2.2 kb was obtained and there was no amplification in control genomic DNA (cgDNA) under similar conditions (Fig. 3.2b). To strengthen the above results PCR analysis of genomic DNA using specific primers for *npt II* gene, a primer specific to PA internal region and CaMV35S promoter was also carried out and a desirable amplification product of approximately 0.7 kb, 2.0 kb was obtained only in transformed plants (Fig.3.2a & c). Extensive PCR studies were carried out to screen the plants thoroughly at an early stage. All the plants grown on selection medium were detected PCR positive, which corroborates the use of high selection pressure during plant regeneration.

Analysis of total soluble protein for detection of the expression of PA in plants

A modification of the extraction protocol and buffer composition was made due to rapid degradation observed in the total soluble protein (TSP). High amount of alkaloids and polyphenolic compounds in tobacco could be one reason for rapid degradation of TSP. The contribution of the foreign gene expression is ruled out since a similar pattern was observed with the control plants also. The harvested leaves were instantaneously dipped in liquid nitrogen before storing them at –70 °C. The composition of protein extraction buffer was also optimized to check the protein degradation. Best results were obtained using a combination of chelating agents and protease inhibitors in protein extraction buffer. The total soluble protein thus extracted was run on 12 % SDS-PAGE and subjected to immunoblot analysis using antibodies raised against purified recombinant protective antigen in rabbits. The TSP obtained from transformed plants exhibited a distinct band with the expected molecular weight of the protective antigen (83 k Da). There was no crossreactivity of these antibodies with TSP extracted from control plants (Fig. 3.3). The intensity of the bands from different plants indicating foreign protein expression levels varied because of the probable random site of insertion of the

transferred DNA into the cellular chromosomal DNA ELISA results showed that the expression levels varied from 0.08- 0.5 % of TSP.

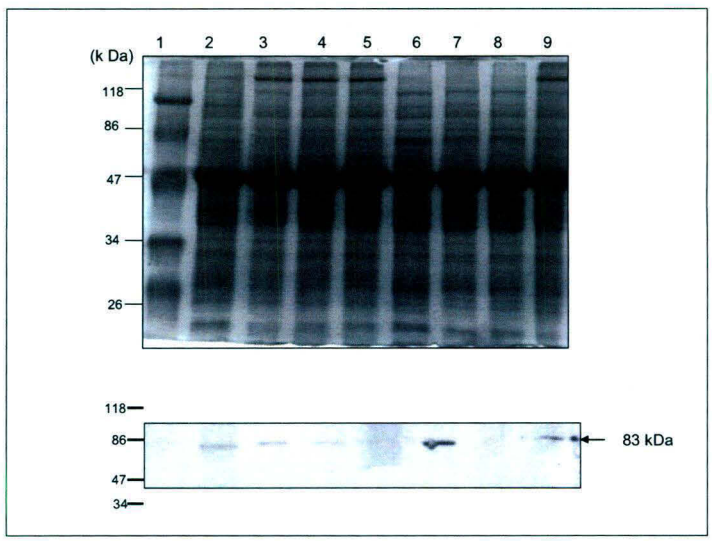


Fig. 3.3)
SDS PAGE AND IMMUNOBLOT ANALYSIS
TSP extracted from transgenic plants of *Brassica juncea* was analyzed by SDS PAGE and Western blotting.
Lanes 2 -7 and 9 Transgenic *Brassica juncea* samples, Lane 8- wild type sample.

PA produced in Brassica juncea is biologically active-Evaluation by In vitro Cytotoxicity assay

To determine the biological activity of the PA expressed in plants, we performed the cytotoxicity assay. Macrophage like cell line J774A.1, which is sensitive to anthrax lethal toxin was used. Total soluble protein extracted from different plants was added to the cells in combination with LF (1 μg/ml) and incubated for 3 h. After 3 h, viability was determined by adding MTT dye. Live cells oxidized the dye to formazon crystals while the dead cells did not. The precipitate was solubilized and optical density was read at 540 nm from which percentage cytotoxicity was determined. It was observed that plant expressed PA, along with LF was able to lyse the macrophage cells and showed biological activity comparable to that of recombinant PA purified from *E. coli*. Different

levels of cell killing ranging from 26 to 81 % were observed (Fig.3.4). This variation can be due to the differential expression level of PA in different plants as evidenced by immunoblot analysis. The effect of extraction buffer alone and TSP isolated from control plants were assessed as negative controls. The negative controls showed 100 % cell viability.

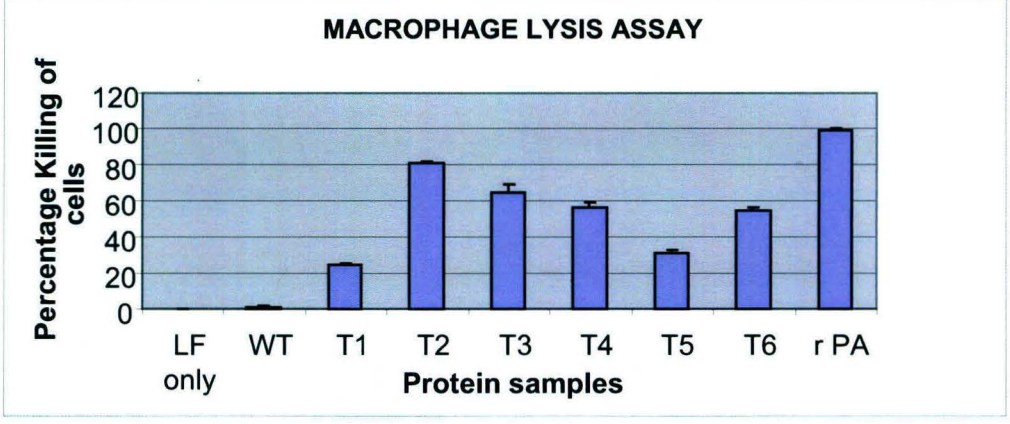


Fig.3.4)
MACROPHAGE LYSIS ASSAY.
Percentage killing of cells with respect to protein samples derived from different plants. WT-Protein extracted from untransformed *Brassica juncea.*
T1-T6: Protein extracted from transformed *Brassica juncea*, rPA: Recombinant Protective antigen. Total Soluble protein was incubated with 1 µg/ml LF. The percentage killing was determined by MTT dye assay. TSP showed 26-81 % killing owing to different expression levels in plants.

DISCUSSION

Anthrax was seen for long as a disease affecting animals specially the herbivores, though carnivores and primates are also occasionally prone to the disease. The late 2001 bioterrorist attacks and resurgence of the disease in endemic areas has drawn considerable attention towards developing effective means to combat the disease. The disease being zoonotic can take its worst shape emerging and remerging from the engraved animal carcasses. Therefore, providing solutions which could effectively prevent the disease at the root level in animals is the need of the hour. Sterne strain attenuated vaccine used today is enveloped with problems such as local inflammation at the site of inoculation, incomplete protection in the vaccinated herd (Krishna P S *et al.,* 2007) and the development of disease itself in some animals e.g. llamas and mice (Cartwright M E, 1987). Moreover, the current vaccines are difficult to use in its recommended subcutaneous route in the developing rural world and in wild life where the disease is prevalent. Subunit vaccine approach proved to be a better means by utilizing the major immunogenic protein the 'protective antigen'. Our accomplishments in this regard include over expression of PA in *E. coli* (Gupta *et al,.* 1999, Chauhan *et al.,* 2001). This vaccine has successfully completed safety and efficacy studies and currently undergoing human clinical trials.

The impinging problems pertaining to delivery especially for veterinary applications still remained a question of concern. The concept of plant derived vaccines has brought immense relief in this regard (Haq *et al.*1995). In an endeavor to develop plant based vaccines against anthrax, we have pioneered the expression of PA in tobacco and tomato (Aziz *et al.,* 2003, 2005). Further, the interest in developing an edible vaccine for both veterinary and human applications has encouraged us to express protective antigen in *Brassica juncea*. We have successfully expressed the PA molecule in its functional form in mustard plants. 6 day old hypocotyl explants of *Brassica juncea* were transformed with pBin*pag* by Agro bacterium-mediated method. Essential care was taken to ensure that the agrobacterium cells harvested for infection were at logphase. Hypocotyl explants were very sensitive to co-cultivation and prone to necrosis very easily. This was overcome by pre-conditioning of the explants on regeneration media for 3 days. This pre-conditioning of the explants before co-cultivation helped in inhibiting necrosis and increased the

transformation efficiency. Improvement in transformation frequency upon preconditioning of the explants has been reported in *Arabidopsis thaliana* (Sangwan *et al.,* 1992), sugarbeet (Jacq *et al.,* 1993), tobacco (Sunilkumar *et al.,* 1999) and watermelon (Choi *et al.,* 1994). Sensitive hypocotyls were also prone to contamination. Silver nitrate was used at a concentration of 100 mg/litre to effectively counter the problem of contamination (Fei S *et al,* 2000). MS media supplemented with BAP (1 mg/l) and NAA (1 mg/l) and kanamycin (40 mg/l) was optimized as selection medium. Augmentin (300 mg/l) was added through out selection to prevent residual growth of agrobacterium. Shoot regeneration occurred after 4-5 weeks on the selection media. Regenerating shoots were transferred routinely after every 15 days to fresh selection plates to avoid contamination and encouraging false positives. Shoots were transferred for rooting on MS media supplemented with IBA (1 mg/l) and Augmentin (300 mg/l).

We were able to successfully regenerate transgenic mustard plants and transfer them to pot stage in greenhouse. Extensive PCR based screening of putative transgenic plants was carried out with different combinations of primers to get detailed information regarding the integration of PA and *nptII* gene in the nuclear genome. Internal PA gene primers were used to select the putative transgenic plants carrying transgene. Specific primers were also used to amplify the *nptII* gene from the genomic DNA of the mustard leaves. The total soluble protein was extracted from mustard leaves using 20 mM HEPES, 5 mM EGTA, 2 mM EDTA 2 mM PMSF, DTT and protease inhibitor cocktail. The Western blot results were encouraging in terms of better yields, as 1:1000 dilution of primary antibody was able to detect the presence of PA in TSP. Further, higher dilutions of antibodies couldn't detect the presence of PA in the plant samples. The amount of PA in different samples varied and the results of cytotoxicity assay further corroborated this notion. Though, the results obtained by transformation of mustard lay a strong basis for the generation of an edible vaccine against anthrax the study promotes scope for a designing more efficient methods to increase the expression yield for generation of uniform expression levels in different transgenic lines.

Chapter 4

Expression of 'Domain-IV' of Protective antigen gene in Tobacco (Nuclear transformation) & *E coli.*

INTRODUCTION

The concept of subunit vaccine concerning the vaccination against anthrax has mainly focused on protective antigen component of anthrax toxin. It is now a universally accepted fact that immunization with PA provides complete protection against anthrax spore challenge. Several studies have supported this idea with experiments in various animal models including humans (Pitt, M L 2001, Reuveny, S *et al.,* 2001, Williamson, E. D *et al.,* 2005). The recent failure by Vaxgen inc. to supply PA based vaccine to FDA, due to rapid degradation of protein, the concerns raised regarding the introduction of potential toxin subunit in persons at risk of being exposed to *Bacillus anthracis* (Aulinger, B. A *et al.,* 2005) have proved that the pursuit for an ideal anthrax vaccine is still not over. Issues concerning the stability of the protein and the safety of the vaccine are very critical for vaccine development. The crystal structure of PA has revealed that it possesses four domains of which Domain-IV (596-735aa) is the receptor binding moiety of the molecule (Santelli, E *et al.,* 2004). Deletion of D-IV from *Bacillus anthracis* genome resulted > 10, 000 fold decrease in virulence (Brossier, F M *et al.,* 2000). Also, studies have shown that the carboxyterminal of PA (D-IV) provides complete protection in mice against anthrax (Flick-Smith, H. C., *et al.,* 2002). Therefore; D-IV may serve as an effective and safe vaccine candidate for next generation anthrax vaccine. The problems associated with stability can be tackled by expressing the antigens in plant systems as discussed in previous chapters. The conventional means of vaccine development which used bacterial, yeast or mammalian expression systems are slowly being taken over by plant production units owing to tremendous advantages related to yield, safety and efficacy. Hence, D-IV expressed in plant system may be suitable for vaccine.

Our studies initially focus on expression of D-IV in plant system by Agrobacterium-mediated transformation. Tobacco has been the choice of crop for transformation. Tobacco is the model crop for plant transformation system. There are several attributes of tobacco crop, which makes it a model plant for initial studies. The ease of regeneration, availability of standard transformation protocols, large biomass, acclimatization of the crop to adverse environmental conditions and self pollinating nature makes tobacco an

ideal crop for commencing any transgenic plant based experiments. Nearly, all the pioneering studies covering different aspects of transgenic plants were initially carried out in tobacco. The first breakthrough in plant based vaccines was also reported in tobacco plants (Haq *et al.,* 1995). Also, D-IV will be expressed in *E .coli* and purified. The efficacy of plant expressed D-IV will be compared with that of recombinant counterpart.

MATERIALS AND METHODS

Reagents and Supplies

Tobacco seeds (*Nicotiana benthamiana*) were purchased from the Indian Agricultural Research Institute (IARI), Pusa, New Delhi. The primers used for cloning were obtained from Microsynth GmbH, Balgach, Switzerland. The enzymes and chemicals used for DNA manipulation were purchased from MBI Fermentas, NEB (USA). The oligonucleotides were obtained from Microsynth (Switzerland). DNA purification kit was obtained from Qiagen (Germany). Growth Media and its components were from Hi-Media Laboratories (India). Plant hormones were procured from Sigma Chemical Co. (USA). Agarose, Acrylamide, Ampicillin, Bovine Serum Albumin (BSA), Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, Glucose, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Potassium acetate, HEPES, PMSF, SDS, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium bicarbonate, Penicillin, Streptomycin, Imidazole, Tris, Tween-20, Protein determination dye and other chemicals were purchased from USB Chemicals, USA. Absolute alcohol and Isopropanol were purchased from E. Merck, India. Ni-NTA slurry was purchased from Quiagen(Germany). Cell culture plastic wares were obtained from Corning, USA. Fetal Calf Serum (FCS) was from Biological Industries (Israel). RPMI 1640, 3-(4,5-dimethylthiazol-3-yl)-5-diphenyltetrazolium bromide (MTT), Phenyl methylsulfonyl fluoride (PMSF), HEPES, NaCl, EGTA, EDTA and other chemicals were purchased from Sigma Chemical Co. (USA). *E. coli* strain DH5α and J774A.1, a macrophage like cell line, were obtained from ATCC (American Type Culture Collection; USA).

Strains and culture medium

E. coli DH5α was used as the host for cloning and propagation of plasmids. *E. coli* DH5α was cultured in Luria-Bertani broth supplemented with kanamycin (50 μg/ml) for the D-IV clones, in Pet-28a for expression in *E. coli* and in plant expression vector pCAMBIA-1303. *Agrobacterium tumefaciens* strain GV2260 was routinely grown in YEM (Refer to Appendix for composition.) supplemented with kanamycin (50 μg/ml) and rifampicin (10 μg/ml) when required. For plant transformation MS media with phytohormones (NAA and BAP) was used (Refer to appendix for composition).

EXPRESSION OF D-IV IN TOBACCO PLANTS

Cloning of Domain -IV in pCAMBIA vector

A pCAMBIA vector pCAMBIA1303, which employs constitutive CaMV35S promoter for gene expression and also carries bacterial *hptII* gene, (which codes for hygromycin phosphotransferase that confers hygromycin selection) was used to clone D-IV of protective antigen gene. Plasmid pCAMBIA-1303 was purified using DNA purification kit as described in the manual (Qiagen). The D-IV region of PA gene coding region (421 bp) was amplified by PCR using pXO1 as template. The PCR reaction was carried out using a forward primer and a reverse primer that included a *Bgl* II site at its 5' and 3' end.

Forward Primer: 5' CGG GAA GAT CTG TTT CAT TAT GAT AGA AAT AAC ATA G 3'

Reverse Primer: 5' CCG AAG ATC TAC TCC TAT CTC ATA GCC 3'

The amplification was standardized by a gradient PCR with varying melting temperatures from 50-60 °C. PCR reaction was carried out in Biorad iCycler under the following conditions:

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	60 °C
Extension	1 min	72 °C
Final extension	7 min	72 °C

The amplified PCR product and pCAMBIA-1303 were digested with restriction enzyme *Bgl* II. The digested products were separated on 1 % agarose gel. The bands were excised and the DNA was eluted using the gel extraction kit (Qiagen). The digested PCR product and the vector were ligated overnight at 16 °C and transformed into *E. coli* DH5α competent cells. Preparation and transformation of competent *E. coli* cells were performed according to procedure described later. The resultant ligated product – pCAM-D-IV after restriction analysis was mobilized into *Agrobacterium tumefaciens* (GV2260) using freeze thaw procedure.

Tobacco Seed Germination

1. Seeds were washed with 0.01 % tween 20, and then rinsed several times with autoclaved milli Q water to remove excessive detergent.
2. Seeds were washed in 70 % ethanol for 1 minute with continuous shaking.
3. Ethanol was drained. Seeds were then washed with 0.02 % of Mercuric chloride for 5-10 minutes with continuous shaking. $HgCl_2$ was removed by decanting.
4. Seeds were washed 4-5 times with autoclaved distilled water.
5. 4-5 seeds were germinated in ½ MS media and kept in the culture room with a photoperiod of 16 h (16 h light, 8 h of dark).

Agrobacterium mediated Leaf disc transformation

1. Agrobacterium carrying the cloned D-IV gene was inoculated in YEM medium with appropriate antibiotics [kanamycin (50 mg/L) and rifampicin (10 mg/ L)].
2. The culture was incubated at 28 °C on a shaker at 200 rpm for 16 –24 h.
3. 1 ml of culture was harvested in a microfuge tube and centrifuged at 13,000 rpm for 1 minute.
4. The pellet was suspended in liquid MS (minus sucrose) medium.
5. Young tobacco leaves are cut into small pieces (3 mm x 3 mm) and kept on regeneration (0.1 mg/L NAA + 1 mg/ L BAP) media for 12-14 h to acclimatize.
6. The cut leaf explants were transferred to liquid MS media in a sterile Petri-plate and added suspended *Agrobacterium* cells.
7. Agroinfection was carried for 15 minutes with continuous shaking.
8. Explants were removed from the infection media and dried on sterilized whatman paper and later kept on regeneration media (0.1 mg/L NAA + 1 mg/ L BAP)
9. After 36 h infected leaves were transferred to selection media supplemented with Cefotaxime (500 mg/L) and hygromycin (30 mg/L).
10. Subculturing of the shoots was done at regular intervals.
11. A control of untransformed cut tobacco leaf explants were also kept on selection media.

Tissue Culture of Tobacco leaf explants

The infected leaf explants were grown on selection medium until a callus was obtained and single shoots were seen sprouting. These single shoots were carefully excised from the callus and transferred to fresh selection medium in jam bottles. After a few more rounds of subculturing, the single shoots were transferred to rooting medium (selection medium devoid of hormones) in the jam bottles. In a few batches silver nitrate was also added to the medium. After the rooting of the fully differentiated single shoots, the plants were transferred to the pots agropeat. These plants that come out from the bottled stage, are fragile and more susceptible to contamination, and thus, kept covered in a polybag and given sterile MS liquid solution without sucrose. Slow process of hardening is carried out initiating with the removal of cover and gradual increase in temperature. Finally, the plants were transferred to the green house for further growth and flowering, ultimately leading to the formation of fully grown tobacco plants.

Extraction of Plant Genomic DNA

1. 20 ml of DNA extraction Buffer (DEB; for composition refer annexure) was taken in autoclaved Oakridge tubes and incubated at 65 °C with slow shaking in a water bath.
2. 5 g of leaf tissue was taken and ground in a pre-cooled mortar with liquid nitrogen, until a fine powder is obtained.
3. The ground homogenate was transferred to Oakridge tubes containing DEB at 65 °C and incubated for 30-45 minutes with occasional mixing by gentle swirling.
4. After cooling at room temperature, 2/3rd volume (approx. 15 ml.) of chloroform: *Iso*-amyl alcohol (24:1) was added and mixed gently for 20 minutes.
5. The sample was centrifuged at 12,000 rpm at room temperature for 10 minutes and the supernatant was transferred to a fresh sterile tube.
6. 2/3rd volumes of chilled isopropanol was added and gently mixed to precipitate the nucleic acids.
7. This precipitate of DNA to a microfuge tube and the remaining sample was centrifuged at 11,000 rpm at room temperature for 10 minutes.

8. The supernatant was discarded and the pellet was washed with 70 % ethanol and allowed to dry.
9. The pellet was dissolved in autoclaved Milli-Q water.

PCR analysis

Genomic DNA was isolated from these selected plants by CTAB method (Nickerent *et.al.,* 1994). Presence of the D-IV gene in the plantlets was confirmed by PCR using primers specific to the gene.

Forward Primer: 5' CGG GAA GAT CTG TTT CAT TAT GAT AGA AAT AAC ATA G 3'

Reverse Primer: 5' CCG AAG ATC TAC TCC TAT CTC ATA GCC 3'

PCR reaction was carried out in Biorad iCycler under the following conditions:

Step		**Recommended conditions**
Denaturation	**5 min**	**95 °C**
3-step cycling (30cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	56 °C
Extension	45 sec	72 °C
Final extension	**7 min**	**72 °C**

Polyacrylamide Gel Electrophoresis

Total soluble protein was extracted from tobacco leaves as mentioned in chapter 3. This protein was resolved by SDS-PAGE prior to immunoblot detection of D-IV. A 15 % separating gel was utilized for the electrophoretic analysis of the proteins. For stacking of proteins 5 % gel was used. Protein samples for SDS-PAGE were prepared by the addition of the 6X loading buffer to the final concentration of 1X, boiled for 5 minutes followed by centrifugation at 12,000 rpm for 5 minutes at room temperature. Proteins were analyzed by SDS-PAGE according to the method of Laemmli at a constant voltage of 100 V in a Bio-Rad mini gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The resolved proteins were visualized by staining the gel with Coomassie Brilliant Blue R-250 for 30 minutes followed by destaining the gel to remove excess stain.

Immunoblot detection and Protein estimation

The detection of D-IV in the total soluble protein extracted from tobacco leaves was carried out following the same protocol as in the case of mustard. Higher dilutions of primary antibodies were used whereas the dilution of secondary antibody was kept same. The amount of D-IV expressed in tobacco was determined by ELISA from TSP as discussed in the previous chapter.

CLONING EXPRESSION AND PURIFICATIONOF D-IV IN *E.COLI*

PCR amplification of Domain-IV

The D-IV fragment of PA was amplified by PCR using pXOl plasmid of *B. anthracis* (Sterne strain). The primers used for amplification were as follows.

Forward Primer: 5' CGC GGA TCC TTT CAT TAT GAT AGA AAT AAC 3'

Reverse Primer: 5' CCC AAG CTT TTA TCC TAT CTC ATA GCC 3'

The amplification was standardized by a gradient PCR with varying melting temperatures from 50-60 °C. PCR reaction was carried out in Biorad iCycler under the following optimized conditions.

Step		**Recommended conditions**
Denaturation	**5 min**	**95°C**
3-step cycling (30cycles)		
Denaturation	1 min	95°C
Annealing	1 min	58°C
Extension	1 min	72°C
Final extension	**7 min**	**72°C**

Restriction Digestion and ligation of DNA

The gel purified PCR product and pET-28a vector were digested with *Bam* HI and *Hind* III enzymes. One unit of enzyme was used per µg of DNA and the reaction mixture was incubated at 37 °C for 3 h. Subsequent to the digestion, the enzymes were inactivated

by incubating the reaction mixture at 60 °C for 20 minutes. The digested fragments were resolved on 1 % agarose gel. Ligation was set in 10 µl reaction volume with vector insert molar ratio of 1:3. The ligation reaction mixture was incubated overnight at 16°C.

Competent cell preparation

E. coli DH5α and *E. coli* BL21 competent cells were prepared as follows. The competent cells were prepared using the protocol described by by Cohen *et al.,* (1972) with slight modification.

1. The host cell culture (*E. coli* DH5α) was streaked on a LB agar plate from the frozen glycerol stock kept at -70 °C. A single colony was inoculated into 3 ml LB broth.
2. One ml of the overnight grown culture was further inoculated into 100 ml LB and allowed to grow for 2-3 h until A_{600} reached 0.5.
3. The culture was chilled on ice, transferred to ice cold 50 ml polypropylene tubes and centrifuged at 4000 rpm for 10 min in a Sorvall SS34 rotor.
4. The supernatant was decanted and the pellet was resuspended gently in 10 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 30 min.
5. The cells were then centrifuged at 4000 rpm in Sorvall SS34 rotor. The pellet was resuspended in 3 ml of ice cold 0.1 M $CaCl_2$. Chilled 0.1 M $CaCl_2$ + 20 % glycerol was added to the cells kept at 4 °C for 12-16 h.
6. About 200 µl aliquots were taken for checking viability, contamination and efficiency of transformation. The rest of the cells were stored in aliquots of 200 µl at – 70 °C.

Screening of Recombinant D-IV clones

The transformants were screened for presence of insert by mini-preparations of plasmid DNA and their restriction analysis. The method used for preparations of plasmid DNA is a modification of the method of Birnboim and Doly (1979). Plasmid DNA obtained by mini-preparation was digested sequentially with *Bam* HI and *Hind* III restriction

enzymes. The digestion mixtures were run on 1 % agarose gel to check for the presence of insert. Glycerol stocks were prepared for positive clones and stored at -70 °C. This construct was designated as pET28a –D IV.

Expression of D-IV

To check the expression of pET28a-DIV, it was transformed into *E. coli* BL21 (λDE3) competent cells. Cells bearing pET28a- DIV were grown at 37 °C in Luria Bertani (LB) broth containing 100 µg/ml of ampicillin, at 200 rpm. When the A_{600} reached 0.8, isopropyl-l-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h of induction, the cells were harvested by centrifugation at 5,000 rpm, at 4 °C for 10 min. To localize the expression of recombinant protein, the cytosolic and inclusion body fractions were prepared as follows.

Cytosolic localization

1. The pellet from 100 ml culture was resuspended in 10 ml of sonication buffer (50mM Na-phosphate pH 7.8, 300 mM NaCl).
2. Sonication was done at 4 °C (30 seconds burst/30 seconds cooling/200-300 W) for 5 cycles.
3. Sonicated sample was centrifuged at 10,000 g for 15 minutes at 4 °C.
4. Supernatant was collected and the pellet was saved for analyzing the localization of the protein in inclusion bodies.

Inclusion body localization

1. The pellet from the cytosolic fraction was resuspended in 8 M urea, 0.1 M Na-phosphate pH 7.8 and 300 mM NaCl and incubated at 37 °C for 1 h.
2. Centrifuged at 13, 000- rpm for 30 minutes at room temperature.
3. Supernatant (inclusion bodies) was collected. The cytosolic and inclusion body fractions were subjected to SDS-PAGE analysis.

Polyacrylamide Gel Electrophoresis

Proteins were resolved by SDS-PAGE according to method of Laemmli (1970). A 15 % SDS polyacrylamide gel was utilized for the electrophoretic analysis of proteins. For stacking of proteins, 5 % gel was used. 1 ml of culture aliquots was taken and the cells were harvested by centrifugation at 5000 rpm for 5 minutes at room temperature. The pellet as re-suspended in 100 µl of 1X lysis buffer, the samples were then boiled for 10 minutes and centrifuged at 12000 rpm for 10 minutes at room temperature. 10 µl of the supernatant was analyzed on the gel. The resolved proteins were visualized by staining the gels with Coomassie brilliant blue followed by destaining the gels to remove excess of stain.

Western Blot Analysis

Protein samples were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membrane at a constant voltage of 100 V for 2 h at 4 °C. Blocking was carried out using 3 % BSA in 1X PBS for 1 hour followed by three washings with PBST (1X PBS and 0.1 % Tween). For probing the blot with antibody, the membrane was incubated for 1 hour with anti-PA antibody/anti-6Xhis antibody, at 1: 10,000 dilution. Blot was washed thrice with PBST and then probed with anti mouse Ig G alkaline phosphatase conjugated secondary antibody (1: 10,000) for 1 hour. The membrane was washed three more times with PBST and then developed by addition of substrate solution of NBT/BCIP.

Purification by affinity chromatography

The selected plasmid pET28a-D-IV construct was transformed into *E. coli* BL21 (λDE3) competent cells and protein was purified as follows. In brief, cultures were grown in LB medium containing 100 µg/ml ampicillin. When OD_{600} reached 0.8, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, the cultures were grown for an additional 4 h and bacterial cells were pelleted at 5000 rpm for 10 min at 4 C. The pET28a-D-IV protein was purified using metal-chelate affinity chromatography under denaturing conditions as follows. The pellet from 500 ml culture was resuspended in 20 ml of denaturing buffer containing 100 mM sodium phosphate

buffer, 300 mM sodium chloride and 8 M urea (pH 8.0). The resuspended pellet was incubated at 37 °C for 1 h on a rotary shaker. The cell lysate was centrifuged for 30 min at 12,000 rpm at room temperature and the supernatant was mixed with 3 ml Ni^{2+} -NTA slurry. Slurry was packed into a column and allowed to settle. The flow through was reloaded on the column to allow maximal protein binding. Ni^{2+}-NTA matrix was washed with 50 ml denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein using 8–0 M urea gradient. The protein was eluted in elution buffer, 100 mM sodium phosphate of pH 8.0 with 250 mM imidazole and 300 mM sodium chloride. Purified protein was analyzed on 15 % SDS-PAGE. Fractions containing the protein were collected, pooled, and dialyzed against 10 mM HEPES buffer containing 50 mM NaCl.

RESULTS

Construction of plant transformation vector & transformation of tobacco plant

A 421 bp D-IV gene was cloned in a pCAMBIA-1303 vector binary vector. The schematic representation of constructing pCAM–DIV is described in figure.4.1 Restriction analysis resulted in 421bp fallout fragment (Fig.4.3). Further, the presence of D-IV gene in the vector was confirmed by sequencing of the gene. The sequenced gene was free of any mutations and thus, the construct was ready for transformation. Leaf disc transformation of tobacco plants was carried out using *Agrobacterium tumefaciens* harboring pCAM-DIV. Hygromycin (30 mg/l) was used for selection along with growth hormones in the medium to avoid regeneration of false positive plants.

Leaf discs from wild type tobacco plants were also kept under selection of hygromycin to check for the levels of hygromycin and find out if the antibiotic concentration was sufficient to prevent regeneration of untransformed tobacco leaf discs. A set of leaf discs were kept on regeneration medium served as positive control for the experiment. Shoots emerged from leaf discs after 2-3 weeks. Selected shoots were transferred to rooting media which comprised of FMS and hygromycin (30 mg/l) (Fig.4.4). Plants were subcultured every 20-25 days so that they are not deployed of nutrients. These bottled plants were taken out of sterile environment and planted in autoclaved agropeat, which consists of balanced nutrients and the essential constituents simulating the soil microcosm. Hardening of tobacco plants was not very difficult as tobacco plants are known to easily adapt to tropical conditions. The acclimatized plants were able to endure extreme conditions of temperature and humidity. Control plants were grown (without antibiotic) along with the transformed ones under similar conditions to monitor and compare the morphology.

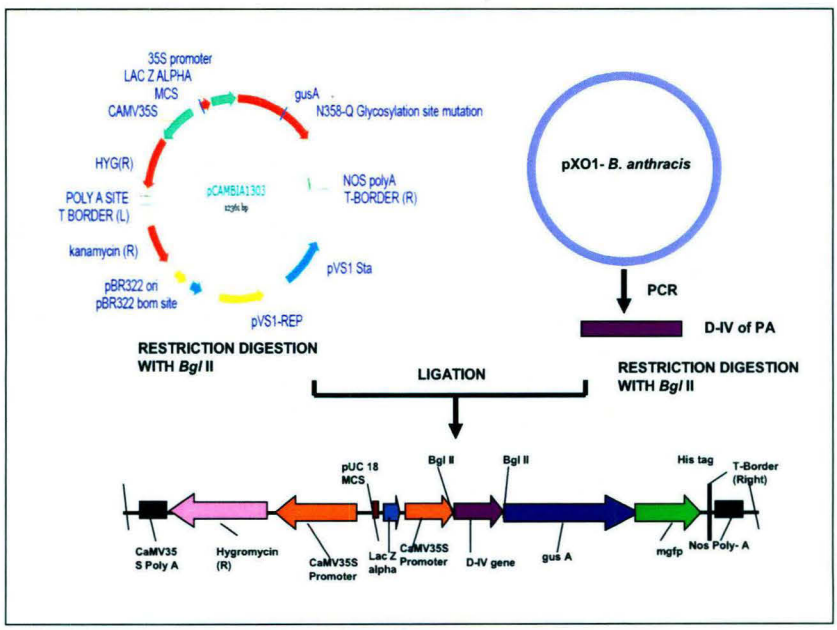


FIG.4.1) SCHEMATIC REPRESENTATION OF CLONING OF D-IV IN PCAMBIA-1303

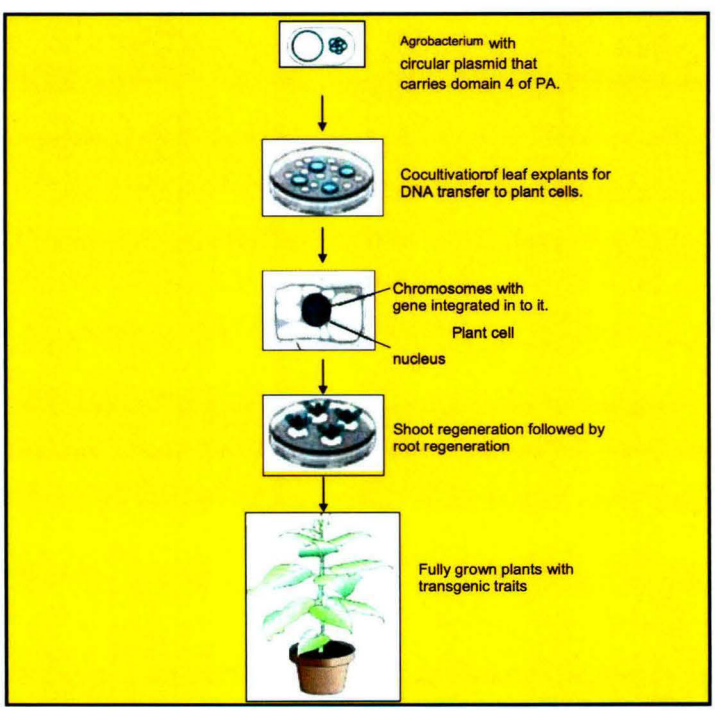


FIG 4.2) SHCEMATIC REPRESENTATION OF AGROBACTERIUM-MEDIATED TRANSFORMATION IN TOBACCO.

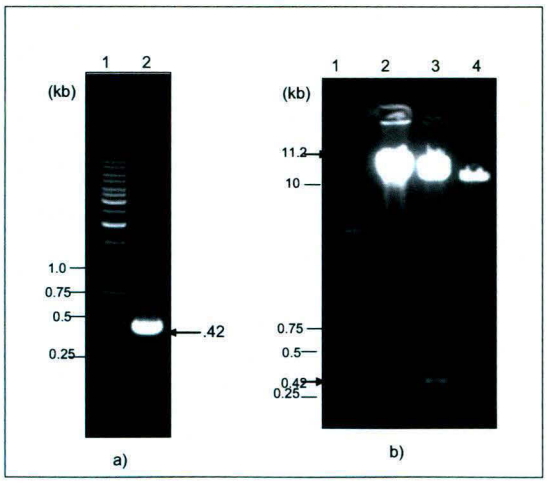


Fig. 4.3)
CLONING OF D-IV IN PCAMBIA 1303 VECTOR.

a) PCR amplification of Domain-IV (D-IV) gene from *Bacillus anthracis* genomic DNA.
Lane 1- 1 kb ladder, Lane 2- 0.42 kb Domain-IV PCR amplicon.

b) Restriction digestion of the clone pCAM-D-IV with *Bgl* II enzyme.
Lane 1- 1 kb ladder MBI Fermentas, lane 2- Linearized clone, Lane 3- Digestion with Bgl II enzyme showing fallout of 0.42 kb, Lane 4- linearised vector pCAMBIA-1303.

Fig. 4.4)
GENERATION OF TRANSGENIC TOBACCO PLANTS EXPRESSING D-IV OF PA.
a) Regeneration of leaf explants on regeneration medium (left Control). Regeneration of explants on selection medium after Agrobacterium-mediated transformation (Right). b) Putative transgenic tobacco plants containing Domain-IV gene at bottle stage in rooting media with hygromycin (30mg/l). c,d) Domain-IV transgenic plants at pot stage in the greenhouse to get acclimatized to external conditions.

Molecular analysis of putative transgenic plants

The plants regenerated on selection medium were subjected to molecular analysis to ascertain the gene integration and expression of a fully functional form of D-IV.

Detection of D-IV of PA gene using PCR

Genomic DNA was extracted from leaves of regenerated tobacco plants and samples were used as template in PCR reactions of 50 µl the same primers used to clone. The expected amplification product of approximately 421 bp was obtained and there was no amplification in control genomic DNA (cgDNA) under similar condition (Fig. 4.5a)

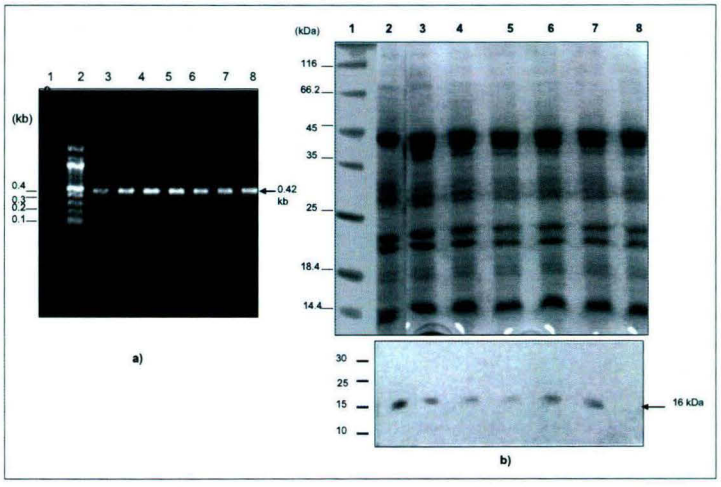


Fig. 4.5)
MOLECULAR ANALYSIS OF TRANSGENIC D-IV PLANTS.

a) PCR amplification of Domain-IV (D-IV) gene from transgenic tobacco genomic DNA. Lane 1- Amplification from wild type plant, lane 2- 1 kb ladder, lane 3-9 PCR amplicon o f 0 .42 kb obtained with D-IV specific primers.

b)TSP extracted from tobacco D-IV transgenic plants of was analyzed by SDS PAGE and Western blot analysis.
Lane 1- Unstained marker (SDS PAGE), Lane 2, 3, 4, 5, 6, 7 transgenic tobacco samples, Lane 8- wild type sample.

Immunoblot detection and ELISA

The expression of D-IV protein was checked using antibodies (polyclonal) raised against purified recombinant protective antigen. Higher dilutions of Primary antibody were able

to show the bands, which in turn indicate better expression levels. A dilution of 1:1500 was able to detect the D-IV expression. The absence of any band in lanes carrying total soluble protein extracted from untransformed tobacco plants ruled out the possibility of cross- reaction of antibodies with other plant proteins. The amount of D-IV was 0.3 to 0.8 % of TSP as determined by ELISA (Fig. 4.5b).

CLONING, EXPRESSION & PURIFICATION OF D-IV IN *E. COLI*

Cloning of D-IV gene in pet 28 a vector

Domain-IV the most immunogenic molecule of PA from 596-735 amino acids of matured PA was amplified with primers possessing restriction sites *Bam* H I and *Hind* III at 5' end and 3' end respectively. A single band of 421 bp obtained by PCR was gel eluted so that the product is free from residual polymerase enzyme. The purified PCR product is then digested with *Bam* H I and *Hind* III enzymes. Simultaneously, pET28a vector DNA was also digested with the same enzymes. Ligation reaction was performed at 16 °C for 16 h with vector insert molar ratio of 1: 3. The reaction mixture was transformed initially in to competent cells of *E. coli* DH5α. The transformants obtained were screened for presence of insert by mini-preparations of plasmid DNA and their restriction analysis (Fig.4.7). Positive clones were selected on the basis of a fall-out of 421 bp after digestion with *Bam* Hl and *Hind* III enzymes. Further, sequencing results confirmed that the clone is free from mutations. The obtained clone was designated as pET28a -DIV. *E. coli* BL21 (λDE3) cells were transformed with pET28a- DIV. Cells were grown and induced by 1mM IPTG. Samples were collected before and after induction. SDS-PAGE was run with uninduced and induced samples. A significant increase in the protein levels was noticed 5 h post induction. Only D-IV was induced indicating the tight regulation of the protein under the influence of T7-promoter. Gels were stained, destained and analyzed (Fig.4.8a). Development of western blot with anti-His antibody and anti-PA antibody confirmed the presence of the recombinant protein (Fig.4.8b). For analysis of localization of the protein, cytosol and inclusion body fractions were prepared. These fractions were subjected to SDS-PAGE and electroblotted.

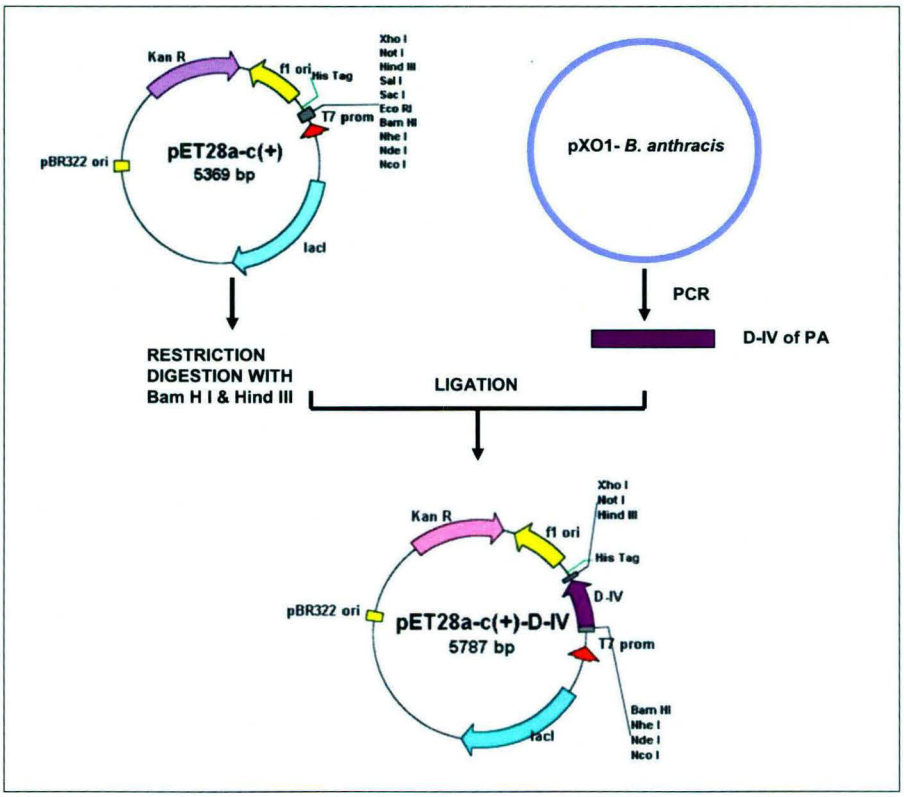


FIG. 4.6) SCHEMATIC REPRESENTATION OF CLONING OF D-IV IN pET28a-c

Purification of recombinant pET28a –DIV

The recombinant pET28a-D-IV was purified from the cytosol under denaturing conditions using Ni^{2+}-NTA affinity chromatography. Elution was done with 250 mM imidazole. The fractions were analyzed on a 15 % polyacrylamide gel. The purified recombinant pET28a-D-IV was found to be more than 95 % pure (Fig.4.8). The fractions containing the purified protein were extensively dialyzed against 10 mM HEPES (pH 8.0) and 50 Mm NaCl. The dialyzed samples were aliquoted and stored at -70 °C. Protein estimation was carried out by Bradford's method. The yield of recombinant D-IV was ~ 28 mg/lit. D-IV expressed in *E coli* was stable for at least 6 months at 4 °C.

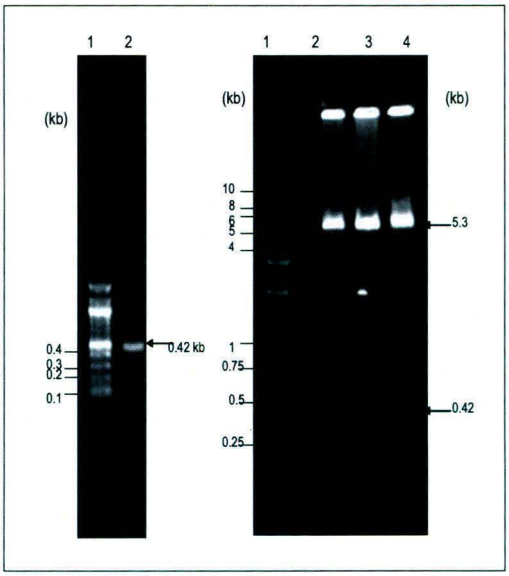


Fig. 4.7)
CLONING OF D-IV OF PA IN PET28A-(C+) VECTOR.
a) PCR amplification of D-IV from *Bacillus anthracis* genomic DNA. Lane 1- 1 kb ladder, lane 2- PCR amplicon of 0.42 kb obtained with D-IV specific primers.
b) Restriction Digestion of D-IV clones. Lane 1- 1 kb ladder (MBI Fermentas), lanes 2, 3 and 4 showing 5.3 kb of vector backbone and 0 .42 kb of insert.

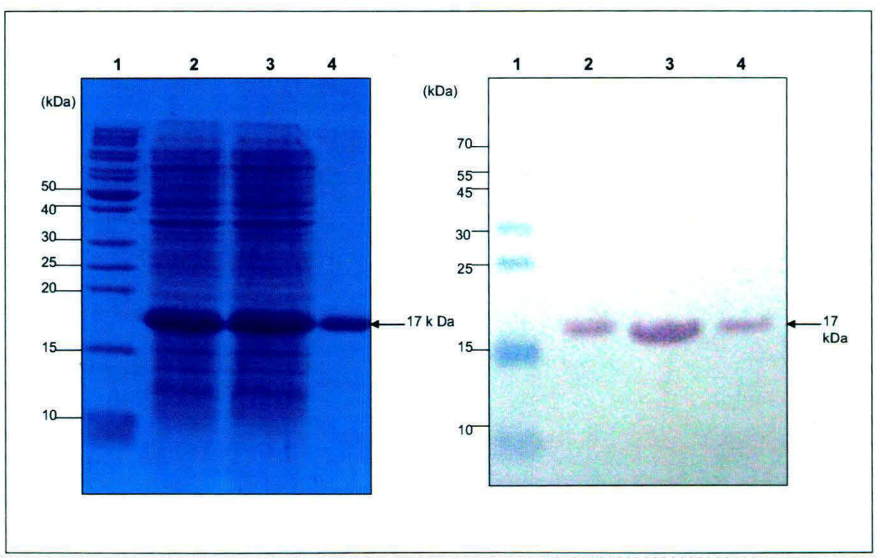


Fig. 4.8)
PROTEIN EXPRESSION AND PURIFICATION OF D-IV IN *E COLI.*
a) SDS PAGE analysis of D-IV expression. Lane 1- Prestained protein molecular weight marker, lane 2- Uninduced expression in *E coli,* lane 3- expression of D-IV after induction with IPTG, lane 4- purified-IV.
b) Western blot of the contemporary gel with prestained molecular weight marker.

DISCUSSION

Protective antigen is the central component of the anthrax toxin. The current anthrax subunit vaccines have PA as the major or only component of the vaccine. Although antibodies generated against PA provide complete protection against the disease, it has been proven that only 'D-IV' of PA, 'the receptor binding element' is sufficient for complete protection. Presently, we do not know much about the interaction of toxins with PA which is delivered by immunization during anthrax infection. Therefore, these vaccines are limited by their implication to individuals during or shortly after infection (Aulinger, B. A *et al.,* 2005). This shortcoming is of serious concern especially in case of sudden mass attack by anthrax infection where there is a strong need for effective prophylaxis but is not sure if the person is actually infected. Such mass calamities due to anthrax infection can be forestalled effectively by administration of Domain-IV (D-IV) of protective antigen alone. In the initiation of infection, the intoxication process mediated by PA and LF is prohibited by D-IV as it competitively binds to anthrax toxin cell receptors. D-IV can thus, also be used for effective vaccination therapy. Studies carried out in this light include administration of salmonella based vectors and adenovirus vectors expressing D-IV of PA (Strokes G M *et al.,* 2007). D-IV has also been expressed in spinach via plant viral vectors (Sussman H E *et al.,* 2003). Stable recombinant expression of D-IV by nuclear or chloroplast transformation provide safe and reliable means for vaccine development.

Initially, we have chosen to express D-IV in tobacco by agrobacterium transformation method; and simultaneously D-IV was expressed in *E. coli.* D-IV was PCR amplified from p-XO2 plasmid of *Bacillus anthracis* Sterne strain with restriction sites for *Bgl* II enzymes at 5' and 3' ends to be cloned in pCAMBIA-1303 plant expression vector. Dephosphorylation of the vector after digestion has ensured that both ends of the vector are not self ligated. Right orientation of the clone was ensured by PCR amplification using forward primer from vector and reverse primer from D-IV. Sequencing results further confirmed the accuracy of the D-IV gene sequence. Agrobacterium leaf disc transformation was carried out for generation of transgenic plants. The selection medium comprising of the hormones for shoot regeneration and MS components provided a nutrient rich environment for plant regeneration. The selection gene *hpt* II encoding

resistance to hygromycin, is driven by a double-enhancer version of the CaMV35S promoter. Thus, use of hygromycin resistance gene for selection was effective to prevent false positives. Addition of silver nitrate protected the explants from necrosis (Fei S *et al.,* 2000), while cefatoxime (300 mg/L) prevented further growth of residual agrobacterium. Repeated rounds of subculturing ensured that the explants are not depleted by nutrients at any point throughout the experiment. Rooting was carried out in MS media with cefatoxime (300 mg/L) and hygromycin (40 mg/L) to ensure that only transgenic plants survive on selection medium. Polymerase chain reaction ensured the integration of D-IV transgene in the genome of plants. Further, electroblotting with anti-PA antibodies confirmed the expression of D-IV in tobacco plants. A single band 16 k Da corresponding to D-IV confirmed that there was no cross reactivity of antibodies with other plant proteins. Foreign proteins when expressed in plants by nuclear transformation are often prone to post translational modifications such as glycosylation (Hood *et al.,* 1997, Karnoup *et al.,* 2005). This could often lead to increase in the molecular weight of the protein and also affect the efficacy of bacterial antigens when expressed in plant system. There was no increase in the size of D-IV protein when expressed in tobacco confirming that post translational glycosylation did not occur. Various bacterial antigens were successfully expressed in plants *via* nuclear transformation (Haq T A *et al.,* 1995, Chikwamba R K *et al.,* 2002, Aziz *et. al.,* 2003, 2005, Yu J *et al.,* 2001). D-IV was also expressed in *E. coli* simultaneously and upon purification yielded a single 17 k Da band that reacted with anti PA antibodies. There was no cross reactivity with any other bacterial proteins. There was no degradation of the *E. coli* expressed D-IV for at least 6 months when stored at 4 °C., while the plant expressed proteins were seen to degrade rapidly within 2 days when stored at 4 °C and 14 days when stored at – 20 °C. D-IV expressed in *E. coli* was pure (>95 % purity) hence, was stable at lower temperatures. The plant expressed D-IV was stored as TSP, the alkaloids and polyphenolic compounds present in tobacco must have led to rapid degradation. The low and varied levels of D-IV expression (0.3-0.8 % of TSP) obtained in plants suggested random integration of D-IV in the genome of plants and emphasize the need for a more consistent and stable integration of the gene which would lead to increased yields.

Chapter 5

Expression of Protective Antigen Gene & its Domain-IV in Tobacco Chloroplasts

INRODUCTION

Plastid transformation technology has emerged as an important tool in plant biotechnology in recent years. Its extensive implication in basic science in elucidating the structure, function and evolution of the plastid genome and its application in expressing recombinant proteins for industrial, agronomical and biomedical purposes have made it the most promising transformation tool ever available. Due to high expression levels, ability to process polycistronic mRNA, and gene containment through the lack of gene pollen transmission, plastid transformation technology is fast replacing the conventional means of Agrobacterium transformation.

Plastids of higher plants are cell organelles with their own genome and transcription machinery. The plastid genome is a circular double stranded DNA 120-160 kb in size and encoding nearly 120 genes. Each cell contains 10,000 identical copies of the plastid genes. A significant feature of the plastid genome of higher plants is the presence large and small copy regions (LSC & SSC) and 2 inverted repeats (IR_A and IR_B) ~ 25 kb in size. The term 'plastid' encompasses various kinds of plastids such as, 'proplastids' the plastid progenitors, 'chloroplasts' the green plastids, and the nongreen plastids such as chromoplasts, amyloplasts, etioplasts etc. The description of 'plastid' transformation technology throughout chiefly underlines the transformation of 'chloroplasts' the green plastids. The transformation in non green plastids has not been very fruitful because their gene regulatory mechanisms are very different from chloroplasts and not much genome analysis has been done so as to effectively localize any foreign gene in to that region.

The process of plastid transformation involves the introduction of foreign DNA with selectable marker by biolistic method or polyethylene glycol treatment, integration of transforming DNA by homologous recombination, and concomitant reduction of wild type genome copies through repeated cell divisions on selection medium. The gradual progression from heteroplasmy to homoplasmy results in homogeneous population of stable genetically transformed plastids. The critical factors for efficient plastid transformation are the development of efficient plastid transformation vectors and recruiting appropriate tissue culture regeneration methods for various crop species.

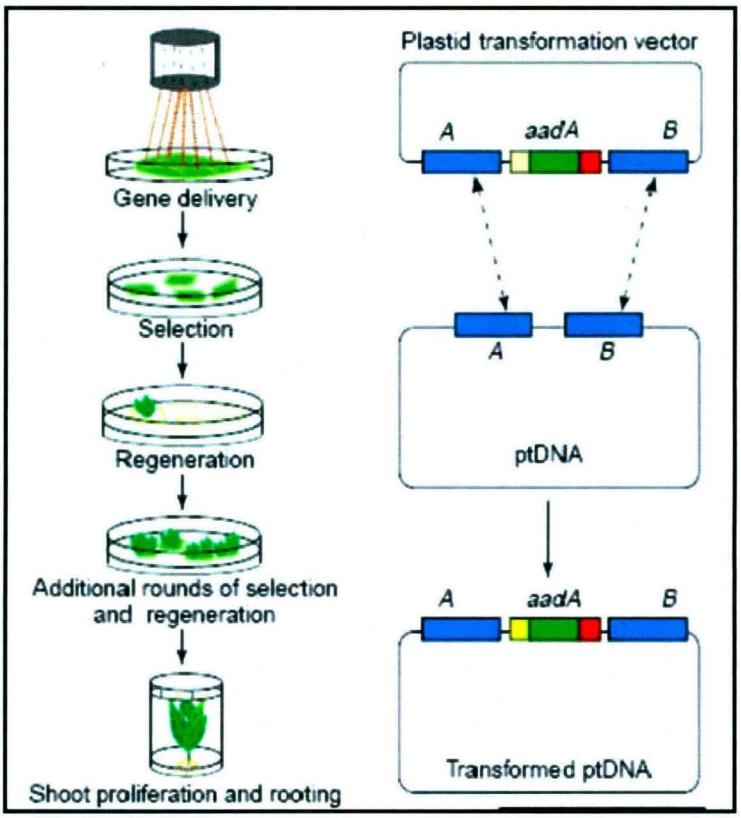


Fig 5.1) SCHEMATIC REPRESENTATION OF CHLOROPLAST TRANSFORMATION PROCESS. Generation of transplastomic plants left); Integration of foreign gene (*aad A*) via homologous recombination in to plastid genome.

The initial approach of plastid transformation used agrabacterium binary vectors (De Block M *et al.,* 1985). The explicit design of the binary vectors to target to the nucleus can very well explain its failure to serve as plastid vector (Ward D V *et al.,* 2002). This was followed by the design of shuttle vectors for episomal maintenance in *E .coli* and plastids. The episome with origin of replication sequence for its maintenance and a selectable marker in plastids could sustain on the selective medium but was rapidly lost in the absence of any selection (Staub J M and Maliga P 1994). Plastid targeting vectors offered the most plausible tool to efficiently target to a precise location in the plastid genome.

Typical Plastid targeting vectors are marked by the presence of left and right plastid targeting regions (LTR and RTR) that flank the expression cassette on either side. LTR

and RTR in the process of transformation replace the plastid genome by two recombination events. Plastid targeting fragments approximately 1-2 kb in size are homologous to the chosen target site. Transcriptionally inactive regions are chosen for insertion of foreign DNA which might otherwise affect the functionality of any vital gene of the plastid genome. Over 16 different insertion sites distributed in different regions of the plastid genome have been successfully used to develop various plastid targeting vectors. So far, only two vector families the pPRV series which target *trnv-rps12* intergenic region and pRBvectors which target to *trnM-trnG* region have been suitably constructed by removing unnecessary restriction sites and introducing multiple cloning sites to facilitate convenient cloning of passenger genes. Read through transcription facilitates the expression of foreign genes with Ribosome Binding Sequence (RBS) through the native promoter in the genome itself. Insertion of genes in *trn I* and *trn A* intergenic region has introduced the concept of universal vectors (Daniell *et al.,* 2001). Universal vectors though, have been used for transformation of closely related crop species, the transformation efficiency was drastically reduced when the homology of the plastid nucleotide sequence among the related species was less than 100 % (Sidorov VA *et al.,* 1999, Ruf S *et al.,* 2001, Zubko M. K *et al.,* 2004). With the availability of chloroplast sequence information for at least 35 crop species and development of appropriate regeneration techniques, tailoring of species specific vectors is far from a dream (Verma D and Daniell H 2007).

The expression cassettes of the plastid targeting vector consist of a 5′ regulatory region and a 3′ regulatory sequence centered by a gene of interest in the MCS region. The promoter and 5′UTR or the promoter and 5′ translational control sequence (TCR) constitute the 5′ regulatory region. The TCR contains 5′UTR and amino-terminal sequence of the coding region. The mRNA of 5′UTR forms a stem-loop structure that stabilizes mRNA and contains sequences that facilitate loading of mRNA on to ribosomes. Changes in the 5′UTR and the N-terminal sequence have shown to radically modify the expression levels (Ye G N *et al.,* 2001). The 3′ regulatory region comprising of the 3′UTR also forms a stem-loop structure required for mRNA stability (Monde R A *et al.*, 2000) and functions as inefficient terminator. Most commonly used T (terminator) cassettes derive from the plastid *psbA, rbcL* and *rps16* genes. The *psbA* and *rbcL* 3′UTR

appear to yield more stable mRNAs than the *rps16* 3'-UTR. Usage of alternative 3' UTR's resulted in modest improvement in the expression levels. Promoter choice is usually not a variable because most laboratories use the strong plastid rRNA operon (prrn) promoter.

The preliminary requirement of the gene of interest in the construct is fulfilled by the presence of antibiotic resistance gene. The most frequently used selectable marker is spectinomycin resistance marker, based either on integration of 16S-rDNAnucleotide sequences containing point mutations or on the expression of *aminoglycide-3'-adenyltransferase* (*aad* A gene). Selection of plastid transformants based on kanamycin resistance, i.e.; expression of *neomycin phosphotransferase* (*npt II* gene) (Huang F C *et al.,* 2002) has also been reported. Recently, betaine aldehyde dehydrogenase (BADH) gene from spinach was used as selectable marker. Reporter genes of *chloromphenicol acetyltransferase*(CAT)(Daniell H *et al.,* 1990), β-*glucorinidase* (*uid*A, GUS) (Liu CW *et al.,* 2007) and green flouroscent protein (GFP) (Khan M S and Maliga P 1999) have been transiently or stably transformed in plastids.

Stable transformation has been proved highly efficient only in tobacco (*Nicotiana tabaccum*). Plastid transformation has also been accomplished in Arabidopsis (Sikdar S R. *et al.,* 1998), Potato (Sidorov V A *et al.,* 1999), and tomato (Ruf S *et al.,* 2001) but encountered with problems of low transformation efficiencies, or the transgenics obtained were not fertile. Recently, lesequerella (Skarjinskaia M *et al.,*2003), cotton (Kumar S *et al.,* 2004), soybean (Zhang X H *et al.,* 2001, Dufourmantel, N. *et al.,* 2004), carrot (Kumar S *et al.,* 2004), oilseed rape (Hou B K *et al.,*2003) and lettuce (Kanamoto H *et al.,* 2006) have also been successfully been transformed. Rapid advancement in genome sequencing methods and efforts to employ efficient tissue culture techniques is promoting towards better understanding of various plastid genomes and development of more efficient plastid expression vectors to extend this technology to recalcitrant crops as well.

The focus of this chapter will be directed towards development of efficient plastid targeting vector for tobacco. The functionality will initially be assessed in *E. coli* with visual expression marker GFP as both share similarity in transcription machineries. Further, the Protective antigen gene and its most immunologically significant portion the 'Domain-IV' will be expressed individually in chloroplasts to achieve enhanced

expression levels over the conventional nuclear transformation methods. Finally, the biological activity of PA will be evaluated *in vitro* on J774A.1 mouse macrophage like cell line.

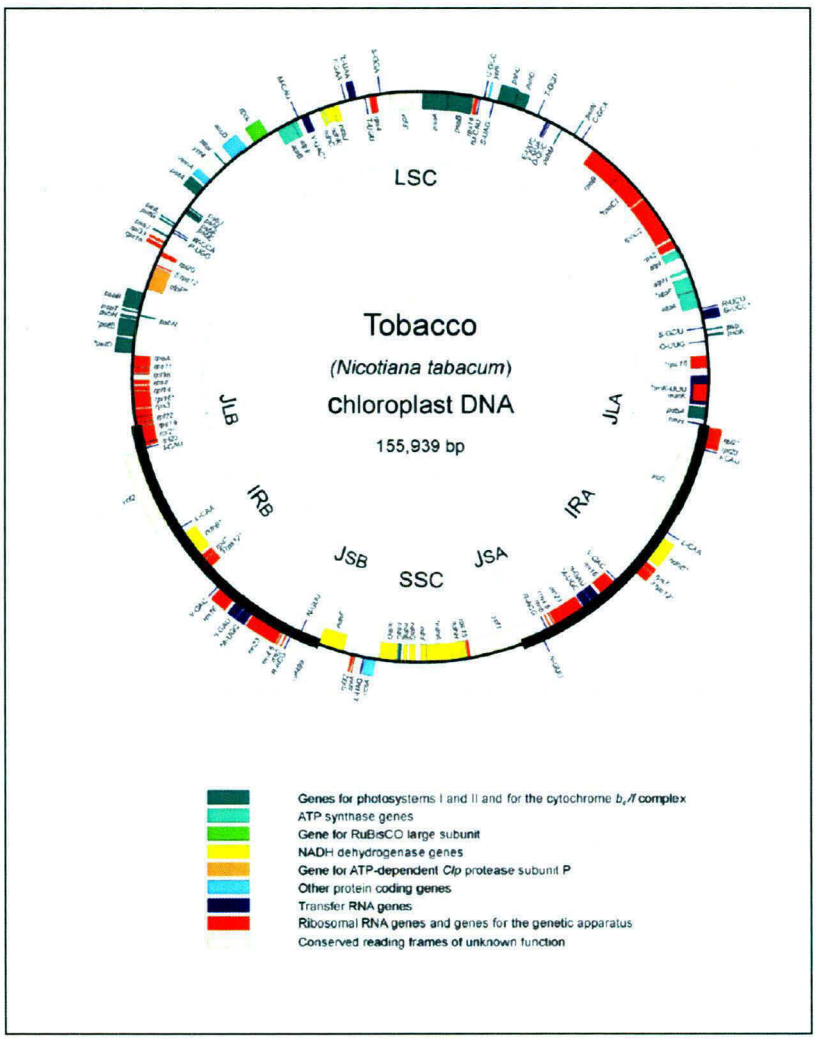


Fig. 5.2) ORGANIZATION OF TOBACCO CHLOROPLAST DNA

MATERIALS AND METHODS

The primers used for cloning were obtained from Microsynth GmbH, Balgach, Switzerland. The enzymes and chemicals used for DNA manipulation were purchased from New England Biolabs (UK) and MBI Fermentas (USA). The oligonucleotides were obtained from Microsynth (Switzerland). DNA purification kit was obtained from Qiagen (Germany). Agarose, Acrylamide, Ampicillin, Bovine Serum Albumin (BSA), Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, Glucose, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Potassium acetate, HEPES, PMSF, SDS, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium bicarbonate, Penicillin, Streptomycin, Imidazole, Tris, Tween-20, and other chemicals were purchased from USB Chemicals, USA. Protein determination dye was from Bio-Rad (USA). Absolute alcohol and Isopropanol were purchased from E. Merck, India. Nitrocellulose membrane was purchased from Amersham Pharmacia Biotech, UK. Cell culture plasticwares were obtained from Corning, USA. Fetal Calf Serum (FCS) was procured from Biological Industries (Israel). RPMI 1640, 3-(4,5-dimethylthiazol-3-yl)-5-diphenyltetrazolium bromide (MTT), Phenyl methylsulfonyl fluoride (PMSF), NaCl, EGTA, EDTA and other chemicals were purchased from Sigma Chemical Co. (USA). *E. coli* strain DH5α and RAW264.7, a macrophage like cell line, were obtained from ATCC (American Type Culture Collection; USA). Tobacco seeds (*Nicotiana benthamiana*) were purchased from the Indian Agricultural Research Institute (IARI), Pusa, New Delhi. Growth Media and its components were procured from Hi-Media Laboratories (India). Plant hormones were procured from Sigma Chemical Co. (USA). Accessories and components of Gene gun PDS-1000/He were purchased from its sole distributor, BioRAD. UV microscope, Nikon SMZ800 with GFP attachment was used to view the expression of GFP in bombarded leaf samples.

Bacterial strains and culture medium

E. coli DH5α was used as the host for cloning and propagation of plasmids and was cultured in Luria-Bertani broth supplemented with kanamycin (50 μg/ml) for the Domain-IV (D-IV) clones.

CONSTRUCTION OF CHLOROPLAST VECTOR

Designing vector backbone (pGEM-pBS)

Plastid vector backbone was designed by cloning of backbone region from pBSK (pbluescript) vector into pGEMT vector (promega) by TA tailing .1011-2961 bp region of pBSK+ vector that included origin of replication, ampicillin resistance (Amp) marker was PCR amplified using specific primers. The PCR reaction was carried out using a forward primer that included *Nru* I site and a reverse primer that included a *Mlu* I at its 5' end.

Forward Primer: 5' ACG TCG CGA GGC GGT TTG CGT ATT GGG CGC 3'

Reverse Primer: 5' CG ACG CGT G TGG CAC TTT TCG GGG AAA TG 3'

PCR reaction was carried out in Biorad iCycler under the following conditions:

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	56 °C
Extension	2 min	72 °C
Final extension	7 min	72 °C

The amplified PCR product was eluted using the gel extraction kit. The eluted PCR product and the vector were ligated by incubating overnight at 16°C and transformed into *E. coli* DH5α competent cells by procedure described in previous chapter. The putative recombinant clones were confirmed for the presence of desired fragment of 1.9 kb by restriction analysis with enzymes *Nru* I and *Mlu* I.

Cloning of chloroplast intergenic sequences in pGEMT vector (pGEM-(trnv-rps12))

Genomic DNA from tobacco (*Nicotiana tabaccum)* was isolated by standard CTAB method. A 2.5 kb fragment from plastid intergenic sequence (110,000-112,444 bp) of chloroplast genome was PCR amplified using specific primers. The PCR reaction was

carried out using a forward primer that included *Nru* I site and a reverse primer that included a *Mlu* I at its 5' end.

Forward primer: 5'ACG TCG CGA ATA ATC AGG CTC GAA CTG ATG ACT TCC ACC 3'

Reverse primer: 5' CG ACG CGT CAG TAC CTC GAC GTG ACA TGA GCG 3'

PCR reaction was carried out in Biorad iCycler under the following conditions:

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	60 °C
Extension	2 min 30 sec	72 °C
Final extension	7 min	72 °C

Ligation reaction was performed as described above and obtained transformants were confirmed for the presence of desired fragment of 2.5 kb by restriction analysis..

Cloning of Prrn cassette in pGEMT vector (pGEM-prrncass)

Prrn cassette which comprises of plastid ribosomal RNA promoter, *aad* A gene (*aminoglyside adenyl transferase*) which confers resistance to spectinomycin, and *psba* 3'UTR was PCR amplified with specific primer. Both forward and reverse primers included restriction site *Afl* II at the 5' end and 3' end respectively.

Forward primer: 5' GCA CTT AAG GCT CCC CCG CCG TCG TTC AAT GAG 3'

Reverse primer: 5' GCA CTT AAG AA CAA ATA CAA AAT CAA AAT AGA 3'

PCR reaction was carried out in Biorad iCycler under the following conditions:

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	95 °C
Annealing	30 sec	55 °C
Extension	1 min 20 sec	72 °C
Final extension	7 min	72 °C

Ligation reaction was performed as described above and obtained transformants were confirmed for the presence of desired fragment of 2.5 kb by restriction analysis.

Generation of pBS-trnvt-rps12

The pBSK backbone from pGEM-pBS was eluted after restriction digestion with *Nru* I and *Mlu* I respectively. Similarly, the chloroplast homologous sequences from [pGEM-(trnv-rps12)] were digested with *Nru* I and *Mlu*. Both the eluted fragments were ligated incubating overnight at 16 °C and the positive clones were confirmed by restriction analysis. The Prrn cassette from pGEM-prrncass vector was obtained by restriction digestion with *Afl* II. Chloroplast sequences have a single restriction site *Afl* II at position 1229/1233 bp of the 2.5 kb extracted sequence of the chloroplast intergenic sequence *trnv-rps12*.

pBS-trnvt-prrncass

pBS-(trnvt-rps12) was digested with *Afl* II, dephosphorylated at 37 °C 1h, and the DNA was precipitated. pBSK-*trnvt* clone was first linearised with *Afl* II. The linearised vector was gel eluted with Qiagen gel extaction kit. 1 µg of eluted DNA was added to 1X dephosphorylation buffer provided with 1 unit of calf intestinal phosphatase. Volume was made to 50 µl with autoclaved distilled water. The reaction was set at 37 °C for 1 h. The dephosphorylated DNA was column purified to remove excess of salt and the phosphatase enzyme itself which may otherwise hinder the ligation reaction. The Prrn cassette was also digested with *Afl* II and set for ligation in the vector: insert ratio of 1:3 at 16 °C overnight. Positive clones were confirmed by colony PCR and restriction analysis of the clones with *Afl* II. Thus, the final functional chloroplast vector was obtained. This was designated as pBS-trnvt-prrncass.

Introduction of MCS into pBS-trnvt-prrncass

Though, pBS-trnvt-prrncass was complete in all aspects fulfilling the basic characteristics of a chloroplast vector, it is limited by the absence of appropriate restriction sites to clone any gene of interest. For this purpose a stretch of nucleotides with appropriate unique restriction sites were designed. MCS was introduced after *aad* A gene sequence. The

MCS possessed *Not* I site at its 5′ end & *Xho* I site at its 3′ end. Vector backbone was PCR amplified with these restriction sites, digested with the above enzymes and ligated with MCS. The obtained colonies were screened for the presence of 64 bp MCS fragment. This vector thus designed was designated as pCHV-RKB.

Multiple Cloning Site (MCS)

Not I, *kpn* I, *Apa* I, *Xho* I, *Sal* I, *Hin*d III, *Sma* I, *Hpa* I, *Nhe* I, *Sac* I, *Xba* I

The nucleotide sequence of the MCS is as follows

Forward primer: GC GGCCGC GGT ACC GGG CCC CCC CTC GAG GTC GAC AAG CTT CCC GGG GTT AAC GCT AGC GAG CTC TCT AGA

Reverse primer: TCT AGA GAG CTC GCT AGC GTT AAC CCC GGG AAG CTT GTC GAC CTC GAG GGG GGG CCC GGT ACC GCG GCC GC

Cloning of green flouroscent protein (*Mgfp 5*) in a chloroplast vector pCHV-RKB

Trvt and rps12 homologous sequence are available which facilitates the integration of foreign gene in chloroplast genome by an event of homologous recombination. **Green flouroscent protein (*Mgfp 5* gene)** was amplified from pCAMBIA-1302 plasmid using specific primers. Forward primer was designed to introduce ribosomal binding site (GGGAG), spacer (TTTAT), initiation codon (AUG) and a restriction site for *Eco*R V enzyme. Reverse primer was designed to introduce *Xho* I site at 3′ end of GFP sequence.

Forward primer: 5′ GGC GAT ATC AGG TTT ATA TGA GTA AAG GAG AAG AAC TTT TC 3′

Reverse primer: 5′ CCG CTC GAG TTA TTT GTA TAG TTC ATC CAT GCC ATG 3′

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	58 °C
Extension	1 min	72 °C
Final extension	7 min	72 °C

The PCR amplified fragment of ~ 728 bp and vector DNA were digested with *Not* I and *Xba* I enzymes in compatible buffers. The digested vector and insert DNA was gel purified using Qiagen gel extraction kit and ligated at 4 °C for 16 h. The transformants were screened by plasmid isolation and restriction analysis.

Cloning of D-IV of Protective antigen gene in a chloroplast vector

D-IV of Protective antigen gene was cloned in a vector that was specifically designed to drive expression of transgene using chloroplast transformation technology. *trvt* and *rps12* homologous sequences are available which facilitate the integration of foreign gene in chloroplast genome by an event of homologous recombination.

D-IV of PA gene was amplified from pXO1 plasmid of *B. anthracis* (Sterne 34 F2 strain) using specific primers. Forward primer was designed to introduce ribosomal binding site (GGGAG), spacer (TTTAT), initiation codon (AUG) and a restriction site for *Not* I enzyme. Reverse primer was designed to introduce *Xba* I site at 3′ end of D-IV.

Forward primer: 5′ AAA AGG AAA AGC GGC CGC AGG AGGTTT AT ATG TTT CAT TAT GAT AGA AAT AAC ATA GCA GTT GGG 3′

Reverse primer: 5′ CCG CTC GAG TTA GTG ATG GTG ATG GTG ATG TCC TAT CTC ATA GCC 3′

PCR amplification was carried out under the following conditions

Step		**Recommended conditions**
Denaturation	5 min	95 °C
3-step cycling (30 cycles)		
Denaturation	1 min	95 °C
Annealing	1 min	60 °C
Extension	30 sec	72 °C
Final extension	7 min	72 °C

The PCR amplified fragment of ~ 421 bp and vector DNA were digested with *Not* I and *Xho* I enzymes in compatible buffers. The digested vector and insert DNA was gel purified using Qiagen gel extraction kit and ligated for 16 h at 4 °C in a stoichiometric ratio of Vector: Insert, 1:3.

Expression of pCHV- RKB-D-IV in *E. coli*

The above prepared construct was transformed in competent XL1 Blue *E. coli* cells for checking the expression of D-IV. The cell culture was grown to an OD_{600} at 1.0 and the cells were harvested by centrifugation at 5000 rpm for 10 min. The supernatant was collected in a separate tube and the pellet was suspended in 8 M urea. 100 µl of the suspended cells were mixed with 6X loading buffer and boiled in a water bath for 5 min. followed by a brief spin. The supernatant from the boiled sample were analyzed by resolving the proteins on a 12 % SDS-PAGE. The expression of D-IV was confirmed by carrying out western blot using polyclonal antibodies generated against purified protective antigen. The dilution of primary antibody used was 1:5000. The expression level was determined by densitometric analysis using Quantity One software.

GENERATION OF TRANSPLASTOMIC PLANTS EXPRESSING PA & D-IV BY BIOLISTICS

Particle Bombardment

Preparation of microcarriers

1. Weighed 50 mg of particles (Au) in an sterile microfuge tube (This amount is more than the recommended 30 mg to compensate for the losses during washing)
2. 1 ml of 70 % v/v ethanol was added & vortexed on a platform vortexer for 1 min.
3. The particles were allowed to settle for 1 min followed by a brief spin at 3000 rpm.
4. The supernatant was removed and discarded.
5. Repeated the following wash steps three times:
    - Add 1ml of sterile water
    - Vortex vigorously for 1min
    - Allow the particles to settle for 1min.
    - Pellet the microparticles by a brief spin
    - Remove the supernatant and discard
6. After the third wash, 500 µl sterile 50 % glycerol was added to bring the microparticles concentration to 60 mg/ml.

7. Gold microcarriers can be stored for one month but tungsten should be used immediately. Aliquots of gold microparicles were stored at -20 °C.

Coating of DNA onto microcarriers

The protocol used for coating microparticles sufficient for six bombardments is outlined below. Continuous vortexing was needed throughout the coating protocol. A platform vortexer was used for the purpose.

1. Microcarriers prepared in 50 % glycerol were vortexed for 5min.
2. 50 µl (3 mg) of microcarriers to a microfuge tube were removed.
3. While vortexing vigorously, the following components were added in strict order
   i. 0.5 µl of ultrapure DNA (1 µg/ul)
   ii. 50 µl $CaCl_2$ (2.5 M)
   iii. 20 µl Spermidine (0.1 M)
4. Continued vortexing for 2-3 min.
5. The microcarriers were allowed to settle for 1 min.
6. Pelleted the microcarriers by a pulse spin.
7. Removed the supernatant and discarded
8. Added 140 µl of 70 % v/v ethanol without disturbing the pellet
9. Removed the liquid and discarded.
10. Added 140 µl of 100 % ethanol without disturbing the pellet
11. Removed the liquid and discarded
12. Added 48 µl of 100 % ethanol
13. Gently resuspended the pellet by tapping the side of the microfuge tube several times and then by vortexing at low speed for 2-3 min.
14. Pipetted out 6 µl aliquot and transferred them to the center of a macrocarrier.

Preparation of Osmoticum plates and target tissue

The addition of an osmoticum (i.e. a supplemental agent increasing osmolarity) to the bombardment medium can dramatically increase the rates of transformation. Elevated osmoticum concentration may work by protecting the cells from leakage and bursting (low turgor pressure) and may also improve particle penetration itself. In case of tobacco

plastid transformation, the standardization of an osmoticum composition was carried out using sorbitol, mannitol and sucrose. The bombardment was performed according to the instructions given in the manual (BioRad He-2000).

Standardization of Bombardment parameters

The efficiency of a bombardment in transporting the DNA coated microcarriers inside the plant cells and then landing up in chloroplast involves a concerted interplay of several factors. The following parameters pressure, nature of the microcarriers, distances (Gap distance, microcarrier flight distance and target distance), composition of the osmoticum, selection of antibiotic concentration, age of the leaf sample and the choice of single or multiple bombardments were optimized to achieve successful transformation of plastids. Green fluorescent protein (GFP) was taken as a standard to accelerate the process of standardization. A GFP construct was used along with the *pag* A construct (from previous studies), D-IV gene construct and the control vector to study the transformation event after the bombardments.

Generation of tobacco plants with transgenic plastids and molecular analysis

The explant used for particle bombardments was a fully grown 3-4 week old tobacco leaf. This leaf was kept with its abaxial surface facing the DNA coated microprojectiles. One leaf was kept on each plate containing solidified osmoticum for 12-16 h. before bombardment. After the bombardment, the leaves were kept in dark for 48 h. Bombarded leaves were cut into small pieces of approximately 5 x 5 mm. and kept on selection medium that contained MS salts and phytohormones along with spectinomycin (500 mg/litre). Two rounds of selection were carried out at an interval of 2-3 months. Control bombardments with only vector DNA were also carried out. The molecular analysis of transplastomic *pag A* and D-IV plants was carried out using PCR, Immunoblot analysis and ELISA. The activity of plant expressed PA was studied on J774A.1 mouse macrophage cell line as described in previous chapters.

RESULTS

Designing the vector backbone (pGEM-pBS)

1.9 kb region was amplified from pbluescript (pBS) vector which has essential regions of vector backbone the origin of replication, ampicillin resistance for antibiotic selection with *Nru* I and *Mlu* I at 5′ and 3′ ends respectively. The amplicon after elution was ligated in to pGEMT vector. The ligated product was transformed in to *E. coli* DH5α cells in addition with IPTG (Isopropyl thiogalactoside) and plated on LB medium containing X gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) with appropriate antibiotics. The blue white selection enabled the selection of positive colonies which were white in colour. The presence of 1.9 kb clone was confirmed by colony PCR and restriction digestion with *Nru* I and *Mlu* I (Fig.5.3 a, b).

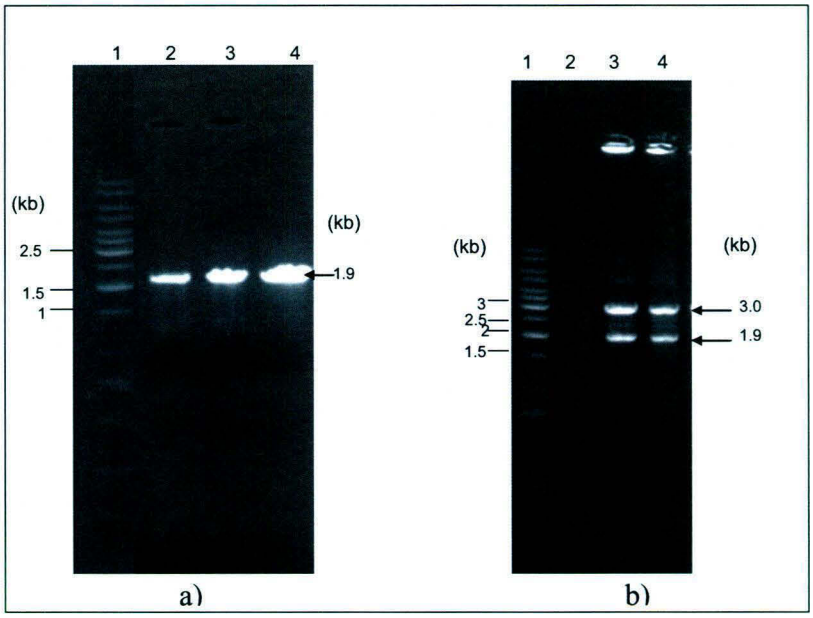


Fig. 5.3)
CLONING OF pBSK VECTOR BACKBONE IN pGEMT-EASY VECTOR
a) PCR amplification of 1.9 kb pbsk+ backbone.
Lane 1 – 1 kb ladder, lane 2, 3, 4 – 1.95 kb amplicon. Lanes 2,3,4 showing gradient PCR at Tm - 55 °C, 55.8 °C, 57.3 °C respectively.
b) Restriction analysis of clones p GEM - pBSK+
Lane 1- 1 kb ladder, lane 2- nil, lanes 3 & 4 show 1.95 kb insert fragment as well as the 3.0 kb pGEMT vector back bone.

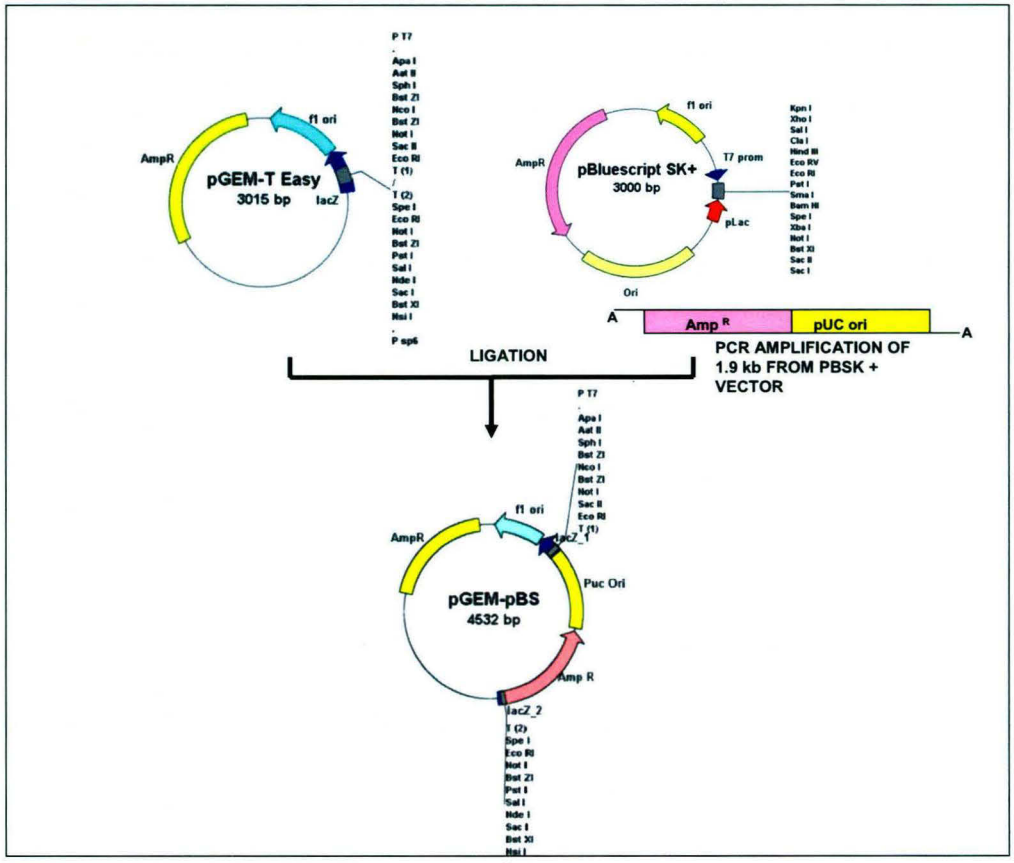


Fig.5.4) SCHEMATIC REPRESENTATION OF CLONING OF pBSK VECTOR BACKBONE IN pGEMT- EASY VECTOR.

Cloning of chloroplast intergenic sequence in pGEMT vector (pGEM-trnv-rps12)

The basis of chloroplast transformation is the integration of gene of interest at a specific site. Intergenic sequences of the chloroplast genome are effectively utilized for the construction of chloroplast vectors so that targeted integration of the gene occurs via homologous recombination. For this purpose intergenic sequence *trnvt-rps12* (110,000-112,444 bp) was amplified from tobacco chloroplast genome and was cloned in pGEMT vector. Positive clones were identified by restriction digestion with *Nru* I and *Mlu* I (Fig 5.5 a, b).

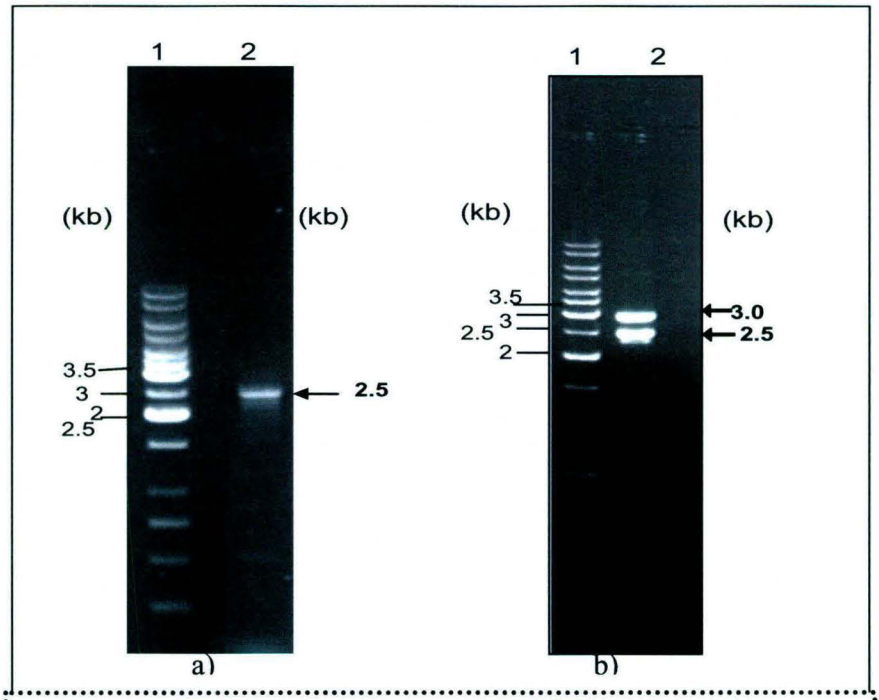


Fig. 5.5)
CLONING OF *trnv- rps12* INTERGENIC SEQUENCES IN pGEMT-EASY VECTOR
a) PCR amplification of 2.5 kb *trnv- rps12* intergenic sequences from tobacco. Lane 1 – 1 kb ladder, lane 2 – 2.5 kb amplicon. Genomic DNA was isolated from tobacco leaves and chloroplast intergenic sequence was PCR amplified
b) Restriction analysis of clone pGEM-trnvt
Lane1- 1kb ladder, Lane 2 shows 2.0 kb insert fragment as well as 3.0 kb vector back bone upon digestion with *Nru* I and *Mlu* I.

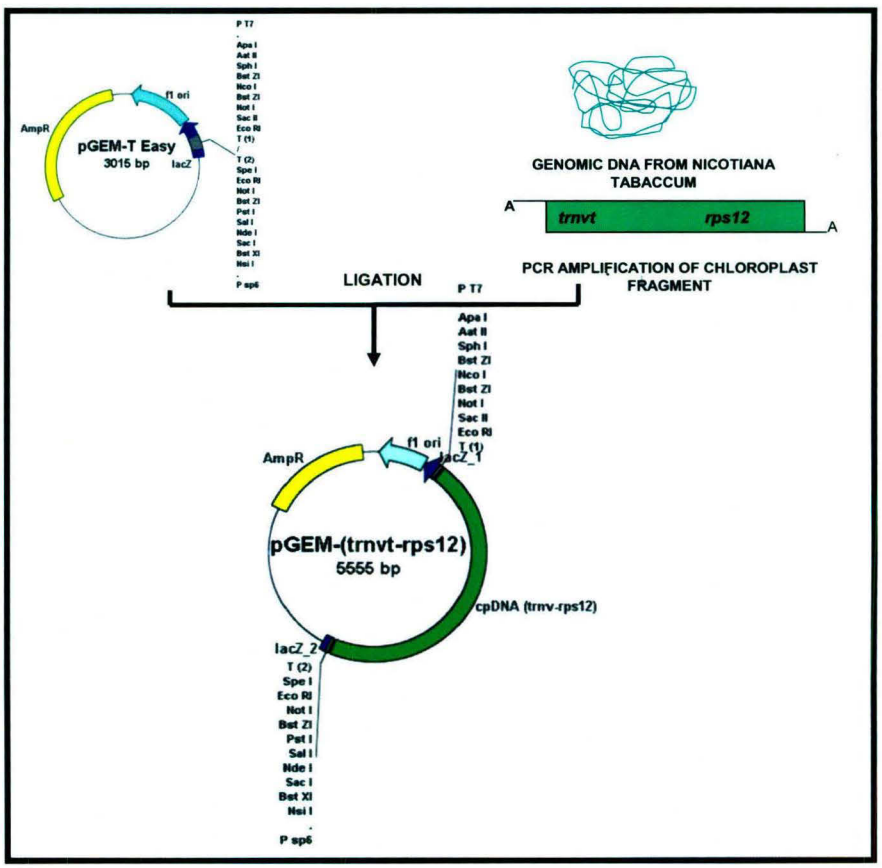


Fig. 5.6) SCHEMATIC REPRESENTATION OF CLONING OF Cp-DNA IN pGEMT- EASY VECTOR.

Cloning of prrn cassette in pGEMT vector (pGEM-prrncass)

The prrn cassette was synthesized by microsynth. The plastid ribosomal RNA promoter, *aad* A gene (*aminoglyside adenyl transferase)* which confers resistance to spectinomycin, and *psba* 3′UTR constitute the expression cassette. The expression cassette was cloned with restriction sites *Afl* II at the 5′ end and 3′ end respectively (Fig.5.7 a, b).

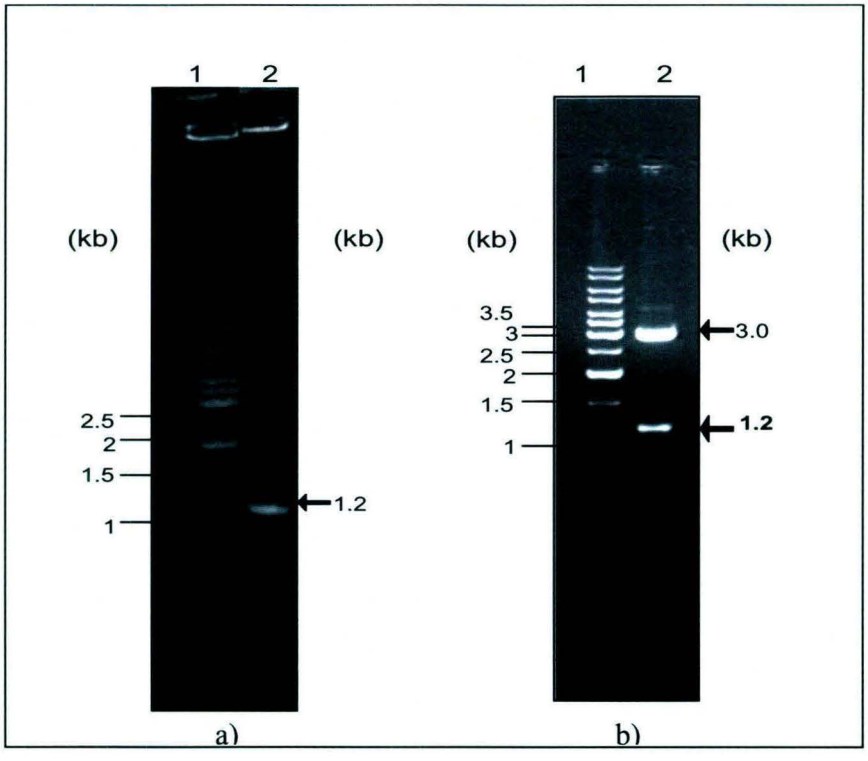


Fig.5.7)
CLONING OF PRRN CASSETTE IN pGEMT-EASY VECTOR
a) PCR amplification of 1.2 kb prrn casette.
Lane 1 – 1 kb ladder, lane 2 – 1.2 kb amplicon of prrn cassette.
b) Restriction analysis of clone pGEM-prrncass.
Lane1- 1kb ladder, Lane2 shows 1.2 kb insert fragment as well as 3.0 kb vector back bone upon digestion with *Afl* II.

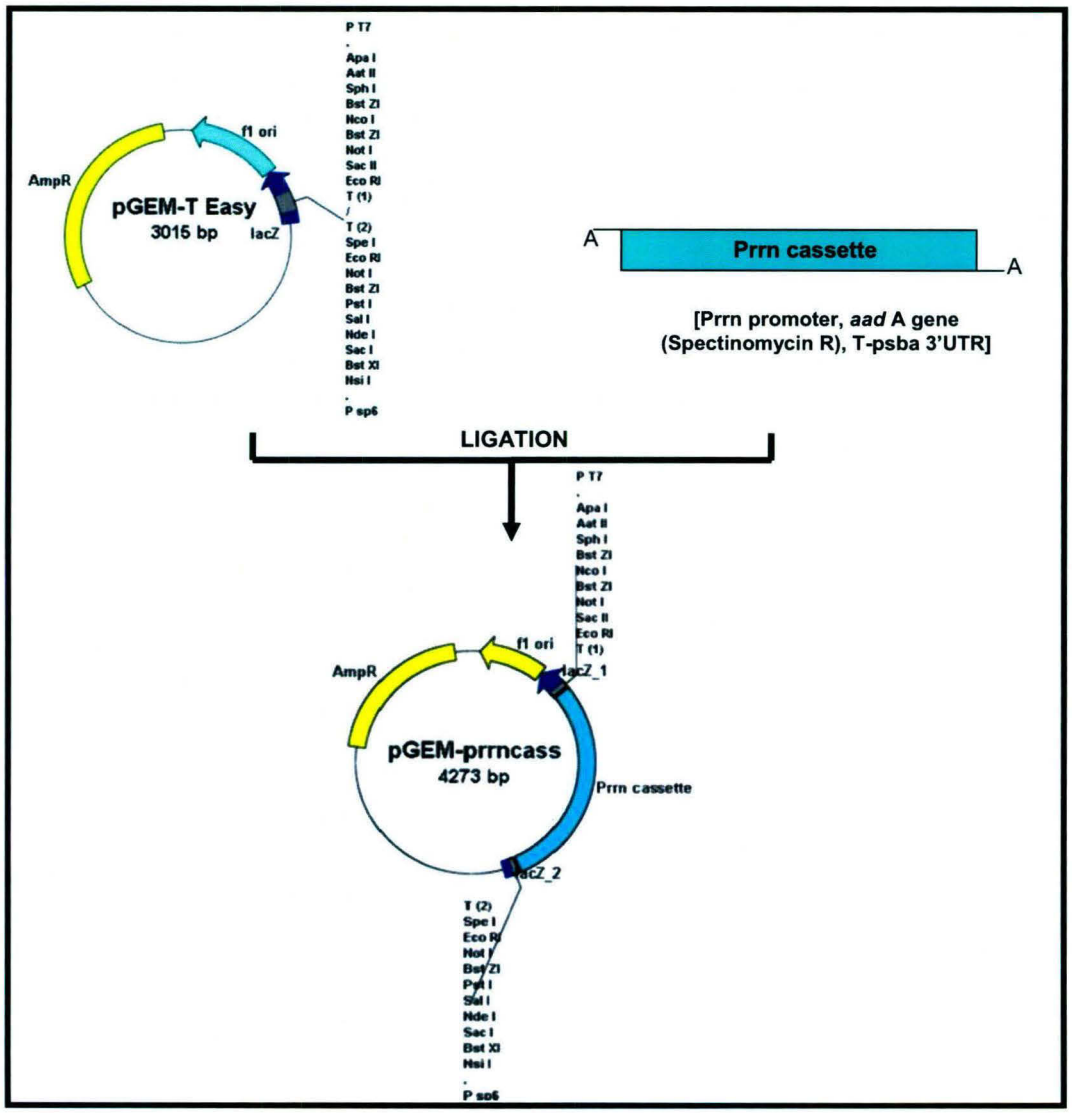


Fig. 5.8) SCHEMATIC REPRESENTATION OF CLONING OF prrn CASSETTE IN PGEMT- EASY VECTOR.

Cloning of pbsk-(trnvt-rps12)-prrncass

The pBSK- vector backbone from clone pGEM-PBS and the chloroplast homologous sequences from pGEM-(trnvt-rps12) were eluted after restriction digestion with *Nru* I and *Mlu* I, respectively. Ligation of both the above eluted fragments resulted in the positive clones which were confirmed by restriction analysis. The prrrn cassette from pGEM-prrncass was obtained by restriction digestion with *Afl* II. Chloroplast sequences have a single restriction site *Afl* II at position 1229/1233 bp of the 2.5 kb extracted sequence of the chloroplast intergenic sequence *trnv-rps12*. Therefore, the prrn expression cassette with *aad* A gene encoding for aminoglycide phosphotransferase for spectinomycin resistance was digested with *Afl* II and cloned in *Afl* II site of pbsk-trnvt clone resulting in a functional chloroplast vector pbs-trnvt-prrncass. This basic functional chloroplast vector obtained was 5748 bp. Restriction digestion with enzyme *Afl* II gave a 3.7 kb back bone comprising of the 1950 bp pbsk sequence and 2540 bp of *trnvt-rps12* chloroplast intergenic sequence. Further, restriction digestion with *Nru* I and *Mlu* I resulted in 1950 bp pbsk backbone and 3798 bp insert which comprised of 1258 bp prrn cassette and 2540 bp chloroplast intergenic sequence. Thus, the enzyme profiling results were in concordance with the hypothetical vector length. The colonies of the final vector were also tested for spectinomycin resistance. The positive clones were selected on ampicillin (100 µg/ml) and spectinomycin (100 µg/ml). Therefore, the expression of *aad A* gene driven by Prrn promoter which provides resistance to spectinomycin has also been confirmed (Fig 5.9).

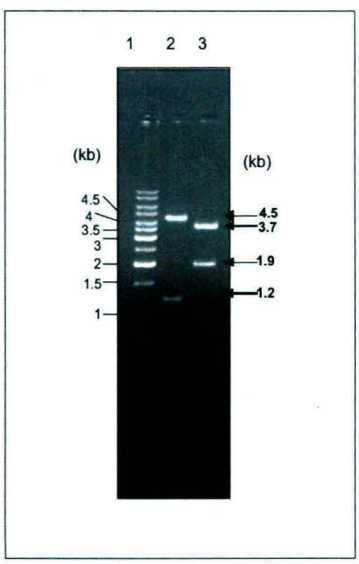


Fig. 5.9)
Restriction analysis of the basic chloroplast vector Pbs-trnvt-prrncass
The basic functional vector was 5748 bp.
Lane 1- 1kb ladder, lane 2- digestion of the vector with *Afl* II to obtain fragment of 1.2 kb prrn cass fragment and 4.5 kb backbone, Lane 3- restriction digestion with enzymes *Nru* I and *Mlu* I releases 1.9 kb insert and a 3.7 kb backbone as expected.

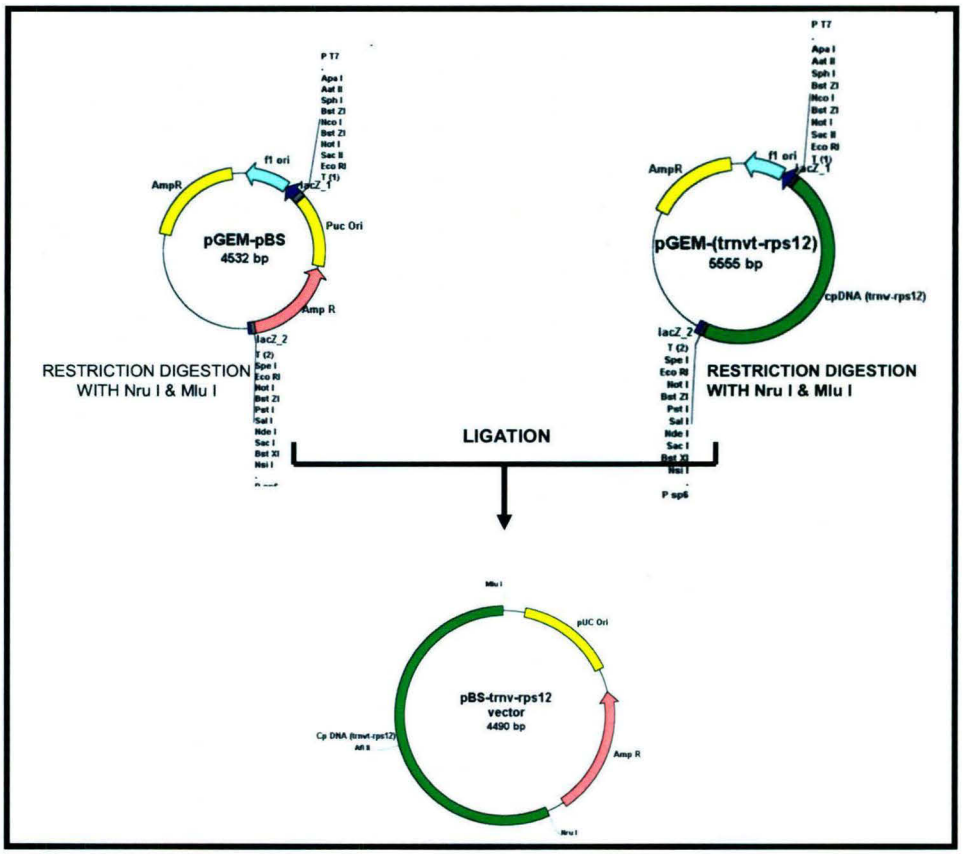


Fig. 5.10) SCHEMATIC REPRESENTATION OF CLONING OF Cp DNA and VECTOR BACK BONE.

Introduction of MCS into pbsk-(trnvt-rps12)-prrneass

A 64 bp Muliple cloning site with restriction sites *Not* I, *kpn* I, *Apa* I, *Xho* I, *Sal* I, *Hind* III, *Sma* I, *Hpa* I, *Nhe* I, *Sac* I, *Xba* I was cloned in to the basic chloroplast vector after the *aad* A gene. The 64 bp stretch of nucleotides was synthesized by microsynth with *Not* I and *Xba* I overhangs at 5′ and 3′ ends for ease of cloning. The MCS was introduced to clone any gene of interest using any of the sites according to the compatibility of the gene. Since the insert is very small in size we confirmed the clone by linearizing the vector with *Sal* I and also sequencing of the clone (Fig. 5.11 a, b).

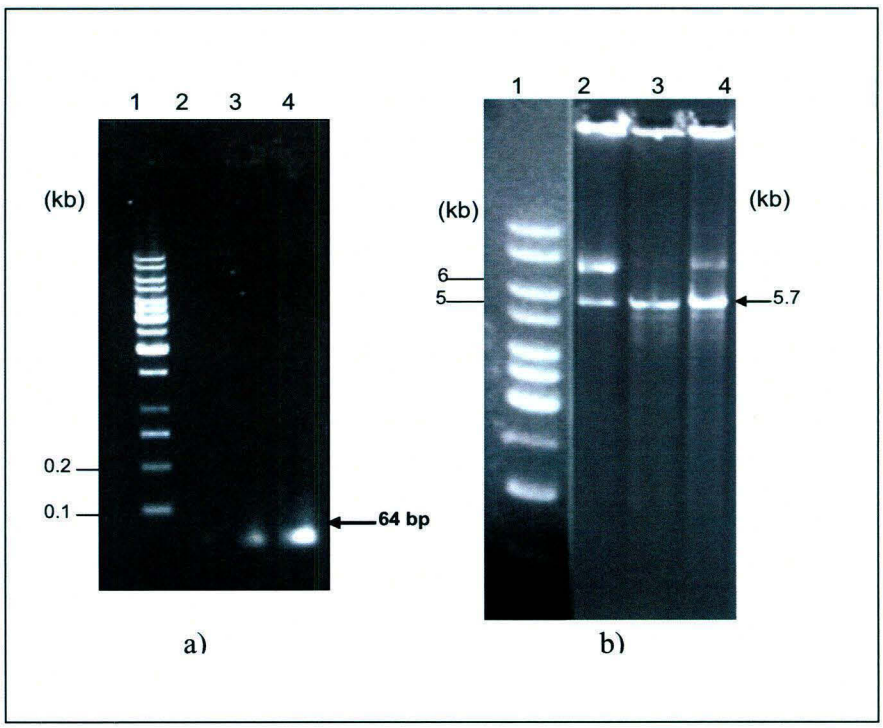


Fig. 5.11)
CLONING OF MCS IN PCHV.
a) Synthetic MCS after annealing on the gel.
Insertion of 64 bp MCS in to the chloroplast vector, Lane 1: 1 kb ladder, lane 2, 3, 4 – 64 bp annealed fragment.
b) Restriction analysis of clone pCHV-RKB
Lane1- 1kb ladder, Lane 2, 3, & 4 digestion with *Sal* I. Only the clone in which MCS has been inserted was linearised (lane-3).

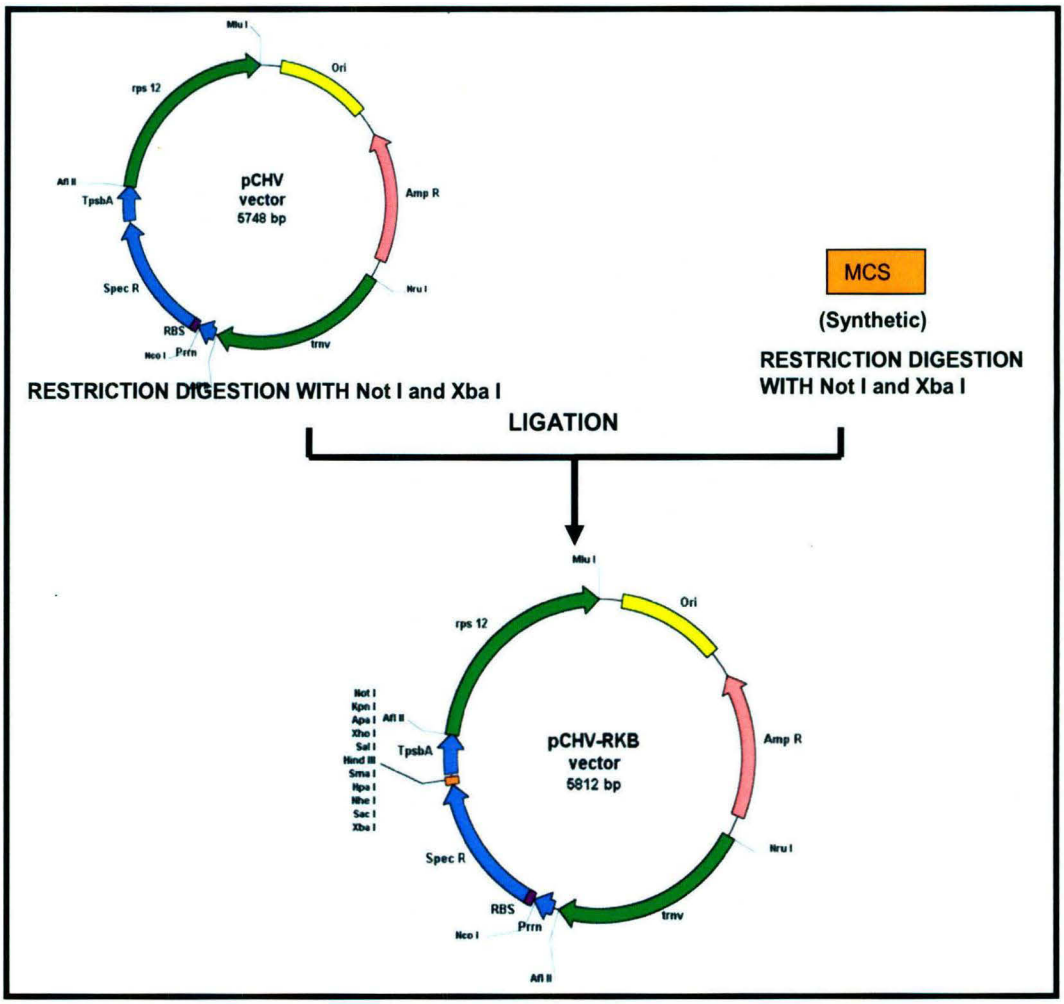


Fig. 5.12) SCHEMATIC REPRESENTATION OF CLONING OF MCS IN CHLOROPLAST VECTOR pCHV.

Cloning of green flouroscent protein (gfp) in a chloroplast vector pCHV-RKB

Green flouroscent protein (*gfp* gene) was amplified from pCAMBIA 1302 plasmid using specific primers and cloned between *Not* I and *Xba* I. The gene was facilitated with ribosomal binding site (GGGAG), spacer (TTTAT), initiation codon (AUG) to aid in efficient transcription and translation. Putative clones obtained after transformation were screened for the presence of gene by colony PCR. Restriction digestion of plasmids further confirmed the presence of the gene. The obtained clones were sequenced to ensure that gene had no mutations. We envisaged checking the expression of GFP gene under the effect of *Prrn* promoter in *E. coli*. Owing to the similarity of plastid transcription machinery with prokaryotic systems, the expression of GFP could easily be checked in *E. coli* itself. Expression of the construct in *E. coli* was confirmed by observation under UV microscope (Fig. 5.13).

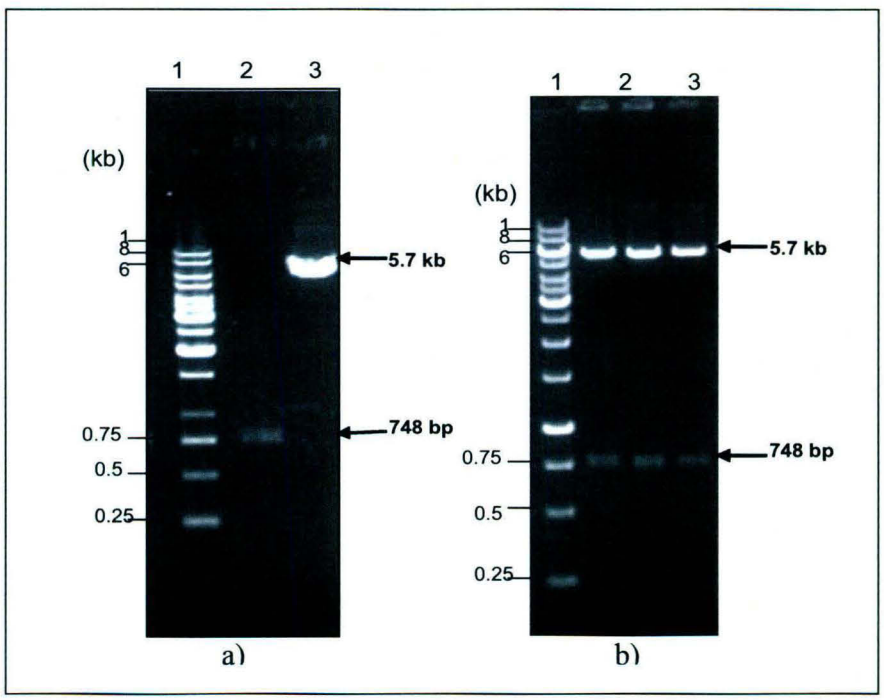


Fig. 5.13)
CLONING OF *GFP* FROM PCAMBIA 1302 VECTOR.
a) PCR amplification of *gfp*
Lane 1- 1 kb ladder, PCR amplicon of *gfp* after restriction digestion with *Not* I and *Xba* I, Lane 3- Vector back bone after digestion with *Not* I and *Xba* I.
b) Restriction analysis of clone pCHV-RKB-GFP
Lane1- 1kb ladder MBI Fermentas, Lane 2 shows 0.75 kb insert fragment as well as 5.7 kb vector back bone.

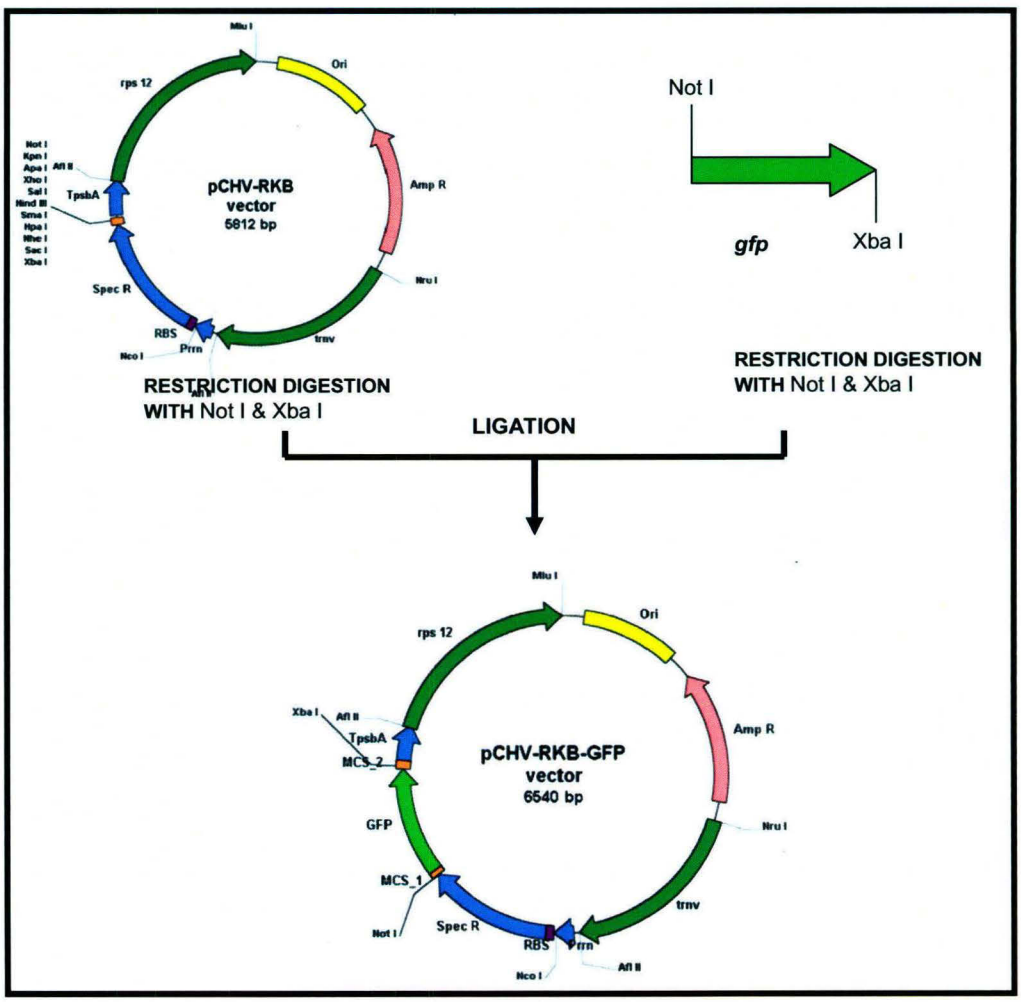


Fig. 5.14) SCHEMATIC REPRESENTATION OF CLONING OF GFP IN CHLOROPLAST VECTOR pCHV-RKB.

Cloning of D-IV gene in chloroplast transformation vector

Chloroplast translation machinery needs a set of promoter, terminator, homologous sequences and antibiotic resistance marker gene for expression of a transgene. A specific chloroplast transformation vector containing all these necessary features was used to express D-IV of PA gene in tobacco chloroplast. *Not* I and *Xba* I site available in the MCS were used to clone the D-IV gene. Histidine tag was also added to the gene at the C- terminal. The amplification of D-IV gene was done using *pfu* polymerase enzyme and the transformants thus obtained were analyzed for the presence of D-IV gene using restriction analysis with *Not* I and *Xba* I. Restriction digestion of the positive clone resulted in a fallout of 452 bp (D-IV- 421bp+ His tag-18 bp + RBS-spacer and start codon-13 bp) fragment of D-IV thus confirming the presence of D-IV.

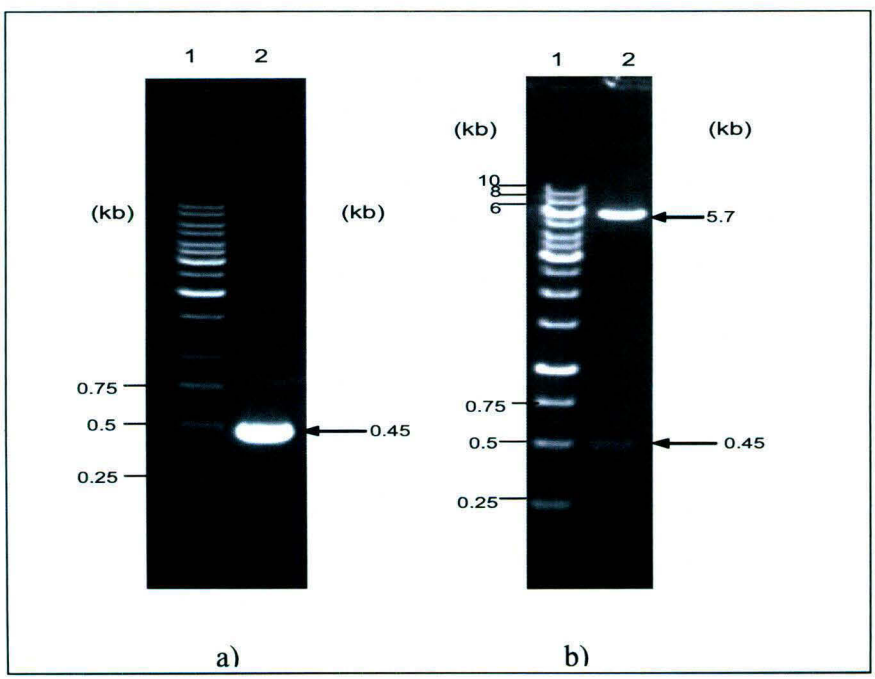


Fig. 5.15)
CLONING OF DOMAIN –IV OF PA GENE.
a) PCR amplification of Domain –IV of PA gene.
Lane 1- 1 kb ladder MBI Fermentas, PCR amplicon of *D-IV* after restriction digestion with *Not* I and *Xba* I.
b) Restriction analysis of clone pCHV-RKB-D-IV
Lane1- 1kb ladder MBI Fermentas, Lane 2 shows 0.45 kb insert fragment as well as 5.7 kb vector back bone (insert includes the RBS site + D-IV sequence and His tag).

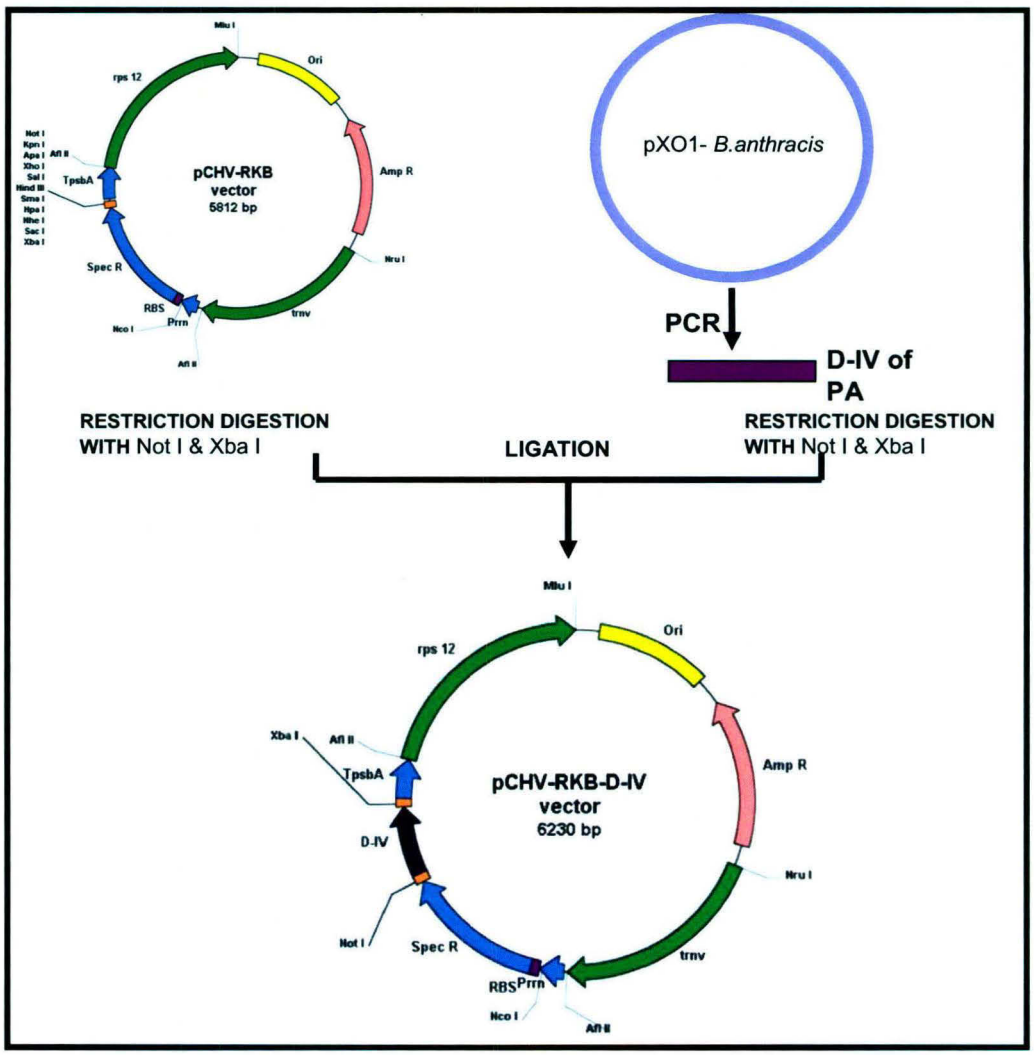


Fig. 5.16) SCHEMATIC REPRESENTATION OF CLONING OF D-IV IN CHLOROPLAST VECTOR pCHV-RKB.

Standardization of parameters for particle bombardment

The basis of standardization of bombarding parameters was to keep a control DNA carrying gene for GFP expression while studying the effect of different variables. This is to facilitate quick monitoring of the bombardment efficiency under a particular set of conditions. Our results describing comparative bombarding efficiency in a set of condition was based on this visual marker. The helium driven system is functional from 600 to 2400 psi. We tried 1100 and 1350 psi pressures for driving the microcarriers. 900 psi was found to be suboptimal and 1350 caused severe injury to the leaf tissue. 1100 psi was found to be the pressure most optimal for our bombardments. Both tungsten and gold micro carriers of 0.6 μ and 1.0 μ were used and 0.6 μ gold particles were found to perform the best. Tungsten has already been reported to be toxic in plants cells and usually results in the formation of uneven sized particles, which reduces the reproducibility of the experiments. A gap distance of ¼", microcarrier flight distance of 11 mm and target distance of 9 cm were the optimum distances to impart requisite momentum to the microprojectiles.

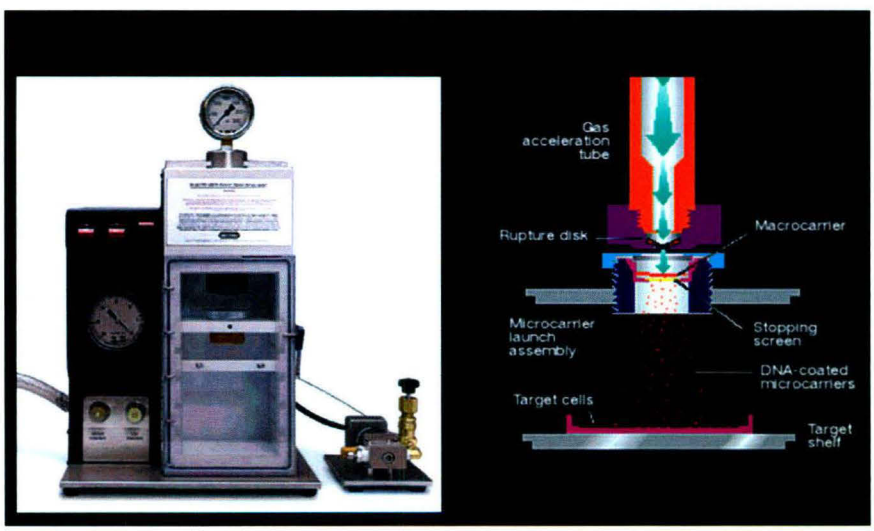


Fig. 5.17) PICTURE OF GENEGUN (RIGHT), DIAGRAM SHOWING MECHANISM OF DNA DELIVERY BY USING GENEGUN (LEFT).

PRESSURE	PARTICLE USED	GAP DISTANCE	FLIIGHT DISTANCE	TARGET DISTANCE	OSMOTICUM	AGE OF LEAF	NUMBER OF BOMBARDMENTS	ANTIBIOTIC CONCENTRATION
1100 psi	0.6 μ Au	¼"	11 mm	9 cm	MS + 2% Sucrose+ 0.5 M sorbitol + 0.5 M Mannitol	3-4 week old	Two per leaf	500 mg/l

STANDARDIZED BOMBARDMENT PARAMETERS USED FOR TRANSFORMING CHLOROPLAST

The selection was carried out at 500 mg/litre of spectinomycin. The leaf samples of 3-4 weeks age were found to show best performance. The idea of bombarding the same leaf twice or thrice at different positions was intended to transform as many numbers of cells as possible in a single leaf. It was observed that two bombardments per leaf gave better results than one or three with *pag* A and D-IV constructs.

Regeneration of bombarded leaf explants and their molecular analysis

The bombarded leaf explants were incubated in dark for 48 h followed by their cutting into small squares and subjecting them to selection medium with appropriate conditions (8 h. light 16 h. dark at 26 °C). Each transformed small explant initially grew into a callus after 6-7 weeks we could see the emergence of tiny new tobbaco shoots from the bombarded leaves. The untransformed leaf explants turned white to brown in colour without forming a callus. The obtained shoots were allowed to grow in selection for another 2 weeks, then the shoots were carefully transferred to the rooting medium which contained MS medium supplemented with 500 mg/l spectinomycin. 2-3 transformed shoots were obtained in about 12 bombarded leaves. The DNA extracted from these plants was subjected to PCR analysis using primers corresponding to 5′ and 3′ termini of *pag* A and D-IV gene for respective transplastomics. All the bombarded leaf explants that received the transgene exhibited amplification of the appropriate size when subjected to PCR, but control explants failed to show any such amplification.

Fig. 5.18)
GENERATION OF PA TRANSPLASTOMICS
a) Regeneration of shoots from explants SM. b) Putative transgenic plants at the bottle stage in SM. c) Explants from the transgenic plants on selection medium for second round of selection to achieve homoplasmy. d) Transgenic plants in agropeat. e) Fully grown transgenic plants in greenhouse. f,g) Germination of T1 seeds on spectinomycin(500 mg/l) media.

Fig.5.19)
DEVELOPMENT OF D-IV CHLOROPLAST TRANSGENIC PLANTS
a) Transformation with plCHV-DIV construct show regeneration (Right).b) Regeneration of shoots from explants SM. Putative transgenic plants at the bottle stage in SM c) Explants from the transgenic plants on selection medium for second round of selection to achieve homoplasmy. d) Transgenic D-IV plants in agropeat in greenhouse to acclimatize to the external environment. e) Fully grown transgenic D-IV plants in greenhouse.

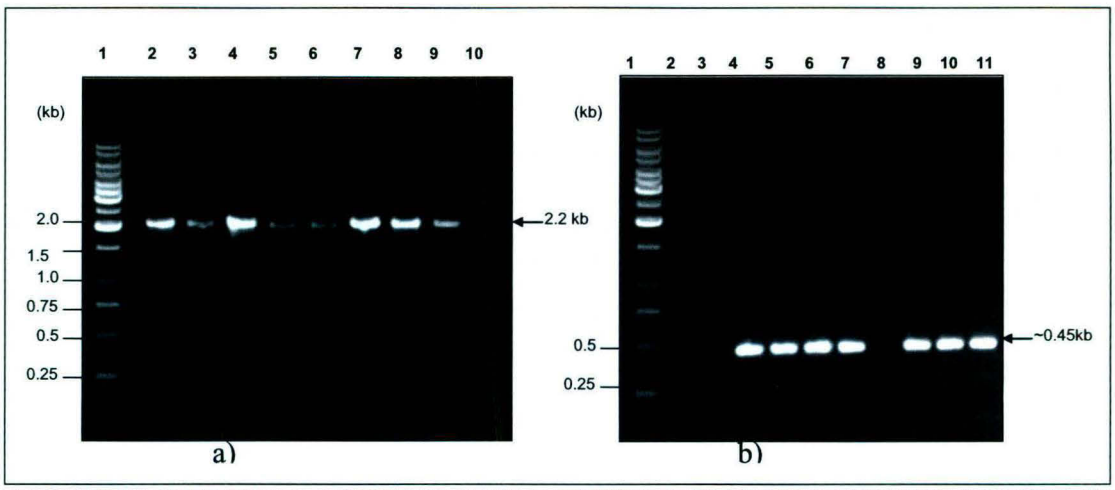


Fig. 5.20)

a) PCR amplification of *pag* A gene.

Lane 1- 1 kb ladder, Lanes- 2-9 PCR amplicon of *pag* A gene from transplastomic plants. Lane 10- PCR amplification from wild type tobacco plant showing no amplification.

b) PCR amplification of *D-IV*.

Lane 1- 1kb ladder MBI Fermentas, Lane 2 shows amplification from wild type tobacco plants, Lanes 3-11 0.45 kb amplicon from D-IV transplastomic tobacco plants (amplicon includes the RBS site + D-IV sequence and His tag).

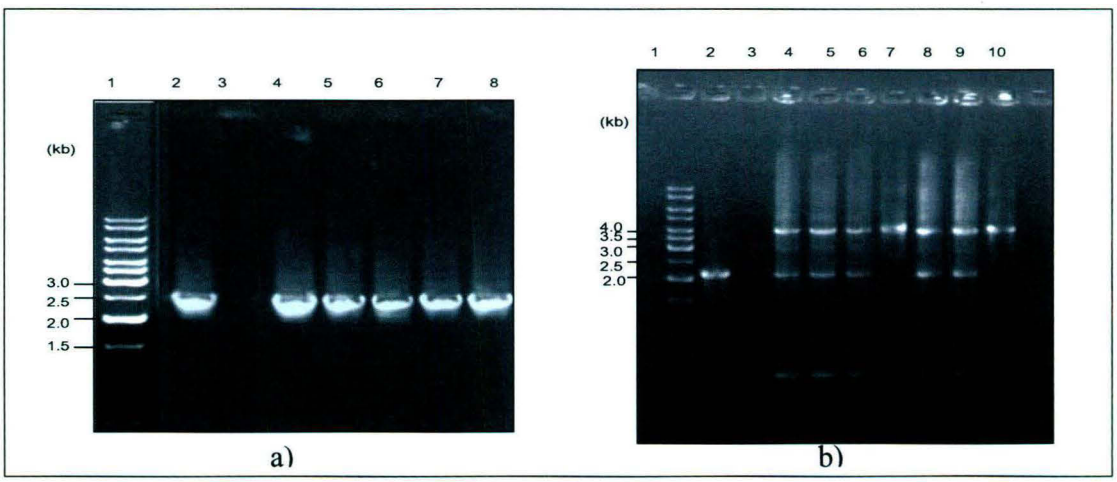


Fig. 5.21) SITE SPECIFIC INTEGRATION- HOMOPLASMY ASSESSMENT BY PCR AMPLIFICATION.

a) PCR amplification with primers internal to *pag* A gene and the flanking sequence.

Lane 1- 1 kb ladder MBI Fermentas, Lanes- 2, 4-8 PCR amplicon of 2.5 kb was obtained. Lane 3- PCR amplification from wild type tobacco plant showing no amplification as there is no integration of *pag* A gene.

b) PCR amplification for D-IV integration with primers corresponding to flanking sequences of chloroplast genome.

Lane 1- 1kb ladder MBI Fermentas, Lane 2 shows amplification from wild type tobacco plants, Lanes 3-10 PCR amplifiation from D-IV transplastomic tobacco plants. Lanes 4,5,6,8,9 show 2.3kb as well as 4.0kb amplicon indicating heteroplasmy, while lanes 7 and 10 show 4.0 kb amplicon indicating homoplasmy of the transplastomic D-IV plants.

Immunoblot detection & Quantification of PA & D-IV from Total Soluble Protein

The total soluble protein extracted in 1X phosphate buffer saline mixed with protease inhibitor cocktail was analysed on SDS-PAGE followed by western blotting. Polyclonal antibodies raised against purified recombinant protective antigen were used as primary antibodies (1:5,000) to carry out immunoblot detection. Higher dilutions of antibodies were able to detect the PA and D-IV present in plant extract, which is indicative of higher yields. The presence of a distinct band in coomassie stained SDS-PAGE gel which was missing in the total soluble protein extracted from control samples is yet another assertion to claim better yields of PA and D-IV using chloroplast transformation technology (Fig. 5.22 a, b). There was no such band visible in the TSP extracted from the nuclear transformed pants as discussed in chapter 3. These results were further corroborated by the ELISA results which indicate a presence of 2.5-4. % of PA and 3.5 to 5.3 % D-IV in total soluble protein in mature leaves The cytotoxicity assay showed 93-96 % killing with PA from transplastomic plants.

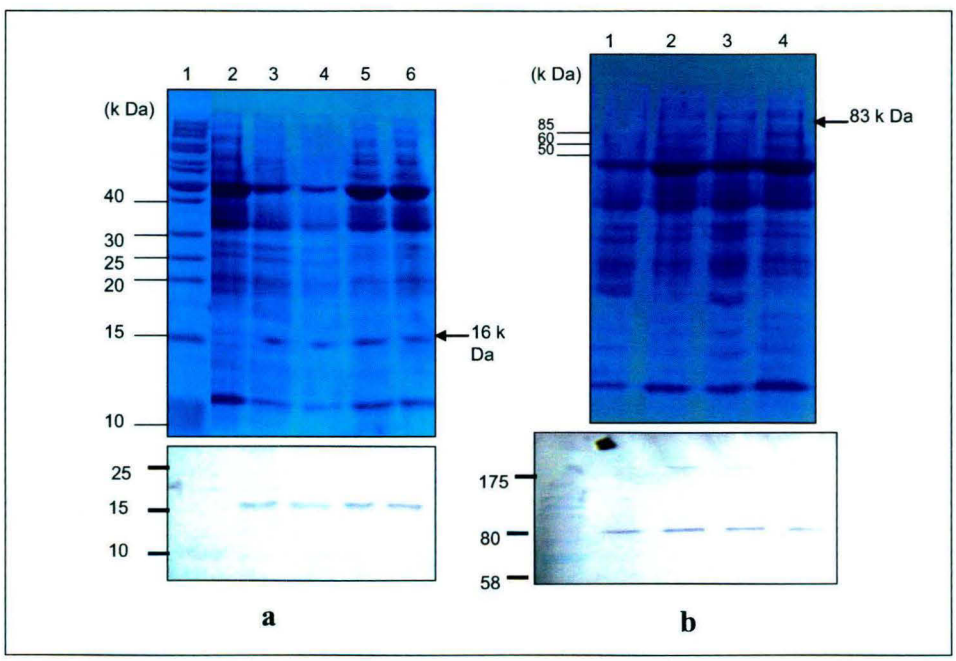
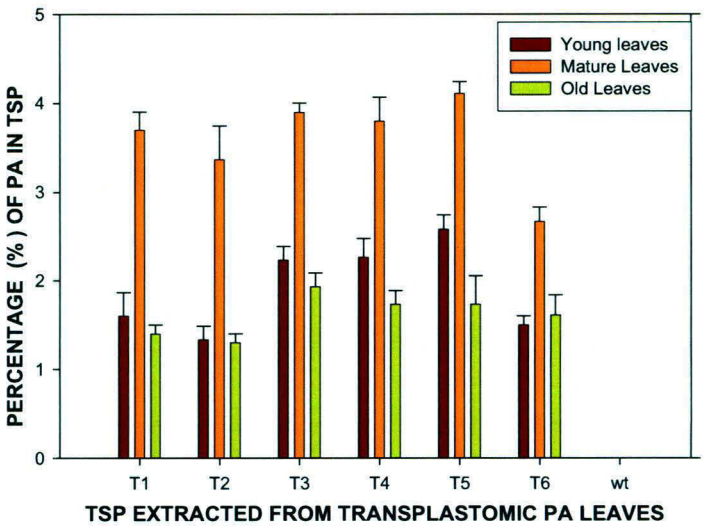


Fig. 5.22)
IMMUNOBLOT ANALYSIS OF TRANSPLASTOMIC PA AND D-IV PLANTS.
a) Immunoblot analysis with antibodies specific to D-IV.
Lane 1- Unstained marker MBI Fermentas, Lanes- 2, TSP from the wildtype plants, Lane 3-6 TSP from transplastomic D-IV plants showed 16 kDa band as expected.
b) Immunoblot analysis with antibodies specific to PA.
Lanes- 1-4, TSP from transplastomic PA plants showed 83 kDa band as expected



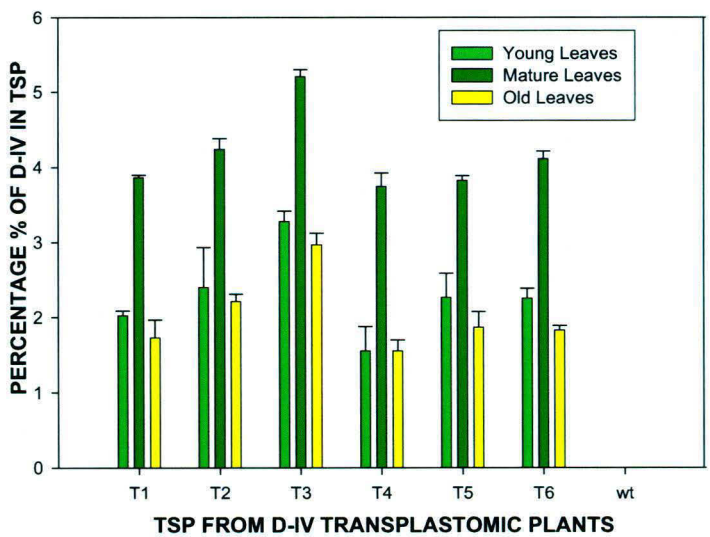


Fig.5.23)
AMOUNT OF PROTEIN IN PA AND D-IV TRANSPLASTOMIC PLANTS AS OBTAINED BY ELISA RESULTS.
PA Expression ranged from 2.5- 4 % of TSP in mature leaves
D-IV Expression ranged from 3.5-5.3 % of TSP in mature leaves. Mature leaves expressed greater amount of protein as compared to young and old leaves.

Selection of T_1 generation plastid transformants on Spectinomycin MS media

Seeds from PA and D-IV plastid transformed tobacco plants were collected, properly dried and put for germination on Murashige & Skoog media with spectinomycin (500 mg/l). The transgenic plants obtained were transferred to green house and assessed for stable transfer and expression of PA and D-IV gene in T_1 generation. Stable transgene integration was reconfirmed by PCR and site specific integration using the primers from flanking regions of *pag* A and D-IV which included sequences from chloroplast genome itself (Fig 5.24).

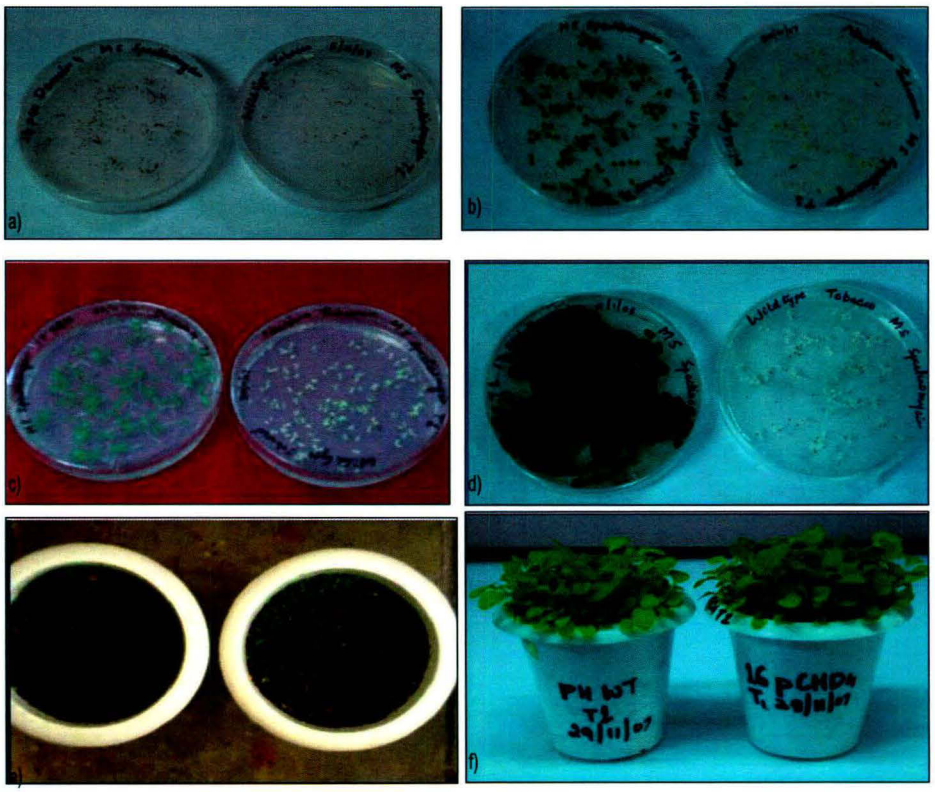


Fig. 5.24)
GENERATION OF T_1 D-IV PLANTS.
a) Wild type T_1 (right) and D-IV T_1 (left) seeds on MS spectinomycin media for germination.b, c, d- Germination of D-IV T_1 seeds on MS spectinomycin media. While the seeds from transgenic plants germinate to give rise to green shoots, the T_1 wild type bleach at cotyledonary stage itself.
e,f-Transgenic seeds germinated in soil are also healthy without any phenotypic anamoly.

DISCUSSION

Chloroplast transformation technology has received great attention over the past decade as it offers several advantages. High expression yields, multigene engineering, versatility, an environmentally friendly approach has spurred interest in plant biotechnologists to promote this technology for the expression of a wide range of proteins in a various crop species. The basis of chloroplast transformation is the site specific integration of the transgene in the chloroplast genome. The integration occurs via homologous recombination of flanking sequences governing the expression cassette. Based on this principle the chloroplast transformation vectors are designed to target the gene to a desired location.

The transformation is most effected by the availability of efficient chloroplast transformation vectors. With the advent of genomic era chloroplast genome sequences of at least 35 species are available making species specific vectors feasible. Currently, only few research groups are involved in vector design and none of these vectors have been commercialized for wide spread use. To harness the fruits of chloroplast transformation technology, we have first embarked on tailoring patentable chloroplast vectors for tobacco. Vector design is a delicate multistep process involving fishing out essential segments required to achieve efficient transformation, followed by careful assembly of these segments in to a concrete vector by series of clonings. Vital elements of the vector include a vector backbone for propagation in *E. coli*, the chloroplast targeting region which aid in homologous recombination, and the expression cassette that includes transgene governed by regulatory elements.

The vector backbone sequences can be taken from any of the available basic cloning vectors that possess origin of replication and an antibiotic resistance gene. The backbone sequences were drawn from pBSK vector from nucleotides 1011-2961 bp, which included ampicillin resistance gene for selection of recombinant clones, and the origin of replication, that enables to procure large copy numbers of the plasmid. These sequences included restriction sites *Nru* I and *Mlu* I at 5′ and 3′ end, respectively. The sequences were initially cloned in pGEMT-easy vector by TA tailing, and the clones were selected by blue white screening.

The choice of chloroplast targeting sequences plays a significant role in defining

transformation efficiencies, stability and yield of the product. Improper insertion sites may destabilize the chloroplast transcripts and can lead to dysfunction of endogenous genes. The main criteria that need to be considered when choosing a target site for vector design are:

1) Homology and species specificity

2) The nature of the targeting site

3) The location of the region within the chloroplast genome

4) The size of the flanking region.

Intergenic regions of the chloroplast genome are used as flanking sequences to the transgene to the target location as they could least perturb the native genes and also prevent the read through transcription of polycistrons. The insertion position in vector pPRV and its derivatives is located in the *trnV-rps7/12* intergenic region, precisely at the *Sca I* site between *trnV* gene and open reading frame *70B* (*Orf70B*) (Zoubenko *et al.,* 1994); while in vector pLD-CtV, the insertion site is situated within the space region of two intron-containing tRNA genes (*trnI* and *trnA*) individually flanked by two conserved rRNA genes (*16S rRNA* and *23SrRNA*) (Daniell *et al.,* 2001). From the gene structures of a few of available chloroplast genomes in higher plants, it is noteworthy that most chloroplast genes are clustered into transcriptional operons, the t-RNA genes are interspersed inside of operons or within the space region of two chloroplast transcriptional units (operon or single chloroplast gene). Therefore, it is suggested that tRNA transcripts might act as a stabilizing element to promote RNA stability of primary transcripts. Hence, in most cases these regions are sorted for insertion sites. Our vector uses the *trnV-rps7/12* intergenic region as insertion site from nucleotides 112444-11000 extracted from *Nicotiana tabaccum var. bemthamiana* chloroplast genome (or plastome). Plastome generally contains two identical inverted repeat regions (IR_A and IR_B), therefore, a transgene inside this region is undoubtedly duplicated by copy correction in each plastome. Thus, such an integration site within the inverted repeat is thought to be contributive to highest expression of foreign valuable gene in chloroplasts. All the intergenic sequences chosen thus far are drawn from the IR region (Verma & Daniel H 2007). It is envisaged that for the recombination to occur the flanking sequence must be at least 1 kb in size. *Afl* II restriction enzyme divides the region at position 1229/1233 bp

of 2.4 kb fragment leaving 1.2 kb on each side.

The Expression cassette

The transgene is governed by the promoter and the regulatory elements form the expression cassette. The expression cassette includes the plastid ribosomal RNA promoter, the *aad* A gene to confer spectinomycin resistance, followed by *psba* 3′UTR for transcript stability. The *Prrn* promoter is the strongest promoter and has been the choice in most laboratories. The plastid rrn operon promoter in higher plants is transcribed by the plastid-encoded RNA polymerase (PEP), the multi-subunit plastid RNA polymerase from Prrn P1, a σ^{70}- type promoter with conserved –10 and –35 core promoter elements (Vera and Suguira, 1995). The *psba* 3′UTR known to provide stability to the transcrpit follows the *aad* A gene. The *aad* A gene encodes the enzyme aminoglycoside 3-adenylyltransferase that inactivates spectinomycin and streptomycin by adenylation and prevents binding to chloroplast ribosomes (Svab and Maliga, 1993). The expression cassette was synthesized with restriction sites *Nru* I and *Mlu* I at 5′ and 3′ ends respectively. The product was cloned in pGEMT vector. Selection of colonies on ampicillin and spectinomycin discouraged the false positive clones. In order to construct minimal functional vector, the vector backbone and the intergenic sequences eluted from pGEMT vector were ligated initially followed by cloning of expression cassette at *Afl* II site. Spontaneous mutations in 16S rRNA region also can develop spectinomycin resistance as evidenced by the use of mutant 16s rRNA as selection marker prior to the use of *aad* A gene. Positive results with PCR analysis with *aad* A gene specific primers ensured the successful transformation. Sustainence of the positive colonies on spectinomycin (100 mg/l) showed that aad A is functional.

Chloroplast system owing to its prokaryotic resemblance in regulatory machinery is able to process polycistronic mRNAs. A gene of interest can be cloned downstream to the *aad* A gene with its own ribosome binding site to produce a functional protein. The assembled vector designed above lacks appropriate MCS to clone any gene of interest. A stretch of MCS was introduced down stream of 3′UTR and the vector is designated as pCHV-RKB. GFP gene (718 bp) was cloned with ribosomal binding site GGGAG for optimal expression in chloroplast and the spacer (TTTAT) was also introduced. The GFP

clones (plCHV-GFP) emitted flouroscence under UV light thus, demonstrating the efficient processing of polycistronic mRNAs. The GFP chromophore forms autocatalytically in the presence of oxygen and fluoresces green when absorbing blue or UV light (Hanson M C *et al* 2001). GFP has been used to detect transient gene expression (Gray J C *et al.,* 1999) of tobacco leaf explants. GFP construct after bombardment with pCHV-RKB-GFP on tobacco leaves also showed flouroscence proving the achievement of successful integration transgene in to the plastid genome. With the groundwork of developing an efficient plastid transformation tool we further pursued on utility of this vector to clone and express anthrax vaccine candidate in tobacco plastid.

D-IV the most immunogenic molecule of protective antigen is cloned in with *Not* I and *Xba* I sites of MCS. A ribosomal binding site GGGAG optimal for expression in chloroplast was introduced along with the spacer (TTTAT). The expression of construct pCHV-RKB-D-IV protein expression was checked in *E. coli* before proceeding to plastid transformation. A single band obtained at 16 kDa confirmed the expression of D-IV in *E .coli* and was a proof of concept for resemblance of plastid transcription machinery with that of prokaryotic counterpart. This preliminary examination also confirmed the expression of D-IV in plastid vector. *pag* A gene construct from previous studies was also used to transform chloroplasts. Purified plasmid DNA of pCHV-RKB-D-IV and *pag* A construct was delivered in to leaf explants separately via particle bombardment. The process involved optimization of various physical and biological parameters effecting transformation. Explants were placed on abaxial facing the particles. Young tobacco leaves were used for transformation as they were more amenable for transformation. Gold particles of 0.6 μ were more suitable for transformation over 1 μ sized particles probably because small sized particles can penetrate easily through the double membranes of the plastid and lead to less damage of the explant. Incubation of explant on regeneration media for 24 h rather than direct placement on SM after bombardment enabled it to recover from any damage by the bombardment of coated DNA or the stress on osmoticum. The transgenic plants obtained were analysed for integration of PA gene and D-IV. PCR analysis with *pag* A specific primers showed 2.3 kb and D-IV gene showed ~ 450 bp amplicon. Further, site specific integration of the transgene was confirmed by primers of which one anneals to the chloroplast genome and the other to the

transgene itself. Mutants and nuclear transgenic plants are not expected to produce a PCR product with these primers. The leaf pieces from PCR-positive shoots are further selected for a second round to achieve homoplasmy. The regenerated shoots are rooted with the same level of selection. Immunoblot detection with anti-PA antibodies confirmed the espression of PA and D-IV in respective transgenic plants. A distinct band of 83 kDa corresponding to PA and 16 kDa corresponding to D-IV was found. There was no cross reaction with other plant proteins in immunoblotting that shows the specificity of the antibody to detect the plant expressed antigen as well. With the increase in expression levels of D-IV in transplastomic samples, we were able to visualize distinct D-IV bands in SDS-PAGE gels stained with coomassie blue. This fact was further corroborated by the ability of higher dilution antibodies (1:5000) to detect PA and D-IV in TSP. The higher levels of PA and D-IV in TSP were validated by results of direct ELISA. A comparison of absorbance obtained with TSP and recombinant D-IV protein gave an estimate of 3.5 to 5.3 % D-IV yield. Higher yields of protein were obtained from mature leaves of the plant as compared to the young and old leaves. Young and old leaves gave a percent as low as 1.3 % of TSP in *pag* A plants and 1.6 % of TSP in D-IV plants, while mature leaves yielded about 4 % with *pag* A plants and 5.3 % with D-IV plants. The large contribution of PA and D-IV from the mature leaves is due to high number of chloroplasts in mature leaves and the high copy number of chloroplast genomes (up to 10,000 copies per cell). The functional activity of PA was assessed on J774A.1 cell line. The activity of PA was comparable to recombinant counterparts. The increased activity as compared to the nuclear transformants could be due to increased expressional levels. Seeds from PA and D-IV plants were collected and plated on MS medium with spectinomycin (500 mg/l). Transgenic seeds germinated on selection medium generating green shoots while the wild type seeds produced small bleached leaves thus, confirming successful transfer of transgene to T_1 generation.

Chapter 6

Studies on Protective efficacy of plant expressed immunogens (PA & D-IV) in mice animal model.

INTRODUCTION

Protective antigen is the major component in all the anthrax vaccines currently available. Apart from the toxin neutralization potential, the role of anti-PA -antibodies in retarding germination and enhancing spore clearance by phagocytic cells (Welkos *et al.,* 2002) highlight the significance of the molecule. Immunity induced by PA has been shown to be effective in various animal models against anthrax spore challenge (Pitt *et al.,* 1999, Ivins *et al.,* 1997). Most of the earlier studies pursued with PA have focused on injectable vaccines with alum that generated systemic immunity only (Ivins BE *et al.,* 1997, Singh Y *et al.,* 1998). In concern with protecting a large population at risk, cost effective needle free approaches with mucosal vaccines would be more preferable. Transcutaneous (Matyas GR *et al.,* 2004, Kenney RT *et al.,* 2004, Peachman KK *et al.,* 2006,), nasal (Gaur R *et al.,* 2002, Boyaka PN *et al.,* 2003, Sloat BR *et al.,* 2007) and oral (Bielinska AU *et al.,* 2007, Stokes *et al.,* 2007 Baillie LW *et al.,* 2008, Aloni-Grinstein R *et al* 2005) methods have been extensively studied with the rational to combat the disease at the portal of entry, as the anthrax spores penetrate via skin, the nasal or the oral mucosal route manifesting in to three deadly forms the cutaneous, the respiratory and the gastrointestinal anthrax. Unlike parenteral vaccines which contribute to systemic immunity alone, mucosal vaccines are proficient in arming the body with mucosal as well as systemic immunity (Freytag *et al* 2005). The significance of targeting the antigens to mucosal surfaces also underlies the fact that 80 % of all the immunocytes are contributed by mucosal immune system (MIS). Also, the amazing nature of mucosal immune system allows the activation of the immunocytes that are distant from the inductive sites. Apart from the generation of humoral responses MIS is adept in promoting cell mediated immunity. Though, humoral response has been shown to be effective in generating protection against *Bacillus anthracis* spore challenge (Bielinska AU *et al.,* 2007), recent reports confirmed the protective role of cell mediated adaptive immunity against capsulated *B. anthracis* (Glomski I J *et al.,* 2007).

Oral immunization is the simplest way to evoke protective mucosal immune response. Several heterologous expression systems and delivery vehicles have been employed to ensure protection from digestive enzymes in the gut. As already discussed in the previous chapters, edible plant based vaccines could provide solution to current injectable vaccines

in promoting stability, being cost effective and ensuring protection in G I (gastro intestinal) tract due its natural bioencapsulation. The first step for the induction of mucosal immunity begins with uptake of antigens by specialized epithelial cells (M cells) in mucosal inductive sites and presented to APC'S (antigen presenting cells). The sensitized lymphocytes transit through the lymph, enter the circulation and seed at the mucosal effector sites. The toxin neutralization at the site of induction is marked by the production of s- Ig A antibodies.

Although, oral immunization scheme is more promising it is limited by lack of proper adjuvants to appropriately present to immunocytes and enhance the immune response. Enterotoxins such as; cholera toxin from *vibrio cholerae* and *E. coli* heat labile enterotoxin are the best studied and have been extensively used as mucosal adjuvants (Freytag R C *et al.,* 2005) but their toxic nature limits the practical applicability to humans. Till date, no mucosal adjuvant has been approved for human or veterinary applications. Several edible vaccines generated in plants were able to generate immune response even in the absence of an adjuvant (Arlen PA *et al.,* 2008). Considering the repertoire of factors that affect the mucosal immunity we focused to study the effect of the plant expressed antigen under *in vivo* conditions in mice animal model. CT has been used in the experiments exclusively to compare the immune response generated with the non adjuvanted counterparts.

Earlier lab studies of parenteral immunization with plant expressed protective antigen have generated neutralizing antibodies in mice (Aziz *et al.,* 2005). To further the work we decided to check feasibility of systemic as well as mucosal immunity with plant expressed protective antigen and Domain-IV. All the experiments were carried out in mice. Mice represent the primary species used in research, comprising 67 % of all animals used in biomedical research and testing. Their short lifespan, proclivity for reproduction, known genetic background and minimal expense of maintenance have made them a desirable animal model. The studies on efficacy of plant expressed antigens in mice models would provide new insights to extrapolate to other animal models and humans.

MATERIALS AND METHODS

Reagents and Supplies

Inbred BALB/c mice of 4-6 weeks age and an average weight of 18-20 gm were used for the experiments. The animals were procured from National Center of Laboratory Animal Sciences, NIN and Hyderabad. For oral gavaging experiments, BALB/c mice of 3-4 weeks age were obtained from animal house facility, Jawaharlal Nehru University, New Delhi. All the experiments were conducted strictly in accordance with the regulations of Institutional Animal Ethics Committee (IAEC). Requirements of sterile hypodermic needles, oral gavaging needles and syringes were fulfilled by the local dealers. Antibodies were purchased from Sigma Chemical Company. ELISA plates were obtained from Falcon, USA. Alhydrogel was purchased from Superfos Biosector (Denmark) and Cholera Toxin (CT) was from Sigma chemicals.

Mice immunization

BALB /c mice were randomly sampled into groups of five to six animals each. Recombinant PA & D-IV purified from *E. coli* and plant extracts expressing protective antigen or D-IV were used in immunization experiments. The antigens were delivered by two routes of immunization intraperitoneal and oral immunization. Control groups of mice were injected with non-transgenic plant extracts and PBS (Phosphate Buffer Saline) only. Mice were orally immuninized on day 0, 7, 14, 21 and 28. A final booster was given by intraperitoneal injection of recombinant PA or D-IV purified from *E. coli* in all the mice groups except the control groups on day 175.

Immunization plan for Protective antigen and Domain-IV

NO	GROUP	ANTIGEN AND IMMUNIZATION MODE
1	A	Transgenic mustard PA(2.5g)+ Corn starch + CT (Oral feed)[TM+CT]
2	B	Transgenic mustard only(Oral feed) [TM]
3	C	Recombinant PA + CT (Gavage)[rPA+CT]
4	D	Recombinant PA only(Gavage)[rPA]
5	E	Wild type TSP +CT(Gavage)[WT+CT]
6	F	Wild type TSP (Gavage)[WT]
7	G	Recombinant PA + Alhydrogel(IP)[rPA+Al.]
8	H	Transgenic mustard PA TSP + Alhydrogel (IP)[TM-PA+Al]
9	I	Tobacco PA TSP + CT (Gavage)[NT-PA+CT]

NO	GROUP	ANTIGEN AND IMMUNIZATION MODE
1	I.	Recombinant D-IV+CT(Gavage)[rD-IV+CT]
2	II.	Recombinant D-IV(Gavage)[rD-IV]
3	III.	Recombinant D-IV+ Alhydrogel(IP)[rD-IV+ Al.]
4	IV.	Tobacco D-IV TSP + CT (Gavage)[NT-D-IV+CT]
5	V.	Tobacco D-IV TSP (Gavage)[NT-D-IV]
6	VI.	Tobacco D-IV TSP + Alhydrogel (IP) [NT D-IV+ Al.]
7	VII.	PBS Only

PA: Protective antigen; **D IV**: Domain 4; **TSP**: Total Soluble Protein; **CT**: Cholera Toxin

I P: Intraperitoneal; **PBS**: Phosphate Buffered Saline.

Intraperitoneal injections

The peritoneal lining of the abdomen provides a large surface area for absorption of injected materials, allowing injection of larger volumes. A 26- gauge 3 / 8- to -1-inch. needle was used for the injections. For intraperitoneal injections total soluble protein from transgenic plants expressing protective antigen or D-IV was injected along with alhydrogel 20 % v/v. The antigen contained in the extracts was quantitated to nearly 10 µg per dose. The needle was inserted at a shallow angle (almost parallel to the long axis of the body) to the left or right of the ventral midline of the abdomen at the cranial aspect

of the lower abdominal quadrants. It's not necessary to insert the needle to its hub; rather it needs only to be inserted enough to penetrate the skin and abdominal muscle layer for entry into the abdominal (peritoneal) cavity, which for these small laboratory rodents is a small distance. Over insertion of the needle will increase the likelihood of penetration of the bowel or other major abdominal organs, causing significant injury, illness, or death of the animal. A 100 µl sample was injected at a single site. Groups of mice that received 10 µg of r PA or r D-IV with alhydrogel 20 % v/v served as positive controls throughout the experiments.

Oral immunization

For oral immunization each mouse in the group was fed with 2.5 g of crushed mustard leaves expressing protective antigen mixed in edible 1g corn starch and 0.5g sugar. In a separate group cholera toxin 5 µg was supplemented as an adjuvant along with the feed to observe for the augmentation of immune response. All mice were caged separately for feeding. In case of gavaging experiments the total soluble protein from plants expressing protective antigen or D-IV was given through the gavage needles. Control mice received wild-type plant material. In some groups, plant material was supplemented with 5 µg of CT as an adjuvant. Blood and fecal matter were collected 7 days after each immunization. Sera and fecal pellets were stored at -80 °C for further analysis. Immunization plan is tabulated in table 6.1.

Serum and Fecal Sample preparation

The blood and fecal matter was collected at various immunization points once a week prior to each immunization and once before the mice were challenged with vegetative ST-1 cells. Immediately after collection of the blood from the retroorbital plexus of the mice the blood was allowed to clot and separate the serum by incubation at 37 °C for 20 min. The samples were then centrifuged at 12,000 rpm for 20 min and the sera was collected and stored at – 20 °C until further analysis. Fecal pellets for each group of mice were weighed and dissolved in 1X PBS buffer supplemented with protease cocktail inhibitor and 1mM PMSF. 10 µl of PBS was added for every mg of dry fecal matter. The

samples were vortexed and soaked overnight at 4 °C and then centrifuged at 12,000 rpm for 20 min and the supernatant was used to determine the IgA antibody levels.

ELISA

The presence of PA specific antibodies in the sera from immunized mice was determined by ELISA using standard protocols. Briefly, 96 well round bottomed microtiter plates were coated with 0.5 µg of *E. coli* derived recombinant purified PA or D-IV diluted in 1X PBS / Carbonate buffer pH 9.6 and incubated at 37 °C for 1 h. The plates were incubated overnight at 4 °C and washed with PBS containing 0.1 % Tween 20. Blocking was done in 1X PBS supplemented with 2 % BSA for 1 h followed by washing with PBST using ELISA washer. The serum obtained at various immunization points was serially diluted and added to the plates 100 µl/well and incubated at 37 °C for 1 h and subsequently washed thrice with PBST. The plates were then incubated with peroxidase conjugated goat antimouse IgG for 1 h at 37 °C and washed with PBST. To check the IgA antibody levels in serum and fecal matter peroxidase conjugated rabbit anti-mouse IgA antibody was used. For subtyping the PA specific antibody peroxidase conjugated rabbit anti-mouse IGg1 and IGg2a antibody was used. TMB substrate (BD BioSciences) was added at 100 µl/well. The reaction was stopped after 20 min by 1M H_3PO_4 and absorbance was recorded at 450 nm on Micro ELISA reader at 450 nm. End point titers were expressed as the maximum dilution of the sample giving an absorbance of or more than 0.2 A_{450} units after the subtraction of the absorbance due to non specific binding detected in the sera obtained from negative control mice immunized with wild type plant material expressing the immunogens PA or D-IV. The results are presented as the reciprocal of the mean antibody titers done in triplicates for each sample.

Toxin Neutralization Assay

The titer of toxin neutralizing antibody in immune sera was determined based on the ability of the serum to inhibit the cytotoxicity of lethal toxin (PA 250 ng/ml + LF 100 ng/ml). J774A.1 murine macrophage cells were harvested and plated out at 3 x 10^4 cells /well in 96 well microtiter plate. Cells were incubated at 37 °C CO_2 incubator for 16 h. Antisera were diluted in separate 96 mirotiter well plate and 100 µl of anthrax toxin was

added (PA 250 µg/ml and LF 100 ng/ml) and incubated for 1 h at 37 °C. The growth media from the cells was removed and serum anti toxin mixture was added at 100 µl /well and incubated at 37 °C for 4 h. The serum antitoxin mixture was removed and 100 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide) 0.5 mg/ml was added and incubated for 45 min. Solubilization buffer [0.5 % (wt/vol) SDS and 25 Mm HCl in 90 % vol/vol isopropanol] at 100 µl /well. Cell death was assessed as described in previous chapters. Cells receiving no lethal toxin were used to determine 100 % cell viability. Lethal toxin neutralizing titer [NC50] was defined as the dilution resulting in 50 % protection of the cells. A positive control which resulted in 50 % protection of the cells as derived from the standard curve was also included in the experiment.

Toxin challenge

The acapsular *B. anthracis* Sterne strain vegetative cells were used to challenge the mice as described previously (Shoop W L *et al.,* 2005).The acapsular *B. anthracis* Sterne strain was obtained from and maintained at -70 °C. The frozen material was thawed and a loop of material was streaked on an agar plate and incubated for 18 h at 37 °C. Colonies were inoculated in 20 ml of LB Broth and grown to 0.8 O.D. The cells were pelleted, suspended in PBS, vortex mixed vigorously, and diluted to achieve an inoculum to give 10^8 colony forming units per 0.2 ml. The mice were challenged i.p. with 10^8 colony-forming units of *B. anthracis* Sterne strain.

Statistical Analysis

All data were expressed as mean ± SD and are representation of at least 2 different experiments. Student's *t* test was done to derive p values and a p value < 0.05 was considered statistically significant. Sigmaplot 11.0 and Graph pad prism version 5.0 for windows (Graphpad Software, Sandiego, CA) were used to perform statistical analysis.

RESULTS

Immunization with plant expressed PA and D-IV generates serum anti PA Ig G antibodies

Protective antigen produced in *Brassica juncea* and *Nicotiana tabaccum* were used for immunization experiments. Sera was obtained from the mice immunized on day 0, 7, 14, 21, 28, 35 and 189. Induction of serum antibodies was seen on day 7 in recombinant groups immunized with adjuvant alhydrogel or CT. In other groups that were immunized with only the recombinant PA and the plant PA with or with out adjuvant the antibodies were detected on day 14. Further, there was progressive increase in the antibody titers in all the groups with each dose of immunization. The antibody titers reached the highest peak by the 5^{th} bleed. Highest serum anti PA IgG antibody titers up to 325000 at 5^{th} bleed and 300000 before challenge (BC) (p <0.001[5^{th} & BC]) were detected in group [rPA+Al.] closely followed by the group [rPA+ CT] which generated peak titers up to 100000 & 125000 (p <0.001[5^{th} & BC]). PA produced in plants both *Brassica juncea* and *Nicotiana tabaccum* could generate significant anti PA IgG titers upon oral or intraperitoneal immunization. The plant groups in which adjuvant CT or Alhydrogel was added, induced better antibody titers as compared to the non adjuvanated groups. Transgenic mustard expressing PA when given along with feed (cornstarch and sugar) including CT [TM+CT] and the corresponding non adjuvanted group [TM] induced significant antibody titers up to 30000 & 33500 (p <0.001[5^{th} & BC]) and 25000 & 28500 (p <0.001[5^{th} & BC]) respectively. The antibody titers among the orally fed mustard adjuvanted and non adjuvanted group were not statistically significant (p>0.5[5^{th} & BC]). Gavage experiments and intraperitoneal injections were also performed with TSP from transplastomic tobacco plants with or with out adjuvant. Oral gavage experiments with CT [NTpag+CT] induced higher serum antibody titers up to 35000 & 50000 (p <0.001[5^{th} & BC]) while its non adjuvanted counterpart [NT pag only] generated a titer of 15000 & 25000 (p <0.05at 5^{th} & p <0.001BC). Oral gavage experiments with rPA+CT induced titers of 100000 & 137500 (p <0.001[5^{th} & BC]) and rPA generated 15000 & 27000 (p <0.05at 5^{th} & p <0.001BC). An antibody titer of 75000 & 100000 (p <0.001[5^{th} & BC]) was detected in the group [TMpag+Al]. All the groups were given a final booster dose with recombinant purified PA 10 μg i.p injection on day

175. Antibody titers were assessed 14 days after the final booster. Final booster was given for all the groups previously immunized except for the control groups. All the groups immunized either maintained similar antibody titers or showed increase in the antibody titers with the final booster dose (Fig.6.1a, b). Serum IgG antibody patterns were similar in D-IV groups also. Recombinant groups with adjuvant showed highest antibody titers with [rD-IV+Al] up to 200000 & 225000 (p <0.001[5^{th} & BC]) and followed by [rD-IV+CT] 52500 & 77500 (p <0.001[5^{th} & BC]). Immunization experiments were also performed D-IV expressed in chloroplasts. Both, oral gavage experiments and *i.p* injections were carried out. Antibody titers in these groups were further enhanced after final booster dose. Significant serum antibody levels were detected in the groups [NT D-IV+ Al.] 50000 & 75000 ($p < 0.001$[5^{th} & BC]) and [NT D-IV+ CT] 23750 & 40250 ($p < 0.05$ 5^{th} & $p < 0.001$ BC). The titers obtained in orally immunized [rD-IV] group and [NT-D-IV] group were not statistically significant by 5^{th} bleed and last immunization ($p > 0.5$) however, [rD-IV] titers were significant 20000 (p <0.5) when the last booster dose rD-IV 10 μg was given (Fig 6.1 a, b).

Generation of serum Ig A and mucosal Ig A in orally immunized mice groups

Serum anti PA Ig A antibodies were detected at 5^{th} bleed and before challenge with the toxin. All the groups orally fed / gavaged induced IgA serum antibodies. Although, the titers obtained in the last bleed showed increase in antibody levels, the rise was not statistically significant. Antibody levels were higher in the groups in which adjuvant was added. Higher serum IgA titers was detected in the recombinant purified PA or D-IV was delivered along with adjuvant (Fig.6.2 a, b).

Ig A antibody that is secreted at the mucosal surfaces plays an important role in affording significant protection against the pathogen. Oral immunization leads to protective immune responses at the mucosal surfaces. S-IgA released at the gut surface can be detected by measuring the fecal secretory IgA antibody levels. Anti PA s IgA responses were detected in only in the groups [TM] [TM+ CT];[rPA+CT];[rD-IV+CT] at a dilution of 1:125 at the 5^{th} bleed. Remaining groups that were immunized orally did not show any measurable levels of anti PA s-IgA antibody.

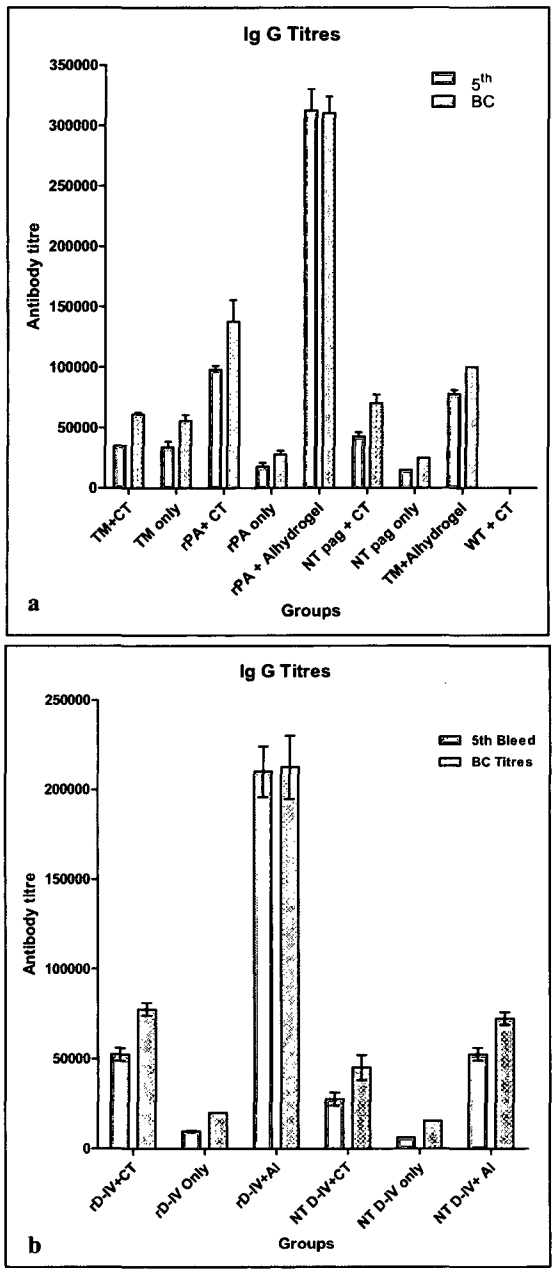
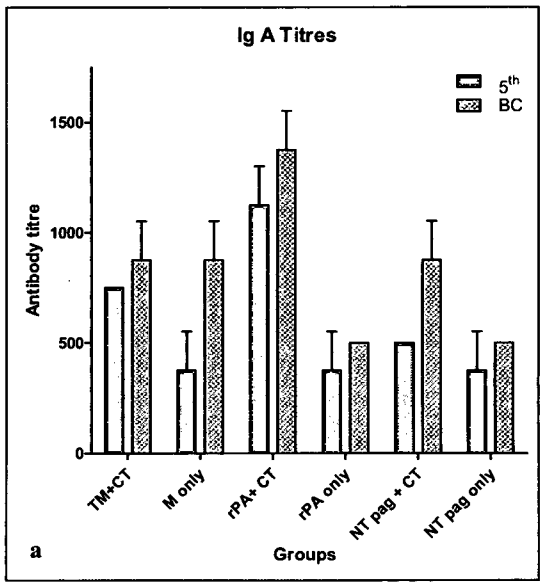


Fig. 6.1)
QUANTIFICATION OF SERUM IgG ANTIBODIES IN IMMUNIZED MICE.
a) IgG antibodies in mice immunized with various PA combinations.
The graph compares the immune responses obtained in various groups of mice immunized with plant PA.
TM, Transgenic mustard expressing PA; rPA Recombinant PA purified from *E coli*; NT pag, PA expressed in *Nicotiana tabaccum*;CT, cholera toxin.
b) IgG antibodies in mice immunized with various D-IV combinations.
The graph compares the immune responses obtained in various groups of mice immunized with plant D-IV.rD-IV Recombinant D-IV purified from *E coli*; NT D-IV, D-IV expressed in *Nicotiana tabaccum*; CT, cholera toxin.



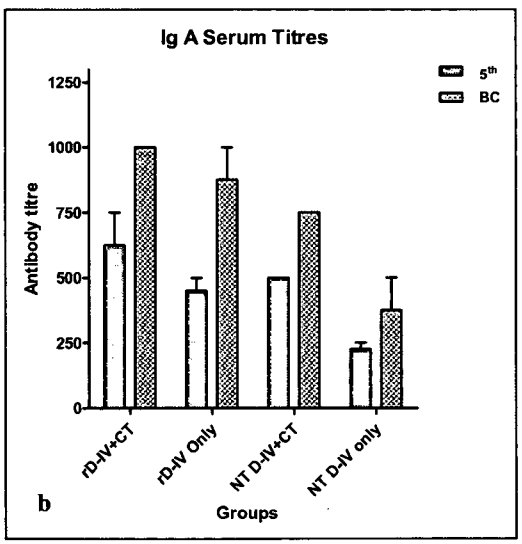


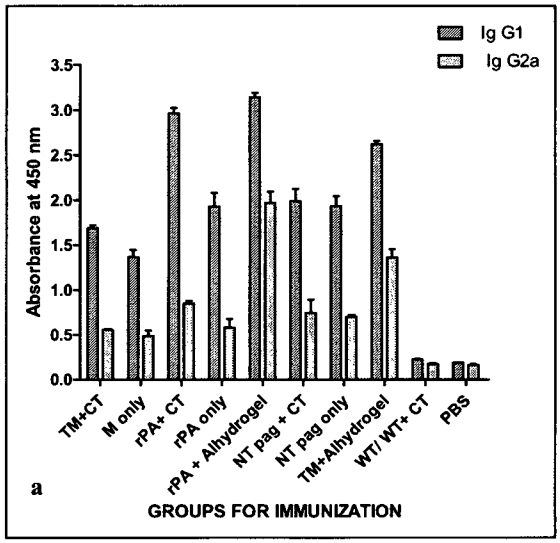
Fig. 6.2)
QUANTIFICATION OF SERUM IgA ANTIBODIES IN IMMUNIZED MICE.
a) IgA antibodies in mice immunized with various PA combinations.
The graph compares the immune responses obtained in various groups of mice immunized with plant PA. TM, Transgenic mustard expressing PA; rPA Recombinant PA purified from *E coli*; NT pag, PA expressed in *Nicotiana tabaccum*;CT, cholera toxin.
b) IgA antibodies in mice immunized with various D-IV combinations.
The graph compares the immune responses obtained in various groups of mice immunized with plant D-IV. rD-IV Recombinant D-IV purified from *E coli*; NT D-IV, D-IV expressed in *Nicotiana tabaccum*; CT, cholera toxin.

Plant expressed PA and D-IV generate strong Th2 type immune response

The pattern of IgG subtype response predicts the type of protective immune response induced. Increased production of IgG1 antibody over IgG2a in all the immunized groups predicted a polarized Th2 type response. Previous studies have shown that protective antigen induces a strong Th2 type response (Williamson E D *et al.,* 1999). Plant expressed PA and D-IV with or with out adjuvant also showed a strong bias towards Th2 response. Therefore, the native proteins of the plant did not influence the type of immune response obtained. It is evidenced from studies that mucosal adjuvant CT promotes essentially strong Th2 response with minimal or no IgG2a antibodies (Marinaro M H F *et al.,* 1995). The groups in which CT was administered as mucosal adjuvant showed very less Ig G 2a antibodies (Fig 6.3 a, b).

Generation of robust lethal toxin neutralizing antibodies

Lethal toxin (Letx) induced mortality of J774A.1 cells can be prevented by the neutralizing activity of anti PA antibodies. Sera from immunized mice when assessed for the toxin neutralizing potential by *in vitro* toxin neutralization assay were able to prevent Letx mediated killing. NC 50 titers were derived by linear regression analysis of serial serum dilutions. Neutralizing antibody titers with an NC 50 of > 10^3 were generated in the groups [rPA+Al.]; [rPA+CT]; [rD-IV+Al.] [rD- IV+CT]. In groups immunized with plant expressed PA and D-IV the NC 50 titers of > 10^2 were detected. The neutralizing titers were examined both at 5^{th} bleed and after the last immunization. An increase in NC 50 titers was observed with the final boost in all the experimental groups although the rise was not statistically significant. Mucosal neutralizing antibodies were detected NC 50 of 10^1 in the groups [rPA+CT]; [rD-IV+CT] [TM+CT][TM] and [NTpag +CT] when fecal extracts were examined. Sera from the negative control groups [WT]/ [WT+CT]/ [PBS] did not generate any Letx neutralizing antibodies (Fig. 4.a, b).



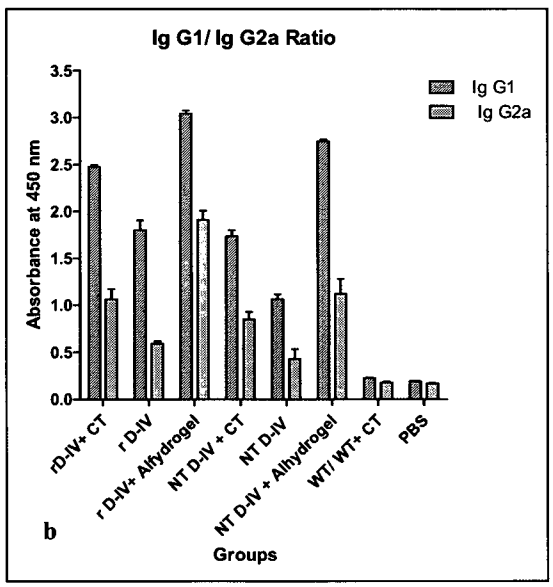


Fig. 6.3)
ANTIBODY ISTOTYPE PROFILE IgG1 VS. IgG2a

a) IgG1 Vs. IgG2a antibody isotype profile in mice immunized with various PA combinations.

The graph compares the serum IgG1 vs. IgG2a immune responses obtained in various groups of mice immunized with plant PA.TM, Transgenic mustard expressing PA; rPA Recombinant PA purified from *E coli*; NT pag, PA expressed in *Nicotiana tabaccum*;CT, cholera toxin.

b) IgG1 Vs. IgG2a antibody isotype profile in mice immunized with various D-IV combinations.

The graph compares the serum IgG1 vs. IgG2a immune responses obtained in various groups of mice immunized with plant D-IV. rD-IV Recombinant D-IV purified from *E coli*; NT D-IV, D-IV expressed in *Nicotiana tabaccum*; CT, cholera toxin.

Immunized mice survive lethal toxin challenge

All the immunized mice were challenged with a lethal dose of Sterne strain vegetative cells. 10^8 cfu /200 µl when the cell culture was grown to O.D =0.8 was found to be completely lethal in BALB/c mice with a mean time death of 62-75 h. All the challenged mice were followed up to 14 days. Immunized mice demonstrated survival percentages ranging from 20-100 % and 0-100 % in the groups immunized with PA and D-IV respectively (Fig.6.5 a, b). The graphical representation of % survival is depicted by Kaplan mere curves.

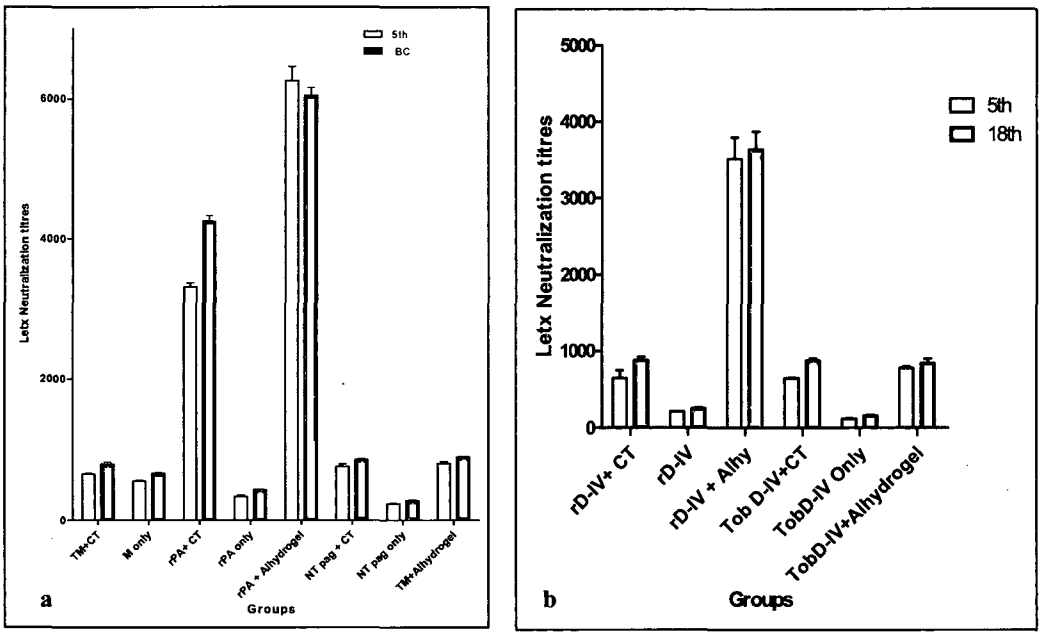


Fig. 6.4)
***IN VITRO* LETHAL TOXIN NEUTRALIZATION ASSAY.**
a) NC 50 LeTx neutralization titers in mice immunized with various PA combinations at 5^{th} bleed & last immunization.
TM, Transgenic mustard expressing PA; rPA Recombinant PA purified from *E coli*; NT pag, PA expressed in *Nicotiana tabaccum*;CT, cholera toxin.
b) NC 50 LeTx neutralization titers in mice immunized with various D-IV combinations at 5^{th} bleed & last immunization.
rD-IV Recombinant D-IV purified from *E coli*; NT D-IV, D-IV expressed in *Nicotiana tabaccum*; CT, cholera toxin. All the control groups WT/WT+CT/PBS only did not generate any neutralization titers

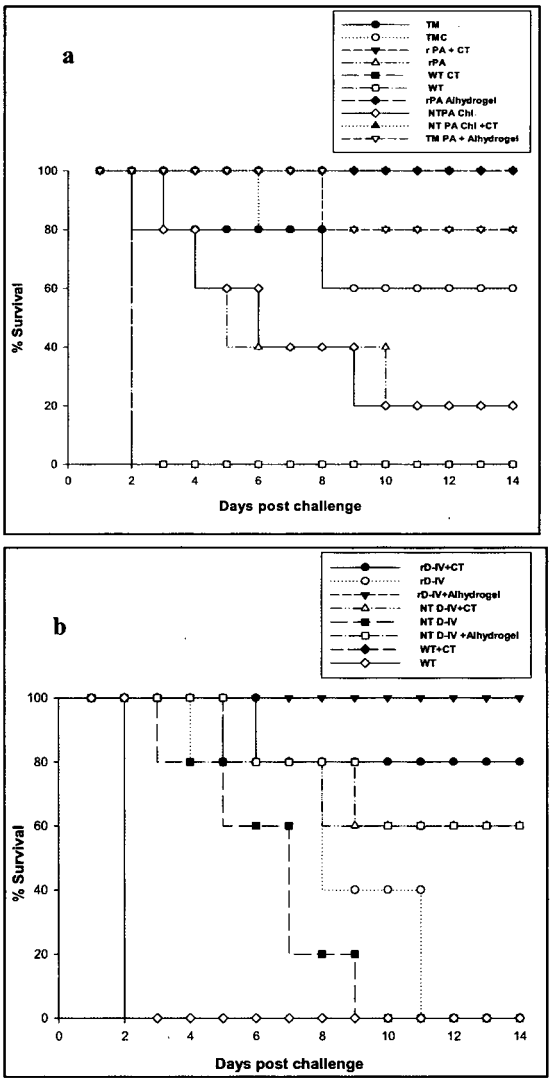


Fig. 6.5)
% SURVIVAL AGAINST TOXIN CHALLENGE.
a) Mice immunized with various PA groups were challenged with vegetative Sterne cells.
Average percent survival rate is represented by Kaplan mere curves. TM, Transgenic mustard expressing PA; rPA Recombinant PA purified from *E coli*; NT pag, PA expressed in *Nicotiana tabaccum*;CT, cholera toxin.
b) Mice immunized with various D-IV groups were challenged with vegetative Sterne cells
Average percent survival rate is represented by Kaplan mere curves; rD-IV Recombinant D-IV purified from *E coli*; NT D-IV, D-IV expressed in *Nicotiana tabaccum*; CT, cholera toxin.

DISCUSSION

The most rational approach to achieve complete protection against anthrax in humans and animals is to strive towards activating the body's defense mechanisms at the portal of entry by mucosal immunization. Since, the anthrax spores enter in to the host via skin, respiratory or the gastrointestinal tracts, a robust immune response at these primary sites would encounter the pathogen at initiation site and also prevent the pathogen from easily gaining access to the systemic milieu. Mucosal immunity also efficiently arms the systemic compartment by activating the systemic immune system if the pathogen overcomes the host's primary defense mechanisms. Protective antigen, though, is endowed with antispore and antitoxin properties (Welkos S *et al.,* 2001, 2002); the exclusion of systemic environment from the mucosal compartments precludes its antispore potential unless administered mucosally. The secretory IgA antibodies exclusively generated at the mucosal sites effectively counter the pathogen at the early infection. Edible plant based vaccines could offer appetizing solutions especially to meet the present day vaccine priorities as discussed previously. Therefore, we initiated to test the efficacy of plant expressed anthrax protective antigen and it's D-IV in mouse model. We have demonstrated for the first time the mucosal immunity in anthrax with oral plant based vaccine.

We have obtained protective immune response when plant expressed protective antigen and D-IV has been orally gavaged or fed to mice. We also report here that co-administration of a musosal adjuvant CT with recombinant or plant protective antigen when orally gavaged, significantly enhanced the immune responses in mice. Previous studies on oral immunization with PA based vaccines expressed in *Lactobacillus* (Zegers *et al.*, 1999) and *Salmonella* could not generate complete protection against anthrax infection (Strokes M G *et al.,* 2008). While, on the other hand mucosal immunization (nasal) with appropriate mucosal adjuvants was seen to augment the protective immune response (Flick Smith H C *et al.,* 2002, Du Bois A. B *et al.,* 2007). Other studies with oral spore vaccines of Sterne strain (Krishna P *et al.,* 2007) and non toxigenic oral spore vaccine (Aloni-Grinstein *et al.,* 2005) expressing PA have been evaluated without adjuvants also have stressed the need to incorporate adjuvants. Our results obtained as evident from the data are in agreement with the previous findings and emphasize the need

for a potent mucosal adjuvant. Till date, research on recombinant oral 'PA' based vaccines and oral plant based vaccines with a strong adjuvant has not been pursued. Our results demonstrate that oral administration of very low quantities of PA 10 μg and 5 μg CT is able to induce protective mucosal and systemic immune responses in mice.

The immunization studies with PA showed highest serum antibody titers > 10^5 in G group [rPA + alhydrogel] closely followed by C [rPA +CT] group. The high antibody levels can be attributed to the fact that recombinant protein was purified and gastric neutralization was carried on one hour before delivering the antigen. Group A [Transgenic mustard PA (2.5g) + Corn starch + CT (Oral feed)], B [Transgenic mustard only (Oral feed)], H [TM PA TSP + Alhydrogel (IP)] and I [Tobacco PA TSP + CT (Gavage)] generated titers >10^4 while, there was no antibody response with TSP from wild type plants. Low titers with plant expressed PA with alhydrogel (H) and CT (I) as compared to the recombinant counterparts is due to the presence of high alkaloid compounds present in TSP as reported for systemic plant based PA vaccines in previous studies (Watson J 2004 and Aziz *et al.,* 2005). The antibody titers obtained in GP A[TM+CT] and GP B [TM] were not significantly different. This can be attributed to two important reasons. Firstly, CT was mixed in the feed which contained crushed leaves mixed with oral feed and mice were able to consume the food with in 3-4 h, therefore, the probability that the externally added CT could have degraded is high. Secondly, CT is not intact with PA therefore, would not have been presented along with CT to the immunocytes of gut mucosa.

Immunological studies in anthrax have shown that the receptor binding component of PA 'Domain-IV 'alone can confer complete protection against anthrax infection (Flick Smith *et al.,* 2002). Studies pertaining to D-IV mucosal strategies (oral) have been carried out using salmonella based vectors (Galen J E *et al.,* 2004, Margaret G M Strokes *et al.,* 2007). To investigate the efficacy of a purified recombinant *E. coli* and plant based D-IV oral vaccine, we performed immunizations in mice model. Similar to the results obtained with PA immunization studies, robust IgG antibody titers > 10^5 were obtained in Grp 1 [Recombinant D IV + CT (Gavage)]and 3 [Recombinant D IV+ Alhydrogel(IP)]. Group 2 [Recombinant D IV only (Gavage)], 4 [Tobacco D IV TSP + CT (Gavage)], 5 [Tobacco D IV TSP (Gavage)] and 6 [Tobacco D IV TSP + Alhydrogel (IP)] generated

immunological titres > 10^4. The low antibody tires with tobacco expressed D-IV over the recombinant purified D-IV can be attributed to impurities and alkaloids present in tobacco. An important inference that could be drawn from this study is that plant expressed anthrax antigens are immunologically active and contribute to significant antibody titers.

Having confirmed the establishment of systemic IgG immune responses with the above sets including PA and D-IV a step ahead, we decided to study the nature of IgG subclass Ab responses upon mucosal and parenteral immunization. In fact, the pattern of IgG response reflects the type of cellular adaptive immunity generated by Th subsets which further are known to control distinct host protection mechanisms (Finkelman *et al.,* 1990). It has been demonstrated that immunization with recombinant PA results in Th2 directed immune response (Williamson E D *et al.,* 1999). Further, the administration of adjuvants also influences the type of immune response. Both, Alhydrogel and CT have a tendency to shift the immune paradigms to Th2 side (Freytag *et al.,* 2003). The Th2 responses are marked by the increased production of IgG1 over IgG2a subclass. The results obtained in this study with PA and D-IV along with alhydrogel and CT adjuvants indicated a strong Th2 polarized immune response. More over, the immune responses obtained with plant expressed antigens also showed similar patterns reflecting that plant expressed antigens behave immunologically alike the recombinant counterparts. To date, no studies have been executed to dissect the type of immune response generated with anthrax mucosal plant based vaccines.

The next important question to address in this study was whether oral immunization with anthrax antigen could generate effective mucosal immune responses? It has been shown that s- IgA antibody response generated at mucosal sites contributes to effective mucosal immunity and also possess immunomodulatory properties to stimulate systemic immunity. Studies have shown that IgA antibody titers measured from the mucosal sites are good indicators of mucosal immunity. Mucosal immunity can be induced at sites distant to the site of initial induction. Oral immunization generates immune responses in the oropharyngeal, adenoids, and the intestinal tract. The simplest way to assess the production of IgA upon oral immunization is to check for the antibodies in fecal extracts. Antibody levels though at low titers were found in the groups A[TM+CT], B[TM],

C[rPA+CT] and I [TM+CT gavage] of the PA sets and group 1 [rD-IV+CT] and 4[NT-D-IV+CT] of D-IV sets in which CT administered as adjuvant. Very poor or no antibody titers were detected in other sets. Antibody titers obtained when only PA was administered though very poor, indicate that PA alone could generate mucosal immune response. The fact that IgA antibodies were detected in groups immunized with plant expressed antigens indicate that plant expressed PA & D-IV contribute to effective mucosal immune response. A repertoire of factors contribute to the low s-IgA titers obtained in the fecal extracts. The antibodies in the fecal extracts do not give a dynamic picture of the antibodies secreted in the intestine, in fact they are the residual antibodies carried out in the feces (Chikwamba R *et al.,* 2002). Therefore, the actual amount of secretion may be much higher than what we obtained from our data. We also analyzed the serum IgA antibodies in all the sets. Serum IgA titres were greater than those of mucosal IgA titers. Better serum levels were observed in the groups administered with CT., thus the serum IgA immune responses may represent the appropriate presentation of antigen at the gut mucosal lining transforming into effective mucosal as well as systemic IgA responses.

The protective efficacy of both serum and mucosal antibodies generated was tested *in vitro* by lethal toxin neutralization assay on J774A.1 mouse macrophage like cell line. Neutralizing antibody titers of the order 10^3 and above were acquired in recombinant PA and D-IV groups administered with an adjuvant. Antibodies generated in orally immunized groups indicated both mucosal and systemic mucosal neutralizing antibodies. In groups that were orally immunized with PA alone poor neutralizing titers were detected. Further, immunized mice were tested for protection against toxin challenge with vegetative cells of Sterne strain. All the control mice succumbed to death within 62-75 h. Though, serological titers correlated well to the immune protection levels by and large in various groups immunized with both PA and D-IV, Letx neutralization titers served as better correlates of protection as reported in previous findings (Reuveny *et al.,* 2001, Marcus H *et al.,* 2004). Letx neutralization titers obtained by the immunizations with plant expressed PA and D-IV also revealed similar correlates. The immunized groups that demonstrated *in vitro* TNA titers > 10^3 (Gp C, G, I), also afforded complete protection in *in vivo* challenge experiments. Mice immunized with various plant expressed proteins

also generated NC 50 > 10^2 titers and contributed to 20- 80% survival rates in various groups (fig 6.5). Therefore, plant expressed PA and D-IV antibodies display immunoprotective properties as evidenced by *in vitro* and *in vivo* toxin challenge experiments in mice.

To summarize, we have investigated the potential of anthrax protective antigen and D-IV to impart protection in mice upon oral immunization. Significant levels of protection were obtained with oral administration of PA and D-IV. Further, we examined if the plant expressed immunogens PA and D-IV delivered intraperitoneally or orally generated protective immune response against anthrax. Our results indicated significant protection levels with plant PA and D-IV as evidenced by *in vitro* and *in vivo* Letx challenge experiments. Oral immunization experiments demonstrate generation of Systemic and mucosal immunoprotective responses in mice. Thus, our study demonstrates for the first time the feasibility of PA based plant oral vaccine against anthrax.

Chapter 7

Summary

The present study aims at addressing crucial issues concerning the current vaccination strategies against anthrax in humans and animals. It emphasizes on exploring the plants as better alternative systems to express anthrax vaccine candidates. The choice of *Brassica juncea* as an expression system was to implement the idea of edible vaccine concept. The quest for increased production has always been a bottleneck in order to materialize the plant based vaccines. Chloroplast transformation technology was improvised to overcome this hurdle. The lack of availability of effective tool kit to execute this technology impelled us to devise a comprehensive chloroplast transformation vector. Concomitantly, we focused on the search for an 'ideal vaccine candidate' that would reflect on both pre exposure and post exposure vaccination therapies. In pursuit of this goal, we expressed protective antigen and Domain-IV of protective antigen in tobacco. Finally, the assessment of these antigens *in vitro* and *in vivo* is the most important step in evaluating their efficacy. Key points in immunological studies stress on needle free vaccination delivery, generation of mucosal and systemic immunity to provide complete protection against anthrax. Important milestones of this thesis work are summarized below in a sequential order:

- **Transgenic *Brassica juncea* plants were generated using Agrobacterium mediated transformation method.**

- **High level of antibiotic selection pressure aided in reducing false positives.**

- **Transgenic *Brassica juncea* plants were grown under controlled conditions and hardenening ensured adaptation to external environment conditions.**

- **The presence of *pag* A and *npt* II in transgenic mustard plants was studied comprehensively using PCR based techniques.**

- **The extraction protocol and buffer composition was optimized to circumvent the problem of extensive degradation observed in total soluble protein extracted from putative mustard transgenic plants.**

- **Immunoblot detection of PA was carried out to ascertain the expression of the antigen in plants. A maximum expression level of 0.5 % of TSP was detected *Brassica juncea* plants expressing PA.**

- **Cytotoxicity assays done on mouse macrophage like cell line J774A.1 concluded that plant expressed PA was functionally active. 26-81% of killing was achieved with TSP from *Brassica juncea* plants.**

- **Transgenic tobacco plants were regenerated from leaf discs infected with pCAM-Domain-IV by Agro bacterium-mediated transformation.**

- **Presence of Domain-IV was detected in total soluble protein of transgenic tobacco using polyclonal antibodies generated against recombinant PA. The expression levels of 0.3-0.8 % of TSP were achieved in pCAM-D-IV transgenic plants.**

- **Domain-IV was cloned in pET-28 vector, transformed initially in *E. coli* DH5α cells and then to BL-21 (λDE3) cells.**

- **Expression localized in inclusion bodies was purified then purified by Ni^{2+}-NTA chromatography after induction with 1 M IPTG. 28mg/ L of protein was obtained with a purity levels above 95%.**

- **Immunoblot detection using polyclonal anti PA antibodies confirmed the expression of 16.5 k Da Domain-IV.**

- **A comprehensive chloroplast transformation vector for tobacco was designed. The vector included 3 major portions 1) the vector backbone with ampicillin resistance gene and origin of replication, 2) chloroplast intergenic sequences to target to plastome via homologous recombination and the expression cassette comprising of plastid ribosomal RNA promoter, a**

selectable marker gene (*aminoglycide 3- adenyl transferase*),and 3'UTR for transcript stability. Multiple cloning Site was introduced downstream of *aad* A gene for ease of cloning any gene of interest.

- ***GFP* (green flouroscent Protein) was cloned down stream to *aad* A gene with its ribosome binding site and a spacer to visually assess the functionality of the vector.**

- **Taking a cue from conserved *Escherichia coli* and plastid PEP transcription machineries, the expression of GFP was checked in *E. coli* for fluorescence under UV microscope to assess the functionality of the vector designed.**

- **Domain-IV of Protective antigen gene was cloned in the chloroplast vector downstream of *aad* A gene.**

- **Expression of Domain-IV was checked in *E. coli* before embarking upon a more cost intensive and operationally tedious task of particle bombardment.**

- **PA and Domain-IV were expressed in plant system by Chloroplast transformation. The experiment involved a thorough standardization of both physical and biological parameters to obtain tranplastomic plants.**

- **The successful integration of *pag* A and *domain-IV* was ascertained by polymerase chain reaction in corresponding transgenic plants. Multiple rounds of selection ensured the homoplasmy of plastids.**

- **SDS PAGE and immunoblot detection ensured the presence of 83 k Da PA and 16 k Da D-IV in transplastomic tobacco plants.**

- **Enzyme linked immunosorbent assay was carried out to measure the expression levels of protective antigen in total soluble protein. An expression level up to 3.1 % of PA and up to 4.3 % of D-IV was obtained.**

- **The yield of protein was highest in mature leaves as compared to young and old leaves in tobacco plants.**

- **Subsequent selection of transplastomic T_0 seeds of PA and D-IV on spectinomycin ensured successful transfer of transgenes to T_1 generation.**

- **Functional activity of plant expressed PA was assessed by macrophage lysis assay. In conjunction with LF (lethal factor purified from *E. coli*) transplastomic PA induced 90 % mortality when assessed on J774A.1 mouse macrophage like cell line.**

- **Comprehensive immunization studies with the total soluble protein extracted from transgenic plants was carried out.**

- **The antibody titers generated via oral immunization with adjuvant were less those achieved via intraperitonial immunization with both TSP from plants and recombinant protein counterparts.**

- **Coadministration of a mucosal adjuvant led to augmentation of immune response with recombinant as well as plant expressed PA and D-IV upon oral immunization in general.**

- **Both adjuvanted and non adjuvanted groups of mice orally fed with *Brassica juncea* generated similar antibody levels.**

- **Both parenteral and oral immunization with recombinant or plant expressed PA with or with out adjuvants generated a polarized Th2 type response in mice.**

- **Generation of both systemic and mucosal immunity was found *via* oral immunization with plant expressed PA and D-IV and the corresponding recombinant *E. coli* expressed counterparts.**

- **The antidodies generated with both recombinant and plant produced PA and Domain-IV were able to neutralize lethal toxin (PA and LF) on macrophage cell lines.**

- **The toxin neutralization titers obtained from immunized animals correlated well to the protection levels achieved by toxin challenge in mice. Letx neutralization titers NC50 > of 10^3 endured complete protection and NC 50 titers > 10^2 showed partial protection when mice were challenged with vegetative Sterne strain cells.**

FUTURE DIRECTIONS

- **Codon optimization of gene when required to over express protective antigen gene in a palatable crop e.g. Banana**
- **Fusion of antigenic candidates with genes for adjuvants so that they interact better at the mucosal lining.**
- **Multigene engineering to enable administration of more than one vaccine in a single dose.**
- **Fruit specific expression of protective antigen and Domain-IV.**
- **Comprehensive study on effect of different parameters on bombarding efficiency for transforming recalcitrant crops**
- **Designing of more user friendly, transformation vectors and regeneration techniques to accelerate the speed of generating transgenic plants especially for edible crops.**
- **Optimization of oral immunization with adjuvant that is safe.**

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Appendix

PREPARATION OF BACTERIAL CULTURE MEDIA

LB medium (Luria Broth)

Dissolve 20 gms of LB powder (Hi Media) in double distilled water. Sterilize the media by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

Dissolve 35 gms of LB agar powder (Hi media) in double distill water. Sterilize the media by autoclaving for 20 minutes at 15 lb/sq.in. Allow LB agar to cool and pour in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allow to solidify.

ANTIBIOTICS SOLUTION

Ampicillin

Prepare 100 mg/ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C.

Kanamycin

Prepare 50mg/ml stock solution in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at - 20°C.

SOLUTIONS FOR PLASMID ISOLATION AND PURIFICATION

Solution I

50mM	Glucose
25mM	Tris-Cl (pH 8.0)
10mM	EDTA

Prepare Solution I in batches of 100ml, autoclave for 20 minutes at 15 lb/sq.in. and store at 4°C.

Solution II

0.2 N	NaOH (freshly diluted from 10 N stock).
1%	SDS

Solution III

5M Potassium acetate	60 ml
Glacial Acetic acid	11.5 ml.
Dd H_2O	28.5 ml

The resulting solution is 3M with respect to potassium and 5 M with respect to acetate. Autoclave at 15 lb/sq.in. for 20 minutes. Store at -4°C.

STOCK SOLUTION OF COMMONLY USED REAGENTS

1M Tris

Dissolve 121.1 gms of Tris base in 800 ml of double distilled water and adjust the desired pH (6.8, 7.4, 8.0) with concentrated HCl. Make up the volume to 1 liter and autoclave.

0.5 M EDTA

Add 186.1 gms of disodium EDTA.2 H_2O in 800 ml of double distilled water. Stir vigorously on a stirrer, adjust the pH to 8.0 with NaOH (about 20 gms of NaOH pellets), make up the volume to1liter and autoclave.

3M sodium acetate

Dissolve 204.5 gms of $C_2H_3O_2Na$. $3H_2O$ in 400 ml of ddH_2O, Adjust the pH to 5.3 with glacial acetic acid. Make up the volume to 500 ml and autoclave.

10% SDS

Dissolve 10 gms of electrophoresis grade SDS in 70 ml of ddH_2O heat at 60°C to dissolve and make up the volume to 100 ml.

10 mg/ml Ethidium Bromide

Dissolve 10 mg of ethidium bromide in 1 ml ddH_2O. Store in a dark bottle.

30% Acrylamide Stock

Dissolve 29.2 gms of acrylamide and 0.8 gms of bis acrylamide in 50 ml of ddH_2O. Make up the Volume to 100 ml, filter the solution through Whatman no. 1 paper, degas and store in a dark bottle.

0.1 M Calcium Chloride

Dissolve 147.0 gms of $CaCl_2.2H_2O$ in 100 ml of ddH_2O and sterilize by autoclaving.

1M IPTG

Dissolve 238 mg of IPTG in 1 ml of ddH_2O. Filter Sterlize and store at -20°C.

1M Sodium Phosphate

Monobasic

Dissolve 138 gms of $NaH_2HPO_4.H_2O$ in 800ml of ddH_2O and make up the volume to 1liter.

Dibasic

Dissolve 268 gms of $Na_2HPO_4.7H_2O$ in 700 ml of ddH_2O and make up the volume to 1 liter.

10%Ammonium persulfate (APS)

To 1 gm of ammonium persulfate add 10 ml of ddH_2O the solution may be stored for several weeks at 4°C.

100mM Phenyl methyl-sulfonyl fluoride (PMSF)

Dissolve 17.4 mg of PMSF in 1 ml of isopropanol. Divide the solution in aliquots and store at –20°C.

BUFFERS

50X TAE buffer (Tris acetate, EDTA)

Dissolve 242 gms of Tris base in 700 ml of ddH_2O and add 57.1 ml of glacial acetic acid and 100 ml of 0.5 EDTA pH 8.0. Make up the final volume to 1liter.

10X TBE buffers (Tris borate, EDTA)

Dissolve 8 gms of Tris base, 55 gms of boric acid and 9.3 gms $Na_2EDTA.H_2O$ in 700 ml ddH_2O and make up the final volume to 1 liter.

Phosphate Buffer Saline (PBS)

Dissolve 8 gms of NaCl, 2 gms of KCl, 1.44 gms of Na_2HPO_4 and 0.2 gms of KH_2PO_4 in 800 ml of ddH_2O. Adjust the pH to 7.4 with HCl. Make up the final volume to 1 liter and sterilize by autoclaving at 15 lb/ sq.in for 20 minutes and store at room temperature.

SDS-PAGE electrophoresis buffer

Dissolve 3 gms of Tris base, 14.4 gms of glycine and 1 gm of SDS in 1 liter of ddH_2O.

Protein transfer buffer

Dissolve 5.8 gms of Tris base, 2.9 gm of glycine and 0.33 gms of SDS in 0.5 liter of ddH_2O. Add 200 ml of ethanol and make up the final volume to 1 liter.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

100mM	Tris-Cl (pH6.8)
200mM	DTT
4%	SDS
0.2%	Bromophenol blue
20%	Glycerol
10%	β-mercaptoethanol

DNA loading dye (6X)

Dissolve 0.2 gms bromophenol blue, 0.2 gms xylene cynol and 30 ml of glycerol and make up the volume to 100 by autoclave ddH2O.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

4.0 ml	30% acrlyamide solution
2.5 ml	1.5 M Tris-Cl pH 8.8
3.3 ml	ddH_2O
100µl	10% SDS
100µl	10% APS
10µl	TEMED

Composition of stacking gel (5%) (5.0 ml)

0.83 ml	30% acrlyamide solution
0.68 ml	10M Tris.Cl pH 6.8
3.4 ml	ddH_2O
50 µl	10% SDS
50 µl	10% APS
5µl	TEMED

Staining solution

Dissolve 1 gm of coomassie blue in 450 ml of methanol. Add100 ml of glacial acetic acid and make up the volume to 1 liter by double distilled water. Filter through Whatman no. 1 and store at room temperature.

Destaining solution

Add methanol: Water: Acetic Acid in the ratio of 45:45:10. Store at room temperature.

ANIMAL TISSUE CULTURE MEDIUM RPMI 1640

Dissolve the following components in 100 ml of double distilled water

RPMI salt	4.16 gms
$NaHCO_3$	0.8 gms
HEPES	4.8 gms
Glutamine	0.12 gms
Penicillin	0.0248 gms
Streptomycin	0.04 gms

Adjust the pH 7.2- 7.4 with HCl and make up the volume to 400 ml. Filter sterilize the media and store at 4°C.

PREPARATION OF SOLUTIONS FOR PLANT TISSUE CULTURE AND TRANSFORMATION

Yeast Extract Medium (YEM) for *Agrobacterium* Growth

Yeast extract	1 g/liter
Mannitol	10 g/liter
NaCl,	0.1 g/liter
$MgSO_4.7H_2O$	0.2 g/liter
K_2HPO_4	0.5 g/liter

MS MEDIA (Murashige and Skoog Media)

Composition	for 1 L
MS synthetic salts	4.3 g (HIMEDIA)
$CaCl_2$	0.44g
Vitamins	1 ml
Myo- inositol	0.1 g
Sucrose	30 g

Water added to make up the volume to 1L. pH was adjusted between a range of 5.5 to 5.8 with 1 N NaOH and agar was added at 0.8 % concentration.

Osmoticum

MS media + 2% Sucrose + 0.5 M sorbitol + 0.5 M Mannitol

Water added to make up the volume to 1L. pH was adjusted between a range of 5.5 to 5.8 with 1 N NaOH and agar was added at 0.7 % concentration.

Phytohormones

6-benzyl amino purine(BAP) (1 mg/ml)

Naphthalene Acetic acid (NAA)(1 mg/ml)

Dissolve the hormones in the minimum amount of 1 M NaOH and then make up the final volume with water.

Ampicillin

Prepare 100 mg/ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C.

Kanamycin

Prepare 50 mg/ ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C.

Cefatoxime

Prepare 300 mg/ ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C. Use at a concentration of 300mg/ Litre.

Augmentin

Prepare 200 mg/ ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C. Use at a concentration of 200mg/ Litre.

Hygromycin

Prepare 30 mg/ ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C. Use at a concentration of 30mg/ Litre.

Rifampicin

Prepare 10 mg/ ml stock in DMSO or 100% Ethanol. Store by freezing at -20°C. Use at a concentration of 10mg/ Litre for agro bacterium culture.

Spectinomycin

Prepare 100mg/ml of stock by dissolving in autoclaved milli Q water. After filter sterilizing use at 100 mg/ Litre concentration for bacterial selection with chloroplast vector clones. For selection of transplastomics use 500mg/L in MS media.

DNA Extraction Buffer (DEB)

Composition	for 100 ml
2% w/v CTAB	12.5 ml
1.4 M NaCl	28 ml
0.2 % β Mercaptoethanol	200 µl
20 mM EDTA (pH-8.0)	4 ml
Tris-Cl (pH-8.0)	10ml

The final volume should be made to 100 ml with autoclaved milli Q water.

Protein Extraction Buffer

20 mM HEPES, 5 mM EGTA, 2 mM EDTA, 2 mM Phenylmethyl sulfonyl fluoride, DTT and protease inhibitor cocktail (Cat # P9599), Sigma chemicals per gram of leaves.

PLASMIDS AND STRAINS USED IN THIS STUDY

Strains of plasmid	Description	Reference/ Source
Bacterial Strains		
Escherichrea coli		
DH5α, XL1 Blue	Host strains for plasmid maintenance and propagation	Lab Stock
BL 21 DE3 λ	Host strain for expression in *E.coli* cells.	Lab Stock
Agrobacterium tumefaciens		
GV2260	Host strain for leaf disc transformation	Lab Stock
Plant strains		
Nicotiana tabbaccum var Petit Havana	For generating transgenic plants by nuclear as well as plastid transformation.	Pusa, IARI
Brassica juncea var Varuna	Nuclear transformation by using hypocotyl explants	Pusa, IARI
Plasmids		
pBINAR	Plasmid Binary vector	Lab Stock
pBIN-*pag*	Bianry *pag A* construct	Lab Stock
pMW-*pag*	Vector containing pag A construct	Lab Stock
pET-28a	Expression vector for expression in *E. coli*	Lab Stock
pET-28a-D-IV	Domain –IV of PA in pET 28-a vector	This study
p-CAMBIA-1303	Plant transformation vectors for nuclear transformation	Commercially Procured
pCAM-D-IV	Domain –IV of PA expressed in CAMBIA vector	This study
pBSK+	For construction of plastid vector	Lab stock
pGEMT-EASY	For construction of plastid vector	Commercially Procured
pGEM-pBSK	To clone plastid vector back bone	This study
pGEM-(*trnv-rps-12*)	To clone plastid intergenic sequences	This study
pBS-(trnv-rps-12)	For construction of plastid vector	This study
pGEM-prrn cass	For construction of plastid vector	This study
pCHV	For construction of plastid vector	This study
pCHV-RKB	Basic functional plastid vector	This study
pCHV-RKB-GFP	GFP in plastid vector	This study
pCHV-RKB-D-IV	D-IV in plastid vector	This study

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Induction of cytotoxic T lymphocyte response against Mycobacterial antigen using domain I of anthrax edema factor as antigen delivery system

Subhash Chandra, Manpreet Kaur, Shuchi Midha, Jyotsna Gorantala, Rakesh Bhatnagar *

School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

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Abstract

We have investigated the efficiency of N-terminal 1–260 residues of Edema factor (EFn) as a delivery system for ESAT-6, an antigenic protein of *Mycobacterium tuberculosis* $H_{37}R_v$, into the cytosol of mammalian cells. The EFn.ESAT-6 recombinant protein was obtained by genetic fusion of EFn and ESAT-6 DNA. Our data shows that in the presence of PA, EFn.ESAT-6 fusion protein is internalized into the cytosol of antigen presenting cells, and the splenocytes produced both Th1 and Th2 cytokines *in vitro*. Further, EFn.ESAT-6 elicited effective cytotoxic T lymphocyte (CTL) response in an *in vitro* CTL assay. This study for the first time demonstrates that EFn can be used as a vehicle to deliver heterologous proteins of therapeutic importance.

Keywords: Edema factor; ESAT-6; Fusion protein; CTL; Cytokines

Humoral and cellular responses constitute the two main arms of immune system. One of the key functions of cellular immunity is to generate cytotoxic T lymphocytes (CTLs) for destruction of cells expressing intracellularly processed antigens on their surface. The CTLs recognize and kill tumor and other diseased cells, which display non-self peptides on their surface [1,2]. These peptides arise from various sources, such as infectious agents or aberrant expression of self-proteins, and mark defective cells for CTL recognition. Proteins within the cytosol are processed by multi-catalytic proteosome to generate small peptides, which are then displayed by class I major histocompatibility molecules (MHC-I) on the cell surface. Recognition of foreign peptide–MHC-I complexes by $CD8^+$ cells leads to activation of specific CTLs, which clear the defective cells expressing foreign peptides or harboring pathogen [1,2–5]. Activated CTLs lyse infected cell, secrete cytokines, proliferate and differentiate. Vaccines that prime such memory CTLs, provide protection to the host, upon subsequent exposure to similar antigen displaying cells [5].

Development of vaccines with the ability to generate specific CTLs is hindered due to paucity of delivery systems of antigenic CTL epitopes into the cytosol of host cells. Several approaches to this problem have been reported [1] including the use of attenuated viruses, intracellular bacteria, bacterial toxins, naked DNA, electroporation, heat shock protein, polycationic peptides, non-ionic triblock copolymer, and adjuvants [6–11]. Each of these methods have inherent problem of safety and/or efficiency. Non-infectious, non-toxic, modified bacterial toxins for delivery of heterologous proteins have been reported in the past [6,8,12–17].

Bacillus anthracis produces a bipartite exotoxin comprising of two toxins namely edema toxin (ET) and lethal toxin (LT). Both ET and LT contain a protective antigen (PA) component along with either edema factor (EF) or lethal factor (LF), respectively. Individually, PA, EF, and LF are non-toxic. PA mediates entry of EF and LF into the

* Corresponding author. Fax: +91 11 26 71 70 40.
E-mail addresses: rakbhat01@yahoo.com, rakeshbhatnagar@mail.jnu.ac.in (R. Bhatnagar).

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cytosol by specific receptor on the susceptible cells [18,19]. EF acts as Ca^{2+}/calmodulin dependent adenylate cyclase that causes edema by elevating cyclic amp levels abnormally in the host cells; [20] and LF, which has zinc metalloprotease activity, causes death by inactivating key molecules like MAPK kinase of signaling pathway [21,22]. Mutational studies in our laboratory have demonstrated that in the N-terminal half of EF, residues 136 VYYEIGK142 are critical for binding to PA and subsequent translocation into cytosol [23]. Additionally, the N-terminal domain I (EFn), (1–260 residues) lacks cytotoxicity, associated with the carboxyl terminal domain of EF, and therefore can be used safely for intracellular delivery of heterologous proteins for generation of CTL response.

Here, we report for the first time, utilization of EFn for delivery of ESAT-6, an antigen of *Mycobacterium tuberculosis* $H_{37}R_v$ into the cytosol of macrophages for induction of cell mediated immune response against ESAT-6.

Material and methods

Construction, expression, and purification of EFn.ESAT-6 fusion protein. EFn fusion protein containing ESAT-6 antigen was produced using expression vector pET23a (Novagen). The DNA sequence encoding EFn was amplified by PCR and cloned in pET23a vector using *Bam*HI and *Sac*I restriction sites. A *Kpn*I site was added by PCR amplification at 3′ end, just before *Sac*I site. A DNA sequence encoding ESAT-6 was amplified by PCR with *Kpn*I and *Sal*I sites at the 5′ and 3′ ends, respectively, and ligated to the above construct at *Kpn*I site, such that ESAT-6 was fused at the C-terminal of EFn. This construct was sequenced to confirm the sequence.

pET23a construct containing EFn.ESAT-6 was transformed into competent cells of BL21 (DE3), Codon Plus strain of *Escherichia coli* and recombinant protein was obtained as follows. In brief, cultures were grown in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (60 μg/ml) to an OD_{600} of 0.8. Protein expression was induced by 1 mM isopropyl α-D-thiogalactoside for 4 h. Cells were pelleted and EFn.ESAT-6 protein was purified using Ni–NTA affinity chromatography under denaturing conditions. The cell lysate containing the denaturant was mixed with 3 ml Ni–NTA slurry and loaded onto a column. Ni–NTA matrix was washed with 50 ml of denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein using 8–0 M urea gradient. The protein was eluted with elution buffer containing 250 mM imidazole (pH 8.0). Purified fusion protein was analyzed on 12% SDS–PAGE and then dialyzed against 10 mM Hepes buffer containing 50 mM NaCl.

Purification of PA and LF. Recombinant PA and LF proteins were purified from culture supernatant of *E. coli* M15 cells as described previously [24,25].

Construction, expression, and purification of ESAT-6 protein. ESAT-6 protein was produced using the expression vector pQE30 (Qiagen). To produce ESAT-6, PCR amplified ESAT-6 gene was cloned using *Kpn*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The plasmid pQE30.ESAT-6 was transformed into *E. coli* M15 cells, and transformants were selected on plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). *E. coli* M-15 culture containing plasmid pQE30.ESAT-6 was induced with 1 mM IPTG and ESAT-6 was purified as described above for EFn.ESAT-6.

Competitive inhibition of lethal toxin activity by EFn.ESAT-6. Binding of EFn.ESAT-6 to PA was tested on J774A.1 macrophage cell line by adding increasing concentrations of fusion protein in combination with PA (1 μg/ml) and LF (1 μg/ml) and incubating at 37 °C for 3 h. At the end of incubation period, cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye assay. MTT dissolved in RPMI 1640 medium was added to the cells at a final concentration of 0.5 mg/ml. Incubation was continued for additional 45 min to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 100 μl of 0.5% SDS/25 mM HCl in 90% isopropanol, and plates were vortexed. Absorbance was read at 540 nm using a Microplate Reader (Bio-Rad).

Mice immunization. All the proteins used for immunization were tested for LPS, which was below 0.3 ng/μg of protein. Six- to eight-week-old female BALB/c (H-2^d) mice were obtained from Panacea Biotec Ltd. and maintained in pathogen free environment. A group of 10 BALB/c mice were injected intraperitoneally on day 0 and on day 14 with 2 μg of ESAT-6, EFn.ESAT-6, and PA+EFn.ESAT-6 in Phosphate buffer saline (PBS).

Isolation of splenocytes. Splenic effector cells were prepared by grinding spleens between frosted slides, followed by aspiration through 22-gauge needle to prepare single cell suspensions. Erythrocytes were lysed by ammonium chloride treatment. Remaining spleen cells were washed twice with complete RPMI medium and viability was determined by trypan blue exclusion. Splenocytes were suspended in complete RPMI medium containing 10% heat-inactivated FBS (Hyclone) and kept in CO_2 incubator for further use.

Cytokine ELISA. Mice were injected with PA+EFn.ESAT-6 and splenocytes were isolated after 7 days. Total splenocytes were cultured and stimulated *in vitro* by different antigens. Quantification of murine cytokines; IL-2, IL-12, IL-4, and IFN-γ present in the culture supernatant of activated splenocytes was determined using BD Opt EIA™ kits according to manufacture's protocol (Pharmingen).

Preparation of stimulator cells. For the preparation of stimulator cells, BALB/c (H-2^d) mice derived macrophage J774.1 cells were treated with the same antigen (1 μg/ml) as used for immunization [26]. ESAT-6 or EFn.ESAT-6 were either incubated with or delivered into J774A.1 cells by osmotic shock as described by Okada and Rechsteiner [27]. PA mediated delivery of EFn.ESAT-6 fusion was carried out as follows. Confluent J774A.1 macrophages in 75-cm^2-tissue culture flask were incubated with PA and EFn.ESAT-6 in RPMI-1640 glutamine minus medium for 4–5 h. The medium was removed, and extracellular protein/antigens were rinsed with complete RPMI 1640 medium. Macrophages were scraped off with a sterile rubber policeman and centrifuged at 200*g* for 5 min. Washed cells were suspended in 5 ml of complete RPMI 1640 medium with mitomycin C (35 μg/ml) and incubated at 37 °C for 45 min with 5% CO_2. Stimulator cells were washed by centrifugation four times with complete RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum. Trypan blue exclusion test was used to count macrophage cell numbers and to determine viability. As control of internalization, LF was also introduced by osmotic shock.

Preparation of effector cells. To obtain effector T cells, splenocytes from immunized mice and stimulator cells prepared with homologous antigen were mixed in 1:1 ratio and incubated in the presence of 30 U/ml murine recombinant IL-2 for 5 days at 37 °C with 5% CO_2. After 5-day incubation, cells were collected and live effector cells were obtained by removing dead cells by density gradient centrifugation over histopaque®-1119 (Sigma–Aldrich). Cell number and viability were determined by the trypan blue exclusion test.

Preparation of target cells. The protocol used to prepare different target cells was same as that for stimulator cells, except the mitomycin C step was omitted. J774A.1 cells were treated with 1 μg/ml each of PA, EFn.ESAT-6, and ESAT-6, PA+EFn.ESAT-6 for 4 h and washed twice with RPMI-1640 medium.

Colorimetric CTL assay. A previously described non-radioactive assay procedure [26,28] was followed with some modifications. This was preferred over the radioactive ^{51}Cr release CTL assay as being more sensitive and safe. Different combinations of effector and target cells were mixed at 10:1 ratio, respectively, and incubated for 16 h at 37 °C. After one wash with warm PBS (pH 7.2–7.4), 200 μl of 0.036% neutral red solution (prepared by diluting a 1% (wt/vol) stock solution to 0.036% in warm PBS (pH 7.2) just before use) was added to stain unlysed target cells. After 30 min, the cells were thoroughly washed and then lysed with 0.22 ml of 0.05 M acetic acid-0.05% sodium dodecyl sulfate solution. The amount of dye released was measured by taking optical density (OD) readings at

570 nm. The percentage of specific lysis was established by applying the formula specific lysis = (OD of control − OD of experimental group)/OD of control × 100.

Results

Plasmid construction

The amino terminal domain of EF required for binding to PA and translocation into the cell cytosol was genetically fused to ESAT-6. The DNA construct expressed a fusion protein in which 1–260 residues of EF are fused to 95 residues of ESAT-6 and 6× His tag. The addition of residues at amino and carboxyl terminal ends of EFn (1–260) do not appear to affect functioning of the fusion protein with respect to binding to PA and protein translocation. The resulting fusion protein has 382 amino acids corresponding to molecular mass of 43.07 kDa (Fig. 1A) and calculated pI of 5.25. The yield of fusion protein was about 40 mg/l.

The molecular mass of recombinant ESAT-6 protein corresponded to12 kDa based on electrophoretic mobility on SDS–PAGE (Fig. 1B) and calculated pI of 6.12. The refolded ESAT-6 protein exhibited multimer formation (Fig. 1B), as already described in the literature [29]. The yield of ESAT-6 protein was about 7 mg/l.

Quantification of fusion protein activity by competitive inhibition of lethal activity of LF

Since EFn.ESAT-6 has no toxic activity on cells, binding and internalization of the fusion protein was determined by indirect competitive inhibition of killing activity of LF. The assay is based on the fact that N-terminal 1–260 amino acids of EF and LF are very similar (56% similarity and 37% homology) and contain an identical PA binding motif VYYEIGK, used for binding to PA and subsequent internalization. Increase in concentration of the fusion protein competitively inhibits binding of LF to PA, resulting in decrease in cytolysis of J774A.1 cells. Thus, progressive inhibition of toxicity of LF+PA by the EFn.ESAT-6 gives an indirect measurement of the EFn.ESAT-6 binding and internalization into the J774A.1 cells. Our results show that about 90% protection of the cells occurred when the fusion protein was used in 2.25-fold excess over LF (Fig. 2), indicating that the fusion is able to compete with LF for binding to PA on the cells.

Cytokine ELISA

T helper cells (Th1/Th2) play an important role in eliciting both humoral and cellular responses via expansion of antigen-stimulated B cells and $CD8^+$ T cells or CTLs, respectively. Therefore, the levels of Th1 cytokines (IL-2, IL-12, and IFN-γ) and Th2 cytokines (IL-4) were measured as parameters of polarization of immune response. The Th1 specific marker cytokine IFN-γ production by splenocytes from mice immunized with ESAT-6 alone is known to be very poor [30]. Hence, we examined the comparative cytokine production profiles of the splenocytes isolated from PA+EFn.ESAT-6 immunized mice, after stimulating with different antigens *in vitro*. The time course of synthesis of cytokines by the splenocytes from immunized mice was

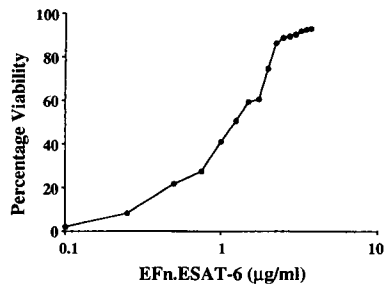

Fig. 2. Competitive inhibition of lethal toxin activity by EFn.ESAT-6. J774A.1 macrophage cells were incubated with PA (1 μg /ml) and LF (1 μg /ml) and increasing concentrations of EFn.ESAT-6 (0–4 μg /ml) for 3 h at 37 °C. Toxicity was determined by MTT assay.

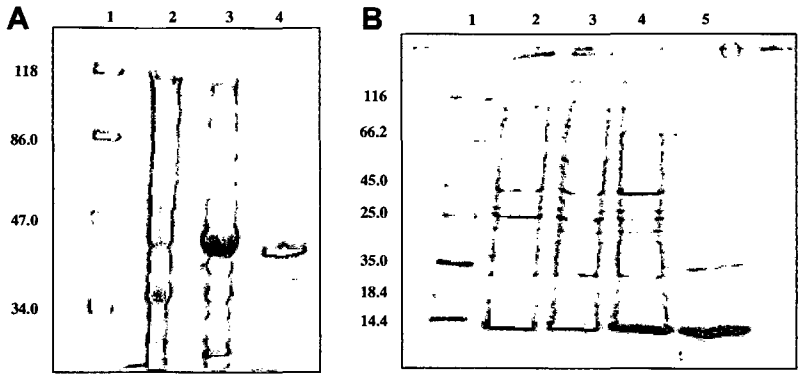

Fig. 1. Expression and purification of recombinant proteins; EFn.ESAT-6 (A) and ESAT-6 (B) in *E. coli* BL-21 (DE3) Codon Plus and *E. coli* M-15 cells respectively. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie brilliant blue; (A) Lane 1, molecular weight marker; Lane 2, Uninduced pET23a.EFn.ESAT-6; Lane 3, induced pET23a.EFn.ESAT-6; Lane 4, Purified EFn.ESAT-6. (B) Lane 1, molecular weight marker; Lane 2, *E. coli* M-15 host cells, Lane 3, Uninduced pQE30.ESAT-6; Lane 4, induced pQE30.ESAT-6, Lane 5, Purified recombinant ESAT-6 (showing both monomeric and dimeric form).

determined at 24, 48, and 72 h. The mean variation of levels of Th1 cytokines (IL-2, IL-12, and IFN-γ) and Th2 cytokine (IL-4) with time are presented in Fig. 3A, B, C, and D, respectively. A substantial increase in the levels of IL-2, IL-12, and INF-γ compared to the control (unstimulated) splenocytes indicated antigen presentation by MHC I pathway and activation of Th1 response. Further, cytokine production by cultured splenocytes was time and antigen dependent. The pattern of cytokine secretion by splenocytes activated with all the antigens was essentially same. An increase of about 2000 pg/ml of IFN-γ was observed in the presence of PA+EFn.ESAT-6 compared to ESAT-6 alone at 72 h, while IL-2 and IL-12 levels were not significantly different in the presence of various antigens. The cytokine levels measured in the supernatant represent the cumulative amounts of each type secreted by different subsets of immune cells present in the splenocytes mixture, therefore it may not accurately reflect the efficiency of antigen delivery. The IL-4 levels were suppressed in the presence of PA+EFn.ESAT-6 as compared to control and also EFn.ESAT-6 fusion, supporting Th1 mediated response in the former. Based on the results it can be inferred that antigens were Th1 response dominant.

CTL assay

The degree of cytolysis caused by effector T cells obtained from mice immunized with either ESAT-6 or EFn.ESAT-6 alone was not more than 20% irrespective of whether the target cells were obtained by incubating the J774A.1 cells in presence of different antigens or by introducing ESAT-6 or EFn.ESAT-6 into cytosol of J774A.1 cells through osmotic shock (data not shown); this was similar to the basal level of cytolysis observed with non-primed target cells (data not shown).The splenocytes form mice immunized with PA+EFn.ESAT-6 produced highest T cell mediated cytotoxicity, when tested in an *in vitro* CTL assay. Maximum cytolysis (60–70%) ($P < 0.05$) occurred when effector T cells from PA+EFn.ESAT-6 immunized mice were incubated with target cells prepared by identical method of antigen delivery i.e PA+EFn.ESAT-6 (Fig. 4-bar 6 A, B, and C). The positive target cells which were prepared by introducing the ESAT-6 or EFn.ESAT-6 into cytosol of J774A.1 through osmotic shock showed 38–45% ($P < 0.05$) level of lysis and, the equivalent lysis showed by both target cells also confirmed that CTLs generated by immunizing with PA+EFn.ESAT-

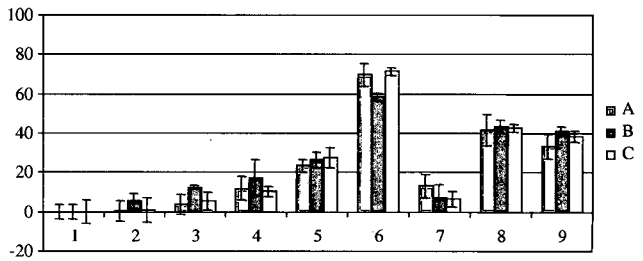

Fig. 4. CTL-mediated lysis of J774A.1 target cells. Splenocytes were isolated from mice immunized with PA+EFn.ESAT-6. Stimulated *in vitro* by stimulator cells prepared by osmotic shock with (A), ESAT-6; (B), EFn.ESAT-6 and (C) by incubating with PA+EFn.ESAT-6. Cytolytic activity of the effector T cells was determined by incubation with differently primed target cells. The effector-to-target-cell ratios (E:T ratios) examined were 10:1. (1), J774A.1 cells (no effector cells); (2), J774A.1; (3), J774A.1 treated with PA; (4), J774A.1 treated with ESAT-6; (5), J774A.1 treated with EFn.ESAT-6; (6), J774A.1 treated with PA+EFn.ESAT-6; (7), J774A.1 given osmotic shock without any antigen; (8), J774A.1 given osmotic shock with ESAT-6; (9), J774A.1 given osmotic shock with EFn.ESAT-6. Data are expressed as means ± SEM of triplicates samples.

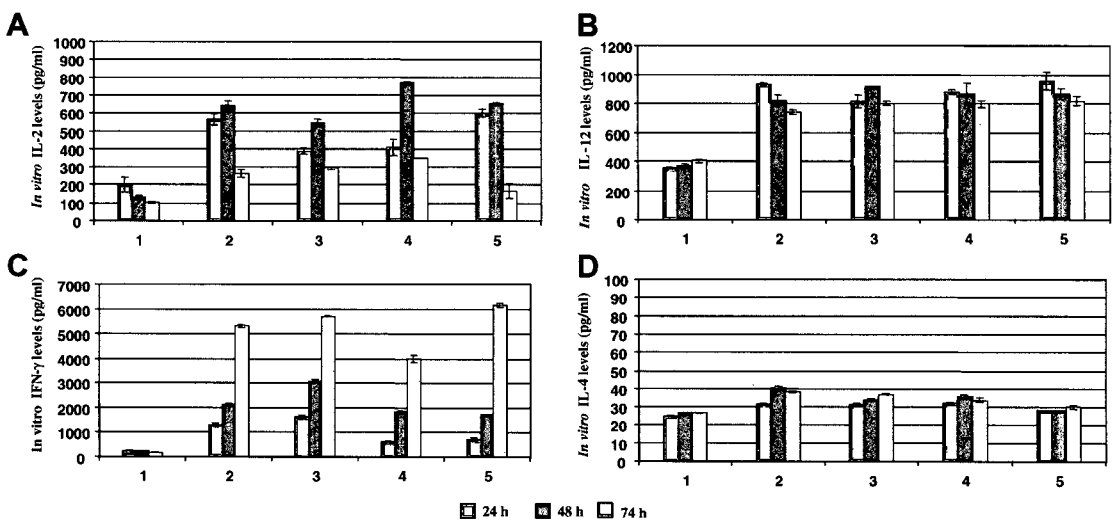

Fig. 3. Concentration of IL-2, (A); IL-12, (B); IFN-γ, (C); and IL-4, (D) in culture supernatants of BALB/c mouse splenocytes. Splenocytes (5×10^5 cells/ml) were stimulated with 10 μg/ml of PA+EFn.ESAT-6 up to 72 h, culture supernatants were collected at 1 h intervals and analyzed by capture ELISA for different cytokines. Splenocytes from four mice were included in each experiment. Data are expressed as means ± SEM of triplicates samples (1), control; (2) PA; (3), EFn.ESAT-6; (4), ESAT-6 and (5), PA+EFn.ESAT-6.

6 were specific to ESAT-6. Further, the method of preparation of stimulator cells *in vitro*, target cells and CTL assay indicated that the route of delivery of ESAT-6 in the antigen presenting cells, proteosomal processing and presentation of epitopes to naïve T cells is critical factor for eliciting the CTL response. Thus, improved efficiency of antigen delivery by PA+EFn.Antigen delivery system resulted in enhanced display of T-cell epitopes on the target cell surface, leading to higher cytotoxicity. The overall results of the CTL assay indicated that CTLs generated by immunization with PA+EFn.ESAT-6 were specific to ESAT-6. In short the data demonstrates that PA mediated delivery of EFn.ESAT-6 fusion protein can elicit effective/desired CTL response.

Discussion

A number of strategies have been used experimentally to deliver an antigen into cytosol for presentation on the cell surface in association with MHC I molecule. Delivery into cytosol and presentation through MHC I molecule is a necessary step for elicitation of CTLs against the host cells expressing foreign peptide [1,10]. ESAT-6 is an important T-cell antigen of *Mycobacterium* and is being investigated as a potential subunit vaccine candidate in several studies [29–31]. Due to relatively low inherent immunogenicity of ESAT-6 [29], all the efforts are directed towards enhancing its ability to evoke a robust T cell response, crucial in the context of tuberculosis infection [30,31].

The mechanism of generation of ESAT-6 specific CTL by PA+EFn.ESAT-6 system can be rationalized as occurring through entry of ESAT-6 into the cytosol of antigen presenting cells in a manner closely resembling its delivery by mycobacterial cells during natural infection. Direct entry of ESAT-6 into the cytosol would induce macrophages and dendritic cells to secrete IL-12, causing differentiation of $TCD4^+$ into Th1 cells. The cytokines produced by the Th1 cells would eventually stimulate differentiation and proliferation of antigen primed $TCD8^+$ cells into cytotoxic T lymphocyte. Synthesis of Th1 type cytokines (IL-2, IL-12, and IFN-γ) by the splenocytes after *in vitro* stimulation supports the proposed mechanism of generation of CTL response. The results of this study demonstrate that an important T cell specific antigen ESAT-6 of *M. tuberculosis*, which is not able to generate adequate protective immunity against infection when used alone [30], worked very efficiently when introduced in the cytosol by this strategy. Thus, detoxified anthrax toxin components can be exploited to deliver protective T cell antigens not only of *M. tuberculosis* but of other intracellular pathogens also. We have experimentally shown that EFn.ESAT-6, when administered along with PA, results in generation of specific CTL response, against ESAT-6. The importance of this study lies in the fact that EFn is able to deliver a target protein into the cytosol of antigen presenting cells in the mice model, for eliciting T cell response essentially required for protection in *M. tuberculosis* infection. This is the first time EF component of anthrax toxin is successfully used as a delivery vehicle.

It is well established that ESAT-6 protein is a potent T-cell antigen [29] and is a vaccine candidate in DNA and subunit vaccines against tuberculosis [31]. In our study, the most likely mechanism of processing of the fusion protein in the mice appears to be PA mediated delivery of the latter in the cytosol followed by proteosomal processing of EF.ESAT-6 and presentation of T cell epitopes by class I MHC complex leading to elicitation of ESAT-6 specific CTL response. The immune response generated by the EFn.ESAT-6 fusion strongly supports the role of EFn as a carrier to deliver specific antigens of *M. tuberculosis*. Further, due to extensive polymorphism of MHC I molecule from individual to individual, protective T-cell epitopes of an antigen also vary from host to host. The ability of EFn to deliver a whole protein molecule into cytosol might be useful in developing vaccine for intracellular pathogens that require T cell specific protective immunity. Multiple epitopes can also be fused to develop more efficient T-cell vaccines against intracellular pathogens. The EFn fusion-mediated delivery has important implications for development of vaccines against infections requiring protective T-cell immunity.

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