DEVELOPMENT OF AN EDIBLE VACCINE AGAINST PNEUMOCOCCAL DISEASES USING TRANSGENIC PLANT

Ph.D. THESIS

BY

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SESSION 2009-10
(RE. 2015-16)

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DHAKA, BANGLADESH

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DEVELOPMENT OF AN EDIBLE VACCINE AGAINST PNEUMOCOCCAL DISEASES USING TRANSGENIC PLANT

A DISSERTATION
SUBMITTED TO THE UNIVERSITY OF DHAKA
IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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IN
MICROBIOLOGY

BY

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Dedicated

To

My Husband

And

Loving Son

CERTIFICATION

This is to certify that thesis entitled **"Development of an edible vaccine against pneumococcal diseases using transgenic plant"** was carried out by **Shahina Akter,** Registration No. 118, Session 2009-10 (Re-Registration 2015-16), for the fulfillment of the degree of **Doctor of Philosophy** from the Department of Microbiology, Faculty of Biological Science, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of the Ph.D. degree.

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DECLARATION

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-The Author

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ABBREVIATIONS

The following abbreviations have been used throughout the text:

%	:	Percentage
+ve	:	Positive
µ	:	Micron
µg	:	Microgram
µl	:	Micro liter
µM	:	Micromole
1 N	:	1 Normal
2, 4-D	:	2, 4-dichlorophenoxy acetic acid
2-ME	:	2-Mercaptoethanol
A.	:	*Agrobacterium*
ACIP	:	Advisory Committee on Immunization
Ap	:	Antigenic propensity
ANOVA	:	Analysis of Variance
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
BSA	:	Bovine serum albumin
bp	:	Base pair
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
cDNA	:	Complementary DNA
CD	:	Cluster designatio

CH	:	Casein hydrolysate
cm	:	Centimeter (s)
CTAB	:	Cyle tetramethyl ammonium bromide
Conc.	:	Concentration
BPP	:	Bacteremic pneumococcal pneumonia
CAP	:	Community-acquired pneumonia
CAPAMIS	:	Community-acquired pneumonia, acute myocardial infarction stroke
CAPiTA	:	Community-acquired pneumonia immunization trial in adults
CD4+	:	Cluster of differentiation 4 positive
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony-forming unit
COPD	:	Chronic obstructive pulmonary disease
dNTP	:	Deoxy nucleoside tri-phosphate
DMSO	:	Dimethyl sulphoxide
DMRT	:	Duncan Multiple Ranked Test
DNase	:	Deoxyribonuclease
Et. Br.	:	Ethidium bromide
e. g.	:	Example gratia, for example
et al.	:	et alil and others
etc	:	et cetra, and the rest
EPI	:	Expanded Program on Immunization
EDTA	:	Ethylene diamine tetra acetic acid
FAO	:	Food and Agriculture Organization
FDA	:	Food and Drug Administration
FAOSTAT	:	FAO Corporate Statistical Database
Fig/s	:	Figure / Figures
FW	:	Fresh weight

g	:	gram (s)
GM	:	Genetically modified
GUS	:	ß-glucoronidase
Ha (s)	:	Hectare
$HCCL_3$	:	Chloroform
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
Hr (s)	:	Hour (s)
HIV	:	Human immunodeficiency virus
HLA	:	Human Leukocyte Antigen
i. e.	:	id est = which to say in other words
icddr,b	:	International Centre for Diarrhoeal Disease Research
IEDB	:	Immune Epitope Database
IFN-γ	:	Interferron-Gamma
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
IgA	:	Immunoglobulin A
IgG	:	Immunoglobulin G
IPD	:	Invasive pneumococcal disease
Kan	:	Kanamycin
Kb	:	Kilo base pair
KD	:	Kilo Daltons
Kcal	:	Kilocalorie
Kn	:	Kinetin (6- furfurylaminopurine)
KNO_3	:	Potassium nitrate
l	:	Litre
LB	:	Liquid Broth
LT-B	:	Heat-labile enterotoxin

LD50	:	Lethal dose 50
lb /sq. inch	:	Pound per square inch
m	:	Meter (s)
M	:	Molar
mg / l	:	Milligram per liter
mg	:	Milligram
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
mm	:	Milimeter
mM	:	Millimolar
min.	:	Minute
MS	:	Murashige and Skoog Medium 1962
MHC	:	Major Histocompatibility Complex
MT	:	Metric tonne
MW	:	Molecular weight
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
NaCl	:	Sodium Chloride
NH_4NO_3	:	Ammonium nitrate
NBPP	:	Non bacteremic pneumococcal pneumonia
nm	:	Nanometer
ng	:	Nanogram
No.	:	Number
NOS	:	Nopaline synthase
*npt*II	:	Neomycine phosphotransferasae II
OD	:	Optical density

PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
PBS	:	Phosphate-buffered saline
PPT	:	Phosphinothricin
PCV	:	Polysaccharide conjugate vaccine
PPV	:	Polysaccharide vaccine
PsaA	:	Pneumococcal surface adhesin A
PspA	:	Pneumococcal surface protein A
PspC	:	Pneumococcal surface protein C
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
rpm	:	Rotation per minute
RT	:	Reverse transcription
sec.	:	Second
SEM	:	Standard error of mean
Sp. / Spp.	:	Species
SPSS	:	Statistical Package for the Social Sciences
t	:	Ton
T- DNA	:	Transfer DNA
T_0, T_1	:	Transgenic lines (First generation inbreed progeny)
TAE	:	Tris acetate EDTA
TAP	:	Transporters Association with Antigen Presentation
Tc/CTL	:	Cytotoxic T cell
TE	:	Tris EDTA
Th	:	Helper T cell
US	:	United States
US$	:	United States dollar
USDA	:	United States Department of Agriculture.
UV	:	Ultraviolet Wavelength

v / v	:	Volume by volume
Var. (s)	:	Variety (s)
Vir	:	Virulence region
Viz	:	Namely
VE	:	Vaccination effectiveness
w / v	:	Weight by volume
Wt.	:	Weight
Zein	:	Fusion protein
α-zein	:	Alpha zein
γ-zein	:	Gamma zein
Ds-red zein	:	Red fluorescent protein
YEP	:	Yeast Extract Peptone
YMB	:	yeast extract Mannitol Broth
WHO	:	World Health Organization.

ABSTRACT

Streptococcus pneumoniae, also known as pneumococcus, is one of the leading causes of respiratory tract infections, meningitis and septicemia in Bangladesh. It is a major cause of morbidity and mortality, especially in children and the elderly. The emergence and progressive increase in incidence of antibiotic resistance coupled with the less-than-adequate polysaccharide vaccine warrants further investigations of protective pneumococcal protein antigens. In recent years, the pneumococcal surface protein A (PspA) has been suggested to be a promising candidate, alone or with other immunogens, to be included in a future vaccine. On the other hand, plant-based edible vaccines are recombinant protein vaccines. Ideally, the choice of plant species used to produce the selected antigen should allow for oral delivery in the form of an edible vaccine. Plant-based edible vaccines have been introduced as a revolutionary cost-effective vaccination modality. It offers a number of advantages over traditional vaccines: they eliminate high-cost purification processes, exhibit long-term stability at room temperature, reduce the risk of disease caused by killed/attenuated organisms and are resistant to gastrointestinal enzymatic degradation.

In this study, immune-informatics tools have been used to identify *in silico* four predicted epitope peptides within PspA. This computational approach was adopted to identify a multi epitope vaccine candidate against PspA that could be suitable to trigger a significant immune response. Sequences of the spike proteins were collected from a protein database and analyzed with an *in silico* tool, to identify the most immunogenic protein. B cell immunity were checked for the peptides to ensure that they had the capacity to induce both humoral and cell-mediated immunity. Four epitope peptide sequences of 33, 10, 14 and 7 amino acids were found as the most potential B cell epitopes. Furthermore, conservancy analysis was also done using *in silico* tools and showed a high conservancy for all epitopes. The sequences of *S. pneumoniae* epitopes were analyzed and placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, respectively. Three constructs were produced using three different inserts namely alpha, gamma and Ds-red construct. *Agrobacterium* strain LBA4404 containing plasmid harboring *npt*II (neomycin phosphotransferase) was used for selection in all cases for the integration of gene of

interest. Antigens of *S. pneumoniae* fused to either α-zein, γ-zein or Ds-red zein in *Nicotiana tabacum* and *Lycopersicon esculentum* were generated using *Agrobacterium*-mediated genetic transformation protocols, and several positive transgenic plants were identified for each of the three constructs. The preparation of the constructs were successfully done. As a prerequisite of the transformation protocol, an efficient *in vitro* regeneration system was established for both *Nicotiana tabacum* and *Lycopersicon esculentum* and the transformation through *A. tumefaciens* were confirmed by kanamycin selection and PCR analysis with the specific primers.

Transgenic plant material containing epitope sequences of PspA were the superior means of inducing a primary immune response. Mice fed transgenic leaves produced PspA-specific antibodies that exceeded the protective level and, on parenteral boosting, generated a strong long lasting secondary antibody response. It have also shown the effectiveness of oral delivery by using a parenteral prime-oral boost immunization schedule. Immunized (fed transgenic leaves) and control mice were challenged with *Streptococcus pneumoniae* serotype 7F, and on average 88% immunized mice survived while 50% survived in case of control. The demonstrated success of oral immunization for pneumococcal with an ''edible vaccine'' provides a strategy for contributing a means to achieve global immunization for pneumococcal prevention and eradication. These immunogenic hot spot within PspA has the potential to serve as an attractive candidate for the development of a novel pneumococcal vaccine.

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1. INTRODUCTION

1.1 Background

1.1.1 Pneumococcal disease; the major infectious disease

Pneumococcal diseases are major public health problems of developing countries including Bangladesh. The etiological agent, *Streptococcus pneumoniae* (Pneumococcus) is surrounded by a polysaccharide capsule and is the major cause of morbidity and mortality all over the world [298]. Acute respiratory infections are still among the leading causes of childhood mortality in developing countries, including Bangladesh [8].

Pneumonia is the primary cause of child mortality, causing 19% of 1.6 million deaths among children < 5 years of age or million deaths per year in 2000–2003; *S. pneumoniae* (pneumococcus) contributes substantially to this disease burden. Pneumonia is also the primary cause of childhood death in Bangladesh. Published comparisons of incidence between developing and developed countries indicate that 90%5 to 95%6 of clinical pneumonia occurs in developing countries [298].

Pneumonia is the commonest cause of childhood mortality, particularly in countries with the highest child mortality, and it has been identified as the major "forgotten killer of children" by the United Nations Children's Fund (UNICEF) and WHO [423] Almost all (99.9%) child pneumonia deaths occur in developing and least developed countries, with most occurring in sub-Saharan Africa (1 022 000 cases per annum) and South Asia (702 000 cases per annum). Of all pneumonia deaths, 47.7% occur in the least developed countries [423], most of which are eligible to get support for the purchase of vaccines and development of their immunization programmes through the GAVI Alliance [36].

Although various pathogens may cause pneumonia, either singly or in combination, the available evidence, including the effectiveness of case management, suggests that two

bacteria are the leading causes: *Haemophilus influenzae* type b (Hib) and *S. pneumoniae* (pneumococcus) [326]. WHO estimates that in 2000, Hib and pneumococcus together accounted for more than 50% of pneumonia deaths among children aged 1 month to 5 years [374, 235]. Who estimates that about 1.6 million people, including up to 1 million children under 5 years old, die every year of pneumococcal pneumonia, meningitis, and sepsis. In population with high child-mortality rates, pneumonia is the leading infectious cause of mortality and accounts for about 20-25% of all child deaths. In these populations, *S. pneumoniae* is identified consistently as the leading cause of bacterial pneumoniae, and pneumococcal disease 20-40 fold, and antibiotic resistance makes treatment difficult and expensive. Thus pneumococcal disease is a major global-health issue [220].

S. pneumoniae is a Gram-positive encapsulated diplococcus, and its polysaccharide capsule is an essential virulence factor. At least 94 serologically distinct pneumococcal serotypes have been identified [56]. The distribution of these serotypes varies significantly between countries and populations. In Europe, the most frequent serotypes are 1, 3, 7F, 14 and 19A [166], while in China, 19F, 23F, 19A, 6B, 14, 6A and 15B are most frequent [356]. A worldwide surveillance program undertaken in 2008 showed that the most common serotypes in children were 19A (28%), 19F (10%) and 14 (9%), whereas in adults the most common serotypes were 19A(13%), 3(7%), 6A (7%) and 7F(7%) [148].

In general, pneumococcal diseases can be classified as either invasive (IPD) or non-invasive pneumococcal disease. The incidence of IPD is bimodal, with one peak in infants<2 years of age and the other in older adults ≥ 65 years of age [113].The World Health Organization (WHO) reported that in 2015, pneumonia (due to all causes, but *S. pneumoniae* is the most common cause) killed ~920 K children ≤ 5 years of age, accounting for 16% of all deaths in this age group [430]. Pneumonia mortality was especially severe in South Asia and sub-Saharan Africa. In China, approximately 30 K children under 5 years of age die from IPD every year [274] In the US in 2014, the IPD morbidity and mortality rates for older adults aged 65–74 years were 19.1 cases and 2.41 deaths per 100,000 population, respectively, while in those ≥ 85 years of age, the rates were 42.6 cases and 8.01 deaths per 100,000 population [415]. In developing countries, the incidence rates for IPD are several times higher than they are in industrialized countries,

and existing data probably underestimate the true disease burden because of the insufficient diagnostic capacity and extensive antibiotic use.

In addition to affecting young children and older adults, *S. pneumoniae* often attacks persons with high-risk conditions: e.g., human immunodeficiency virus (HIV) infection, pregnant women, and patients with cancer, influenza and diabetes. For example, individuals with diabetes may have three- to six-fold increased risk of IPD compared with healthy individuals [202]. For many persons who are immune compromised, the risks are even greater.

1.1.1.1 History

In 1881, the organism, known later in 1886 as the pneumococcus [287] for its role as a cause of pneumonia, was first isolated simultaneously and independently by the U.S. Army physician George Sternberg [350] and the French chemist Louis Pasteur [283].

The organism was termed *Diplococcus pneumoniae* from 1920 [393] because of its characteristic appearance in Gram-stained sputum. It was renamed *S. pneumoniae* in 1974 because it was very similar to streptococci [386, 287].

S. pneumoniae played a central role in demonstrating that genetic material consists of DNA. In 1928, Frederick Griffith demonstrated transformation of life turning harmless pneumococcus into a lethal form by co-inoculating the live pneumococci into a mouse along with heat-killed virulent pneumococci [143]. In 1944, Oswald Avery, Colin MacLeod, and Maclyn McCarty demonstrated that the transforming factor in Griffith's experiment was not protein, as was widely believed at the time, but DNA [17]. Avery's work marked the birth of the molecular era of genetics [216].

The pneumococcus has been an integral and important part of early discoveries in the fields of microbiology, immunology, and microbial genetics. Although probably first observed and reported by Klebs in 1875 [434], the pneumococcus was not reportedly isolated until 1881, when Pasteur and Sternberg independently described it. Pasteur isolated the

pneumococcus from rabbits inoculated with saliva from an infant who died of rabies, and Sternberg did the same from rabbits that were injected with his own saliva, which was used as a control in one of his experiments [284, 355]. In the late 1880s the pneumococcus was implicated as the causative agent of pneumonia, meningitis, and otitis media [434]; this report initiated the quest for a better understanding of the workings o this organism. In the next decade a few seminal discoveries followed. In 1891 Klemperer and Klemperer [192] were the first to show passive protection against pneumococcus with serum therapy by demonstrating that protection could be obtained in offspring of immunized animals against challenge with a homologous strain of pneumococcus. These researchers then injected immune serum into patients to demonstrate protection against infection [192]. Metchnikoff was the first to demonstrate agglutination of pneumococci by immune serum and thus the first to establish a method of differentiating pneumococci by serotype [434]. This led to an early differentiation of three types of pneumococci.

In 1884, Christian Gram used the pneumococcus to demonstrate the vital but now routine Gram stain [140]. In 1925, Avery and colleagues discovered that the soluble substance that surrounds the pneumococcus and was used for strain differentiation, was made of polysaccharides, providing the first description of the capsular composition in any bacterial organism [343]. In 1928, Griffith found that when he injected into mice a mixture of virulent, encapsulated, heat-killed pneumococci with live un encapsulated, avirulent bacteria, the un encapsulated strain was capable of being converted to the same serotype as the heat-killed strain.

The limitations of the currently available polyvalent vaccine formulations comprising purified pneumococcal capsular polysaccharide are well documented. The first is that the protection they impart is type-specific. And because of this, a formulation of serotypes that are effective for one population may not be as effective for another if serotype prevalence differs significantly [217]. The second shortcoming of the present vaccines is that even that protection they provide against specifically included serotypes is by no means complete and may be very poor for certain high risk groups, who have a poorer antibody response to polysaccharide vaccines than healthy adults [120].

Again polysaccharides are T-cell-independent antigens and are poorly immunogenic among children under 2 years of age [170]. Thus, it would seem that there are some disadvantages of the polysaccharide vaccines in attempts to elicit its responses. This encouraged the search for substances other than capsular polysaccharides which could serve as antigens and provide protection against pneumococcal infection [267]. To overcome the limitations of polysaccharide vaccines, protein-polysaccharide conjugate or sub-unit vaccines are being evaluated. These vaccines appear to activate T-helper cells, thereby eliciting T-cell-dependent responses that provide a long-term immunological memory [267].

As mentioned earlier, Pneumococcal diseases can cause high mortality in children, the elderly and immune compromised patients. With more than 90 distinct serotypes, the transition from asymptomatic nasopharyngeal carriage of *S. pneumoniae* to invasive pneumococcal disease depends on the balance between the host's defense mechanisms and bacterial adherence ability, nutrition and their replication within the host [45]. Of the available vaccines, 23-valent capsular polysaccharide vaccine (23-PPV) is ineffective in children less than 2 years of age [24], while as 7-valent glyconjugate vaccine (7-PCV) is effective but has limited serotype coverage [81]. Lately two vaccines, 10-valent and 13-valent glyconjugate vaccines has been licensed for use in humans, while as 15-valent vaccine is currently under consideration [27, 288].

1.1.1.2 Epidemiology

S. pneumoniae is identified consistently as the leading cause of bacterial pneumonia in these populations.

S. pneumoniae, or **pneumococcus**, is a Gram-positive, alpha-hemolytic (under aerobic conditions) or beta-hemolytic (under anaerobic conditions), facultative anaerobic member of the genus *Streptococcus*. [425]. They are usually found in pairs (diplococci) and do not form spores and are non-motile [427]. As a significant human pathogenic bacterium *S. pneumoniae* was recognized as a major cause of pneumonia in the late 19th century, and is the subject of many humoral immunity studies (Fig. 1.1).

S. pneumoniae resides asymptomatically in healthy carriers typically colonizing the respiratory tract, sinuses, and nasal cavity. However, in susceptible individuals with weaker

immune systems, such as the elderly and young children, the bacterium may become pathogenic and spread to other locations to cause disease. It spreads by direct person-to-person contact via respiratory droplets and by autoinoculation in persons carrying the bacteria in their upper respiratory tract [433]. It can be a cause of neonatal infections [28].

S. pneumoniae is the main cause of community acquired pneumonia and meningitis in children and the elderly [376] and of septicemia in those infected with HIV. The organism also causes many types of pneumococcal infections other than pneumonia. These invasive pneumococcal diseases include bronchitis, rhinitis, acute sinusitis, otitis media, conjunctivitis, meningitis, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess [336].

S. pneumoniae can be differentiated from the viridans streptococci, some of which are also alpha-hemolytic, using an optochin test, as *S. pneumoniae* is optochin-sensitive. *S. pneumoniae* can also be distinguished based on its sensitivity to lysis by bile, the so-called "bile solubility test". The encapsulated, Gram-positive coccoid bacteria have a distinctive morphology on Gram stain, lancet-shaped diplococci [336]. They have a polysaccharide capsule that acts as a virulence factor for the organism; more than 90 different serotypes are known, and these types differ in virulence, prevalence, and extent of drug resistance [376].

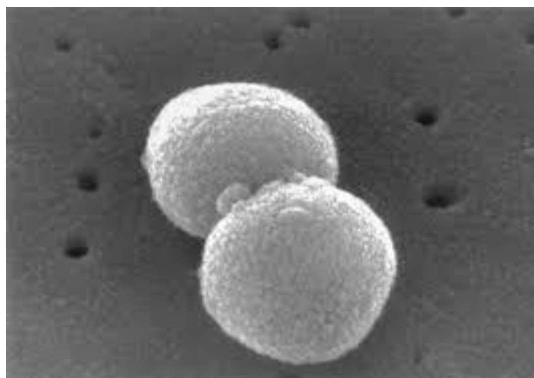

Fig. 1.1: Scanning electron micrograph of *S. pneumoniae*

(Courtesy: CDC)

The pneumococcus is also a major cause of septic infections and meningitis and is occasionally an etiological agent of endocarditis (Fig. 1.2). Worldwide, there are an estimated 4 million deaths in children because of pneumonia; about 1 million of these deaths are caused by *S. pneumoniae*, mostly in children who live in developing countries and are below the age of 1 year [344].

In the US for the year 1999, the pneumococcus was ranked 6th among the top 10 leading causes of disease [6]. In the general population, *S. pneumoniae* infections cause 100,000–135,000 hospitalizations for pneumonia; 6 million cases of otitis media; and over 60,000 cases of invasive disease, including 3,300 cases of meningitis [5]. In children below the age of 5 years, *S. pneumoniae* remains the leading cause of infections, including bacterial pneumonia, bacteremia, and otitis media, [191, 218]; where about 17,000 cases of invasive disease in these children are reported every year including 700 cases of meningitis and 200 deaths [6].

S. pneumoniae has become the most common invasive pathogen in infants and children because of the emergence of antibiotic-resistant strains during the 1990s and because of the fact that the vaccine against *Haemophilus influenzae* type b (Hib) has proven highly effective in eliminating Hib as the primary pathogen in immunized children [40].

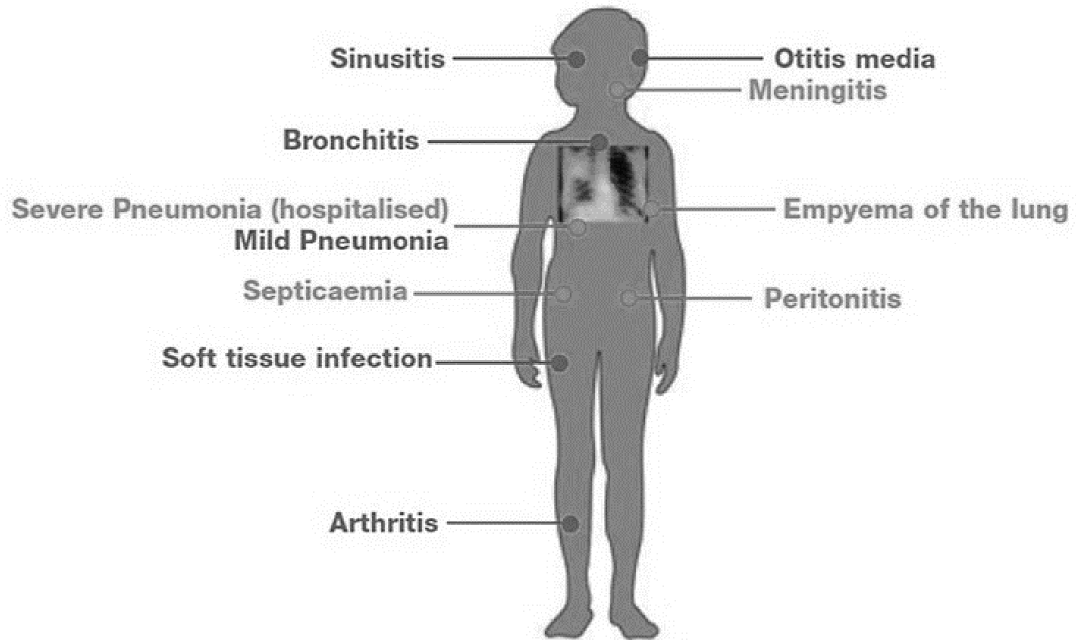

Fig. 1.2: Spectrum of Pneumococcal infections

(Courtesy: Meningitis Research Foundation)

1.1.1.3 Risk factors

Pneumococcal infections increase with age and previous hospitalizations. Workers and residents at nursing homes, workers and children at day-care centers, and persons with underlying disorders like liver disease, heart disease, or chronic obstructive pulmonary disorder are all predisposed to pneumococcal infections [55,227]. Previous infection with influenza virus or other respiratory tract viruses is thought to promote pneumococcal infection [370, 158, 20, 55, 149, 291]. Malnutrition, especially zinc deficiency, increases susceptibility to pneumococcal infection. Also, anatomical factors such as narrow eustachian tubes in children are responsible for increased incidence of otitis media [185, 319].

The polysaccharide capsule of *S. pneumoniae* has been considered the primary virulence factor because non-encapsulated bacteria are essentially harmless compared with the same

encapsulated strain. Recent studies, however, have suggested that certain pneumococcal proteins on the surface of *S. pneumoniae* may also be important virulence factors in the pathogenesis of infection and disease, and potentially for the development of new vaccines. These proteins enable *S. pneumoniae* to evade host defenses and cause invasive disease by concealing and protecting the bacterial surface from host immune defenses and interacting with host tissues to facilitate bacterial colonization; adherence and ultimately pulmonary and extra-pulmonary dissemination, bacteraemia, and systemic disease [174].

1.1.1.4 Transmission, pathogenesis and virulence

Pneumococci are part of the normal microflora of the upper respiratory tract in humans. Five to 10% of healthy adults carry pneumococci in their nasopharynx, but carriage in children is much higher [142]. Carriage rates have been estimated to be as high as 44% among children aged 6 years or younger [407], 60%-80% for children at day-care centers [42, 41, 103], and greater than 70% for children with otitis media [85].

Both colonization and adherence to the respiratory tract are thought to be important at the beginning of the pathogenic process. Nasopharyngeal carriage is reported to be the single most important factor in predisposing children to pneumococcal disease [42].

Still, only a few individuals carrying pneumococci develop symptomatic otitis media, pneumonia, bacteremia, or meningitis. It is not uncommon for individuals to carry more than one strain, and it has been reported that as many as four serotypes can be carried simultaneously by a child [15]. Carriage with a specific strain can last anywhere from a few weeks to 6 months; although prolonged carriage has not been shown to increase the risk of infection, acquisition of new serotypes has been implicated [142, 181]. Infants are thought to acquire a new serotype every 4 months on an average, thereby increasing their chances of developing disease.

1.1.1.5 Mode of infection and immune response to streptococcal antigens

Pneumococcal meningitis is caused by direct spreading from the nasopharynx to the meninges or by hematogenous spread [357]. In hematogenous spread the bacteria usually initially infect the lungs, causing pneumonia. Infection spreads to the blood resulting in bacteremia (Fig. 1.3). From the blood the bacteria is able to traverse the blood-brain barrier

and infect the meninges [327]. The mucosal epithelium of the nasopharynx is the primary site of pneumococcal colonization. Binding to the nasopharynx is dependent upon introconversion between two phenotypes: opaque and transparent. Only the transparent phenotype is able to persist in the nasopharynx *in vivo* [279]. Colonization involves pneumococcal surface adhesins which bind those epithelial cell receptors which display glycoconjugates with the disaccharide GlcNAcl-4Gal [327].

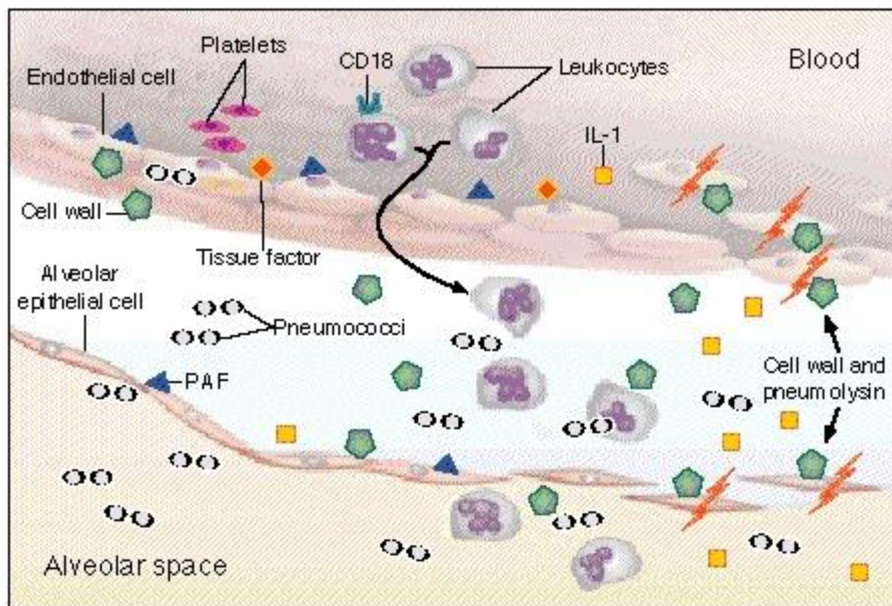

Fig. 1.3: Mechanism of the inflammatory response to pneumococcal cell walls in the lung. (Courtesy: The New England Journal of Medicine)

As stated earlier, *S. pneumoniae* may go directly to the meninges, or it may be aspirated into the lungs, spread to the blood and traverse the blood-brain barrier [327]. The imflammatory response to pneumococal cell walls in case of lung infection is shown in the figure 1.3. The cell wall of pneumococcus bacteria is made of more than a dozen glycopeptides which are continuously inserted and released from the circumferential macromolecule [279]. Pneumococci adhere to platelet activating factor (PAF) receptors on cytokine activated cells by the phosphorylcholine in the teichoic acid component of their cell walls. Phosphorylcholine modulates the bioactivity of PAF, which results in the recruitment of leukocytes and platelets to the area. Leukocytes are also attracted by a selectin-CD18 integrin pathway which becomes active during infection. The bacteria bind

epithelia, endothelia and leukocytes and trigger production of interleukin-1 (IL-1), a key cytokine in the inflammatory response. Among many functions, IL-1 increases vascular permeability and stimulates platelet production [241]. The cell wall is acted upon by components of the acute phase response and has the ability to fix complement. Cell wall components also enhance the permeability of the cerebral endothelia and pulmonary alveolar epithelia, stimulate cytokine production, activate the procoagulant cascade, damage neurons and affect cerebral blood flow and vascular-perfusion pressure [279]. Thus, due to the strong response created to the pneumococcal cell wall, the alternative complement pathway is activated prior to the production of specific anti-capsular antibody. The result is complement fixation to pneumococci and clearance by the reticuloendothelial (RE) system. Once antibody to the capsule is formed, rapid opsonization and removal by the RE system results [327]. Detectable levels of IgM against pneumococcus are observed in infants after infection with *S. pneumoniae*, frequently even in the first year of life. Carriage also sometimes results in the development of anti-pneumococcal IgM in infants [41], although the induction of anti- pneumococcal antibodies by other organisms cannot be excluded. The amount of anti- pneumococcal antibody is age dependent: antibody is present in nearly all older children and adults, but their levels decrease after the age of 50 to 60 years [47]. It is likely that infants can elicit anti-capsular polysaccharide antibody of the IgG class following pneumococcal infection, although they rarely do so in the case of asymptomatic carriage with poorly immunogenic pediatric serotypes 23F and 19F [141].

1.1.1.6 Diagnosis

Diagnosis is generally made based on clinical suspicion along with a positive culture from a sample from virtually any place in the body. An ASO titre greater than 200 units is significant [242]. *S. pneumoniae* is, in general, optochin sensitive, although optochin resistance has been observed [290].

The recent advances in next-generation sequencing and comparative genomics have enabled the development of robust and reliable molecular methods for the detection and identification of *S. pneumoniae*. For instance, the *Xisco* gene was recently described as a biomarker for PCR-based detection of *S. pneumoniae* and differentiation from closely related species [317].

Atromentin and leucomelone possess antibacterial activity, inhibiting the enzyme enoyl-acyl carrier protein reductase, (essential for the biosynthesis of fatty acids) in *S. pneumoniae* [410] Optochin sensitivity in a culture of *Streptococcus pneumoniae*.

1.1.1.7 Genetics

The genome of *S. pneumoniae* is a closed, circular DNA structure that contains between 2.0 and 2.1 million base pairs depending on the strain. It has a core set of 1553 genes, plus 154 genes in its virulome, which contribute to virulence and 176 genes that maintain a noninvasive phenotype. Genetic information can vary up to 10% between strains [377].

1.1.1.8 Transformation

Natural bacterial transformation involves the transfer of DNA from one bacterium to another through the surrounding medium. Transformation is a complex developmental process requiring energy and is dependent on expression of numerous genes. In *S. pneumoniae*, at least 23 genes are required for transformation. For a bacterium to bind, take up, and recombine exogenous DNA into its chromosome, it must enter a special physiological state called competence [75].

Competence in *S. pneumoniae* is induced by DNA-damaging agents such as mitomycin C, fluoroquinolone antibiotics (norfloxacin, levofloxacin and moxifloxacin), and topoisomerase inhibitors [75]. Transformation protects *S. pneumoniae* against the bactericidal effect of mitomycin C [109] Michod et al. [258] summarized evidence that induction of competence in *S. pneumoniae* is associated with increased resistance to oxidative stress and increased expression of the RecA protein, a key component of the recombinational repair machinery for removing DNA damages. On the basis of these findings, they suggested that transformation is an adaptation for repairing oxidative DNA damages. *S. pneumoniae* infection stimulates polymorphonuclear leukocytes (granulocytes) to produce an oxidative burst that is potentially lethal to the bacteria. The ability of *S. pneumoniae* to repair the oxidative DNA damages in its genome, caused by this host defense, likely contributes to this pathogen's virulence. Consistent with this premise, Li et al. [223] reported that, among different highly transformable *S. pneumoniae*

isolates, nasal colonization fitness and virulence (lung infectivity) depend on an intact competence system.

1.1.2 Pneumococcal surface protein antigen (PspA)

Pneumococcal surface protein A (PspA) is one of most abundant surface molecules and a major determinant of protective immunity. Study of the role of PspA in virulence through insertion duplication mutagenesis revealed that PspA is essential for nasopharynx colonization [247]. Addition to its role in lung infection and bacteremia [276], PspA prevents phagocytosis by inhibiting complement-mediated opsonization of the bacterial cells [303]. With high genetic variability, this choline binding protein with molecular size ranging from 67 to 99 kDa, is employed for analyzing the global distribution of pneumococci [163, 80]. On one side where serotype diversity of *S. pneumoniae* complicates the generation of effective vaccines, use of proteins seems advantageous to overcome the limitation with the existing vaccines. To this, PspA is a promising vaccine candidate because genomes of all S. pneumoniae isolates harbor the PspA gene (Fig. 1.4).

Pneumococcal surface protein A (PspA), is one of 12 known choline binding proteins and the gene is present in all pneumococci [80]. PspA has been shown to be required for complete virulence. Strains where PspA has been inactivated show reduced virulence in murine infection models [247]. Immunization with PspA has been shown to protect against pneumococcal infection [51, 50, 358 49, 252, 48, 247 , 248].

PspA, which is a highly conserved, cell wall associated surface protein that plays a major role in pneumococcal virulence by binding human lactoferrin and interferes with complement deposition on the bacterial surface [339, 31].

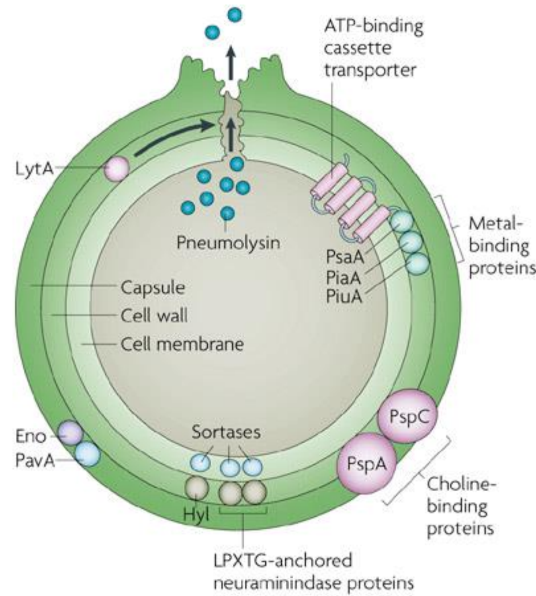

Fig. 1.4: Pneumococcal surface protein (PspA) in *S. pneumoniae*

(Courtesy: Nature Reviews)

1.1.2.1 Biological function of PspA

PspA research was first approached from the protection-eliciting or immunogenic aspect of the molecule. More recently, some functional aspects of the protein have been studied. Many properties have been assigned to PspA, but its actual role in virulence has not been elucidated. Recent findings have suggested that the role of PspA in virulence is to interfere with deposition of complement factor C3b on the pneumococcal surface, inhibiting the formation of a fully functional alternative pathway C3 convertase [373]. Data from the same study showed that the PspA negative strain caused more complement activation in mice, implicating an obstructive interaction between PspA and complement. PspA has also been shown to bind to human lactoferrin, which is produced abundantly during inflammation [150, 151]. Since lactoferrin is known to downregulate the immune system and to block complement activation [237, 186, 383, 44], the elucidation of this interaction may offer more clues about the actual function of PspA during pneumococcal infection.

1.1.2.2 Structure

PspA is expressed on the surface of all pneumococci [80] and varies in size from 65 kDa to about 95 kDa [162]. Knowledge of the structural features of PspA has come from the sequences of PspA/Rx1 and PspA/EF5668 [177, 252] PspA has four structural domains [249]. The α helical region at the N-terminus is thought to take on an anti-parallel coiled-coil conformation. This region contains the surface-exposed, protection-eliciting (albeit variable) region. Major cross-protective epitopes have been mapped to the most C-terminal 100 amino acids of the α -helical (coiled coil) domain [249]. The N-terminal domain comprises the N-terminal half of PspA [Hollingshead 2000], and is followed by the proline-rich region, which in some strains is interrupted with a non-proline block of residues. This region may help to span the cell wall as has been demonstrated in other gram-positive bacteria [101]. After this region is the C terminal end of the molecule, which is composed of 9 to 10 conserved choline-binding repeats of 20 amino acids each; and this region is required for the attachment of PspA to the choline residues of lipoteichoic acids. The C-terminal tail of the molecule consists of a highly conserved 17 amino-acid residue.

1.1.2.3 Role of PspA in virulence and Immunogenicity

Pneumococcal surface protein A attributed virulence to the S. pneumoniae is essential for nasopharynx colonization, an in causing lung infection and bacteremia [276]. PspA elicits a high level of antibodies in humans, as antibodies to PspA were found in the sera of infected individuals [255, 256, 50]. The protective ability of PspA was analyzed when mice were given PspA − and PspA + unencapsulated strain Rx1 and challenged with the strain WU2 [247]. Active immunization with PspA in animal models was found to confer protection against the nasopharyngeal carriage and invasive disease [397]. Mice immunized with DNA vaccine expressing the N-terminal region of PspA were found protected against the intraperitoneal challenge with a strain expressing heterologous PspA [115]. Daniels et al. demonstrated that the proline-rich region of PspA contains surface-accessible epitopes that are protective in both active and passive mouse protection experiments (Fig. 1.5).

Irrespective of the serological variability, PspA expression is observed in all clinically relevant capsular serotypes [80]. Immunization of mice with recombinant PspA elicited antibodies produced in humans passively protects it upon infection with pneumococci [50].

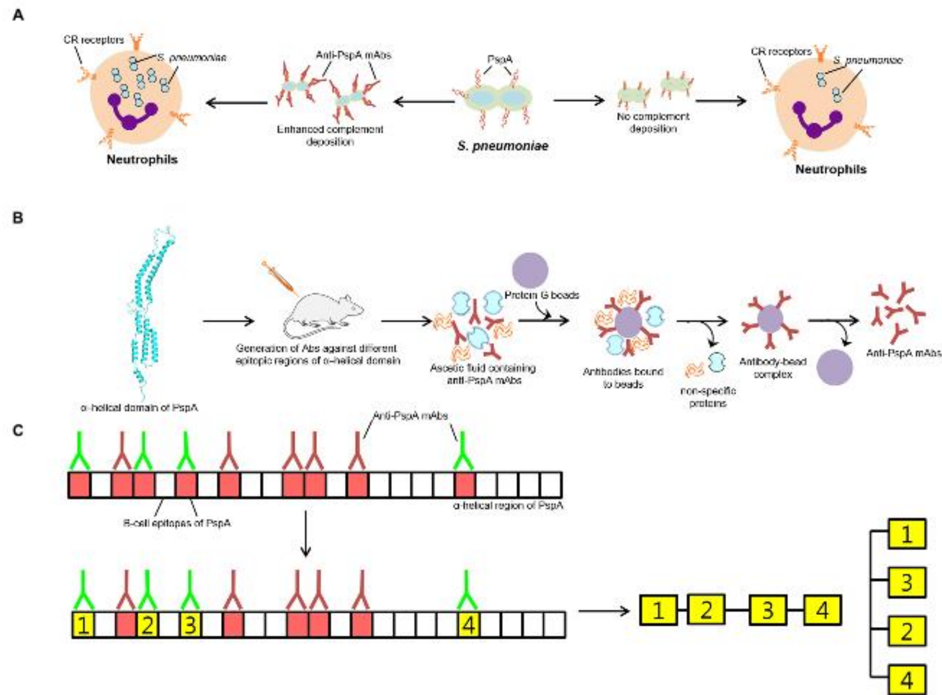

Fig. 1.5: Role of PspA in protection
(Courtesy: Frontiers in Microbiology)

To see whether PspA had any effect on the virulence of pneumococci, the pspA sequence was mutated by insertion duplication mutagenesis and the resulting strains were tested for virulence in a murine sepsis model. PspA negative D39 and WU2 cells had increased lethal dose 50s (LD50) and higher clearance rates [52, 247]. The absence of PspA resulted in more rapid clearance of the pneumococci from the blood of the infected mice [247]. Sequencing of the helical region of PspA from 24 unrelated clinical isolates representing 13 capsular types led to the emergence of two major families, which were subdivided into five clades [162]. The differentiation of the molecule into clades and families is based on the sequence within the clade-defining region (CDR) of the helical domain. An

examination of 2,000 strains from around the world revealed that at least 98% are in families 1 and 2, which are made up of clades 1and 2 and clades 3-5, respectively [79]. Because of the variability of PspA, the ideal formulation for a PspA vaccine has been hypothesized to be one that contains PspAs from the major sub-divisions of PspA families and/or clades. However, most PspAs appear to share conformational epitopes and are adequately cross-reactive to be able to elicit protection against strains of pneumococci that express different PspA serotypes [49].

1.1.2.4 Medical perspective of PspA

Emergence of non-vaccine serotypes poses a great challenge in the management of pneumococcal diseases. These concerns are driving efforts to develop a 'universal' pneumococcal vaccine that is immunogenic in all age groups and broadly cross protective against all serotypes. Efforts are being made to develop a serotype independent protein based vaccine to prevent pneumococcal infections. Rather than targeting a single candidate protein, targeting a complex of proteins based on their roles in bacterial pathogenicity and physiology seems appropriate. Several studies have reported use of two or more proteins for achieving additive and broad protection against pneumococci in mice [66, 53, 209, 276].

The polysaccharide vaccines have several shortcomings which include its limited serotype coverage and poor immunogenicity in high-risk groups. To this, replacement of the vaccine serotypes by other non-vaccine serotypes is currently being perused. However, increasing the number of serotypes in the vaccine increases the cost of preparation that may limit its deployment in uner-developing and developing countries. These concerns are driving efforts to develop universal pneumococcal vaccine that is immunogenic in all age groups and broadly cross protective against all serotypes. As proteins are antigenically conserved across epidemiologically relevant serotypes, it is assumed that coupling proteins with potential to act as effective immunogens in a multi-component protein-based pneumococcal vaccine or as a carrier protein in conjugate vaccine would confer broader resistance to pneumococci [77, 53, 209]. With this, protein based vaccines are considered as a better replacement of capsular-polysaccharide based vaccines.

Study of the prevalence of seven different protein candidates including PspA within global (445 isolates from 26 countries representing four continents) serotype 1 collection of S. pneumoniae, revealed only 68% (305/445) coverage for possible implementation as a vaccine candidate [77, 53].

1.1.3 B-cell epitope

The antigenicity of proteins resides in different types of antigenic determinants known as continuous and discontinuous epitopes, cryptotopes, neotopes, and mimotopes. All epitopes have fuzzy boundaries and can be identified only by their ability to bind to certain antibodies. Antigenic cross-reactivity is a common phenomenon because antibodies are always able to recognize a considerable number of related epitopes. This places severe limits to the specificity of antibodies. Antigenicity, which is the ability of an epitope to react with an antibody, must be distinguished from its immunogenicity or ability to induce antibodies in a competent vertebrate host. Failure to make this distinction partly explains why no successful peptide-based vaccines have yet been developed. Methods for predicting the epitopes of proteins are discussed and the reasons for the low success rate of epitope prediction are analyzed [244].

B-cells recognize solvent-exposed antigens through antigen receptors, named as B-cell receptors (BCR), consisting of membrane-bound immunoglobulins, as shown in figure - 1.6 (Fig. 1.6). Upon activation, B-cells differentiate and secrete soluble forms of the immunoglobulins, also known as antibodies, which mediate humoral adaptive immunity. Antibodies released by B-cells can have different functions that are triggered upon binding their cognate antigens. These functions include neutralizing toxins and pathogens and labeling them for destruction [421].

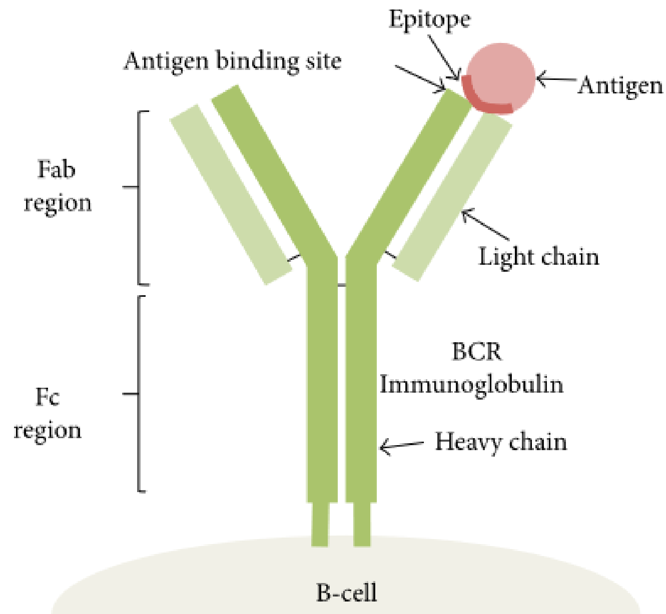

Fig. 1.6: B-cell epitope recognition; B-cell epitopes are solvent-exposed portions of the antigen that bind to secreted and cell-bound immunoglobulins. B-cell receptors encompass cell-bound immunoglobulins, consisting of two heavy chains and two light chains. The different chains and regions are annotated. (Courtesy: Marc HV)

1.1.3.2 Prediction of B-Cell epitopes

B-cell epitope prediction aims to facilitate B-cell epitope identification with the practical purpose of replacing the antigen for antibody production or for carrying structure-function studies. Any solvent-exposed region in the antigen can be subject of recognition by antibodies. Nonetheless, B-cell epitopes can be divided in two main groups: linear and conformational. Linear B-cell epitopes consist of sequential residues, peptides, whereas conformational B-cell epitopes consist of patches of solvent-exposed atoms from residues that are not necessarily sequential. Therefore, linear and conformational B-cell epitopes are also known as continuous and discontinuous B-cell epitopes, respectively. Antibodies recognizing linear B-cell epitopes can recognize denatured antigens, while denaturing the antigen results in loss of recognition for conformational B-cell epitopes. Most B-cell epitopes (approximately a 90%) are conformational and, in fact, only a minority of native antigens contains linear B-cell epitopes [233, 349].

MHC I and MHC II molecules have similar 3D-structures with bound peptides sitting in a groove delineated by two α-helices overlying a floor comprised of eight antiparallel β-strands. However, there are also key differences between MHC I and II binding grooves that we must highlight for they condition peptide-binding predictions. The peptide-binding cleft of MHC I molecules is closed as it is made by a single α chain. As a result, MHC I molecules can only bind short peptides ranging from 9 to 11 amino acids, whose N- and C-terminal ends remain pinned to conserved residues of the MHC I molecule through a network of hydrogen bonds [233, 349]. The MHC I peptide-binding groove also contains deep binding pockets with tight physicochemical preferences that facilitate binding predictions. There is a complication however. Peptides that have different sizes and bind to the same MHC I molecule often use alternative binding pockets [234]. Therefore, methods predicting peptide-MHC I binding require a fixed peptide length. However, since most MHC I peptide ligands have 9 residues, it is generally preferable to predict peptides with that size. In contrast, the peptide-binding groove of MHC II molecules is open, allowing the N- and C-terminal ends of a peptide to extend beyond the binding groove [233, 349]. As a result, MHC II-bound peptides vary widely in length (9–22 residues), although only a core of nine residues (peptide-binding core) sits into the MHC II binding groove. Therefore, peptide-MHC II binding prediction methods often target to identify these peptide-binding cores. MHC II molecule binding pockets are also shallower and less demanding than those of MHC I molecules. As a consequence, peptide-binding prediction to MHC II molecules is less accurate than that of MHC I molecules.

Given the relevance of the problem, there are numerous methods to predict peptide-MHC binding. The most relevant with free online use are collected on Table 1. They can be divided in two main categories: data-driven and structure-based methods. Structure-based approaches generally rely on modeling the peptide-MHC structure followed by evaluation of the interaction through methods such as molecular dynamic simulations [401,210]. Structure-based methods have the great advantage of not needing experimental data. However, they are seldom used as they are computationally intensive and exhibit lower predictive performance than data-driven methods [285].

Data-driven methods for peptide-MHC binding prediction are based on peptide sequences that are known to bind to MHC molecules. These peptide sequences are generally available in specialized epitope databases such as IEDB [385], EPIMHC [261], Antijen [340, 371]. Both MHC I and II binding peptides contain frequently occurring amino acids at particular peptide positions, known as anchor residues. Thereby, prediction of peptide-MHC binding was first approached using sequence motif (SM) reflecting amino acid preferences of MHC molecules at anchor positions [84].

1.1.3.2 Sequence-based B-cell epitope detection software tools

For over 30 years, computational methods have been developed for facilitating epitope recognition [108]. In the past, the majority of the *in silico* methods were focused on linear epitopes. Most of these approaches are sequence-based and use amino acid-based propensity scales, such as hydrophilicity, solvent accessibility, secondary structure and flexibility; a score derived from the propensity scales is assigned to each residue, and the whole sequence is examined for high-scoring window fragments, which are then predicted as epitopes [286,193, 281, 107, 183, 161].

A B-cell epitope is defined as a region of an antigen recognized by either a particular B-cell receptor (BCR) or subsequently the elicited antibody in a humoral response [430,176, 9]. A B-cell epitope can be categorized into two types by its spatial structure: liner epitope or conformational epitope. A liner epitope (also called continuous epitopes) is composed of residues that are sequentially consecutive, where as a conformational epitope (also known as discontinuous epitope) consists of sequential segments that are brought together in spatial proximity when the corresponding antigen is folded. It has been reported that more than 90%of B-cell epitopes are discontinuous B-cell epitopes [23,278].

By far, the study of B-cell epitope prediction mainly aimed at predicting linear epitopes [382, 131]. However, since most B-cell epitopes are conformational epitopes, the prediction of liner B-cell epitope has limited application. In recent years, some computational methods were proposed though the number is limited and the performance is not significant [117,156]. Consequently, to improve the performance of cell epitope

prediction, integrating multidisciplinary knowledge and combining different methods become a promising prospective.

Table 1.1: Databases for 3Dstructure of the antigen and epitopes data [352].

Databases	Websites
PDB	http://www.rcsb.org/pdb/home/home.do
CED	http://immunet.cn/ced/
IEDB	http://www.immuneepitope.org/
HIV Molecular Immunology	http://www.hiv.lanl.gov/content/immunology/index

The IEDB Analysis Resource database uses NetMHCpan as prediction method since 2011. This method generates a quantitative prediction of the affinity of any peptide-MHC class I interaction, covering HLA-A and HLA-B for humans as well as chimpanzee, macaque, gorilla, cow, pig and mouse. This constitutes one of the few databases that include this variety of organisms [145].

This site provides a collection of tools for the prediction and analysis of immune epitopes. It serves as a companion site to the Immune Epitope Database (IEDB) , a manually curated database of experimentally characterized immune epitopes.

The tools contained fall into the following categories:

1.1.3.3 T cell epitope prediction tools

This set of tools includes MHC class I & II binding predictions, as well as peptide processing predictions and immunogenicity predictions.

1.1.3.4 B cell epitope prediction tools

The tools here are intended to predict regions of proteins that are likely to be recognized as epitopes in the context of a B cell response.

Analysis Tools

The epitope analysis tools are intended for the detailed analysis of a known epitope sequence or group of sequences [437].

1.1.4 Vaccination against pneumonia

Despite the advent of antibiotics, the incidence of disease caused by pneumococci has not been eradicated. Vaccines for *S. pneumoniae* have been available for a long time, but the emergence of drug-resistant strains has been cause for serious concern in recent years. It is estimated that about one-third to one-half of all the pneumococcal strains recovered from humans have become at least partly resistant to penicillin [93, 7]. New control measures need to be implemented to combat the rise of antibiotic-resistant strains and the subsequent increase in morbidity and mortality caused by *S. pneumoniae*. Wide spread use of the vaccine coupled with judicious administration of drugs will go a long way in stemming disease caused by pneumococci.

1.1.4.1 Pneumococcal capsular polysaccharide vaccines (PCPV): The earliest vaccine for pneumococcus was administered in 1911 in the form of heat-killed whole cells [395]. Soon after World War II, two different 6-valent vaccines became available in the US; however, interest in them waned with the discovery of penicillin as the panacea for bacterial infections. The vaccines were then withdrawn from the market because of lack of demand. A 14-valent vaccine was licensed for use in 1977 in the US, and not until 1983 did the two currently available 23-valent pneumococcal polysaccharide vaccines came into the market. Pneumovax 23 consists of a purified capsular mixture (25 µg of each capsular polysaccharide from the 23 most prevalent invasive serotypes. The capsular types included in the vaccine are 1, 2 ,3 4, 5, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 17F, 19A, 20, 22F, 23F, and 33F. Although the 23-valent vaccine is expected to cover 85%-90% of the serotypes that cause invasive disease in adults and children in the US [39] the polysaccharide vaccine is ineffective in the very young and only about 60% effective in the elderly [330]. One plausible reason for this diminished effectiveness is the poor immunogenicity of the polysaccharide vaccine (because of its T-cell-independent nature) in the elderly and the failure in young children below 2 years of age to make antibodies to

polysaccharides. It is also known that certain serotypes such as 6B, 9V, 19F and 23F elicit relatively weak antibody responses and that the levels fall to pre-vaccination titers rather quickly [322]. Additionally, qualitative differences in the antibodies made have been reported in some vaccinated adults [311].

1.1.4.2 Polysaccharide conjugate vaccines (PCV): The existing polysaccharide vaccine is less than ideal because of its low efficacy in the target population, especially children, and because the protection is afforded only against serotypes present in the vaccine. The success of *H. influenza* type B oligosaccharide conjugate vaccine prompted the development of a more effective pneumococcal conjugate vaccine [16,306]. Invasive pneumococcal disease rates are the highest in children under the age of 2 years. Because pneumococcal polysaccharides are T-cell-independent antigens, antibody responses induced against them are limited. However, because conjugating a carrier protein to the polysaccharide vaccine induces a T-cell-dependent response and can induce higher levels of antibodies in infants, this approach has been tried; however, the problem of serotype switching limits its efficacy [195, 99]. Carrier proteins that have been used include diphtheria toxoid, tetanus toxoid, a non-toxic mutant derivative of diphtheria toxin (CRM197), pneumolysin, and meningococcal outer membrane proteins [331].

1.1.4.3 Protein vaccines (PV): Protein vaccines are of particular interest because they are immunogenic in young children, who do not respond to polysaccharide vaccines, and they are potentially less variable. Some of the well-studied protein vaccine candidates are discussed below. Sixty-nine potential surface proteins have been found by the pneumococ cal-genomic-sequencing project. Based on sequence homology, it was estimated that of these were likely contributors to the pathogen's virulence, capsule synthesis, and colonization of the host [266]. Additionally, these studies have also led to the identification of several potential protein vaccine candidates [394].

1.1.4.4 Pneumococcal protein vaccine candidates (PPVC): Vaccines directed against pneumococcal non-capsular antigens common to all serotypes hold great promise for preventing pneumococcal infections. A number of pneumococcal proteins have been identified as likely candidates, including autolysin, neuraminidase, pneumolysin, PspA, PsaA, and CbpA [277,361]. In addition to providing protection against all pneumococcal

serotypes, these proteins will induce a T-cell-dependent response with immunologic memory. PspA and pneumolysin have been the most extensively studied candidates for eliciting protection against pneumococcal infection. PsaA is another candidate that has naturally immunogenic properties and alone or in concert with PspA or pneumolysin has been shown to be protection eliciting [337, 300].

1.1.5 Plants based edible vaccine

The plant-based vaccine production method works by isolating a specific antigen protein, one that triggers a human immune response from the targeted virus. A gene from the protein is transferred to bacteria, which are then used to “infect” plant cells. The plants then start producing the exact protein that will be used for vaccinations. The flexibility of the plant expressed vaccine system, combined with its low cost and ability to massively scale, may provide vaccine protection to many parts of the world that cannot currently afford vaccines. Subunit vaccines that consist of one or more antigenic epitopes or proteins are often preferred to traditional vaccines made of killed or attenuated organisms. Mammalian, yeast and insect cell cultures are used to produce subunit vaccines because of their ability to process recombinant proteins in a manner similar to that of the native organism. However, expensive media and the purification steps needed for recovering recombinant proteins expressed in these organisms increase the cost of producing these subunit vaccines. In addition, most subunit vaccines produced in these systems are heat sensitive and require parenteral delivery. This restricts use of subunit vaccines in the poorly funded health systems of developing countries [420].

A promising alternative is to transform plants with a gene(s) encoding an immunogenic protein capable of preventing infection by a pathogenic agent. The production of vaccines in transgenic plants overcomes the risk of contamination with mammalian pathogens and can enable oral delivery. These characteristics simplify vaccine delivery and decrease the cost of an immunization program [420].

Creating edible vaccines involves introduction of selected desired genes into plants or animal and then inducing these altered plants or animals to manufacture the encoded proteins. This process is known as "transformation," and the altered plants or animals are called "transgenic plants" or “transgenic animals”. Edible vaccines are similar to

conventional subunit vaccines as they are composed of antigenic proteins and are devoid of pathogenic genes, hence, they cannot cause infection and can be safely used in patients with weak immune system (Fig. 1.7). Immunization is done by feeding animals or humans with food derived from edible parts of transgenic plants or animals in which an orally active antigen of the target pathogen is expressed and accumulated. Immunoglobulin molecules have been successfully synthesized in tobacco plants using the same technology [420].

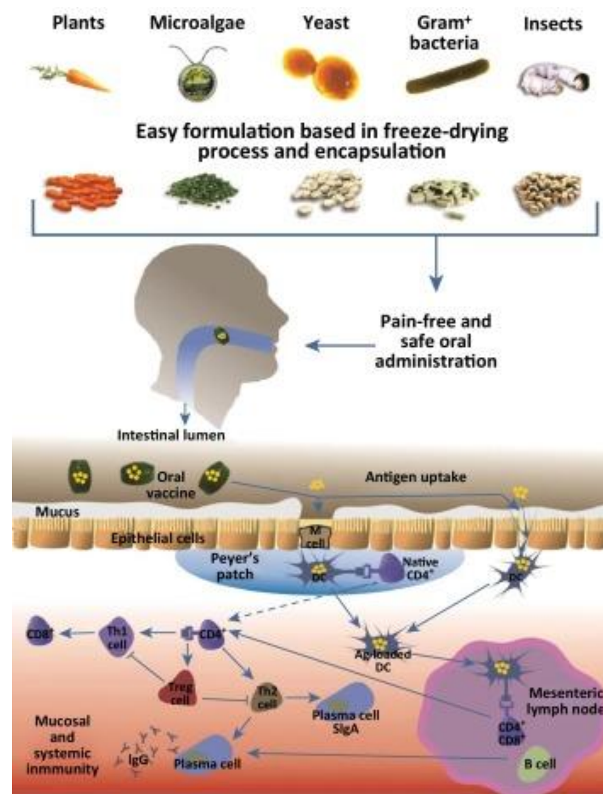

Fig. 1.7: Plant based vaccine provides immunity in mucosal surface

(Courtesy: Trend in Biotechnology)

Plant based vaccines offer a number of advantages over traditional vaccines: they eliminate high-cost purification process, exhibit long-term stability at room temperature [219], reduce the risk of disease cause by needles, killed/atteneuated organisms and dissemination of blood borne disease, and are resistant to gastrointestinal enzymatic degradation [219].

The production of vaccines in edible plant tissues such as leaves, fruit or seeds promotes direct human consumption with limited or no processing [213], and are further advantageous because they can be stored or, in case of seeds with low moisture content or water/oil-based plant extracts, stored for long periods of time, thus circumventing cold-storage issues (the "cold chain").

Vaccine must also be generated in quantities sufficient for immunization and promote appropriate uptake from the intestine [68, 178]. Most importantly, perhaps, one of the benefits provided by plant-based vaccines is that it can induce protective mucosal immunity [405], which is highly effective against infectious agents that enter host cells via invading the mucosal barrier.

1.1.5.1 Edible Vaccines: what are they and how do they work?

The information outlined above highlights the importance of identifying novel, rapid, and concrete solutions for control and prevention. Edible vaccines are of interest as alternative methods of vaccination; as the name suggests, these are foods that provide nourishment in terms of vitamins, proteins, and other nutritional qualities that also act as vaccines to immunize the consumer against a certain disease.

Edible vaccines include all vaccines that are produced in a type of edible format (i.e., part of a plant, its fruit, or subproducts derived from that plant) that, upon oral ingestion, stimulate the immune system [173]. It is worth mentioning that edible does not necessarily mean nutritious, tasty, or organoleptically pleasing, since edible vaccines need only be safe (non-toxic) for human consumption (Fig. 1.8).

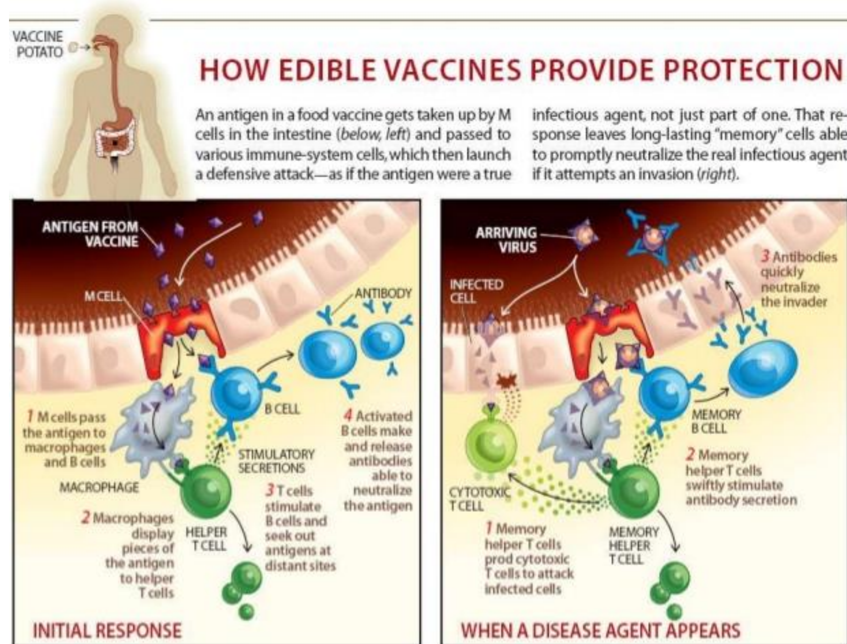

Fig. 1.8: Mechanism action of edible vaccine (Courtesy: Journal of nutrition and food science)

Edible plant-derived vaccines present a better possibility of safer and more efficient immunization in the future. Limitations linked with traditional vaccines, like production, distribution and delivery can be eliminated by the use of edible vaccines through various immunization programs. Edible vaccines successfully embraced the obstacles encountered in rising vaccine technology [178]. Despite restricted global access to health care and much attention still being paid towards complex diseases like HIV, malaria, etc. The time is not so far when there is need for an economical, safer and efficient delivery system to be developed at a larger scale in the form of edible vaccines The ray of hope is based on assumption that edible vaccines may be grown mostly in the developing countries which is basically a fact as in reality they would be used in these countries. Hence, edible vaccines provide a greater opportunity in the near future when no longer injectable needles be used but a fruitful path may be available where an individual get protected from diseases by simply eating a fruit [178].

To create an edible vaccine, the information necessary to produce an antigenic protein must be introduced into the plant of interest by genetic engineering techniques (Fig. 1.9). Once an individual consumes an edible vaccine, the outer wall of plant cells protects the antigens

from degradation by gastric secretion, allowing the antigens to be delivered to the intestinal mucosal surfaces, where they are absorbed by different mechanisms in order to stimulate a strong and specific immune response [178].

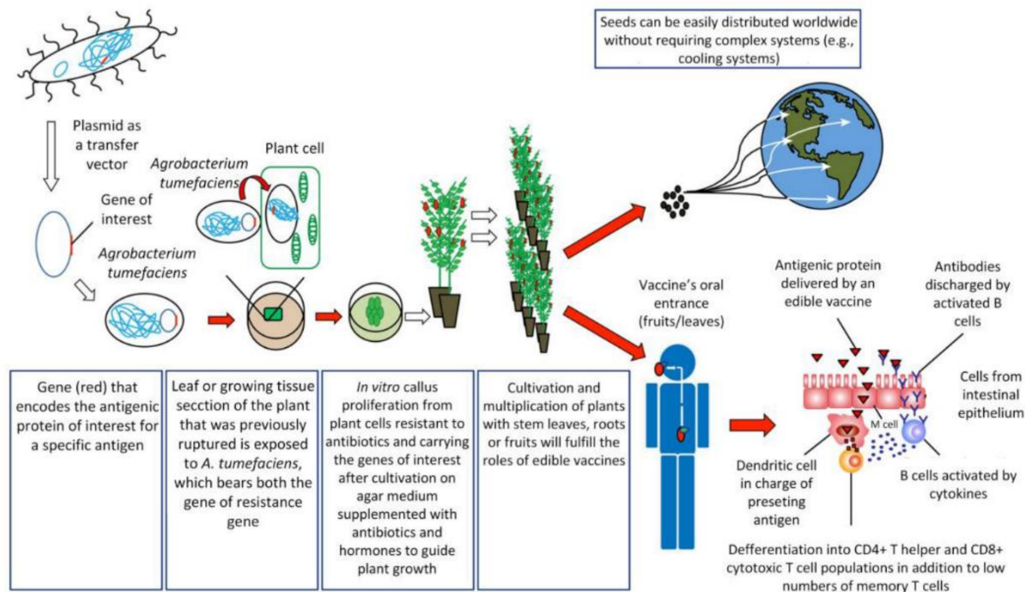

Fig. 1.9: Procedures involved in obtaining an edible vaccine and an immune response; Edible vaccine development begins with the identification of the gene encoding the antigenic protein and its introduction into the plant that will process the food (edible vaccines). [Courtesy: Vaccine]

One of the main routes of antigen capture at the intestinal level is through Microfold (M) cells. M cells represent a small number of specialized follicular-associated epithelium (FAE) enterocytes found primarily in the gastrointestinal tract. These cells efficiently capture a wide variety of macromolecules and microorganisms from the lumen of the small intestine to submucosal antigen-presenting cells (APCs) on Peyer's patches [263]. Among APCs, dendritic cells (DCs) are the most potent antigen-presenting cells in priming naïve T cells to initiate an adaptive immune response [178]. DCs in steady state are found in an immature stage, characterized by high endocytic activity and a low capability to prime naïve T cells. However, under inflammatory conditions, DCs mature, increasing the

expression of co-stimulatory molecules and migrating to T-cell-rich zones in lymph nodes. There, they present antigens together with the release of cytokines facilitating the differentiation of naïve antigen-specific T cells into effector cells and their migration to the specific site of inflammation [90]. Interestingly, intestinal DCs can promote the activation of naïve T cells and the differentiation to follicular T helper cells (Tfh) either by directly promoting Tfh differentiation or indirect by promoting Th17 cells that later will become Tfh [333, 257]. Tfh cells specifically promote the activation of follicular B cells and the generation of IgG and IgA-secreting plasma cells [82]. Then, these activated B cells leave the lymphoid follicles and migrate to the mucosa associated lymphoid tissue (MALT), where plasma cells secreting immunoglobulin A (IgA) antibodies are found [259]. These IgA antibodies are transported across epithelial cells in secretions to the lumen, where they can interact with antigens [259]. It has been recently shown that DCs are critically important in IgA class switching and secretion in B cells [307]. Moreover, DCs can directly capture luminal antigens by projecting dendrites through the epithelial cell layer and into the lumen [308]. Another recent mechanism of antigen capture in the small intestine involved goblet cells, a cell type involved in the production of mucins. By intravital microscopy it was shown that goblet cells can directly capture and deliver antigens to intestinal DCs. An efficient, edible vaccine will stimulate specific T and B cell responses, which will promote long-lasting memory cells for subsequent encounters in which the antigen is presented in the course of an actual infection [259]. However, one of the debates about the oral administration of vaccines has been the development of “oral tolerance”, referring to the phenomenon mediated by T cells that involves a decrease in the specific immune response to antigens previously encountered through the oral route [60]. In the intestinal immune system, the release of antigens occurs in the absence of inflammation (because antigen presentation is not mediated by adjuvants that induce this inflammation), where the antigens are presented to T cells by immature dendritic cells, inducing tolerance [208]. This occurs by the secretion of cytokines, such as IL-10, or by direct cell-to-cell contact, where regulatory T cells interfere with the maturation of dendritic cells, altering their tolerogenic function [208]. Repeated administration of antigens in the mucosa may even result in the suppression of the humoral immune response [208], and it remains difficult to generate vaccines with stable concentrations of antigen in transgenic plants.

Recent studies have applied different strategies to overcome this problem. For example, Kesik-Brodacka et al. use hepatitis B virus (HBV) core protein (HBcAg) as a carrier of the antigen to induce immunogenicity, with promising results. Other strategies involve intramuscular priming before the delivery of the edible vaccine [74]. However, more studies are necessary to efficiently overcome this problem. Alternatively, these particular issues provide the basis for the introduction of edible vaccines in solving problems of autoimmune diseases, based on the selective activation of the autoimmune system to teach the body to tolerate antigenic proteins [259]. Therefore, oral administration of autoantigens could induce tolerance [208].

1.1.5.2 How are edible vaccines developed?

The mechanisms of edible vaccines involve a series of general principles. The first step consists of the identification, isolation, and characterization of the antigen of interest [187]. This antigen must elicit a strong specific immune response [289]. If the latter criterion is met, the gene encoding for this antigen must be cloned into a transfer vector carrying an antibiotic-resistance gene, followed by transformation of the plant of interest. Plant viral vectors appear to be the most promising for expressing foreign proteins in plants [58]. Plant transformation is attained by different methodologies [198]. One of the most commonly used methods for efficiently transferring recombinant DNA into plant cells involves the bacterium *A. tumefaciens* [198]. An *Agrobacterium* strain has been designed to eliminate virulent genes that produce a tumor growing at the base of plants while retaining the genes involved in efficient DNA transfer. The tumor DNA (T-DNA) is contained in a plasmid called the Ti plasmid [239]. The sequence of interest (pathogen) is then inserted into T-DNA to produce the antigenic protein [239]. Once the transgene (T-DNA + antigen DNA) is integrated into the plant genome, the sequence should be expressed and inherited in a typical Mendelian fashion [242], following permanent or temporary (transient) expression of the antigen of interest in the plant or fruit. Later, this genetic line may be propagated by vegetative methods (cutting) or seeds arising from asexual reproduction [359]. This technology is time-consuming, and the scientific infrastructure costs can be a barrier for massive production, especially in low-income countries [116]. However, transient transformation using either *Agrobacterium* or viral vectors is robust, less time-consuming,

easier to manipulate, and offers better opportunities for the industrial production of vaccines or vaccine-related products in a short time. A limitation of transient transformation is that transformation must be repeated if new plant products are required [116]. Ultimately, both transformation systems have their advantages and disadvantages, and the selection of one of these systems depends on the long-term aims and/or urgency of implementing vaccination.

However, the genetic transformation process is not a trivial event. Some agronomically important species (for example, soybeans and most cereal grains) strongly resist *Agrobacterium* transformation. For such plants, a bioballistic method (micromissile bombing) is commonly used, in which gold microparticles are coated with DNA and then blasted into the vegetables using compressed helium gas to attain random transgenic incorporation into the target plant's chromosomal DNA [242]. Due to the random nature of the insertion, there is variability in the percentage of the genetic transformation achieved, and post-transformation diligence is required to select the most vigorous and stable transgenic lines.

Bioballistic methods are also a very efficient alternative when the objective is the plant chloroplast, since more than one copy of the gene of interest can be integrated, thus improving the efficiency of protein expression [87]. In addition, because plastids are not contained in the pollen of most plant species, public acceptance of chloroplast-based transformation seems promising [87]. As mentioned above, edible vaccines can also be generated using viral vectors for expression, by infecting a plant with a virus that is able to replicate independently and transcribe and translate a recombinant protein inserted into the virus genome that corresponds to a characteristic epitope of another pathogenic agent, whether it be from animals or humans [146]. The system is very efficient [146] since the soluble protein is not only expressed in the host plant cells but may also be fused to the capsid of the virus and multiply each time the virus replicates [146]. One of the first edible vaccines developed using the viral vector methodology was a virion that expressed malarial epitopes on its surface [88]; other viruses that have been used include the potato virus, the bamboo mosaic virus, the papaya mosaic virus, and the cowpea mosaic virus [58]. The final step is the oral administration of the vaccine, whether through direct consumption of

the part of the plant that contains the vaccine or by ingesting the part of the plant that carries the vaccine in concentrated pill form. However, as we discussed in the previous section, immune tolerance is a potential problem for edible vaccines, and thus, in order to overcome this immune tolerance, increased concentrations of antigen are needed in the vaccine to stimulate a strong immune response [265]. In fact, studies in the potato in 2005 showed that, although vaccine parenteral administration requires a dose of 40 µg of HBsAg (surface antigen of hepatitis B), oral vaccines require at least three doses of 100 g of potatoes containing a dose of 1 mg of HBsAg to be partially effective. Better results have been obtained through production by viral vectors of up to 295 µg of protein in 1 g of fresh weight of plant tissue [167].

Due to the difficulty of achieving stable vaccine production, compound systems have been developed to generate more stable protein concentration yields in plant tissue systems. These systems combine the integration of *Agrobacterium* DNA with high protein expression of the plant RNA virus and posttranslational capabilities of a plant; this system is called a "launch vector" [270]. In this system, the β-1-3 1-4 glucanase (lichenase) thermostable enzyme, which is stable up to 65°C, is used as a carrier to enhance stability and protein expression [270]. Transient expression using vector methodologies based on viruses or agroinfection in specific parts of a plant can facilitate stabilization via convenience and speed; both viral vectors and systems based on *Agrobacterium* infiltration can produce large amounts of protein in the days after the initial molecular cloning event, in contrast to months for the development of plant and transgene expression [314]. The system based on viral vectors not only enables the expression of the antigenic protein in the particular plant tissue more quickly and efficiently but also results in higher protein concentrations due to expression of this protein in the virus structure that is replicated [214]. First-generation viral vectors retain infectivity in the plant but have raised safety concerns. Second-generation viral vectors maintain a minimum of viral elements required for replication of the vector, and most DNA delivery to the target plant is via non-viral elements. The latter are called viral "deconstructed" vectors and deliver higher performance than the full virus [316, 214]. These types of vectors have been used as an expression system for monoclonal antibodies due to their high and stable levels of protein

expression in plant tissue [157]. An example of this is the production of antibodies for West Nile virus in *Nicotiana benthamiana* developed by agroinfiltration [76].

1.1.5.3 Edible vaccine advantages and disadvantages

During the past 10 years, many studies have been conducted regarding the potential to express antigens in the edible parts of plants, with very promising results [387,228]. It appears possible that this type of oral immunization may become a realistic main strategy in significantly preventing devastating diseases, particularly in low-income countries [11]. Moreover, edible vaccines do not require an extensive framework for their production, purification, sterilization, packaging, or distribution, reducing costs in the long term compared to traditional vaccines [135]. Furthermore, the distribution and maintenance of the vaccine are easier than for conventional vaccines, enabling application of a form of immunization worldwide without the constant cold chains used to preserve conventional vaccines [200]. Consumption of a raw material is another advantage of plant-based vaccines that reduces the cost of processing and purification of antigens [178] as well as the potential degradation of antigens by the gastrointestinal tract due to the protective role of plant cells inside the stomach [321]. Antigen expression in seeds allows maintenance and stability for longer periods, another advantage of edible vaccines [321].

Although edible vaccines are presented as a lower-cost option from a strategic point of view after production of the transgenic plant, this statement is not strictly true. While the administration of an edible vaccine is less complex than conventional vaccine administration because of the use of the oral route, the costs associated with the development and distribution of edible vaccines is a complex issue, particularly for the storage and maintenance of transgenic plants [329]. Additionally, control, purification, and biosafety are the responsibility of pharmaceutical companies, which involves additional costs and presents a barrier to the development of vaccines by small- and medium-size pharmaceutical companies. In that sense, edible vaccines appear to be more promising in terms of animal vaccination [182], although the quality and safety of raw plant materials need to be assured. Another limitation of edible vaccines is the uncertainty related to the calculation of adequate oral administration dosage, which may require several rounds of administration, increasing the final cost of its application [69]. As long as the production

costs remain high and a proper estimation of necessary antigen concentration remains unresolved, the future of edible vaccines will be as uncertain as that of traditional oral vaccines.

Despite these issues, the potential of edible vaccines for immunization is undisputed. A notable example is the outbreak of Ebola virus in Africa in mid-2014, which caused a great number of casualties. No vaccine or globally tested treatment against Ebola virus is available [418]. *Nicotiana benthamiana* plants were used to transiently express three monoclonal antibodies that recognize Ebola virus surface glycoproteins isolated from individuals who survived Ebola infections [171], demonstrating that plants can be effectively used as biopharmacies. The development of an edible vaccine against this lethal disease would be extremely helpful (once the viral antigen that triggers an effective immune response has been identified) in regions where the transportation and delivery of conventional vaccines are difficult. The goal would be to deliver not only vaccines but also “pharmafood”. The objective in creating a vaccine as a food is to create a food source to reinforce health, particularly in underdeveloped countries, where it is difficult to obtain treatments that require complex equipment for their development or conventional vaccines that are difficult to store and transport. However, increased developmental research is essential, as is the need to develop essential legislation as soon as possible, before mass production occurs. Among other concerns, overconsumption of these plants bearing antigens that stimulate the immune system might produce overstimulation of the immune system itself. Moreover, the secondary effects of antigen ingestion should be more thoroughly investigated over the long term, similar to the production of traditional vaccines. Another important factor to be considered is the site where edible vaccine-producing plants are grown. Absolute control should be exercised to protect the environment where such plants are grown to avoid the loss of seeds or pollen during plant removal. The presence of pesticide residues and secondary or toxic metabolites in the plants may pose a major problem [11]. Post-production of the transgenic plant, the risks associated with the use of this plant and its crop are directed to the spread of pollen, seed dispersal, possible horizontal gene transfer, and protein toxicity in herbivores [239]. Contact with insects and release of contaminated water into the environment are also possible mechanisms of transgene escape, though the escape of genes into a food chain is

a more serious concern that cannot be underestimated. However, the likelihood and severity of each risk depends on the plant species and the antigen for each vaccine in transgenic plants [360]. Another important point is that, although, in principle, the development of an edible vaccine has been presented as a solution for the stimulation of the immune response based on the ingestion of a portion of a plant, the process presents difficulties in standardizing antigen concentrations in different plant tissues. The prime difficulty lies with the plants' inherent genetic variability, even in plants propagated by *in vitro* asexual conditions (e.g., somaclonal variation). Here, factors such as growth and fruit development, type, and texture of eatable leaves or roots might influence the availability of antigens [114]. Nonetheless, future prospects also include the possibility of generating vaccines in unicellular green algae, which have many of the same advantages as land plants but much simpler handling and faster mass production [114]. Commercialization of edible vaccine-producing plants might face problems in countries that do not allow transgenic food sales or are not willing to allow the entry or consumption of plants (or parts of plants) that produce edible vaccines (Fig. 1.10). However, the pros and cons of edible vaccines are not restricted to legislation and distribution, as shown by Jacob et al. [171] and Waheed et al. [387], who have presented general summaries of the advantages and disadvantages of edible vaccines.

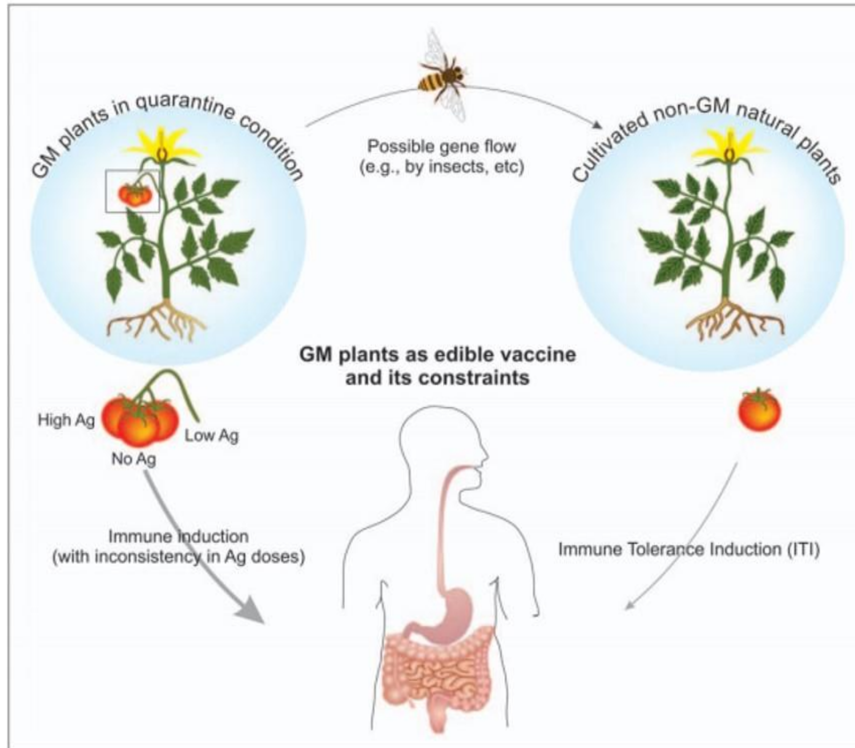

Fig. 1.10: Plant platform limitations of genetic manipulated plant in production of edible vaccines; Plant-based edible vaccines appear to associate with production: vaccine dosage inconsistency, environmental risks. (Courtesy: Internet)

1.1.5.4 Plants already transformed for use as edible vaccines

Most plants studied as edible vaccines have been transformed to express antigens for rotavirus, cholera, gastroenteritis, autoimmune diseases, or rabies [239]. Additionally, most studies have used potatoes for cultivation, but potatoes may not be the best choice for edible vaccines because cooking or boiling may destroy most of the antigenic proteins. Other plants, such as tomatoes, corn, tobacco, bananas, carrots, and peanuts, have a more promising future as edible vaccines, not due to their widespread use but due to the successful development and testing of genetic transformation methods [135, 239].

The plant checklist that follows presents developed edible vaccines that have already been tested in animals and whose use is expected to be authorized in both human and animal medicine. A summary of this checklist is presented in Table -1.2.

Table- 1.2: Transgenic plants used in the therapeutic antibodies.

Antigen	Pathogen or disease	Plant species	Reference
Hepatitis B surface Antigen (Hbs Ag)	Hepatitis B	Tobacco	Mason et al. (1992)
LT-B	*E. coli* heat-labile enteroxin B subunit	Potato, Tobacco	Haq et al. (1995)
SIgA-G	*Streptococcus mutans*	Tobacco	Ma et al. (1995)
Glycoprotein G	Rabies virus	Tomato	McGarvey et al. (1995)
Capsid protein	Norwalk virus	Tobacco, Potato	Mason et al. (1996)
CT-B	*Vibrio cholerae*	Potato	Arakawa et al. (1998a, b)
Insulin	Diabetes (autoimmune)	Potato	Arakawa et al. (1998a, b)
Structural protein VP 1	Foot and mouth disease	Arabidopsis	Carrillo et al. (1998)
Spike (S) glycoprotein	Swine transmissible gastroenteritis coronavirus (TGEV)	Arabidopsis	Gomez et al. (1998, 2000)
s-LT-B	Heat-labile enterotoxin B	Potato	Mason et al. (1998)
Structural protein VP60	Rabbit hemorrhagic disease virus (RHDV)	Potato	Castanon et al. (1999, 2002)
HBsAg	Hepatitis B	Lupin, Lettuce	Kapusta et al. (1999)
Glycoprotein B	Human cytomegalovirus (HCMV)	Tobacco	Tackaberry et al. (1999)
HBsAg	Hepatitis B	Potato	Richter et al. (2000)
F protein	Respiratory syncytial virus (RSV)	Tomato	Sandhu et al. (2000)
S-glycoprotein	TGEV	Tobacco	Tuboly et al. (2000)
Capsid protein 2L21	Canine parvovirus	Arabidopsis	Gil et al. (2001)
Hemagglutinin protein	Measles virus (MV)	Tobacco	Huang et al. (2001)
Human acetyl-choline esterase (AchE)	Organophosphate poisoning	Tomato	Mor et al. (2001)
LT-B	Heat-labile enterotoxin B subunit	Corn	Streatfield et al. (2001)
S	TGEV	Corn	Streatfield et al. (2001)
CT-A2:CFA/I-CT-B:NSP4	Cholera, enterotoxigenic *E. coli*	Potato	Yu and Langridge 2001
Protective antigen	*Bacillus anthracis*	Tobacco	Aziz et al. (2002)

Potatoes

Mason et al. conducted the first assay based on a vaccine produced in potatoes (*Solanum tuberosum*) to combat enteritis produced by *Escherichia coli* strain LT-B in mice [442]. That same year, the effectiveness of antigens produced by potatoes against the pathogen from Norwalk virus capsid and the non-toxic subunit (CT-B) of *Vibrio cholerae* enterotoxin was demonstrated in rats and human volunteers [13]. Tacket's second-phase clinical assay (Phase I considered patients previously vaccinated) involved the study of human immune responses to Norwalk virus capsid expressed in potatoes, with 95% (19 out of 20 volunteers) developing a type of immune response. However, a significant increment was not always obtained [359]. In 2005, Thanavalas's group

proposed that the potato might have a role as an oral reinforcement to the hepatitis B injectable vaccine in humans [362]. Moreover, edible vaccines have also been developed as an oral reinforcement to injectable vaccines for animal protection. For example, an edible vaccine was developed in potatoes to protect minks from diseases caused by mink enteritis virus (MEV) [59]. In wild rabbits (*Oryctolagus cuniculus*), immunization via potatoes producing the protein VP60 provided protection against infection produced by rabbit hemorrhagic virus (RHDV) [59].

Tobacco

First, we want to highlight that tobacco per se is not an edible plant; rather, it is used as a proof-of-concept model species for edible vaccine development. Thus, in 1996, in parallel with the potato studies, transgenic tobacco (*Nicotiana benthamiana)* plants expressing a protein from Norwalk virus capsid that produces gastroenteritis were developed [63] and resulted in antibody, specifically IgA and IgG, development in rats [63]. In 2007, transgenic tobacco expressing the virus VP1 protein from chicken infectious anemia was reported. Other studies in tobacco have demonstrated the ability to express a polypeptide associated with hepatitis B [63]. In this study, it was feasible to stimulate a humoral immune response that produced the HBsAg; such stimulation evoked higher blood T-cell counts, and these results were used to calculate correlations of the immunoglobulin A and G humoral responses with the corresponding vaccine dose [63]. Gómez et al. [138] endeavored to more effectively express the virus antigen in transgenic tobacco. In 2012, transgenic tobacco plants expressing HPAIV H5N1 from avian flu virus gave rise to IgG stimulation when tested in rats [335]. Recently, transgenic tobacco plants expressing a protein from *Eimeria tenella*, the agent that causes coccidiosis [84], and transgenic tobacco plants to combat anthrax [136] were reported. In the latter, the tobacco expressed a protective antigen (PA) that resulted in elevated serum IgA and IgG in murine models.

Tomatoes

An effective vaccine candidate against the coronavirus that causes a highly acute respiratory syndrome (SARS) was developed in the tomato (*Solanum lycopersicum*), [292]. A study in 2006 showed that tomatoes expressing the Norwalk surface virus protein that

were dried outdoors instead of lyophilized before consumption by rats provided immune protection superior to that of potatoes [63]. Tomatoes have also been used to express CT-B protein from *Vibrio cholerae* B toxin, as supported by ELISA and Western blot analysis in leaves, stems, fruits, and other tissues [179]. HBsAg has recently been produced in tomatoes of the Megha variety, as confirmed by qRT-PCR and ELISA, the first report of stable expression of an antigen in tomatoes [63]. In 2008, human beta-amyloid was expressed in the tomato as a potential vaccine against Alzheimer's disease [63]. Another study of transgenic tomatoes included the fusion antigen F1-V from *Yersinia pestis*, a bacterium that is highly pathogenic and causes pneumonic, septicemic, and bubonic plagues. In short, given the wide possibility of indoor as well as outdoor cultivation, tomatoes are currently one of the foods with the greatest potential for use as an edible vaccine.

Lettuce

Experiments focusing on lettuce (*Lactuca sativa*) plants expressing the B subunit of the thermolabile protein of *E. coli*, which is responsible for enteric diseases both in humans and animals, indicate that this vegetable may be a potential edible vaccine. In this experiment, approximately 2% of the total protein detected in the leaves corresponded to the antigen [188]. In 2005, lettuce expressing glycoprotein E2 of the classical swine fear hog pest virus was developed [214]. In Poland, transgenic lettuce plants that produce effects against hepatitis B virus are in the first phase of development [188]. Because this food is mainly consumed raw, it has the greatest potential to be used as an edible vaccine.

Rice

A study in 2007 showed that transgenic rice (*Oryza sativa*) plants expressing the B subunit of *E. coli* induce a considerable amount of antibodies against this subunit [444]. In the same year, transgenic rice expressing the VP2 antigenic protein from infectious bursitis was shown to induce an immune response in chickens. In 2008, functional expression of HBsAg in rice seeds was confirmed by PCR and Southern blot analyses [295]. Furthermore, in 2008, transgenic rice was produced in parallel to express the B subunit of the *E. coli* thermolabile toxin using the bioballistic approach to transform the plant cells; the

expression was verified by PCR [444]. World rice production for 2016/2017 is estimated to be 480 million metric tons, and China and India (the two countries with the largest populations in the world) will produce and consume almost half of that annual production [429]. Thus, any vaccine developed using this plant will have a huge impact on the public health systems not only of these two countries but also other nations where rice is an important part of the daily diet.

Carrots

Transgenic carrots (*Daucus carota*) expressing the B subunit from *E. coli* thermolabile toxin induced IgA and IgG production, and occurred at the intestinal and systemic levels in rats [309]. In 2010, the UreB subunit of *Helicobacter pylori* in transgenic carrots was reported to have potential use as a possible vaccine [408]. Carrots, along with *A. thaliana*, were also utilized in experimental edible vaccines for surface HIV antigen expression, and studies performed in rats showed more positive effects in treated animals compared to non-treated animals. The utilization of carrots to treat HIV appears promising not only because carrots are healthy and delicious but also because the consumption of carrot-derived carotenoids increases lymphocytes, monocytes and other immune defenders in rats [309]. Thus, people with weakened immune systems might benefit from consuming this potentially edible anti-HIV vaccine. Studies in humans must be conducted to confirm the potential of these vaccines.

Soybeans

B subunit expression studies of *E. coli* thermolabile toxin were conducted in the soybean (*Glycine max*) endoplasmic reticulum, in which a total antigen level of up to 2.4% of the soy seeds' total protein was obtained without producing any instability during seed drying for further processing treatment; moreover, oral consumption by rats led to increases in systemic IgA and IgG levels [264].

Alfalfa

In 1999, successful oral immunization was achieved against virulent foot-and-mouth disease (FMDV) in rats, providing the first evidence that long protein chains can be

successfully produced using only raw extracts when sufficient plant quantities are utilized [392]. Transgenic alfalfa (*Medicago sativa*) expressing the antigen eBRV4 from VP4 of hog rotavirus (BVR) was subsequently used as an edible vaccine in a veterinary environment. In 2005, transgenic alfalfa plants were developed to express hog pest virus glycoprotein E2 [214]. In 2009, transgenic alfalfa development was reported in which alfalfa expressed the σC protein from the capsid virus, which causes poultry infections. The same antigen was developed in other plants, for example, *A. thaliana* [398]. In another alfalfa study, Eeg95-EgA31 of *Echinococcus granulosus* was expressed. This protein was purified and was also delivered directly from the leaves to the target organism [402], confirming the huge potential of this plant for veterinary purposes.

Corn

In 2012, transgenic corn (*Zea mays*) plants expressing rabies virus antigenic glycoproteins showed quite promising results as an edible vaccine for both humans and animals [11, 362]. Promising results have been obtained in relation to the development of vaccines against transmissible gastroenteritis coronavirus (TGEV) in pigs [204]. Studies using transgenic corn as a vaccine showed that 50% of treated pigs developed diarrhea, in contrast to 75% of pigs not treated with the vaccine. The study concluded that the transgenic corn conferred partial protection to piglets against clinical disease and experimental challenge with the pathogen [204]. In other studies, oral feeding with transgenic corn expressing the fusion protein of the Newcastle disease virus (NDV) produced immunogenic effects and conferred protective immunity in poultry [144].

Papaya

A vaccine based on papaya (*Carica papaya*) fruit was produced in 2007 by expressing synthetic peptides in 19 transgenic papaya clones to combat cysticercosis caused by *Taenia solium*. This vaccine was tested in rats, and 90% of treated rats showed an immunogenic response [153]. These edible vaccines could provide sweet relief in both humans and pigs, the main two disease carriers, but have not been tested in these systems.

Quinoa

In 2012, an edible vaccine was developed by expressing the VP2 antigen from infectious bursitis virus in quinoa (*Chenopodium quinoa*). The vaccine was developed for poultry veterinary medicine [65].

Bananas

The expression of HBsAg has been reported in banana plants using four different expression cassettes (PHB, PHER, pEFEHBS, and pEFEHER). Expression was studied at various levels using PCR, Southern hybridization and reverse transcription PCR. The expression levels in the crop plants reached a peak of 19.92 ng/g, and the antigen was present in the leaves of the plant [199]. However, the use of this vaccine was rejected due to the long periods of time that it takes for the tree to develop.

Peas

This transgenic plant was developed based on the expression of a capsid protein of Norwalk virus. Protein accumulation of up to 8% of the soluble protein was observed in the unripened fruit, with lower accumulation in red ripened fruits [12,115]. Expression in plant seeds allowed storage of the antigenic peptide and thus generated a plant with a high yield of protein expression; the protein content was estimated at 20% to 40% [12,409], and thus extraction of the pharmaceuticals would not require extensive purification procedures [12].

Pea plants have also been used for expression of the hemagglutinin protein (H), a PA against rinderpest virus. The level of expression was determined by Western Blot and was observed to be between 0.12% to 49% of the total soluble protein in leaves [12]. Thus, further studies to optimize protein expression in transgenic peas are needed [409].

Apples

The gene encoding the F protein of human respiratory syncytial virus (RSV)-F was constitutively expressed in apple leaves using the CaMV35S promoter. Protein expression was considered stable and corresponded to 20 mg/g of plant tissue [211].

Cherry Tomatillos

Lines of transgenic cherry tomatillos were developed for the HBsAg gene of hepatitis B. Gene expression was observed throughout the plant but was highest in the leaves, reaching 300 ng/g fresh weight, with 10 ng/g fresh weight in fresh fruit. Significant immune system activation was observed in rodents [125].

Algae

The green alga *Chlamydomonas reinhardtii* has been used as a model to produce large amounts of proteins related to therapeutic processes in both humans and animals [122]. The use of algae for vaccine production seems promising because algae have a very fast growth rate, their entire structures can be used as a raw material to produce edible vaccines, and there are no limitations in terms of habitat (sea farms) or aspects related to fertility. Moreover, there are no negative concerns about cross-contamination with other field crops [345]. Additionally, algae can be cultivated in bioreactors [122] to further accelerate their already fast growth. Importantly, algal vaccine effectiveness is unaltered after lyophilization, which may facilitate the global distribution of edible vaccines made from algae. In particular, the model alga *C. reinhardtii* contains only one chloroplast, increasing the stability of algal lines expressing the desired antigens [123].

1.1.6 Biotechnology, genetics and bioinformatics

Modern technology is the wave in science. It represents an interface of basic and applied sciences with gradual and subtle transformation of science into technology. Over the past twenty five years, a mere sliver of recorded time, the world of biology and indeed the world in general has been transformed by the technical tools of a field now known as Biotechnology [426].

Biotechnology is defined as the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services. Since the inception of the word 'biotechnology' in 1919, this had made changes in various life science processes to improve existence. Biotechnology comprises a number of technologies based up on increasing understanding of biology at the cellular and molecular levels [426]. Biotechnology has impactful applications on a vast number of fields. Biotech has been

producing innumerable new products that have the possibility to alter our lives for the betterment [413]. Genetically modified crops, transgenic animals, DNA recombinant medicines, genetically engineered microbes for waste management and also biosensors have improved our life. Biotechnology plays a big role in the biofuels industry also.

Enhancing plant and animal behavior by traditional methods like cross-pollination, grafting, and cross-breeding are time-consuming. Biotech advancement has led for specific changes to be made rapidly, on a molecular level through over-expression or removal of genes, or by introduction of foreign genes. Environmental biotechnology gives response to a chemical that helps to measure the level of damage caused or the exposure of the toxic or the pollution effect caused, so it can be regulated accordingly. Even in forensic analysis, biotechnology has brought huge positive changes. With the use of minimum amount of DNA samples from a crime scene or body it is now possible to identify the criminal or determine the percentage and pedigree. All these have been possible because of the knowledge of genetics [413].

Genetics has progressed rapidly in the last few decades. Genetics in general is the study of genes, characters heredity, and genetic variation in living organisms. A distinct part of genetics is genomics, which works on recombinant DNA and DNA sequencing methods. Bioinformatics assemble these sequences and analyze the function and structure of genomes. These activities completely define genomics. Increased understanding of human genetics has the potential to predict how people, depending on their precise genetic makeup, will respond to certain drugs and environment condition. Genomics along with proteomic which deals with protein structures, function and protein interaction in physiological metabolic pathways, now it is possible to develop personalized medicine. Pharmacokinetics analyze the DNA and can tell intolerance or side effects of a drug application may cause. Viewing these importance of human genome sequence New Zealand has established a databank of DNA profiles. It contains over 70,000 DNA profiles [414].

All these achievements have now become possible because of our present understanding of DNA at molecular level. For better understanding of central molecular dogma of life and implementation of biotechnology there is Bioinformatics; a discipline that has been

developed to improve on methods for storing, retrieving, organizing and analyzing molecular data at DNA and protein levels [416]. One of the major activity in bioinformatics is to develop software tools to generate useful biological knowledge. The other activities include deciphering this data to understand the life system at DNA and protein levels, and also to understand the interaction of DNA and proteins and cell signaling.

1.1.7 *Agrobacterium*-mediated genetic transformation

Twenty-five years ago, the concept of using *A. tumefaciens* as a vector to create transgenic plants was viewed as a prospect and a "wish." Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily. In some developed countries, a high percentage of the acreage of such economically important crops as corn, soybeans, cotton, canola, potatoes, and tomatoes is transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium*-mediated, as opposed to particle bombardment-mediated transformation. There still remain, however, many challenges for genotype-independent transformation of many economically important crop species, as well as forest species used for lumber, paper, and pulp production. In addition, predictable and stable expression of transgenes remains problematic. Several excellent reviews have appeared recently that describe in detail various aspects of *Agrobacterium* biology [133,91,71]. In this review, the author described how scientists utilized knowledge of basic *Agrobacterium* biology to develop *Agrobacterium* as a "tool" for plant genetic engineering. He also explore how our increasing understanding of *Agrobacterium* biology may help extend the utility of *Agrobacterium*-mediated transformation. It is his belief that further improvements in transformation technology will necessarily involve the manipulation of these fundamental biological processes.

The transformation of dicotyledons by *Agrobacterium* is well established for many years in the past. Monocots, particularly gramineous crop plants including important cereals like rice and wheat were considered outside the *Agrobacterium* host range. However, transformation methods based on the use of *Agrobacterium* is stilled preferred in many

instances as it is easy to practice, highly efficient and result in more predictable pattern of foreign DNA integration in low copy number.

Binns and Thomashow (1988) reported the *Agrobacterium* mediated transformation as one of the most suitable and efficient methods of transferring cloned genes into plants. The gram negative soil bacterium, *A. tumefaciens* and *Agrobacterium rhizogenes* were considered as natural genetic engineers capable of transforming a range of dicotyledonous plants by transferring plasmid encoded genes into recipient plant genomes [32].

Successful integration of transgene or foreign genes in groundnut was first reported by Lacorte et al. (1991). But they were not able to generate plants from the transformed tissues [203]. Eapen and George (1994) first reported regenerated groundnut plants following *Agrobacterium*-mediated genetic transformation [105]. Since then, many successful genetic transformation protocols have been reported in different plants via *Agrobacterium*-mediated genetic transformation method based on tissue culture [66,367,2, 338,238,169,366, 293,30,4,325, 328,224, and 67]. Transformed plants have also been obtained by non-tissue culture based approach or in planta transformation by inoculating injured embryo axes with *A. tumefaciens* [282,238,310,246]. Physical method like microprojectile bombardment mediated transformation or Biolistic method of transformation also used for development of transformed plants [225,341,72,225].

Agrobacterium-mediated genetic transformation is widely accepted technology in transformation in comparison to other techniques. In this method, bacteria namely, A. tumefaciens or A. rhizogenes are employed to introduce foreign genes into plant cells. *A. tumefaciens* is a soil-borne gram (-) bacterium, which might induce disease so called crown gall in plants by transferring a distinct portion of its DNA [26,64]. This transformation was initially based on tissue culture method and takes less time (usually 4–5 months) to obtain primary putative transgenic plants [128]. In majority of cases, the regeneration capacity depends on genotype, explant and the composition of medium (Fig. 1.11). For induction of morphogenesis in vitro, natural phytohormones and synthetic hormone-like regulators are used which consist of auxins such as IAA, 2,4-D in varying combinations with cytokinins like BA/ kinetin. The cefatoxime, was verified to be effective in regeneration from crop explants.

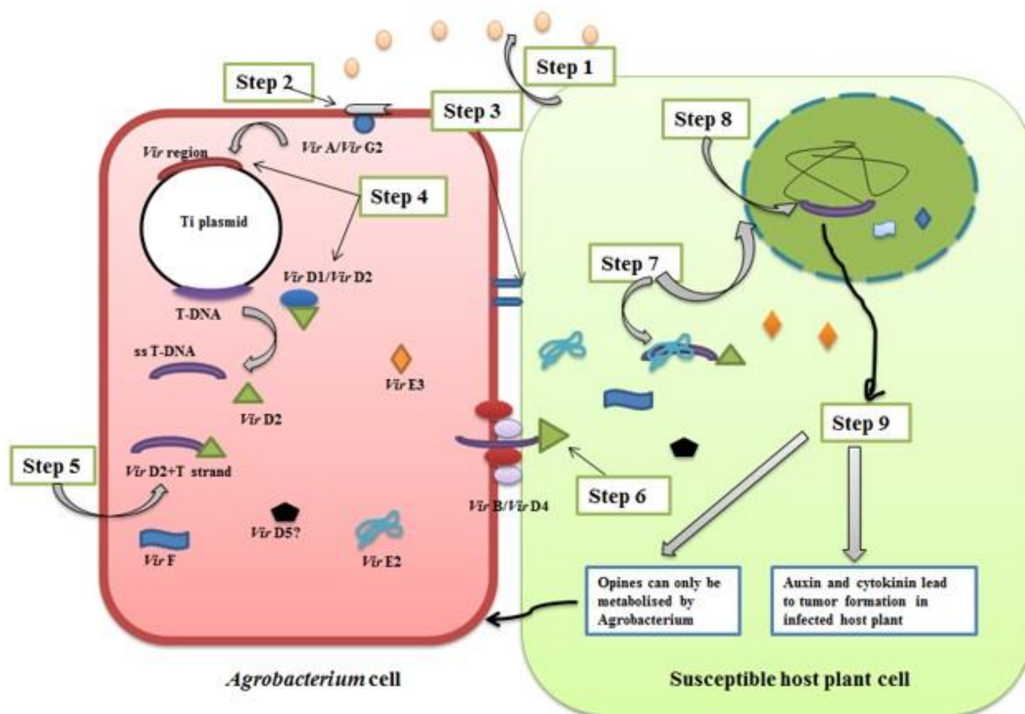

Fig. 1.11: Schematic representation of *Agrobacterium*- mediated transformation; Step 1: Production of signal molecules form wounded plant cell, Step 2: Recognition of signal molecules by bacterial receptors, Step 3: Attachment of *Agrobacterium* to plant cell, Step 4: Activation of *Vir* proteins which process ss-TDNA, Step 5: Formation of immature T-complex, Step 6: T-DNA transfer, Step 7: Assembly of mature T-complex and Nuclear transport, Step 8: Random T-DNA integration in the plant genome, Step 9: Expression of bacterial genes and synthesis of bacterial proteins. (Courtesy: Pacurar DI)

1.1.7.1 *A. tumefaciens* LBA4404

Agrobacterium tumefaciens (updated scientific name *Rhizobium radiobacter*, synonym *Agrobacterium radiobacter*) [432,424,403] is the causal agent of crown gall disease (the formation of tumours) in over 140 species of eudicots (Fig. 1.12). It is a rod-shaped, Gram-negative soil bacterium [343]. Symptoms are caused by the insertion of a small segment of DNA (known as the T-DNA, for 'transfer DNA', not to be confused with tRNA that transfers amino acids during protein synthesis, confusingly also called transfer RNA), from

a plasmid, into the plant cell, [67] which is incorporated at a semi-random location into the plant genome.

A. tumefaciens is an alphaproteobacterium of the family Rhizobiaceae, which includes the nitrogen-fixing legume symbionts. Unlike the nitrogen-fixing symbionts, tumor-producing *Agrobacterium* species are pathogenic and do not benefit the plant. The wide variety of plants affected by *Agrobacterium* makes it of great concern to the agriculture industry [262].

To be virulent, the bacterium must contain a tumour-inducing plasmid (Ti plasmid or pTi), of 200 kb, which contains the T-DNA and all the genes necessary to transfer it to the plant cell. Many strains of *A. tumefaciens* do not contain a pTi.

Since the Ti plasmid is essential to cause disease, prepenetration events in the rhizosphere occur to promote bacterial conjugation - exchange of plasmids amongst bacteria. In the presence of opines, *A. tumefaciens* produces a diffusible conjugation signal called 30C8HSL or the *Agrobacterium* autoinducer. This activates the transcription factor TraR, positively regulating the transcription of genes required for conjugation [262].

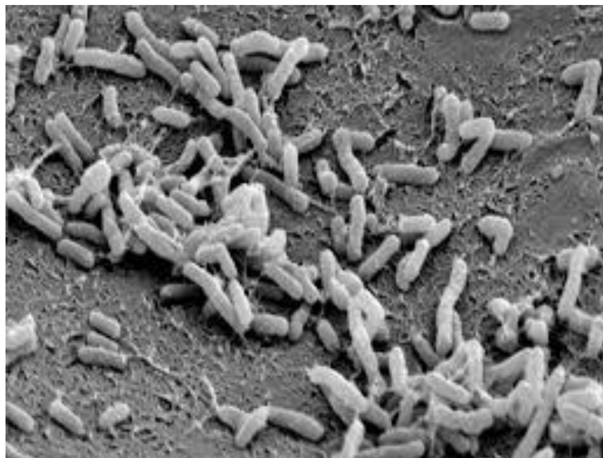

Fig. 1.12: Scanning electron microscopic view of *A. tumefaciens*

(Courtesy: Davidson College)

1.1.7.2 Method of infection

A. tumefaciens infects the plant through its Ti plasmid. The Ti plasmid integrates a segment of its DNA, known as T-DNA, into the chromosomal DNA of its host plant cells. *A. tumefaciens* has flagella that allow it to swim through the soil towards photoassimilates that accumulate in the rhizosphere around roots. Some strains may chemotactically move towards chemical exudates from plants, such as acetosyringone and sugars. The former is recognised by the VirA protein, a transmembrane protein encoded in the virA gene on the Ti plasmid. Sugars are recognised by the chvE protein, a chromosomal gene-encoded protein located in the periplasmic space [353].

At least 25 vir genes on the Ti plasmid are necessary for tumor induction. In addition to their perception role, virA and chvE induce other vir genes. The virA protein has autokinase activity: it phosphorylates itself on a histidine residue. Then the virA protein phosphorylates the virG protein on its aspartate residue. The virG protein is a cytoplasmic protein produced from the virG Ti plasmid gene. It is a transcription factor, inducing the transcription of the vir operons. The chvE protein regulates the second mechanism of the vir genes' activation. It increases VirA protein sensitivity to phenolic compounds [353].

Attachment is a two-step process. Following an initial weak and reversible attachment, the bacteria synthesize cellulose fibrils that anchor them to the wounded plant cell to which they were attracted. Four main genes are involved in this process: chvA, chvB, pscA, and att. The products of the first three genes apparently are involved in the actual synthesis of the cellulose fibrils. These fibrils also anchor the bacteria to each other, helping to form a microcolony [353].

VirC, the most important virulent gene, is a necessary step in the recombination of illegitimate recolonization. It selects the section of the DNA in the host plant that will be replaced and it cuts into this strand of DNA.

After production of cellulose fibrils, a calcium-dependent outer membrane protein called rhicadhesin is produced, which also aids in sticking the bacteria to the cell wall. Homologues of this protein can be found in other rhizobia.

Possible plant compounds that initiate *Agrobacterium* to infect plant cells: (U.S. Patent 6483013)

- Acetosyringone and other phenolic compounds
- alpha-Hydroxyacetosyringone
- Catechol
- Ferulic acid
- Gallic acid
- p-Hydroxybenzoic acid
- Protocatechuic acid
- Pyrogallic acid
- Resorcylic acid
- Sinapinic acid
- Syringic acid
- Vanillin

The various Agrobacterium strains have been used to accomplish the tasks. Of them strain LBA4404 is commonly used strain as because of the availability in the market. Moreover, this strain can hold plasmid for a long time. In a study with the comparative ability of *A. tumefaciens* strains GV2260, LBA4404, C58C1, AGL1 and EHA105 harboring the plasmid pBin19 to transform tobacco were analysed. For plasmid confirmation, colony PCR was performed on selected colonies of different Agrobacterium strains harboring plasmid of interest. Colony PCR amplified bands of ~1.32 kb and 450 bp for beta-glucuronidase uidA and nptII genes respectively which indicated the positive clones ready to be transformed in tobacco [22].

They concluded that, the regenerated shoots transformed by LBA4404 shown best results regarding GUS staining assay followed by GV2260, EHA105, C58C1 and AGL1. The transformation efficiencies of all Agrobacterium strains were significantly different

(P\0.05), except between LBA4404 and GV2260. Hence it was concluded that *A. tumefaciens* strains differ in their ability to transform tobacco plants and LBA4404 remains the best strain for a suitable transformation system in tobacco [25].

1.1.8 Molecular basis of *Agrobacterium*-mediated transformation

1.1.8.1 What is T-DNA?

The molecular basis of genetic transformation of plant cells by *Agrobacterium* is transfer from the bacterium and integration into the plant nuclear genome of a region of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid resident in *Agrobacterium* (Fig. 1.13). Ti plasmids are on the order of 200 to 800 kbp in size [133,119,92]. The transferred DNA (T-DNA) is referred to as the T-region when located on the Ti or Ri plasmid. T-regions on native Ti and Ri plasmids are approximately 10 to 30 kbp in size [215]. Thus, T-regions generally represent less than 10% of the Ti plasmid. Some Ti plasmids contain one T-region, whereas others contain multiple T-regions [21]. The processing of the T-DNA from the Ti plasmid and its subsequent export from the bacterium to the plant cell result in large part from the activity of virulence (*vir*) genes carried by the Ti plasmid [160,129].

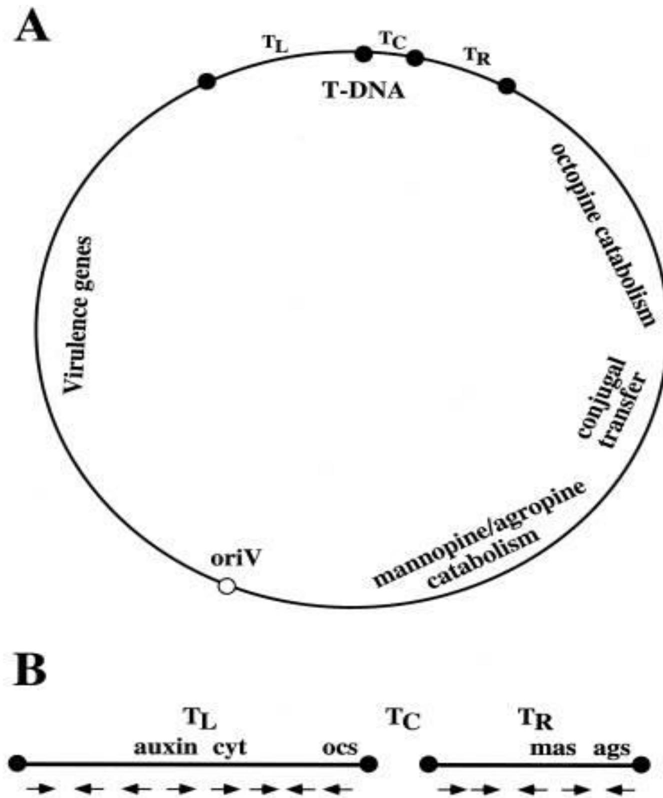

Fig. 1.13: Schematic representation of a typical octopine-type Ti plasmid; (A) and the T-DNA region of a typical octopine-type Ti plasmid (B); (A) The T-DNA is divided into three regions. T_L (T-DNA left), T_C (T-DNA center), and T_R (T-DNA right). The black circles indicate T-DNA border repeat sequences. *oriV*, the vegetative origin of replication of the Ti plasmid, is indicated by a white circle. (B) The various T-DNA-encoded transcripts, and their direction of transcription, are indicated by arrows. Genes encoding functions involved in auxin synthesis (auxin), cytokinin synthesis (cyt), and the synthesis of the opines octopine (ocs), mannopine (mas), and agropine (ags) are indicated. (Courtesy: Internet)

Hormones

To cause gall formation, the T-DNA encodes genes for the production of auxin or indole-3-acetic acid via the IAM pathway. This biosynthetic pathway is not used in many plants for the production of auxin, so it means the plant has no molecular means of regulating it and auxin will be produced constitutively. Genes for the production of cytokinins are also expressed. This stimulates cell proliferation and gall formation [412].

Opines

The T-DNA contains genes for encoding enzymes that cause the plant to create specialized amino acid derivatives which the bacteria can metabolize, called opines [412]. Opines are a class of chemicals that serve as a source of nitrogen for A. tumefaciens, but not for most other organisms. The specific type of opine produced by A. tumefaciens C58 infected plants is nopaline [111].

Two nopaline type Ti plasmids, pTi-SAKURA and pTiC58, were fully sequenced. A. tumefaciens C58, the first fully sequenced pathovar, was first isolated from a cherry tree crown gall. The genome was simultaneously sequenced by Goodner et al. [137] and Wood et al. [396] in 2001. The genome of A. tumefaciens C58 consists of a circular chromosome, two plasmids, and a linear chromosome. The presence of a covalently bonded circular chromosome is common to Bacteria, with few exceptions. However, the presence of both a single circular chromosome and single linear chromosome is unique to a group in this genus. The two plasmids are pTiC58, responsible for the processes involved in virulence, and pAtC58, dubbed the "cryptic" plasmid [396,137].

The pAtC58 plasmid has been shown to be involved in the metabolism of opines and to conjugate with other bacteria in the absence of the pTiC58 plasmid [378]. If the pTi plasmid is removed, the tumor growth that is the means of classifying this species of bacteria does not occur.

Different strains of *Agrobacterium* such as LBA4404, EHA105, EHA101, C58 and A281 were used for the transformation of plant. *Agrobacterium*-mediated transformation protocols have been standardized using different explants including leaf sections, zygotic embryos, cotyledonary nodes, embryo axes, leaflets, de-embryonated cotyledon and hypocotyls [293,365,380,224]. First fertile transgenic plants were developed in New Mexico with Valencia variety in which leaflet explants were infected with *Agrobacterium* strain EHA105 and showed 10 % regenerated on selection medium as GUS positives and also these plants showed stable integration of transgenes (uid A and *npt* II) at T1 generation with 0.2-0.3 % of transformation frequency [67].

1.1.8.2 Genes in the T-DNA

The plasmid DNA has 2 important regions namely the T-DNA and the *vir* region. The T-DNA has a 25bp direct repeat sequence flanking it on either side. This helps in the transfer of any foreign genes cloned into the T-DNA region [406,348]. *Vir* genes, labeled *Vir A* to *vir* G, are required for the virulence and infective capacity of the *Agrobacterium*. Normally the *vir* genes are not expressed in *Agrobacterium* until they become activated by certain plant factors. These factors have been identified as phenolic compounds like acetosyringone (AS) and hydroxy acetosyringone [37].

VirA and VirG proteins function as member of a two-component sensory signal transduction genetic regulatory system. VirA is a periplasmic antenna that senses the presence of particular plant phenolic compounds that are induced on wounding. In coordination with the monosaccharide transport. ChvE and in the presence of appropriate phenolic and sugar molecules, VirA autophosphorylates and subsequently transphosphorylates the VirG protein. VirG in the non-phosphorylated from is inactive, however on phosphorylation the protein helps to activate or increase the level of transaction of the vir genes, mostprobably by interaction with vir-box sequences that form a component of vir gene promoters [37].

Constitutively active VirA and VirG proteins that do not require phenolic inducers for activity or VirG proteins that interact more productively with vir-box sequences to activate vir gene expression, may be useful to increase *Agrobacterium* transformation of ficiency or host range. Vird4 may serve as a `linker` to promote that interaction of the processed T-DNA or VirD2 complex with the VirB encoded secretion apparatus. Most VirB proteins either from the membrane channels serve as ATPases to provide energy for channel assambly or export processes. Several proteins including VirB2, BirB5 and possibly VirB7 make up the T-Pilus. VirB2, which is processed, and cyclized is the major pilin protein [406,348].

LBA4404 was considered as good ‘ordinary strain’ for transformation [159] and EHA101 as the ‘super-virulent’ strain. The vectors were pIG121 Hm (for EHA101 and LBA5505 strains), a derivative of the ‘normal’ binary vector pBIN19 and pTOK233 (for LBA4404 strain), a derivative of a ‘super-binary’ vector pTOK161 [196]. In transformation

experiments, LBA4404 (pTOK233) was slightly more effective than both LBA5505 (pIG121 Hm) and EHA101 (pIG121 Hm) with the Tsukinohikari cultivar. Hiei *et al*. (1994) tested the efficiency of various combinations of two strains and two vectors [445].

Selectable marker genes (SMG), that confer resistance to antibiotics or herbicides, are generally incorporated along with the gene of interest in a transformation process so as to allow recognition of the transformed cells from untransformed ones. Since the production of first transgenic plant [369], antibiotic resistance genes are widely used as marker genes for selection of transgene (Fig. 1.14). Subsequent to the generation of transgenic plants, the presence of these marker genes becomes no more of practical utility and thus arguably a matter of public euphoria, speculating the risk they can pose to the environment and health. The recurrent transformations to pyramid desirable genes are practically difficult with the same selectable markers used [369].

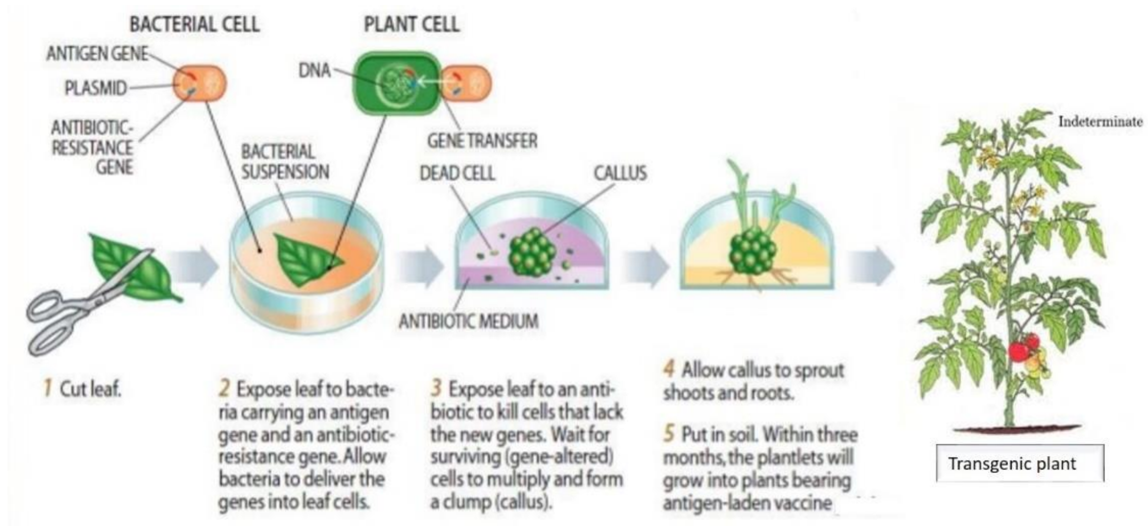

Fig. 1.14: *Agrobacterium*- mediated genetic transformation

1.1.8.3 Tumor-inducing (Ti) Plasmid

Several components of *Agrobacterium* are necessary for transferring the piece of bacterial DNA into the plant cell. One component is the chromosomal virulence A (chv A) gene, which is on the *Agrobacterium* chromosome and activated by sugars. ChvA protein triggers

bacteria to bind to the wounded plant tissue and to respond to a specific chemical (chemotaxis).

The Ti plasmid in bacteria contains the other main components, which are generated or activated efficiently for causing crown gall in host plants after bacteria attach to the plant wound site. The first is T-DNA, which is actually integrated into the plant cell chromosome. The second is the 35-kb virulence (vir) region, which is composed of seven loci (vir A, vir B, vir C, vir D, vir E, vir G, and vir H). Expression of vir genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of Vir proteins are to mediate the T-DNA excision from the Ti plasmid, export of the T-DNA piece from the bacteria, and insert it into the host plant chromosome [353]. These two components are essential for a successful gene transfer. The Ti plasmid also has other components, an opine catabolism region, a conjugal 5 transfer region, and a vegetative origin of replication of the Ti plasmid (ori V). Engler et al. (1981) found that these vir regions have sequence conservation between the octopine and nopaline Ti plasmids [106].

Factors to Increase Gene Expression and Transformation Efficiency Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and vir gene expression, respectively [232,260,152]. Seedling age and pre-culturing of explants have been tested to increase the transformation efficiency. These trials were conducted to determine the best conditions for plant cell infection or increasing the number of dividing plant cells before bacterial infection [3,253]. To increase the virulence of bacteria by inducing the vir gene expression, temperature [97] medium pH [260] and chemical inducers, such as acetosyringone [232], have been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria, as well as between the bacteria and plants.

Manipulation of other factors, such as bacterial density, co-cultivation duration, surfactant, and vacuum infiltration, have also increased transformation efficiency in many experiments [232,260,3,67]. According to previous experiments, inducing vir gene expression seems most important and effective for increasing plant transformation efficiency, regardless of the type of plant being studied. Although low temperature was

very important to induce bacterial pili formation by inducing the vir gene, optimization experiments were conducted at room temperature or over 25∞C for co-cultivation.

1.1.8.4 Zein

Zein, a protein-based polymer found in maize seeds, has been widely used as a carrier because of favorable properties such as biocompatibility, insolubility and low water uptake, mechanical and chemical stability, and its propensity to form coatings and microparticles [222,212,207,388]. Zein is also generally regarded as safe (GRAS) for food use and resists digestion, making it particularly suitable as an encapsulation polymer for oral drugs [1,411,212,139,168].

γ-zein protein, a member of the major prolamin-type storage protein family in maize [332]. Several reports have confirmed that zein-derived sequences induce ectopic PBs when appended to either the N-terminus or the C-terminus of diverse recombinant proteins, including phaseolin [240], enhanced cyanfluorescen tprotein [230], xylanase [229], DsRed [180], and the Human papillomavirus E7 protein [391].

1.1.9 *Agrobacterium*-mediated genetic transformation in *Nicotiana tabacum*

Nicotiana tabacum L., or cultivated tobacco, is an annually-grown herbaceous plant. It is found only in cultivation, where it is the most commonly grown of all plants in the *Nicotiana* genus, and its leaves are commercially grown in many countries to be processed into tobacco. It grows to heights between 1 and 2 meters. Research is ongoing into its ancestry among wild *Nicotiana* species, but it is believed to be a hybrid of *Nicotiana sylvestris*, *Nicotiana tomentosiformis*, and possibly *Nicotiana otophora* [305].

Tobacco is a product prepared from the leaves of the tobacco plant by curing them. The plant is part of the genus *Nicotiana* and of the Solanaceae (nightshade) family. While more than 70 species of tobacco are known, the chief commercial crop is *N. tabacum*. The more potent variant *N. rustica* is also used around the world.

Tobacco contains the alkaloid nicotine, which is a stimulant, and harmala alkaloids [312] Dried tobacco leaves are mainly used for smoking in cigarettes, cigars, pipe tobacco, and

flavored shisha tobacco. They can also be consumed as snuff, chewing tobacco, dipping tobacco and snus (Jorda).

Tobacco use is a risk factor for many diseases, especially those affecting the heart, liver, and lungs, as well as many cancers (Fig. 1.15). In 2008, the World Health Organization named tobacco as the world's single greatest preventable cause of death (WHO report on the) The English word "*tobacco*" originates from the Spanish and Portuguese word "*tabaco*". The precise origin of this word is disputed, but it is generally thought to have derived at least in part, from Taino, the Arawakan language of the Caribbean. In Taino, it was said to mean either a roll of tobacco leaves (according to Bartolomé de las Casas, 1552) or to *tabago*, a kind of Y-shaped pipe used for sniffing tobacco smoke (according to Oviedo; with the leaves themselves being referred to as *cohiba*) [436].

Tobacco has long been used in the Americas, with some cultivation sites in Mexico dating back to 1400–1000 BC [428]. Many Native American tribes have traditionally grown and used tobacco. Eastern North American tribes historically carried tobacco in pouches as a readily accepted trade item, as well as smoking it, both socially and ceremonially, such as to seal a peace treaty or trade agreement (Heckewelder, they smoke with) In some populations, tobacco is seen as a gift from the Creator, with the ceremonial tobacco smoke carrying one's thoughts and prayers to the Creator [172].

Fig. 1.15: Morphology of *N. tabacum*

Leaf disc transformation of tobacco is the paradigm for *Agrobacterium* mediated transformation of plant tissues and subsequent selection and regeneration of transgenic plants. This system combines efficient gene transfer, selection and regeneration together in a simple process.

Tobacco is an excellent host for *Agrobacterium tumefaciens*, and also responds exceedingly well in culture. While the technique is most easily practiced with tobacco, it has been applied to a number of other species [243].

Tobacco usually after aging and processing used in various ways namely, smoking, chewing, snuffing and also for the extraction of nicotine which is the principle alkaloid of tobacco. Tobacco holds an unparalleled position among crop plants in the world. It is one of the most widely grown commercial non-food plant. It holds a high importance in financial and economic policies in many countries. The global production of tobacco is estimated at 6.265 million kg from an area of 4.2 mha. Almost all continents are capable of growing tobacco but the United States, China, India and Brazil are the leading countries to grow tobacco [354].

1.1.10 *Agrobacterium*-mediated genetic transformation in *Lycopersicon esculentum.*

Tomato (2n = 2x = 24) is one of the most widely produced and popular vegetabl es ofthe world. It ranks second to potato in many countries and tops the list in cane-vegetables (Tiwari and Choudhury 1986). Tomato is known as a protective food because of its special nutritive value. Tomatoes are particularly very popu lar since they can be eaten fresh or in a multiple of processed forms. The intensive cultiv ation of tomatoes has led to a significant increase in the farmer′s income in many developing countries (Tiwari and Choudhury 1986).

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable and economic crop worldwide, and it is a model plant for fruit development research works. Tomato is a highly cold-sensitive crop. Its growth is hindered when the temperature drops below 10°C, gradually decreases at 8°C, and completely stops when the temperature reaches 5°C. Because of low temperatures, tomato plants often show a reduced growth period, growth retardation, dwarfism, and yield decreases [446]. In recent years, plant freezing tolerance has been much studied, and researchers have found that cold-induced genes play an important role in plant resistance to low temperature and cold adaptation processes [364].

Across the globe, in 2008, tomato production was about 136.230 million tonnes from an area of 4.837 million hectares with an average yield of 28.16 tonnes/ha [299]. Tomato fruit contains variety of compounds including antioxidants such as lycopene and ascorbic acid, which are important for human health.

Considerable efforts have been exerted to improve the genetics of this valuable plant. Genetic transformation is a fundamental process in plant molecular genetics and plant improvement. *Agrobacterium*-mediated transformation is commonly employed to transfer a foreign gene into the genome of plants, such as tomato [130]. Since the first transgenic tomato was produced nearly 30 years ago, [251] significant progress in the genetic transformation of tomato has been achieved in recent years [190,250]. Efficient

transformation protocols for different cultivars were established, but the factors that affect transformation must be optimized to realize efficient transformation in different genotype cultivars [190, 250].

The Tomato plant is one of the selected plants for transformation as oral vaccine since its fruits are edible fresh (Fig. 1.16). Jani et al. suc-cessfully transferred the ctb gene controlled by the CaMV 35S promoter into tomato plants, and this transgenic plant expressed the CTB subunit in leaves and fruits, which could specifically bind to G(M1)-ganglioside receptor, a special receptor for CTB subunit [175]. A transgene driven by the constitutive CaMV 35S promoter could be expressed in all tissues of a transgenic plant, such as fruit, stem, leaf, and even flowers [320]. If the transgene is expressed specifically in fruit, the amount of expression should be increased. The E8 promoter is a tomato fruit-specific promoter, which drives the expression of the E8 gene [95,93]. Sandhu et al. made RSV-F antigen express specifically in transgenic cherry tomato fruit by using the E8 promoter. The amount of the RSV-F antigen seemed a little higher than that expressed in fruit with CaMV 35S promoter [320]. In this study, we aimed to express the CTB specifically in fruit and to increase the mount of expres- sion in tomato fruit using the E8 promoter.

The immunogenicity of the CTB protein expressed in tomato fruit was also evaluated through determination of the serum and mucosal anti-CTB antibody levels in experi-mental mice.

Fig. 1.16: Morphology of *L. esculentum*

1.2 Statement of purpose

Edible vaccines offer cost-effective, easily administrable, storable and widely acceptable as bio friendly particularly in developing countries. Oral administration of edible vaccines proves to be promising agents for reducing the incidence of various diseases like hepatitis and diarrhea especially in the developing world, which face the problem of storing and administering vaccines [173]. Edible vaccines are obtained by incorporating a particular gene of interest into the plant, which produces the desirable encoded protein. Edible vaccines are specific to provide mucosal activity along with systemic immunity. Various foods that are used as alternative agents for injectable vaccines include cereals (wheat, rice, corn) fruits (bananas) and vegetables (lettuce, potatoes, tomatoes). Thus, edible vaccines overcome all the problems associated with traditional vaccines and prove to be best substitutes to traditional vaccines. [173].

Vaccines have proved to be boon for the prevention of infectious diseases. In spite of the global immunization programme for children against the six devastating diseases, 20% of

infants still remain unimmunized which lead to approximately two million unnecessary deaths per annum, particularly in the far flung and poor parts of the world [205]. This is because of the limitations on vaccine production, distribution and delivery. This problem needs to resolve in order to prevent the spread of infections and epidemics by un-immunized populations in the immunized, safe areas [301]. Immunization for certain infectious diseases, either do not exist or they are unreliable or very expensive like; immunization via DNA vaccines is substitute but is an expensive method, along with some undesirable immune responses. Besides being expensive, these vaccines pose the problem of storage and transportation, as many of them require refrigeration. Hence, there is search for easily administrable, storable, fail-safe and widely acceptable bio friendly vaccines and their delivery systems especially in developing countries. Therefore, as substitutes have to be produced for traditional vaccines, it was envisaged that plants could be promising agents for efficient production system for vaccines, which in turn gave rise to the novel concept of edible vaccines.

Development of edible vaccines involves the process of incorporating the selected desired genes into plants and then enabling these altered plants to produce the encoded proteins. This process is known as transformation, and the altered plants are known as transgenic plants. Edible vaccines like traditional subunit vaccines consist of antigenic proteins and are devoid of pathogenic genes. Despite this advantage, traditional subunit vaccines are unaffordable and technology-intensive, require purification, refrigeration and produce poor mucosal response. Unlikely, edible vaccines would eliminate the need for trained medical personnel required for oral administration particularly in children. Production of edible vaccines is effective process and can be easily scaled up. Edible vaccines offer numerous advantages like they posses good genetic and heat stability and do not need cold-chain maintenance. Edible vaccines can be stored at the site of use thus avoiding long-distance transportation. Syringes and needles are also not required, thus reduces the incidence of various infections [389]. Important advantage of edible vaccines is elimination of contamination with animal viruses-like the mad cow disease, which is a hazard in vaccines developed from cultured mammalian cells, as plant viruses cannot infect humans [173].

The limitations of the currently available polyvalent vaccine formulations comprising purified pneumococcal capsular polysaccharide are well documented. The first is that the protection they impart is type-specific. And because of this, a formulation of serotypes that are effective for one population may not be as effective for another if serotype prevalence differs significantly [217]. The second shortcoming of the present vaccines is that even that protection they provide against specifically included serotypes is by no means complete and may be very poor for certain high risk groups, who have a poorer antibody response to polysaccharide vaccines than healthy adults [118].

Again polysaccharides are T-cell-independent antigens and are poorly immunogenic among children under 2 years of age (CDC 1993). Thus, it would seem that there are some disadvantages of the polysaccharide vaccines in attempts to elicit its responses. This encouraged the search for substances other than capsular polysaccharides which could serve as antigens and provide protection against pneumococcal infection [267]. To overcome the limitations of polysaccharide vaccines, protein-polysaccharide conjugate or sub-unit vaccines or protein vaccine are being evaluated. These vaccines appear to activate T-helper cells, thereby eliciting T-cell-dependent responses that provide a long-term immunological memory [267].

The incomplete protection offered by the current polysaccharide vaccine and the recently developed protein-conjugate vaccine against otitis media, carriage, and bacteremic disease caused by non-vaccine types [304, 57] has increased the importance of studies of other vaccine candidates. Pneumococcal surface protein A (PspA) has been the most extensively examined pneumococcal proteins used for protective immunization in animal models [38,268], although a number of other pneumococcal proteins have been reported [164,269].

At the same time traditional vaccines are expensive, need purification, require refrigeration and produce poor mucosal response. In contrast, edible vaccines would enhance compliance, especially in children, and because of oral administration, would eliminate the need of trained medical personnel. They are cheaper, sidestepping demands for purification, grown locally using standard methods and do not require capital-intensive pharmaceutical manufacturing facilities. Mass indefinite production would also decrease

dependence on foreign supply. They are heat stable, and do not require cold chain maintenance. Non requirement of syringes and needles also decreases chances of infections [259].

Gene encoding antigen from pathogenic organisms (virus, bacteria or parasites) that have been characterized and for which antibodies are available, can be handled in two ways. In one case, the entire structural gene is inserted into a plant transformation vector between 5' and 3' regulatory elements; this will allow transcription and accumulation of coding sequence in plant. In second case, epitope within the antigen are identified, DNA fragment encoding these can be used to construct genes by fusion with a coat protein gene from plant virus e.g. TMV or CMV [259].

In this present investigation, attempt have been taken to develop an edible vaccine through Agrobacterium mediated genetic transformation. Where B-cell epitope predicted peptide sequences were analysed and each of the sequences was fusion with zein protein and after making construct, genetic transformation carried out following immunogenic testing.

1.2.1 Specific objectives

The highest rate of pneumococcal disease occurs in young children and in the elderly population, although all age groups may be affected. In addition, persons suffering from a wide range of chronic conditions and immune deficiencies are at increased risk. In developing countries, infants under three months of age are at high risk, especially for pneumococcal meningitis. *S. pneumoniae* is believed to cause a substantial portion of childhood deaths in Bangladesh. In spite of the availability of antibiotics, the mortality rate of pneumococcal disease remains high and in the last four decades it has remained stable between 25 and 29% [298]. As the pneumococcal surface protein antigen (PspA) showed promising results as a potential candidate for pneumococcal vaccine, thus, this study was undertaken to develop a plant based vaccine by incorporating the gene of the PspA B-cell epitope protein into plant.

So, the main objectives of this research is-

(a) To determine the gene sequence of the B-cell epitope predicted peptide sequence,

(b) To construct plant expression vectors,

(c) To transform targeted plant (tomato and tobacco) and screen putative transgenic plants,

(d) To analyze the gene of interest in transgenic plant tissue,

(e) To check immunogenicity of the transgenic plant part in experimental animals, like mice.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strain:

Streptococcus pneumoniae 7F

Agrobacterium tumefaciens LBA4404

2.1.2 Pathogenic factor:

Pneumococcal surface protein antigen (PspA)

2.1.3 Gene of interest:

Four selected B-cell epitope predicted peptide sequences. These are-

1. ILTSLASVAILGAGFVASQPTVVRAEESPVASQ
2. AQKKYDEDQKKTEE
3. SESEDYA
4. SKAEKDYDAA

2.1.4 Plant materials

The following plants were used in the present investigation:

(i) *Nicotiana tabacum* (Tobacco)

(ii) *Lycopersicon esculentum* (Tomato)

2.1.5 Explants

Different types of explants were used in the present investigation. These are -

1. Immature leaflet,

2. Node,

3. Cotyledonary leaf,

4. Cotyledonary node and

5. Hypocotyle.

2.1.6 Animal model

Swiss Albino BALB/c mice.

2.1.7 *Agrobacterium* strain and vector plasmids

Agrobacterium strain namely LBA4404 was used for the transformation experiments.

Three different genetically engineered constructs were used in transformation experiments. These are-

1. Vector Alpha zein fertig
2. Vector gamma zein fertig and
3. pTRAkt-glyDs-zen

2.1.8 Experimental approach

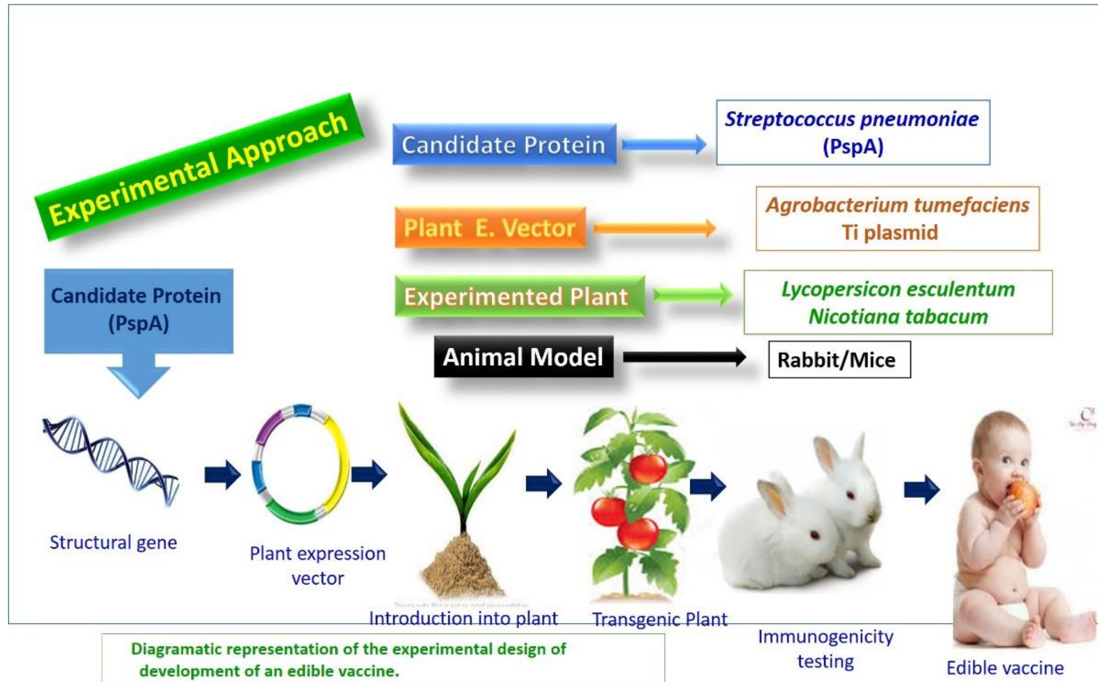

Fig. 2.1: Diagrammatic representation of the experimental design of development of an edible vaccine

2.2 Methods

2.2.1 Working Place

The experiments of this research study were carried out in the Department of Microbiology, University of Dhaka and Tissue Culture Section, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

This section can be divided into three parts:

Part-1: Construction of plasmid vector with B-cell predicted peptide sequences

Part-2: *Agrobacterium* mediated genetic transformation and

Part-3: Mice immunization and challenge.

Part-1 Construction of plasmid vector with B-cell predicted peptide sequences

2.2.2 Bacterial studies

2.2.2.1 Strain collection, maintaining and identification

The *S. pneumoniae* serotype 7F used in this study were obtained from the stock culture of the Department of Microbiology, Dhaka Shishu Hospital. Originally the *S. pneumoniae* strain was collected from the out-and-in patients of Dhaka Shishu Hospital and was serotyped by capsular swelling procedure (quellung reaction) with type specific anti pneumococcal pool, type or group, and factor sera.

The pneumococcal strains were stored at –80°C in 12% glycerol and in -20ºC. From the stock culture it was then transferred to blood agar plates, and incubated at 37°C in 5% CO2 atmosphere overnight. Optochin disc were placed every times to make sure about the organism. Colonies grown on blood agar were used to inoculate liquid growth medium. The organism was grown in Brain heart infusion (BHI) broth supplemented with 10% fetal bovine serum (FBS) at 37°C for 24 hours on a shaker.

However, identity of these strains was reconfirmed by the Gram staining, optochin susceptibility, and bile solubility tests.

2.2.2.2 Gram staining:

The *S. pneumoniae* strains were grown on gentamycin blood agar plate and a Gram staining was performed and observed under a microscope for Gram positive diplococci.

2.2.2.3 Optochin susceptibility:

The bacterial strains grown on gentamycin blood agar and a colony was touched with a sterile loop and streaked onto a fresh blood agar plate. After the loop was streaked across the plate, an optochin disk (Ethylhydrocupreine hydrochloride, and diameter of 6 mm containing 5 μg, BBL, USA) was placed aseptically at the end of the streak where the loop was first placed. The plates were incubated at 37°C for 24 hours and an α-haemolytic growth greater than 14 mm in diameter was identified as *S. pneumoniae* [126].

2.2.2.4 Bile solubility test:

The bile solubility test was performed by taking several loops of the bacterial strains from the growth on a gentamycin blood agar plate and making a suspension in a 1.0 mL of sterile saline. The suspension of cells was divided into two equal volumes (0.5 mL per tube). An amount of 0.25 mL of saline was added into one tube and 0.25 mL of 2% sodium deoxycholate (bile salts) was added to the other. The tubes were gently shaken and incubated at 37°C for up to two hours. Then the tubes were periodically examined for lysis of the cells in the tube containing bile salts. A cleaning of the tube or less of turbidity was considered as a positive result [73].

2.2.2.5 Extraction of surface protein

Water extraction procedure was applied for the isolation of surface materials from the *S. pneumoniae* strains following the method described by Oaks *et al.* [273].

1. A fresh culture was subcultured from the stock culture.

2. A clearly isolated colony was inoculated and the organism was grown in Brain heart Infusion (BHI) broth supplemented with 10% DNA at 37°C for 24 hours in a shaker.
3. The whole culture medium was harvested by centrifugation (10000x g for 20 min) and the supernatant was discarded.
4. The pellet was taken and washed three times in normal saline. (Pellet was suspended in 5-10 mL of normal saline and centrifuged at 10000x g for 10 min. the procedure was repeated for three times.)
5. Cells were then resuspended in 20 volumes of distilled water and were shaken (100 oscillations per min) for 6 hours at room temperature.
6. The suspensions were centrifuged at 10000x g for 30 min and supernatants were collected, filtered through 0.45 µm Millipore membrane filters and were stored at -20°C in aliquots until used.

Sterility was maintained at every step (before Millipore filtration) by growing onto blood agar plate with the optochin susceptibility and bile solubility test.

2.2.2.6 Genomic DNA extraction of *S. pneumoniae*

Geneomic DNA from *S. pneumoniae* using DNA extraction kit TA10 (Pirma, Japan).

1. 1 ml culture was used to harvest the cells into a single eppendorf tube and the supernatant discarded.
2. The cells were re-suspended in 180µl enzyme buffer solution containing 10µl enzyme A and 10µl enzyme B.
3. Incubate for 1 hr at 37ºC.
4. The solution was mixed with 300µl extraction buffer solution.
5. The lysate was than centrifuge for 5 min at 12000 rpm.
6. The supernatant was transferred into a new tube containing 400µl of 100% isopropanol and centrifuged for 5 min at 12000 rpm.
7. The resulting DNA pellet was washed with wash buffer and centrifuge for 5 min at 12000 rpm.
8. The pellet was then dissolve in 160µl of elution buffer and heated for 3 min at 70ºC

9. Centrifuge for 5 min at 12000 rpm and store at -20ºC.

2.2.2.7 Determination of DNA Concentration

The concentration of DNA sample can be checked by the use of UV spectrophotometry. DNA absorb UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5 ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. Using a 1-cm light path, the extinction coefficient for nucleotides at this wavelength is 20. Based on this extinction coefficient, the absorbance at 260 nm in a 1-cm quartz cuvette of a 50µg/ml solution of double stranded DNA is equal to 1. The concentration of the sample DNA can be calculate as follows:

DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (50 µg DNA/ml)/(1 OD_{260} unit)

In contrast to nucleic acids, proteins have a UV absorption maximum of 280 nm, due mostly to the tryptophan residues. The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85.

Procedure for using the to measure OD_{260} and OD_{280}

1. Turn the machine on using the switch in the back of the machine
2. Open the lid on the top of the machine. Choose the small cuvette holder and place it in the holder in front of the light source. The word front should be toward the front of the machine.
3. Click on the UV light key to turn the UV on. Quit the diagnostic screen by clicking on the word quit in the upper right hand corner. The Main Menu will appear Choose Nucleic Acid from the menu. When the nucleic acids analysis menu appears, make sure that the measured absorbances are 260 and 280.
4. Use lens paper to clean the surfaces of the cuvette. Rinse the cuvette chamber with 70% ethanol.
5. Shut the lid of the machine.
6. Click read blank in the bottom left corner of the screen.

7. Prepare a 1:100 dilution of the sample you want to read.
8. After the machine has read the blank, remove the cuvette, remove the blanking solution from the chamber, rinse and dry the chamber and place your diluted sample in it.
9. Click on read samples in the upper left hand of the screen.
10. After the machine has read sample, the data appeared on the screen.
11. Calculate the concentration of your DNA sample and
12. Measured the purity of DNA by the OD_{260}/OD_{280}.

2.2.2.8 Procedure for DNA quantification using Nanodrop

1. The Nanodrop arm was cleaned by using distill deionized water carefully.

2. The machine was initialized by using water

3. Blank was set by using TE buffer

4. 1.0 µ of sample was put in the arm and reading of the sample was taken at 260nm.

5. Ratio of 260/280 and 260/230 was also calculated

6. Clean the arm with distill water and put the machine off.

2.2.2.9 Assessment of purity of the samples

A ratio value of 1.8 indicates a highly pure DNA preparation. Lesser ratio values implied significant presence of contaminants-generally proteins and the ratio of 2.00 indicated high concentration of RNA contamination.

2.2.3 Preparation of vector

Bioinformatics analysis/*in silico*

2.2.3.1 B cell epitope prediction tools description

IEDB analyses: prediction of linear epitopes from protein sequence

Six different tools are provided that predict antibody epitope candidates from amino acid sequences. Five are based on amino acid property scales and a sixth method uses a Hidden Markov Model. Parameters such as hydrophilicity, flexibility, accessibility, and antigenic propensity of polypeptides chains have been correlated with the location of continuous epitopes in a few well-characterized proteins. Based on these observations, amino acid property scales have been developed to predict antigenic determinants. Each scale consists of 20 values assigned to each of the amino acid residues on the basis of their relative propensity to possess the property described by the scale. The following amino acid property scales have been selected and implemented based on their popularity and coverage of different categories.

- Secondary structure - Chou and Fasman beta turn prediction
- Surface exposure - Emini surface accessibility prediction
- Flexibility - Karplus and Schulz flexibility prediction
- Antigenicity - Kolaskar and Tongaonkar antigenicity prediction
- Hydrophobicity/hydrophilicity - Parker hydrophilicity prediction

BepiPred combines the predictions of a hidden Markov model and the propensity scale of Parker et al., (1986) [281]. It is described in Larsen et al. [206].

In this study, Kolaskar and Emini antigenicity prediction method was employed for evaluating the potentiality of the peptides as linear B cell epitopes. In Kolaskar method, 156 antigenic determinants (< 20 amino acids) in 34 different proteins were analyzed [193] to calculate the antigenic propensity (Ap) of residues. This antigenic scale was used to predict the epitopes in sequence. This semi-empirical method uses physicochemical properties of amino acid residues and their frequencies of occurrence in experimentally known segmental

epitopes to predict antigenic determinants on proteins; this method can predict the antigenic determinants with about 75% accuracy.

Another method followed in this study was Emini method. The calculation was based on surface accessibility scale on a product instead of an addition within the window. The accessibility profile was obtained using the formulae Sn = (n+4+i) (0.37) -6 Where Sn is the surface probability, dn is the fractional surface probability value, and i vary from 1 to 6. A hexapeptide sequence, with Sn equal to unity and probabilities greater than 1.0 indicates an increased probability for being found on the surface [107].

The B cell prediction tools can be found at-

http://tools.immuneepitope.org/main/html/bcell_tools.html.

From these analyses four predicted epitopes were selected on the basis of the frequency of their presence. These are-

1. **ILTSLASVAILGAGFVASQPTVVRAEESPVASQ**
2. **AQKKYEDDQKKTEE**
3. **SESEDYA**
4. **SKAEKDYDAA**

2.2.3.2 Prediction of population coverage

For prediction of population coverage, a web-based tool based on MHC-binding and/or T cell restriction data was used. This tool is designed to calculate the cumulative allelic frequency in a population that is covered by the epitope. This calculation is made on the basis of HLA genotypic frequencies assuming non-linkage disequilibrium between HLA loci. HLA allele genotypic frequencies were obtained from dbMHC database (http://www.ncbi.nlm.nih.gov/mhc/).

2.2.3.3 Cross-conservation analysis

Cross-conservation of the identified peptide sequence with the human proteome may not elicit immune responses to vaccine. Therefore, reciprocal BLAST best-hit method was employed to reveal cross-conservation with non-human and other bacterial proteomes as well.

On the other hand all the available nucleotide sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/nucleotide) and translated by using EMBOSS-Transeq tool

(http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The sequence for antigenic epitope determination was selected via multiple sequence alignment by using MEGA.

Mega

A computer program package called MEGA has been developed for estimating evolutionary distances, reconstructing phylogenetic trees and computing basic statistical quantities from molecular data. It is written in C++ and is intended to be used on IBM and IBM-compatible personal **computers**. In this program, various methods for estimating evolutionary distances from nucleotide and amino acid sequence data, three different methods of phylogenetic inference (UPGMA, neighbor joining and maximum parsimony) and two statistical tests of topological differences are included. For the maximum parsimony method, new algorithms of branch-and-bound and heuristic searches are implemented. In addition, MEGA computes statistical quantities such as nucleotide and amino acid frequencies, transition/transversion biases, codon frequencies (codon usage tables), and the number of variable sites in specified segments in nucleotide and amino acid sequences. Advanced on-screen sequence data and phylogenetic tree editors facilitate publication-quality outputs with a wide range of printers. Integrated and interactive designs, on-line context-sensitive helps, and a text-file editor make MEGA easy to use.

The present invention also provides automatic serial analysis and report generation of a gene using a database and tools to calculate codon usage from a raw sequence and graphically report the location of the rare codons along a translated DNA sequence. Where multiple candidate versions of a particular gene are designed, an analysis of all versions is performed to determine the best candidate for synthesis. This comparison, along with a comparison of the candidate versions with that of a reference codon preference, is presented in a useful human-readable format.

2.2.3.4 Codon optimization

The present work includes a synthetic polynucleotide sequence that has been optimized for heterologous expression of PspA. Here after back-translate of the amino acid sequences codon optimization could be done. For this reason specific tables usually have been used.

The sequences were selected after searching many different serotypes in the Genebank and BLAST. For choosing the sequences, the maximum presence of the same sequence in different serotypes and following IEDB Analysis resource were considered, where we found some predicted peptide sequences. The nucleotides of respective peptide sequences which have been obtained after considering codon biasness of *S. pneumoniae* were provide here. Considering these two factors, the selected peptides and nucleotide sequences are given here.

2.2.3.5 Predicted peptides sequences with back translation

Codon usage bias is an essential feature of all genomes. The effects of codon usage biases on gene expression were previously thought to be mainly due to its impacts on translation. The predicted epitope sequences were confirmed after back translation and codon optimization.

(1) ILTSLASVAILGAGFVASQPTVVRAEESPVASQ

Nucleotide:

TTTTAACAAGTCTAGCCAGCGTCGCTATCTTAGGGGCTGGTTTTGTTGCG
TCTCAGCCTACTGTTGTA AGAGCAGAAGAATCTCCCGTAGCTAGTCAG

(2) SKAEKDYDAA

Nucleotide:

TCTAAAGCTGAGAAAGACTATGATGCAGCA

(3) AQKKYDEDQKKTEE

Nucleotide:

GCGCAAAAAAAATATGACGAAGATCAAAAGAAAACTGAAGAG

(4) SESEDYA

Nucleotide:

TCTGAATCAGAAGATTATGCT (Accession No.- NP_357715)

The sequences have been introduced into a plant expression vector (pTRA) and transferred into *Agrobacterium tumefaciens*.

2.2.3.6 Preparation of the constructs

As mentioned above the sequences of *S. pneumoniae* epitopes have been introduced into a plant expression vector (pTRA) and transferred into *Agrobacterium tumefaciens*. Four predicted peptide sequences were selected and those were placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, respectively. For easy detection, the alpha zein construct (construct alpha) contains a flag- and his-tag, the gamma zein construct (construct gamma) contains only the his-tag.

The constructs with alpha and gamma zein. 1,2,3 and 4 are the sequences for the epitops of *Streptococcus pneumoniae*. The HIS- and the FLAG-Tag are important for the detection. NcoI, NotI and Xbal are restriction sites. The constructs are of different size. Construct gamma has about 500 bp which corresponds to 18 kD. Construct alpha is twice the size of construct gamma (1000bpor36 kD). These constructs were applied to a vector which contained an *E. coli*-origin and a resistance gene for ampicillin. For multiplication, the constructs were re-transferred into E. coli Omni Max 2T1R, plated on LB-agar with ampicillin overnight and incubated in 5 ml LB-media with ampicillin. The next step was to purify the vectors using the QIAprep Spin Miniprep kit. After determining the concentration using NanoDrop, the vectors were digested with a NcoI- and Xbal. The digestion mix was loaded on an agarose gel and separated at 70 V for 40 min.

Zein, a protein-based polymer found in maize seeds, has been widely used as a carrier because of favorable properties such as biocompatibility, insolubility and low water uptake, mechanical and chemical stability, and its propensity to form coatings and microparticles [212, 388,155,222].

2.2.4 Primer designing

The general guidelines for primer design were as follows:

- The position of the primers should be chosen in such a way that the size of the PCR products would preferably be <300 bp (preferably 100–300 bp) in order to be able to use paraffin-embedded material;

- A minimal distance to the functional region of preferably >10–15 bp should be taken into account (in order to avoid false-negativity due to the impossibility of the 3′-end of the primer to anneal to the rearranged target because of nucleotide deletion from the germline sequence); and
- Primers preferably should not be too long (eg <25 nucleotides).
- Search for primers should be performed with *moderate* stringency;
- Priming efficiency (PE) value should preferably be ∼400 (and >630, if the primer is to be used as a consensus primer for other gene segments as well);
- The most stable 3′ dimer of upper/upper, lower/lower, or upper/lower primers should not exceed −4 Kcal (moderate search strategy), the most stable dimer overall being less important; and
- In view of multiplex PCR, the following guidelines were taken into account: a common primer would have to be designed in the most consensus region (ie high PE in consensus search), whereas individual primers (family or member) would have to be designed in the least consensus region (ie low PE value of that primer for gene segments that should not be covered) to avoid cross annealing to other gene segments and thereby multiple (unwanted) PCR products.

2.2.4.1 Web application design for Primer

Polymerase chain reaction (PCR) is a basic molecular biology technique with a multiplicity of uses, including deoxyribonucleic acid cloning and sequencing, functional analysis of genes, diagnosis of diseases, genotyping and discovery of genetic variants. Reliable primer design is crucial for successful PCR, and for over a decade, the open-source Primer3 software has been widely used for primer design, often in high-throughput genomics applications. It has also been incorporated into numerous publicly available software packages and web services.

Primer3, the subject of this article, is a popular group of programs, programming libraries and web interfaces that assist researchers with PCR primer design.

2.2.4.2 How to: Design PCR primers and check them for specificity

One or more primer sequences

1. Go to the Primer BLAST submission form.
2. Enter one or both primer sequences in the Primer Parameters section of the form. If only one primer is available, a template sequence is also required. See "A Target Template Sequence..." below.
3. In the Primer Pair Specificity Checking Parameters section, select the appropriate source Organism and the smallest Database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
4. Click the "Get Primers" button to submit the search and retrieve template and specificity information.

2.2.4.3 A target template sequence or accession number

Go to the Primer BLAST submission form.

1. Enter the target sequence in FASTA format or an accession number of an NCBI nucleotide sequence in the PCR Template section of the form. If the NCBI mRNA reference sequence accession number is used, the tool will automatically design primers that are specific to that splice variant.
2. If one or both primer sequences are to be used in the search, enter these in the Primer Parameters section of the form. Primer BLAST performs only a specificity check when a target template and both primers are provided.
3. In the Primer Pair Specificity Checking Parameters section, select the appropriate source Organism and the smallest Database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
4. Click the "Get Primers" button to submit the search and retrieve specific primer pairs.

Reset page Save search parameters Retrieve recent results

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear

Range

From To

Forward primer

Reverse primer Clear

Or, upload FASTA file Browse...

Primer Parameters

Use my own forward primer (5'->3' on plus strand) Clear

Use my own reverse primer (5'->3' on minus strand) Clear

PCR product size Min 70 Max 1000

# of primers to return 5

Primer melting temperatures (T_m) Min 57.0 Opt 60.0 Max 63.0 Max T_m difference 3

Exon/intron selection

A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span No preference

Exon junction match Exon at 5' side 7 Exon at 3' side 4

Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

Intron inclusion Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range Min 1000 Max 1000000

Primer Pair Specificity Checking Parameters

Specificity check Enable search for primer pairs specific to the intended PCR template

Database Refseq mRNA

Organism Homo sapiens

Enter an organism name, taxonomy id or select from the suggestion list as you type.

Add more organisms

Exclusion (opional) Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences

Entrez query (optional)

Primer specificity stringency Primer must have at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end.

Ignore targets that have 6 or more mismatches to the primer.

Misprimed product size deviation 4000

Splice variant handling Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)

Get Primers Show results in a new window Use new graphic view

Advanced parameters

Fig. 2.2: The interface consists of several sections where users can input the PCR template and/or pre-existing primers as well as other user-adjustable parameters.

SnapGene Viewer

SnapGene Viewer allows to create, browse, and share richly annotated DNA sequence files. SnapGene rounds up all the difficult, annoying and unruly aspects of cloning, gives them a stern talking to, and reintroduces them as pleasant and cooperative colleagues. With SnapGene, it takes only a few minutes to figure out which of a few hundred primers anneal with a template. Or to scan through a plasmid's entire cloning history. Or to test which of five different strategies will give you the result you want.

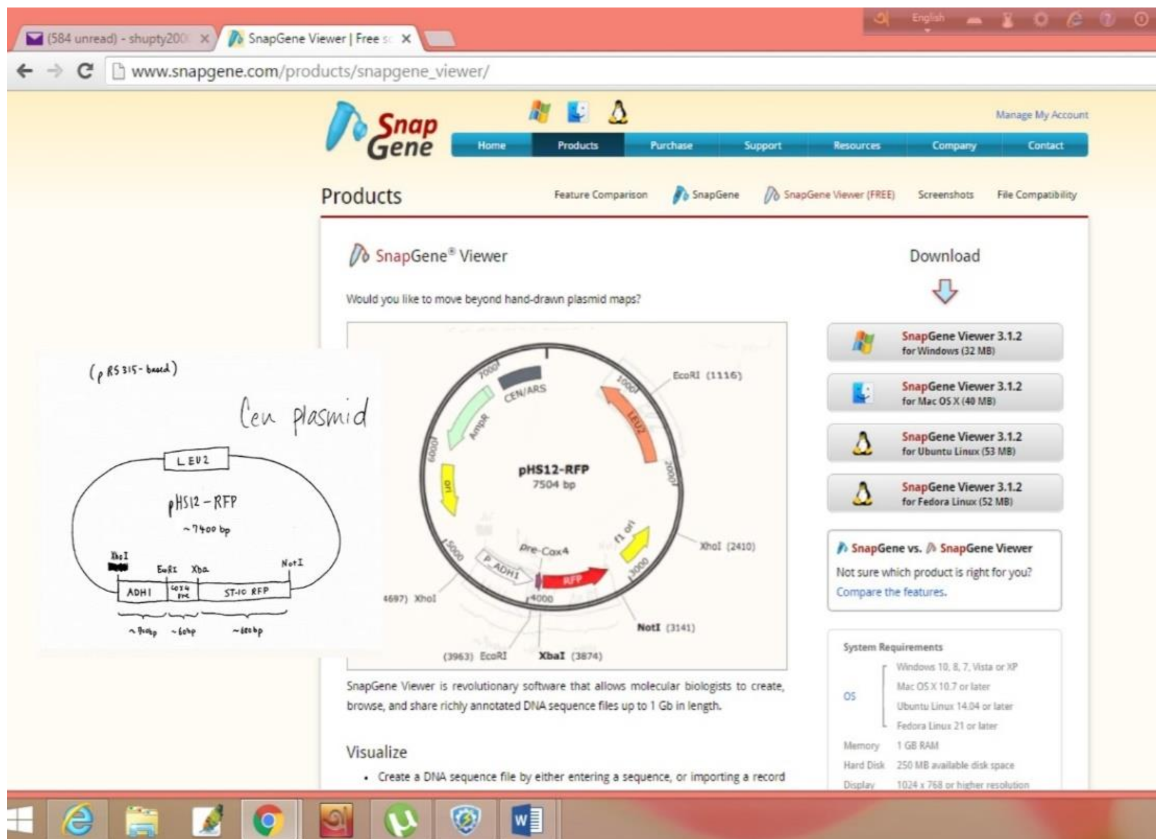

Fig. 2.3: Home page of SnapGene Viewer

SnapGener Viewer allows to visualize and search DNA sequences, annotate them manually or automatically and share your work by exporting a plasmid map as an image, or export an annotated DNA sequence to GenBank format.

Visualize

- Create a DNA sequence file by either entering a sequence, or importing a record from GenBank, or opening an annotated sequence stored in one of many common file formats.
- Browse or print a DNA sequence and its annotations using customizable Map, Sequence, Enzymes, Features, Primers, and History views.
- Search a DNA sequence to match either a DNA query, or a protein translation, or an annotation.
- View and edit DNA sequence traces.

Annotate

- Automatically annotate common features, or manually annotate coding sequences and other features.
- Design and annotate primers for PCR, sequencing, or mutagenesis.
- Identify open reading frames (ORFs) with a single mouse click.

Share

- Export a plasmid map as an image, or export an annotated DNA sequence to GenBank format.
- Freely share data with your colleagues or customers using the universally accessible SnapGene format.

Part-2: *Agrobacterium*-mediated genetic transformation

2.2.5 *In vitro* regeneration

2.2.5.1 Sources of plants

Seeds of *N. tabacum* were collected from Department of Botany, University of Dhaka, Bangladesh. Seeds of *L. esculentum* were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

2.2.5.2 Media used

For *In vitro* regeneration protocol establishment of *N. tabacum* and *L. esculentum*, different culture media were used in the present investigation. The different media used for different purposes are given as follows:

2.2.5.3 Seed germination

For both tomato and tobacco plants, surface sterilized seeds were soaked overnight in sterilized distilled water to be used as a source of explants. For rapid seed germination and subsequent seedling development seeds were placed on cotton bed soaked with autoclaved sterile distilled water and MS (Murashige and Skoog 1962) [254] media parallely.

2.2.5.4 Media for shoot regeneration and elongation

For shoot initiation and their further development MS medium supplemented with different combinations of hormones such as BAP (6-Benzyl aminopurine), Kn (Kinetin), NAA (α-naphthalene acetic acid), IAA (Indole-3-acetic acid) and Zn (Zeatin) were used during the present experiment.

2.2.5.5 Media for root induction

For the induction of roots at the base of the *In vitro* regenerated shoots, half strength of MS medium (half strength of macro, micro, organic nutrients and iron source of MS medium) supplemented with IAA (Indole-3 acetic acid), IBA (Indole-3 butyric acid) or NAA (α-naphthalene acetic acid) were used. Gelrite (Duchefa, Netherlands) was used as the solidifying agent for the rooting media, whereas agar was used in all others experiments.

2.2.6 Plant genetic transformation

2.2.6.1 Identification of A. tumefaciens using Biolog

Isolates exhibiting distinct colonial morphologies were isolated by repeated sub culturing into basal salt medium and solidified basal salt medium until purified strains were obtained. Identi- fication at species level was performed by using Biolog GN microplate according to the manufacturer's instructions. Briefly, a pure culture of the bac-terium was grown on a Biolog Universal Growth agar plate. The bacteria were swabbed from the surface of the agar plate, and suspended to a specified density in GN/GP inoculating fluid. Hundred and fifty microlitre of a bacterial suspension was pipetted into each well of the micro-plate. The micro-plate was incubated at 30° or 35° C depending upon the nature of the organism for 4–24 h according to manufacturer's specification. The micro-plate was read with the Biolog MicroStation TM system and compared to the database. Databases include aerobic and anaerobic bacteria, yeast, and fungi.

Fig. 2.4: The Biolog microbial ID system

2.2.6.2 Media for *Agrobacterium sp.* culture

Two different types of bacterial culture media, namely, YMB (Yeast extract Mannitol Broth) and YEP (Yeast Extract Peptone) with appropriate antibiotics were used to grow genetically

engineered *Agrobacterium tumefaciens*. Liquid YEP and YMB were used to grow A. tumefaciens which were used as bacterial suspension for genetic transformation. Solid YEP and YMB were used as maintenance media for different strains of *Agrobacterium sp*.

2.2.6.3 Co-culture medium

For the purpose of co-cultivation, MS media without hormonal supplement were used.

2.2.6.4 Selection media

For the selection of transformed tissues/plantlets kanamycin (Duchefa, Netherlands) was used, as the constructs in *Agrobacterium* used in the present investigation contained marker gene *npt*II (kanamycin resistance). The prepared regeneration medium after autoclaving was cooled down to 50˚C and appropriate antibiotics were added at a desired rate to a particular regeneration medium inside laminar flow cabinet. Medium was then poured into suitable culture vial and allowed to solidify. Apart from the above antibiotics some other antibiotics such as Carbenicilin and cefatoxime were used to control the overgrowth of bacteria.

2.2.6.5 Antibiotics

The explants of tomato and tobacco were infected by genetically engineered *Agrobacterium* strains for transformation. To prevent the excessive growth of this microbe in the media after co-cultivation, it was essential to add appropriate antibiotics in the media. For this purpose any one of the following antibiotics were used.

(i) Ticarcillin (Duchefa, Netherlands)

(ii) Carbenicilin (Duchefa, Netherlands)

(iii) Cefatoxime (Duchefa, Netherland)

2.2.6.6 Preparation of stock solutions for different culture media and growth regulators

Different stock solutions were prepared as the first step for the preparation of medium for ready use during the preparation of medium.

2.2.6.7 Preparation of stock solutions for MS medium

Since different constituents were required in different concentrations for the preparation of MS medium (Murashige and Skoog 1962), separate stock solutions for macro- and micro-nutrients, vitamins, plant growth regulators, etc. were prepared.

2.2.6.8 Stock solution A (Macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 40 times more than the final strength of the medium in 1.0 liter distilled water. For this purpose, 40 times the weight of different salts required for 1.0 liter of medium were weighed accurately and were sequentially dissolved one after another in a 1.0 liter volumetric flask with 600 ml of distilled water. The last two salts are dissolved in 100 ml of distilled water separately and added serially at last. The final volume of the solution was made up to 1.0 liter by further addition of distilled water. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton, etc. and was poured into a clean plastic container. After labeling, the solution was stored in a refrigerator at 4˚C for several weeks.

2.2.6.9 Stock solution B (Micro nutrients) for MS medium

For this constituent of the medium two separate stock solutions were prepared. These are as follows:

2.2.6.10 Stock solution B_1 (all micro-nutrients except iron)

This part of the stock solution was made with all the micro-nutrients except $FeSO_4.7H_20$ and Na_2-EDTA. This was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton, etc. and was poured into a clean plastic container and stored at 4˚C for future use.

2.2.6.11 Stock solution B_2 (iron chelate solution)

The second solution was made 100 times the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at low temperature until the salts were dissolved completely. Finally the solution was filtered and stored in a refrigerator at 4˚C for future use.

2.2.6.12 Stock solution C (Organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water. This solution was also filtered and stored at 4˚C for future use.

2.2.6.13 Stock solution D (B_5 vitamin) for MS medium

For the preparation of vitamin following components were added in distilled water.

Components	Amount needed for 1000 ml medium
Myo inositol	10.0 gm
Thiamin	1.0 gm
Nicotinic Acid	0.1 gm
Pyridoxin HCl	0.1 gm

This was made 100 times the final strength of necessary components in 500 ml of distilled water as described in the section before. The solution was filtered and stored at 4˚C.

2.2.6.14 Stock solutions for growth regulators

The following different growth regulators and supplements were used in the present investigation.

a. Auxins

Auxins induce cell division and formation of callus. It causes cell division, cell elongation, swelling of tissues and the formation of adventitious roots. The auxins are:

(i) Indole-3-acetic acid (IAA)

(ii) α- naphthalene acetic acid (NAA)

(iii) Indole-3-butyric acid (IBA)

b. Cytokinins

Cytokinins concerned with cell division and modification of shoot differentiation in tissue culture. The most frequently used cytokinins are

(i) 6-benzyl amino purine (BAP)

(ii) 6-furfuryl amino purine (Kinetin/Kn)

(iii) Zeatin (Zn)

(iv) Gibberellic acid (GA_3) etc.

The growth regulators, their solvents, and molecular weight are listed below (Sigma Plant Cell Culture Catalogue, 1992)

Growth regulators	Solvent	Molecular weight
IAA	1N NaOH	175.2
IBA	1N NaOH	203.2
NAA	1N NaOH	186.2
BAP	1N NaOH	225.3
Kinetin	1N NaOH	215.2
2,4-D	Ethanol	221.04

To prepare any of the above mentioned hormonal stock solution, 10 mM stock solutions of each hormones were prepared. For this purpose certain amount of the hormone was weighted and dissolved in required amount of appropriate solvent and then the final volume of the solution was made up 50 ml by addition of distilled water. The solution was then filtered and poured into clean plastic container/another 50 ml in sterilized falcon tube and stored in a refrigerator at 4ºC for future use.

2.2.6.15 Preparation of stock solutions of antibiotics

Different types of antibiotics were used for different bacterial and plant regeneration media in transformation experiments.

a) Kanamycin (Duchefa, Netherland)
b) Rifampicine (Duchefa, Netherland)
c) Carbenicilin (Duchefa, Netherland)
d) Cefatoxime (Duchefa, Netherland)

For the preparation of kanamycin, carbenicilin and cefatoxime stock solutions, 1 gm of each antibiotic was separately dissolved in 10 ml of deionized water. After micro filter sterilization, these solutions are stored in 1.5 ml Eppendorf tubes at - 20ºC in the dark as stock. For Rifampicin 1 gm of each antibiotic was dissolved in 10 ml of DMSO (Dimethylsulfoside).

2.2.6.16 Preparation of medium for *In vitro* culture of explants using MS stocks solutions

To prepare one liter of medium the following steps were carried out successively-

(i) A beaker of two liter capacity were taken.

(ii) 50 ml of stock solution A, 10 ml of stock solution B and 10 ml of stock solution C were added to this 500 ml distilled water and mixed well.

(iii) Required amount of hormonal supplements were taken from the stock solution of hormone and added to the medium either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 10 mM of the chemical in 50 ml of solution, the addition of 100 μl of any hormonal stock solution will make 1 litre of medium resulted in 1μM concentrations of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution to the medium following the similar procedure described earlier.

(iv) For the preparation of MS medium, 30 g of sucrose was dissolved in 500 ml of distilled water in a liter of volumetric flask

(v) The whole mixture was then volume up to 1litre with distilled water.

(vi) pH of the medium was adjusted to 5.8 with a digital pH meter (JENWAY, Japan) with the help of 1[N] NaOH and 1[N] HCL. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86, respectively.

(vii) To solidify either 8.0 g (at 0.8%) of phytoagar (Duchefa, Netherlands) or 2.0 g (at o.2%) of phytagel (Sigma, USA) was added to the medium. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (Butterfly, China).

(viii) To make liquid medium the last step (vi) of media preparation was omitted.

2.2.6.17 Preparation of MS medium for plants regeneration using MS powder

Occasionally commercially available powdered MS medium (Duchefa Biocheme, Netherlands) containing all the constituents of MS medium (inorganic salts, vitamins and

amino acids) except sucrose and agar were used to prepare medium for *In vitro* regeneration of plants. Appropriate amount of media powder is dissolved in distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. Normally 0.8% agar was added to prepare agar solidified media.

2.2.6.18 Preparation of *Agrobacterium* culture medium

Liquid YMB, and YEP were used to grow the genetically engineered A. tumefaciens strain which were later used to obtain bacterial suspension for transformation. Solid YEP and YMB were used as maintenance media for different strains.

2.2.6.19 Preparation of YMB (Yeast Extract Mannitol Broth) medium

For the growth of A. tumefaciens strain, YMB medium was prepared in the following manner:

Components	Amount needed for 100 ml medium
Mannitol	1.0 g
Yeast extract	0.04 g
$MgSO_4.H_2O$	0.02 g
NaCl	0.01 g
KH_2PO_4	0.05 g

All of these ingredients of the medium were added in 50 ml distilled water and mixed properly. Then the final volume was made up to 100 ml by adding distilled water. The pH was adjusted at 7.0 - 7.2.

For the preparation of working culture medium (to make bacterial suspension), liquid medium was used. For preparing maintenance medium 1.5 gm agar was added to the medium before autoclaving.

Liquid medium was stored at 4˚C for future use. Filter sterilized antibiotic kanamycin (50 mg/l), streptomycin (25 mg/l) and rifampicin (25 mg/l) was added to the autoclaved liquid medium (working) prior to bacterial inoculation and to the autoclaved maintenance medium when the medium was cooled down enough. The medium was then poured into Petri plates.

After solidification, the media were ready for bacterial culture. When required these culture plates were stored at 4˚C for further use.

2.2.6.20 YEP medium was prepared in the following composition

Components	Amount needed for 100 ml medium
Bactopeptone	1.0 g
Bacto Yeast extract	1.0 g
NaCl	0.5 g

Preparation procedure for YEP media was same as above media.

2.2.6.21 Preparation of co-culture medium

For co-cultivation (infection of tomato and tobacco explants together with *Agrobacterium*), shoot regeneration medium as well as without hormonal supplement i.e. solidified MS media with a pH 5.8 was prepared.

2.2.6.22 Sterilization

Fixed volume of the medium was dispensed into culture vessels (i.e. test tubes, bottles or conical flasks) or 500 ml screw capped bottles. The culture vessels were plugged with non-absorbent cotton or covered with aluminium foil and marked with the help of a glass marker to indicate the specific media with hormonal supplements. The culture vessels were then autoclaved (HA-300MN, Hirayama, Japan) at 15 lbs/sq inch pressure at 121˚C temperature for 20 minutes.

2.2.6.23 Preparation of selection medium for putatively transformed plantlets

For the selection of transformed shoots antibiotics, namely, kanamycin (Duchefa, Netherlands) was used, as the *Agrobacterium* construct used in the present investigation contained *npt*II (kanamycin resistance) genes as selectable marker gene. After preparation the regeneration medium was autoclaved. The medium was cooled down to 50˚C and appropriate antibiotics were added at a desired rate to a particular regeneration medium inside laminar flow cabinet. Medium was then poured into suitable culture vial and allowed

to solidify. Apart from these other antibiotics such as carbenicilin and cefatoxime was also used to control the overgrowth of bacteria.

2.2.6.24 Precaution to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet (Labtech Co. Ltd., Korea, Forma Scientific, USA, Heraeus, Germany). The cabinet was switched 'on' for at least half an hour before use and cleaned with 70% alcohol to make it free from surface contaminants. The instruments like scalpels, forceps, inoculation loop, Petri dishes and materials like cotton wool, filter papers, Microcentrifuger tubes, etc. were sterilized by steam sterilization methods. During the entire period of work in the cabinet, the scalpels, forceps, and inoculation loop were kept immersed into absolute alcohol containing in a glass jar inside the cabinet. At the time of inoculation and subculture these were sterilized by flaming method from time to time in the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the preparation of explants. After autoclaving bacterial media were poured inside the laminar airflow cabinet to avoid contamination. Antibiotics were filter sterilized with a micro-filter inside the laminar flow hood and stored in sterile Eppendorf tubes. Antibiotics were also added to the medium inside the laminar flow cabinet. After each transformation experiment, used *Agrobacterium* suspension and contaminated Petri dishes, cotton wool, filter papers, instrument, glass cuvettes were autoclaved to destroy genetically engineered *Agrobacterium* as a part of 'biosafety' requirement. Any contaminants and old bacterial cultures were also autoclaved before discarding them.

2.2.6.25 Culture techniques

The following culture techniques were employed in the present investigation-

(i) Axenic culture
(ii) Explant culture (Inoculation)
(iii) Subculture
(iv) Rooting
(v) Transplantation
(vi) *Agrobacterium* culture
(vii) Infection and incubation
(viii) Co-culture

2.2.6.25.1 Axenic culture

To reduce the level of surface organisms the seeds of tomato and tobacco were washed first with running tap water for 3-5 times. The floating seeds were discarded. The seeds were then deepened in 70% alcohol for 1 minutes followed by washing with distilled water. After transferring the seeds in autoclaved flask, final surface disinfection was done with 0.1% $HgCl_2$ solution for 10-15 minutes inside the laminar flow cabinet. During this period, the flask was agitated. Then the seeds were washed four to five times with sterilized distilled water. The surface sterilized seeds were then kept in sterilized distilled water for overnight and sometimes sterile cotton soaked bottle for the collection of explants.

2.2.6.25.2 Explants culture (Inoculation)

Leaf, node, Cotyledonery node, cotyledonary leaf and hypocotyle were used in this investigation as explants. Immature leaflets were excised from aseptically grown 7-8 days old seedlings. The explants were then cultured on MS media supplemented with different concentrations and combinations of BAP, Kn, 2,4-D, NAA, for *In vitro* regeneration of shoots.

2.2.6.25.3 Subculture

After two weeks, regenerated shoots were transferred to fresh medium. Cultures were sub-cultured regularly, at an interval of 21-28 days for maintenance and were routinely examined for different morphogenic developments.

2.2.6.25.4 Rooting

2.5 to 4 cm long shoots were separated and cultured on freshly prepared half strength of MS medium containing different combinations and concentrations of IAA, IBA and NAA for root induction.

2.2.6.25.5 Transplantation

The plantlets with sufficient root system were taken out from the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing sterilized soil. Pots were then covered with transparent perforated polythene bags. Inner side of these bags was moistened with water to prevent desiccation. To reduce sudden shock, the pots were kept in growth room for two weeks, of which polythene covers were

maintained for the first week and without cover for the second week. These plantlets were exposed to environment for 2-8 hours daily and again placed in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, then they were transferred to larger pots for further growth and to get seeds from those regenerated plants.

2.2.6.26 Preparation of *Agrobacterium* culture for transformation

As it has been mentioned earlier, two kinds of culture media were needed for the bacterial strain, one for maintaining *Agrobacterium* stock and the other for infection of explants.
For maintenance, one single colony from previously maintained stocks was streaked into freshly prepared Petri dish containing YMB and YEP medium having appropriate antibiotics. The Petri dish was sealed with Para film and kept in incubator at 28˚C temperature for at least 48 hours. This was then kept at 4˚C to check the growth. The culture was sub cultured regularly every week in freshly prepared media to maintain the stock.
For infection of explants, bacterial suspension was prepared. For this purpose, single colony from the bacterial stock was taken in an inoculation loop and was inoculated in a conical flask containing liquid medium with required antibiotic. This culture was allowed to grow at 28˚C overnight on a rotary shaker to get optimum population for infection and co-cultivation of explants.

2.2.6.26.1 Infection and incubation

The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm and the pellet was resuspended with liquid MS medium (pH 5.8) to make the *Agrobacterium* suspension. This *Agrobacterium* suspension was used for infection and incubation. Prior to this "Optical Density" or OD of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). Following the determination of density, to get suitable and sufficient infection of the explants, cut explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium. Before each cut the scalpel blade was dipped in the *Agrobacterium* suspension. Sometimes overnight grown *Agrobacterium* culture were directly used for the infection and incubation of explants.

2.2.6.26.2 Co-culture

Following infection and incubation, the explants were co-cultured on regeneration medium or hormone free MS medium. Prior to transfer of all explants to co-cultivation media they were soaked in filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-culture medium for 2 - 4 days.

2.2.6.26.3 Culture of *Agrobacterium* infected explants

Co-cultured explants were washed with distilled water three to four times until no opaque suspension was seen, then washed for 10 minutes with distilled water containing 300 mg/l carbenicilin/cefatoxime. Then explants were gently soaked with a sterile Whatman filter paper and transferred to regeneration medium containing 100 mg/l carbenicilin. After 12 - 15 days, the regenerated shoots were then sub-cultured in selection medium containing 50 mg/l kanamycin and 100 mg/l carbenicilin. Regenerated shoots were sub-cultured regularly with an interval of 15 - 21 days and the concentration of selection agents was gradually increased up to 200 -250 mg/l in case kanamycin on selection medium. Shoots survived on selection medium were sub-cultured on root induction medium for fully developed plantlets. As control, non-infected explants were cultured in normal regeneration medium. 7 to 10 days old shoots were sub-cultured in selection medium to detect the effect of selection agents on this control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

2.2.7 Molecular characterization of the integrated gene to confirm of the gene integration:

2.2.7.1 Integration of alpha, gamma and Ds-red zein gene:

To confirm integration of the alpha and gamma zein gene, the control and the transformed tobacco plants were tested by PCR. For this purpose both the plasmid and plant genomic DNA were isolated.

Isolation of plasmid DNA from *Agrobacterium:*

a. Materials

1. 1% SDS + 0.2N NaOH Sterile eppendorf tubes
2. Micropipeter and autoclaved microtips

3. Vortex mixer
4. Microfuge
5. Laminar air flow chamber.
6. Inoculation needle
7. Bacterial culture plate
8. YEP liquid medium
9. Solution I

    50mM glucose

    25mM Tris-Hcl

    10mM EDTA

10. Lysozyme 10mg/mL
11. Solution II
12. Tris saturated phenol
13. Solution III

    3M sodium acetate + acetic acid (pH 4.8)

14. Absolute alcohol and 70% alcohol
15. Chloroform
16. TE buffer

    10mM Tris-HCl (pH 7.5-8.0)

    1mM EDTA (pH 8.0)

17. Sterile double distilled water.

b. Procedure:

1. A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation needle and was grown overnight in 1.0mL YEP broth containing kanamycin (50mg/L) within a sterile microfuge tube.

2. Overnight grown 1.0mL culture was added to 5.0mL YEP broth containing kanamycin and again grown overnight.

3. 1.5 mL of this culture was taken and centrifuged at 5,000 rpm for 2min.

4. The supernatant was poured off and the cell pellet was resuspended in 100µl of solution I + 25µl of lysozyme and incubated for 15-20minutes at 37°C.

5. 200µl of freshly prepared solution II was added, mixed by inverting 5 times and kept on ice for 3-5 minutes.

6. 150µl of solution III was added to the viscous solution and mixed by inverting several times and kept on ice for 5 min.

7. The bacterial lysate was centrifuged for 10 min at 13,000 rpm.

8. The supernatant was poured out into a new tube.

9. The tube was filled with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1) mixture.

10. The tube was centrifuged for 5 min at 13,000 rpm.

11. The supernatant was carefully removed and transferred to a fresh tube. To that 0.6 volumes of isopropanol was added.

12. The DNA was pelletted in a microfuge for 10 minutes at 13,000 rpm.

13. The pellet was dried at 36°C for 1.0 hr.

14. The pellet was suspended in 50 µl sterile water and 1 µg/mL of pancreatic RNase was added and stored at 4°C [318].

Purification of plasmid DNA (Sambrook *et al*., 2001) [318]

- Equal volume of phenol was added and centrifuged at 5,000 rpm for 2 minutes.
- The supernatant was collected and treated with equal volume of chloroform: isoamylalcohol (24:1).

- After centrifugation at 5,000 rpm for 2 minutes the supernatant was collected.
- To the supernatant, equal volume of chilled ether was added and mixed well.
- The sediment was collected and treated with 2 volumes of chilled absolute alcohol and kept at –20°C for 30 minutes.
- To the pellet, 1 ml of 70% alcohol was added and the pellet was dislodged by tapping.
- The solution was centrifuged at 5,000 rpm for 2 minutes and to the pellet 1-2 drops of absolute alcohol was added.

Then it was vacuum dried and resuspended in sterile water.

2.2.7.2 Restriction analysis of the gene construct:

Restriction digestion was carried out for plant binary vector to confirm the specificity of the plasmid carrying Alpha and gamma zein gene along with the CaMV 35S promoter.

a. Materials

1. Sterile1.5mL eppendorf tube
2. Plasmid DNA
3. Enzymes

   *Kpn*I and *Sac*I (New England Biolabs)
4. Assay buffer
5. Sterile water
6. Water bath
7. Micropipette.

b. Procedure

1. Sterile 1.5mL eppendorf tube was taken and 50.0µl of the plasmid DNA was added to it.
2. Using micropipette 1.5µl each of *BamHI* and *EcoRI* (from NEB) were added to the eppendorf tube.
3. 10.0µl of Eco RI buffer (from NEB)was added to it.
4. The volume was made upto 80.0µl by adding 17µl of sterile water.
5. A short spin was given for settlement of the reaction mixture.
6. It was then kept in the water bath at 37^{0}C for 4hrs. followed by incubating at 65^{0}C for 15min. to inactivate the enzymes.

7. The digested product was run on 1.0% agarose gel electrophoresis along with the marker.

2.2.7.3 Plant genomic DNA isolation from putatively transformed plantlets

2.2.7.3.1 Collection of plant sample

To extract genomic DNA, *In vitro* regenerated putatively transient plantlets and their corresponding control plantlets were collected. The materials were washed in distilled water and dried on fresh tissue paper to remove any components of medium nutrients.

2.2.7.3.2 Preparation of stock solutions and working solutions used for DNA isolation

The following chemicals were used for plant genomic DNA isolation.

2.2.7.3.3 1 M stock solution of Tris HCl pH 8.0 (100 ml)

12.14 gm of Tris base was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4ºC.

2.2.7.3.4 0.5 M stock solution of EDTA pH 8.0 (100 ml)

18.61 gm of EDTA was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets was added to adjust the final pH to 8.0. Final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4ºC.

3.14.2.3 5 M stock solution of NaCl (100 ml)

29.22 gm of sodium chloride (NaCl) was dissolved in 75 ml of distilled water. Then it was volume up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at 4ºC.

2.2.7.3.5 β-Mercaptoethanol

β - Mercaptoethanol was available at strength of 14.4 M solution and was stored in a dark bottle at room temperature.

2.2.7.3.6 Ribonuclease A stock solution

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and was store in -20ºC.

2.2.7.3.7 Chloroform:Isoamyl alcohol (24:1) (100 ml)

24 ml of Chloroform and 1 ml of Isoamyl alcohol were mixed properly using vortex mixture. Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, was used only in a fume hood wearing gloves and eye protection.

2.2.7.3.8 70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol.

2.2.7.3.9 Stock solution of TE (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4ºC.

2.2.7.3.10 7.5 M Ammonium acetate pH 5.2 (100 ml)

57.81 g of ammonium acetate was dissolved in 70 ml of ddH_2O and was adjusted to a volume of 100 ml with the addition of ddH_2O and was sterilized by autoclaving.

2.2.7.3.11 Extraction Buffer (Homogenization buffer)

Following components were used for preparing DNA extraction buffer.

Name of the chemical	Molecular Weight	Stock con.	Reference con./working con.	Working Volume	
				100 ml	1000 ml
CTAB			2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (pH 8)	121.1	01 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7 ml

The following steps were performed for the preparation of Extraction Buffer (100 ml):

(i) 10 ml of 1 M Tris-HCl (autoclaved, pH 8.0) was taken in a 250 ml conical flask.

(ii) 28 ml of 5 M NaCl (autoclaved) was added to it.

(iii) 4 ml of 0.5 M EDTA (autoclaved, pH 8.0) was added next.

(iv) Then 0.5 gm PVP and 2/3 gm CTAB was added freshly and kept it at 60º C preheated water bath to dissolve the PVP.

(v) 200 µl β-mercaptoethanol was added prior to use the buffer and mixed it properly by shacking.

2.2.7.4 Isolation of genomic DNA using CTAB method

Plant genomic DNA was isolated according to Doyle and Doyle (1990). The steps of DNA isolation are as follows:

(i) 200 - 250 mg leaf materials of required source were taken and grind in liquid nitrogen (sometimes directly) using pestle and mortar.

(ii) 800 µl of extraction buffer was added and grinded the leaf until it became homogenous paste.

(iii) The paste was transferred to 2 ml centrifuge tube and incubated at 60º C water bath for 30 min.

(iv) 700µl of chloroform:Isoamyl alcohol (24:1) was added to the extract and centrifuged at room temperature for 10 min at 13000 rpm. Sometimes this process was repeated twice to obtain a clear sample.

(v) The supernatant was collected and DNA was precipitated with 2/3 volume chilled Isopropanol and kept the sample overnight in - 20º C.

(vi) Then the suspension was centrifuged for 10 min at 13000 rpm at room temperature.

(vii) Supernatant discarded and the pellet was washed with 70% ethanol for 3 times.

(viii) The DNA was then resuspended in 40 - 50µl of TE buffer.

2.2.7.4.1 Quantification of Isolated DNA from plants

Measurement of isolated DNA concentration can be done by estimating the absorbance of DNA by spectrophotometer at 260 nm or by using Nanodrop photometer . Both the methods were carried out during this investigation.

2.2.7.4.2 Measurement of DNA concentration and quality by Agarose Gel Electrophoresis

2.2.7.4.3 Preparation of stock solutions used for Gel Electrophoresis

50 × TAE Buffer (pH8.3) (1 litre)

242 g Tris base was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added in it. The pH of the solution was adjusted with concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

10 × Loading Dye

For the preparation of 10 ml of 10 × loading dye, 40 mg of bromophenol blue (i.e., the final concentration was 0.4%), 40 mg of xylene cyanol FF (i.e., the final concentration was 0.4%) and 5 ml of 98% glycerol (i.e., the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and was stored at 4°C.

Ethidium Bromide Solution

For 1ml solution, 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile de-ionized distilled water. It was mixed by hand shaking. The solution was transferred to a dark bottle and was stored at room temperature.

2.2.7.4.4 Agarose Gel Electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp *et al.* 1973.

(i) 1.0/0.8 gm of agarose was melted into 100 ml of TAE buffer, ethidium bromide was added (10 μg/ml) and poured into gel-tray fixed with appropriate combs.

(ii) After the gel was solidified it was placed into gel-running kit containing 1× TAE buffer.

(iii) Digested plant DNA solutions were loaded with 6× gel loading dye and elecetrophoresis was continued until DNA fragments were separated well.

2.2.7.5 Polymerase Chain Reaction

2.2.7.5.1 Preparation of the Master Mixture

Master mixture was prepared by mixing all of the PCR component e.g. reaction buffer, dNTPs, Primer- F & R, Taq DNA polymerase etc. except the component template DNA. In each reaction, the volume of PCR buffer was used $\frac{1}{10}$ th of the total reaction volume which was 25µl. After thorough mixing and momentary spin of the master mixture, it was transferred to different PCR tubes. The final volume was made 25µl by adding varying amounts of sterilized ultra-pure water. Template DNA was added afterwards. DNA polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to thermo cycler for the amplification reaction (Eppendorf Mastercycler gradient).

3.15.2 Thermal cycling profile used in PCR

The working concentration of the template DNA was 100 ng for each sample. The PCR condition was used for gene amplification as described below:

The PCR condition for alpha, gamma, Ds-red and *npt*II gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	
Annealing	58°C	1 min	35
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

2.2.7.5.3 Analysis of PCR product

2.2.7.5.4 Visualizing the PCR Product

3-5 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 1.0 - 1.2% agarose gel containing ethidium bromide (0.05µl/ml). Electrophoresis was accomplished at 40 volts and the PCR products were visualized under UV transilluminator and photographs were taken for documentation by gel documentation (Bio.Sci. Tech.Gelsccan, 6.0, professional, Germany).

2.2.7.6 Protein Extraction, SDS-PAGE and Western Blot

A SDS-PAGE (Sodium-DodecylSulfate-PolyAcrylamide-GelElectrophoresis) is a form of a polyacrylamide gel electrophoresis to separate proteins because of their size. This involves SDS and a discontinuous gel. SDS is an anionic detergent that denatures secondary and non-disulfidelinked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Heating the samples to near boiling in the presence of a reducing agent, such as 2-mercaptoethanol, promotes denaturation of proteins by reducing disulfide linkages. This is known as reducing SDS-PAGE. The negative charge of the proteins in proportion to its mass allows a separation in an electric field because of their size. The polyacrylamide gel consists of acrylamide, bisacrylamide, buffer to adjust the pH-value, SDS, APS (ammonium-persulfate), TEMED (Tetramethylendiamin) and distilled water. There are two different parts in the gel, a separating gel and a stacking gel. They differ in the content of polyacrylamide (separating gel - 12%; stacking gel - 4%) and the pHvalue. The stacking gel is used to collect the proteins and due to this effect allow them an equal starting point, which results in improved band sharpness. The separation gel consists of a higher concentration of polyacrylamide and is therefore in charge for the separation of proteins, due to their size. The separation takes place in an electric field induced by BIO RAD PowerPac. Proteins move to the anode due to their negative charge, caused by SDS. After the separation, the proteins are transferred to a membrane (nylon, glass fiber, nitrocellulose) and detected by incubation with a specific antibody conjugated to alkaline phosphatase (AP) that causes a color reaction with a luminescent or chromogenic substrate (like nitro blue tetrazolium

chloride and 5-bromo-4-chloro-3-indolyl posphate). This is called western blotting and is necessary to detect proteins whose concentration is too low for direct detection on the gel.

2.2.7.6.1 Protein Extraction

The first step was to cut out pieces of transformed leaves with the cap of a 2 ml microcentrifuge tube, add a small metallic ball (necessary for crushing of the probes) and fridge them in liquid nitrogen to stop the degradation of the proteins. The probes were ground with a RETSCH MM400 and mixed with 200 µl Laemmli buffer. (contained 0.1% 2-mercaptoethanol, 0,0005% bromophenol blue, 10% glycerol, 2% SDS and 63 mM tris-HCl; pH 6.8) Afterwards, the probes were lysed with a Branson Sonifier 250 and centrifuged for 10 min. The supernatant was transferred to a new tube and boiled for about 10 min before the probes were loaded on the gel.

2.2.7.6.2 SDS-PAGE

First, the gel had to be prepared. Like mentioned above, it composed of a stacking and a separating part. Before that, all instruments had to be cleaned thoroughly and all parts had to be put together so the whole apparatus was tight.

Table 4: The composition of the separating and the stacking gel. All components are for one gel.

	Separating gel	Stacking gel
ddH2O	1.717 ml	0.9 ml
Acrylamide 30%	2 ml	0.245 ml
4x separating buffer	1.250 ml	-
4x stacking buffer	-	0.375 ml
APS	0.020 ml	0.004 ml
TEMED	0.013 ml	0.008 ml.

After that, all components of the gels were mixed together (for the composition see table 1). It was important that it was worked quickly, because after APS and TEMED were added the acrylamide began to polymerize. So first the separating gel was mixed and filled into the apparatus. Isopropanol was applied on top of the separating part to get a straight surface. The polymerization took about 30 minutes. After this half an hour, the isopropanol had to be removed and the gel had to be dried with filter paper. The stacking gel was mixed and added above the separating part. To create wells for probes it was necessary to insert a comb. The duration of the polymerization was again half an hour. After that, the gel was ready to use. The gels were placed into an electrophoresis chamber which was filled with running buffer. The combs were removed and the wells were washed with this buffer. Afterwards the probes and a ladder were filled into these wells and the gel was run at 200V for 40 min.

2.2.7.6.3 Western Blot

After the SDS-PAGE, the proteins were transferred to a membrane of nitrocellulose with an electric field. To this end, the blotting device was filled with a blotting buffer moistened fiber pad and filter paper. On the filter paper the gel and the membrane were placed. The membrane was covered again with wet filter paper. It was important that no air bubbles were enclosed. On the top, there was another fiber pad. The jig was closed and put into the blotting chamber which is filled with blotting buffer. The electric field was applied with 0.16 A/gel and run for 1h 40min.

Meanwhile, the blocking solution was prepared. Per gel 50 ml of 5% blocking solution was needed. So milk powder as weighed in, the beaker was filled up with PBS buffer and mixed for a while to get a homogenous solution. After blotting, the membrane was transferred into a dying tub and incubated with blocking solution for 30 min. This was necessary to prevent that the antibodies were interacting with the membrane. The membrane was shortly washed with PBS buffer and incubated with the first antibody for 1h at room temperature. Afterwards, the solution with the antibody was removed. The solution could be reused several times. The membrane was washed 3 times with PBS buffer and incubated with the second antibody, which bound on the first antibody and was conjugated with AP, over night at 4°C or for 2h at room temperature. The second antibody was removed and the membrane was washed again 3 times with PBS buffer and one time with distilled water. Now, the

developer solution was added and incubated for 10 min at room temperature in the dark. After this 10 min the developer solution was discarded and the membrane was washed with distilled water. The detected proteins, should then become visible.

Part-3: Mice immunization and challenge

2.2.8 Collection and maintaining of Mice

In the present study, mice model system was used to analyze the immunogenicity and protective efficacy of the transgenic plant. For each experiment, 48 of Swiss Albino BALB/c mice (8 to 10 weeks old) were collected from the Animal Resources Branch of International Center of Diarrheal Disease Research, Bangladesh (ICDDR,B). The weights of the mice were approx. 2 gm. The mice were kept in mice isolator cages with free access to commercial food pellets and autoclaved water and housed under standardized conditions with regulated daylight, humidity, and temperature. Male and female mice were kept separately so that no new birth happened. All experiment were done in accordance with the ethical guidelines at ICDDRB. Mice were supervised daily, ensured proper care and maintained in germ free condition.

2.2.9 Preparation of special tablets with transgenic plants used for mice immunization

The experiment of mice immunization were carried out by feeding the mice with transgenic plant tissue. For this purpose, at first some tablets like forms were made by the combination of mice feed pellet and the transformed plant tissue which were survived after selection pressure. The plant tissue which was genetically transformed by Alpha, Gamma and Ds-red zein separately and survived after kanamycin selection were harvested and made them dried. Then the dry tissues were subjected to made powder using lyophilizer. Mice feed pellet and plant tissue were mixed in the ration of 1:1, prepared as tablet and led them dried.

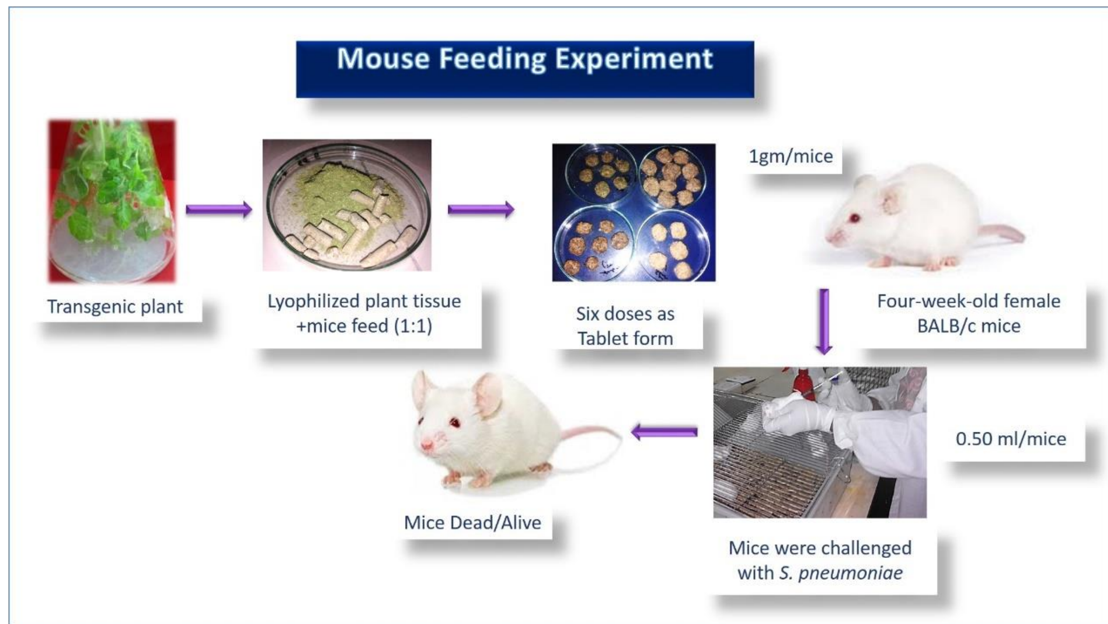

Fig. 2.5: Methods of feeding technique for mice immunization with transgenic plant

2.2.10 Feeding the mice transgenic plant tissue

Mice were immunized at 7 days intervals with transgenic plant tissue with mice feed pellet in the ration of 1:1. Total 6 doses were performed. Mice were divided into four groups which were kept in different mice cages. The group are divided as mention bellow-

Group 1: Immunized with Alpha zein

Group 2: Immunized with Gamma zein

Group 3: Immunized with Ds-red zein and

Group 4: Immunized with control leaves.

Before oral immunization, groups of mice were deprived of food for 3-4 h followed by intragastric administration of 0.2 ml of an isotonic bicarbonate solution (8 parts Hanks' balanced salt solution and 2 parts of 7.5% sodium bicarbonate) to neutralize stomach acidity. Oral dosing was done by using specific tablet contain one gram of transgenic leaves which was half of their body weight. All doses were given orally like feeding. The same process were repeat twice heading by 1st trial and 2nd trial.

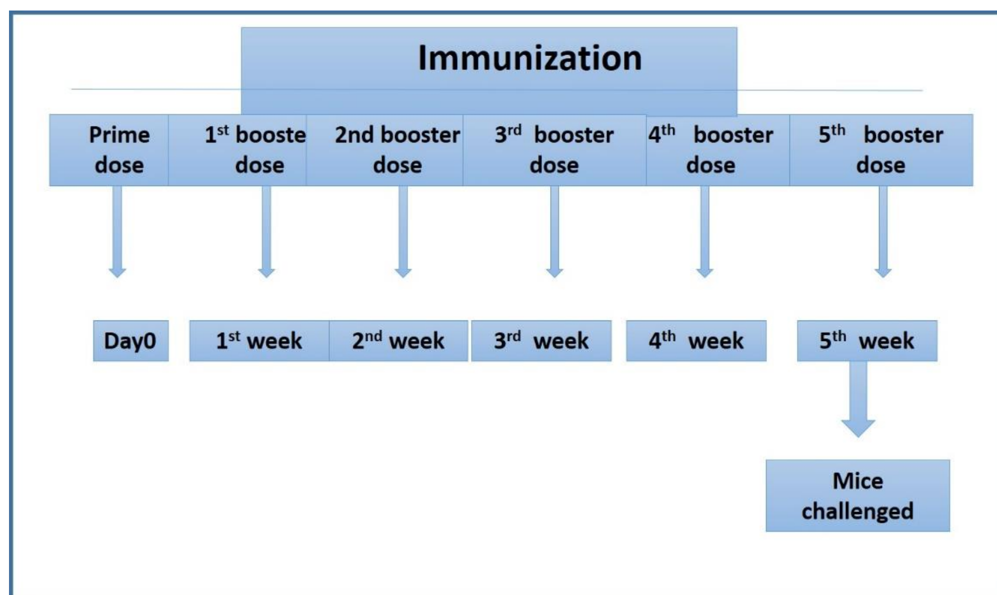

Fig. 2.6: Immunization schedule of mice

2.2.11 Determination of the LD_{50} of *S. pneumoniae* serotype 7F

To determine the LD_{50} of *S. pneumoniae* in mice, each group of mice was injected intra-peritonially with 10^6, 10^7 or 10^8 live pneumoniae type 7F. Four mice (8 to 10 weeks old) were include in each group and death of mice were considered to have survived the challenge [46]. LD_{50} dose were determine by the method of Probites [43].

Virulent strains were defined as those, which caused a median type to death of less than 10 days. The 10 day cutoff was chosen because very few strain show a median time to death of 10 to 30 days post infection [46].

2.2.12 Challenge of mice with *S. pneumoniae* serotype 7F

Mice immunized with transgenic plant tissue and the control mice, which received the control plant along with mice feeding pellet, were challenged intra-peritonially with 10 times of the LD_{50} dose of the virulent *S. pneumoniae* type 7F. Death of the mice was re-coded daily for 30 days.

2.2.13 Statistical analyses

All the tests are performed with SPSS of its version 22.0. SPSS Statistics is a software package used for interactive, or batched, statistical analysis. Long produced by SPSS Inc., it was acquired by IBM in 2009. The current versions are named IBM SPSS Statistics.

The software name originally stood for Statistical Package for the Social Sciences (SPSS), reflecting the original market, although the software is now popular in other fields as well, including the health sciences and marketing.

The results were statistically analyzed for considering the variance of mice challenge experiment with T-test, Oneway ANOVA, Post Hoc Tests etc.

3. RESULTS

3.1 Preparation of vector plasmid

3.1.1 Bacterial Strains

S. pneumoniae serotype 7F was collected from the stock culture of the Microbiology Department of Dhaka Shishu (Children) Hospital and maintained in the Department of Microbiology, University of Dhaka and Plant Tissue Culture Section, BCSIR laboratories, Dhaka. Different *A. tumefaciens* strains harboring construct Alpha zein, gamma zein and Ds-red zein separately were kindly provided by Professor Eva Stöger, University of Natural Resources and Life Sciences, Vienna, Austria.

Identity of *S. pneumoniae* serotype 7F was again reconfirmed by the Gram staining, Optochin susceptibility and bile solubility tests (Table 3.1 and Fig. 3.1, 3.2, 3.3).

Table 3.1: Confirmatory tests of the *S. pneumoniae* strain used in this study

Tests	**Results**
Microscopic examination	Gram positive diplococci
Optochin susceptibility test	Inhibition zone >14 mm
Bile solubility test	Positive

Fig. 3.1: Growth of *S. pneumoniae* on Gentamycin blood agar; plate shows haemolytic colonies and Gram staining showing positive result

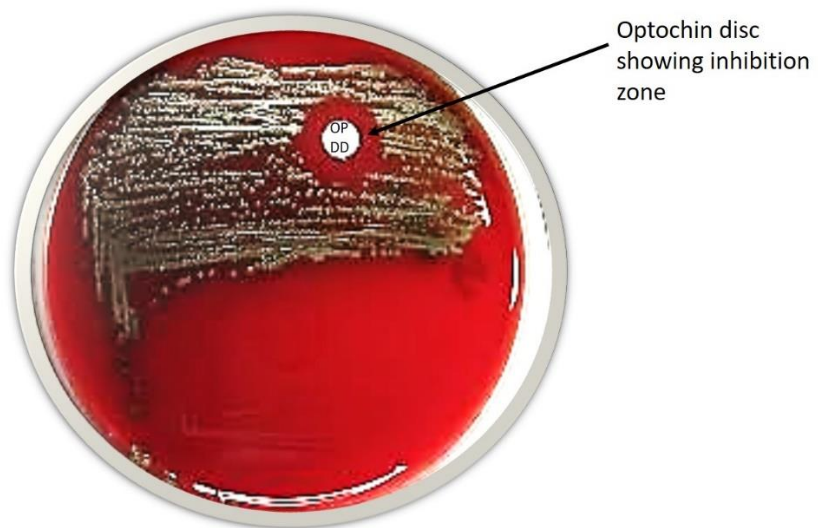

Fig. 3.2: Optochin susceptibility test showing the zone of inhibition

Fig. 3.3**:** Bile solubility test showing the positive result

3.1.2 Preparation of surface protein (WEM)

Surface protein prepared by the water extraction method (WEM) was stored at -20°C. Each and every step was checked for the presence of any sort of contamination by inoculating the sample in the blood agar media and Optochin test. No bacterial growth was observed on the plate after overnight incubation of the protein preparations, indicating water extraction method removed the surface materials from the cells.

3.1.3 Determination of soluble protein concentration

The soluble protein concentration of the surface material of *S. pneumoniae* was determined using bovine serum albumin as the standard and calculated from the standard curve (Fig. 3.4). The concentration of the protein was determined 0.57mg/ml. This concentration was sufficient for SDS-PAGE analysis and subsequent Western Blot experiment.

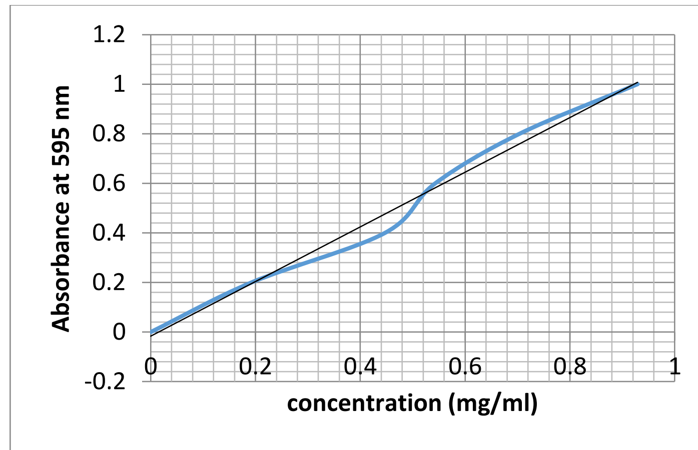

Fig. 3.4: Standard curve for protein concentration estimation.

3.1.4 The BLAST sequence analysis

The comparison of nucleotide or protein sequences from the same or different organisms is a very powerful tool in molecular biology. By finding similarities between sequences, scientists can infer the function of newly sequenced genes, predict new members of gene families, and explore evolutionary relationships. Now that whole genomes or partially are being sequenced, sequence similarity searching can be used to predict the location and function of protein-coding and transcription regulation regions in genomic DNA. Basic Local Alignment Search Tool (BLAST) is the tool most frequently used for calculating sequence similarity which was developed by NCBI. BLAST comes in variations for use with different query sequences against different databases.

The PspA whole and partial genome were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the Basic Local Alignment Search Tool (BLAST) to identify different genotypes of PspA.

3.1.5 Querying and linking the data of PspA

The query sequence is represented by the numbered red bar at the top of the Fig.3.5. Database hits are shown aligned to the query, below the red bar. Of the aligned sequences, the most similar are shown closest to the query. In this case, there are three highscoring database matches that align to most of the query sequence. The next bars represent lower-scoring matches. The remaining bars show lower-scoring alignments. Mousing over the

bars displays the definition line for that sequence to be shown in the window above the graphic.

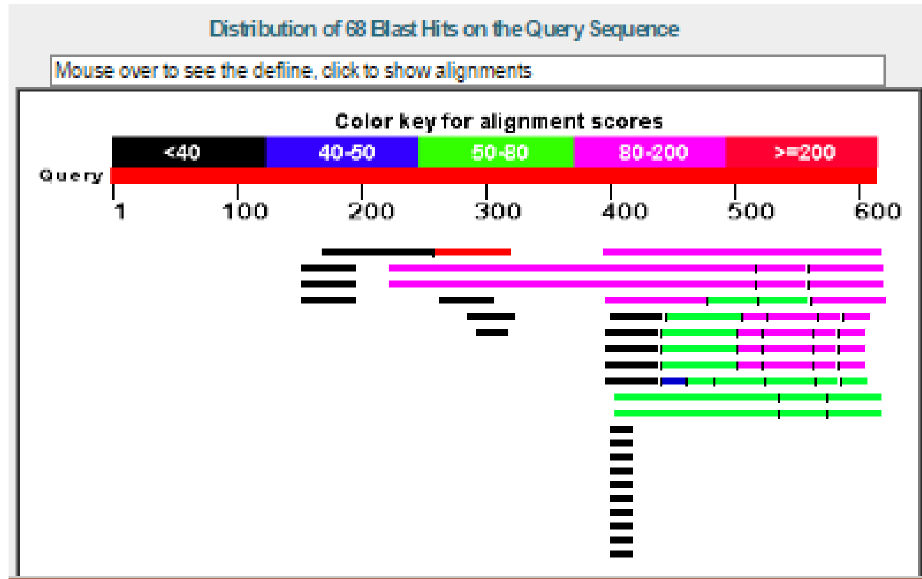

Fig. 3.5 Graphical overview of BLAST results of Pneumococcal surface protein antigen (PspA) of *S. pneumoniae*.

3.1.6 Sequences producing significant alignment

The sequences producing significant alignment of PspA are given in Fig.3.6. For the every hit in the list, the gi number, the database designation is sp (for SWISS-PROT), the accession numbers are given here. Note that the first 5 hits have low E-values (much less than 1). The other database matches have much higher E-values, 0.5 and above, which means that these sequences may have been matched by chance alone.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	24.8	100%	0.90	100%	ABE67228.1
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	24.8	100%	0.93	100%	ABE67236.1
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	24.8	100%	0.93	100%	ABE67229.1
pneumococcal surface protein A [Streptococcus pneumoniae] >gb|ABE67233.1| pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	24.8	100%	0.93	100%	ABE67227.1
Chain C, Crystal Structure Of The Complex Of Human Lactoferrin N-Lobe And Lactoferrin-Binding Domain Of Pneumococcal Surface Protein A >pdb|2PMS|D Ch	24.8	39.9	100%	0.97	100%	2PMS_C
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	38.2	100%	1.0	100%	ACR50682.1
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	36.5	100%	1.1	100%	ABY57196.1
pneumococcal surface protein A [Streptococcus pneumoniae]	24.8	38.2	100%	1.1	100%	ABV50383.1
PspA, partial [Streptococcus pneumoniae WU2]	24.8	39.9	100%	1.1	100%	AAF27710.1
PspA, partial [Streptococcus pneumoniae]	24.8	24.8	100%	1.1	100%	AAF27708.1
hypothetical protein, partial [Streptococcus pneumoniae]	24.8	51.5	100%	1.1	100%	WP_000350453.1
Pneumococcal surface protein A, partial [Streptococcus pneumoniae]	24.8	38.2	100%	1.1	100%	CCP20600.1
M protein repeat family protein, partial [Streptococcus pneumoniae] >gb|EJG76053.1| M protein repeat family protein, partial [Streptococcus pneumoniae 208223	24.8	24.8	100%	1.1	100%	WP_001856842.1
M protein repeat family protein [Streptococcus pneumoniae] >gb|EJG64029.1| M protein repeat family protein [Streptococcus pneumoniae 2061617]	24.8	24.8	100%	1.1	100%	WP_001854147.1
surface protein pspA [Streptococcus pneumoniae R6] >ref|YP_815641.1| surface protein A [Streptococcus pneumoniae D39] >ref|WP_001035315.1| hypothetical	24.8	51.5	100%	1.1	100%	NP_357715.1
hypothetical protein, partial [Streptococcus pneumoniae]	22.3	36.5	85%	6.9	100%	WP_000475110.1
hypothetical protein, partial [Streptococcus pneumoniae]	22.3	36.5	85%	6.9	100%	WP_000450135.1
helicase [Streptococcus pneumoniae] >gb|EJG43005.1| helicase conserved C-terminal domain protein [Streptococcus pneumoniae 2070335]	19.7	19.7	100%	42	86%	WP_001851618.1

Fig.3.6: Sequences of PspA protein producing significant alignment

Table- 3.2: BLAST results for some sequenced samples

Close proximity to the accession number	Identity	Max score	Query cover	E value
ABE67228.1	100%	24.8	100%	0.90
ABE67236.1	100%	24.8	100%	0.93
ABE67229.1	100%	24.8	100%	0.93
ABE67227.1	100%	24.8	100%	0.93
2PMS.C	100%	24.8	100%	0.97
ACR50682.1	100%	24.8	100%	1.0

3.1.7 Pairwise sequence alignment from a BLAST report

The alignment is preceded by the sequence identifier, the full definition line, and the length of the matched sequence, in amino acids. Next comes the bit score (the raw score is in parentheses) and then the E-value. The following line contains information on the number of identical residues in this alignment (Identities), the number of conservative substitutions (Positives), and if applicable, the number of gaps in the alignment. Finally, the actual alignment is shown, with the query on top, and the database match is labeled as Sbjct, below. The numbers at left and right refer to the position in the amino acid sequence. One or more dashes (–) within a sequence indicate insertions or deletions. Amino acid residues in the query sequence that have been masked because of low complexity are replaced by Xs (see, for example, the fourth and last blocks). The line between the two sequences indicates the similarities between the sequences. If the query and the subject have the same amino acid at a given location, the residue itself is shown. These records provide more information about the sequence, including links to relevant research abstracts in PubMed.

```
Alignment of Sequence_1:  [4_Probe 4 gamma1_S9522 A01 microsynth.fasta] with  Seq

Similarity : 444/444 (100,00 %)

Seq_1  1     ATGGGTTCCAAGGCAGAGAAAGACTACGACGCTGCCGCACAGAAGAAATACGATGAGGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     ATGGGTTCCAAGGCAGAGAAAGACTACGACGCTGCCGCACAGAAGAAATACGATGAGGAT  60

Seq_1  61    CAGAAGAAAACAGAGGAAAGCGAATCAGAAGATTATGCAGCGGCCGCAGCTGCCGAAGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    CAGAAGAAAACAGAGGAAAGCGAATCAGAAGATTATGCAGCGGCCGCAGCTGCCGAAGGG  120

Seq_1  121   GGCGGGTCTGGCGGAGGTGGTAGTGGTGGATGCGGATGCCAGCCACCTCCACCAGTCCAC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   GGCGGGTCTGGCGGAGGTGGTAGTGGTGGATGCGGATGCCAGCCACCTCCACCAGTCCAC  180

Seq_1  181   CTGCCACCTCCAGTACATCTTCCACCTCCAGTTCACCTACCACCGCCTGTTCATTTGCCT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   CTGCCACCTCCAGTACATCTTCCACCTCCAGTTCACCTACCACCGCCTGTTCATTTGCCT  240

Seq_1  241   CCTCCAGTTCATCTCCCTCCCCCCGTTCACCTTCCTCCTCCAGTGCACGTCCCTCCACCA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  241   CCTCCAGTTCATCTCCCTCCCCCCGTTCACCTTCCTCCTCCAGTGCACGTCCCTCCACCA  300

Seq_1  301   GTGCATTTACCACCTCCACCTTGTCACTATCCAACTCAGCCTCCTAGACCGCAACCTCAT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  301   GTGCATTTACCACCTCCACCTTGTCACTATCCAACTCAGCCTCCTAGACCGCAACCTCAT  360

Seq_1  361   CCTCAACCCCATCCGTGTCCGTGTCAACAACCGCATCCCTCTCCCTGCCAAGCAGCTGCT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  361   CCTCAACCCCATCCGTGTCCGTGTCAACAACCGCATCCCTCTCCCTGCCAAGCAGCTGCT  420

Seq_1  421   CATCATCATCACCATCACTAATGA  444
             ||||||||||||||||||||||||
Seq_2  421   CATCATCATCACCATCACTAATGA  444
```

Fig.3.7 The alignment of one of the constructs. Seq_1 is the sequenced vector by Microsynth and seq_2 is the theoretical sequence.

3.1.8 B cell epitope identification

3.1.8.1 Kolaskar and Tongaonkar antigenicity analysis

For B cell linear prediction several algorithms are available in IEDB. Among those Kolaskar Tongaonkar algorithm is well recommended and known to predict B cell epitope with 75% fidelity. Kolaskar and Tongaonkar algorithm predicted linear epitope in the 7-39 region.

Kolaskar & Tongaonkar Antigenicity Results
Input Sequences of PspA [*Streptococcus pneumoniae*]

GenBank: AAC62252.1

```
ORIGIN
        1 mnkkkmilts lasvailgag fvassptfvr aeeapvanqs kaekdydaav kkseaakkdy
       61 etakkkaeda qkkydedqkk teakaekerk asekiaeatk evqqaylayl qasnesqrke
      121 adkkikeatq rkdeaeaafa tirttivvpe pselaetkkk aeeatkeaev akkkseeaak
      181 eveveknkil eqdaenekki dvlqnkvadl ekgiapyqne vaelnkeiar lqsdlkdaee
      241 nnvedyikeg leqaitnkka elattqqnid ktqkdledae lelekvlatl dpegktqdel
      301 dkeaaeaeln ekvealqnqv aeleeelskl ednlkdaetn nvedyikegl eeaiatkkae
      361 lektqkelda alnelgpdgd eeetpapapq pekpaeepen papapkpeks adqqaeedya
      421 rrseeeynrl tqqqppkaek papapqpeqp apapkigwkq engmwyfynt dgsmatgwlq
      481 nngswyylns ngamatgwlq yngswyylna ngamatgwlq yngswyylna ngamatgwlq
      541 yngswyylna ngdmatgwlq yngswyylna ngdmatgwak vhgswyylna ngsmatgwvk
      601 dgetwyylea sgsmkanqwf qvsdkwyyvn glgslsvntt vdgykvnang ewv
//
```

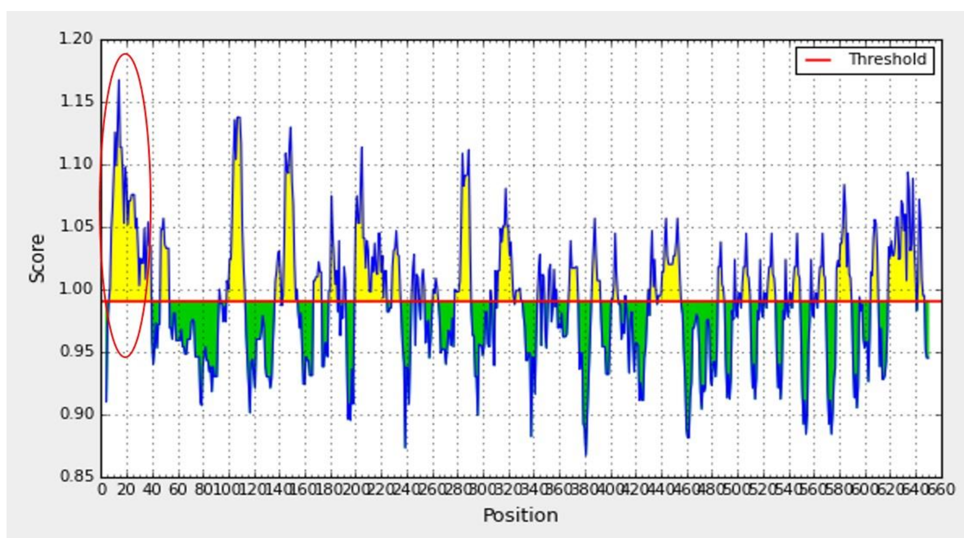

Fig. 3.8: B cell epitope predicted by Kolaskar and Tongaonkar prediction method

(Yellow color indicates the amino acid moieties predicted to harbor B cell epitopes. Red circle indicates selected amino acid starting position 7, and end position 39.)

Table -3.3: Predicted peptide sequences of PspA according to Kolaskar and Tongaonkar

Predicted peptides:

No.	Start	End	Peptide	Length
1	7	39	ILTSLASVAILGAGFVASSPTFVRAEEAPVANQ	33
2	47	53	DAAVKKS	7
3	99	113	TKEVQQAYLAYLQAS	15
4	143	154	RTTIVVPEPSEL	12
5	167	173	EAEVAKK	7
6	179	185	AKEVEVE	7
7	200	224	IDVLQNKVADLEKGIAPYQNEVAEL	25
8	229	235	ARLQSDL	7
9	278	290	DAELELEKVLATL	13
10	312	324	KVEALQNQVAELE	13
11	326	331	ELSKLE	6
12	368	374	LDAALNE	7
13	386	392	APAPQPE	7
14	401	406	PAPAPK	6
15	438	455	AEKPAPAPQPEQPAPAPK	18
16	504	510	SWYYLNA	7
17	524	530	SWYYLNA	7
18	544	549	SWYYLN	6
19	564	569	SWYYLN	6
20	578	589	WAKVHGSWYYLN	12
21	605	610	WYYLEA	6
22	619	640	WFQVSDKWYYVNGLGSLSVNTT	22
23	642	647	DGYKVN	6

3.1.8.2 Sequence 1 of the predicted epitope

Hence, from the above analysis it is observed that the 33 amino acid sequence 7-39 is predicted to have the capabilities to mount Tc, Th and B cell response. Therefore, the first selected sequence of the 33 amino acid is-

1. ILTSLASVAILGAGFVASQPTVVRAEESPVASQ

3.1.8.3 Emini Surface Accessibility prediction

The calculation was based on surface accessibility scale on a product instead of an addition within the window. The accessibility profile was obtained using the formulae $Sn = (n+4+i)(0.37)^{-6}$ where Sn is the surface probability, dn is the fractional surface probability value, and i vary from 1 to 6. A hexapeptide sequence with Sn greater than 1.0 indicates an increased probability for being found on the surface.

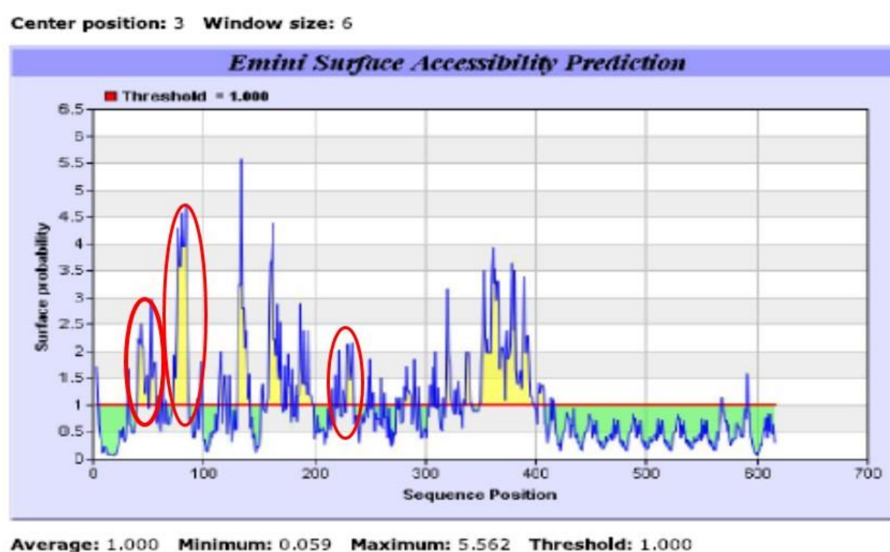

Fig. 3.9: B cell epitope predicted by Emini surface accessibility prediction method

(Yellow color indicates the amino acid moieties predicted to harbor B cell epitopes. Red circle indicates selected amino acid starting and end position for epitope 2, 40-49; for epitope 3, 73-86; and for epitope 4, start and end position is 229-235.)

Table-3.4: Predicted epitope peptide sequences of PspA according to Emini surface accessibility method (red marked are the selected epitopes)

Predicted peptides:

No.	Start Position	End Position	Peptide	Peptide Length
1	40	49	SKAEKDYDAA	10
2	51	56	KDAKNA	6
3	73	86	AQKKYDEDQKKTEE	14
4	130	141	EAKKREEEAKTK	12
5	158	170	ETKKKSEEAKQKA	13
6	185	199	EEAEKKATEAKQKVD	15
7	229	235	SESEDYA	7
8	281	287	AEENNNV	7
9	319	324	EPEKPA	6
10	334	342	APAEQPKPA	9
11	349	398	PAPKPEKPAEQPKPEKTDDQQAEEDYARRSEEEYNRLTQQQPPKAEKPAP	50
12	402	407	TGWKQE	6

Reference: Emini EA, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-s Sep;55(3):836-9.

Scale values: A:0.49, C:0.26, D:0.81, E:0.84, F:0.42, G:0.48, H:0.66, I:0.34, K:0.97, L:0.4, M:0.48, N:0.78, P:0.75, Q W:0.51, Y:0.76

3.1.8.4 Sequence 2, 3, and 4 of the predicted epitope

From the above analysis it is observed that three more sequences were selected from Emini surface accessibility method. The 10, 14 and 7 amino acid sequences posited in 40-49, 73-86 and 229-235 are predicted to have the capabilities to mount Tc, Th and B cell response. Therefore, another three selected sequences are-

2. AQKKYDEDQKKTEE

3. SKAEKDYDAA

4. SESEDYA

All the selected epitopes which have been selected were present in the maximum protein sequences of PspA.

Fig.3.10: FASTA file of PspA where all the selected epitopes are present

3.1.8.5 Epitope cluster analysis

With the help of IEDB analysis resource, four selected epitope cluster results could be found that is presented in the table- 3.5.

Table-3.5: Epitope cluster analysis results

IEDB Analysis Resource

Home | Help | Example | Reference | Download | Contact

Epitope Cluster Analysis Results

Number of epitopes: 4

Number of clusters: 4

Identity threshold: 80%

Cluster number	Number of epitopes in the cluster	Epitope number	Epitope name	Epitope sequence
1	1	1	1	ILTSLASVAILGAGFVASQPTVVRAEESPVASQ
2	1	1	2	AQKKYDEDQKKTEE
3	1	1	3	SESEDYA
4	1	1	4	SKAEKDYDAA

3.1.8.6 Epitope conservancy analysis

Epitope conservancy analysis have been done with the selected epitope sequences with the help of IEDB analysis resource. In all those 4 cases it can be found that maximum identity, minimum identity and percentage of protein sequences matches are 100. This results are summarized in the table- 3.6

Table-3.6: Epitope conservancy analysis results.

IEDB Analysis Resource

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Epitope Conservancy Analysis Result

Epitope #	Epitope name	Epitope sequence	Epitope length	Percent of protein sequence matches at identity <= 100%	Minimum identity	Maximum identity
1	ws-separated-0	ILTSLASVAILGAGFVASQPTVVRAEESPVASQ	33	**100.00% (1/1)**	100.00%	100.00%
2	ws-separated-1	AQKKYDEDQKKTEE	14	**100.00% (1/1)**	100.00%	100.00%
3	ws-separated-2	SESEDYA	7	**100.00% (1/1)**	100.00%	100.00%
4	ws-separated-3	SKAEKDYDAA	10	**100.00% (1/1)**	100.00%	100.00%

3.1.9 PspA is a highly conserved protein

Multiple sequence alignment (MSA) using MEGA 7.0 shows that whole sequences of PspA is a highly conserved protein. All the protein sequences collected and aligned shows no sequence variation. (Fig.3.11). Within this Alignment all the selected epitopes peptide sequences are matched with the sets of PspA whole protein sequences. This results proved that the selected epitope peptides are also highly conserved.

3.1.9.1 ILTSLASVAILGAGFVASQPTVVRAEESPVASQ is a highly conserved epitope

After doing BLAST with the epitope peptide sequence ILTSLASVAILGAGFVASQPTVVRAEESPVASQ in MEGA 7.0, 100% homology was found. This peptide sequence is matched with all the sequences in a set of PspA (Fig. 3.11). The sequence position starts from 7 and end to 39. After the protein name all the identifiers are denoted with the accession number of NCBI.

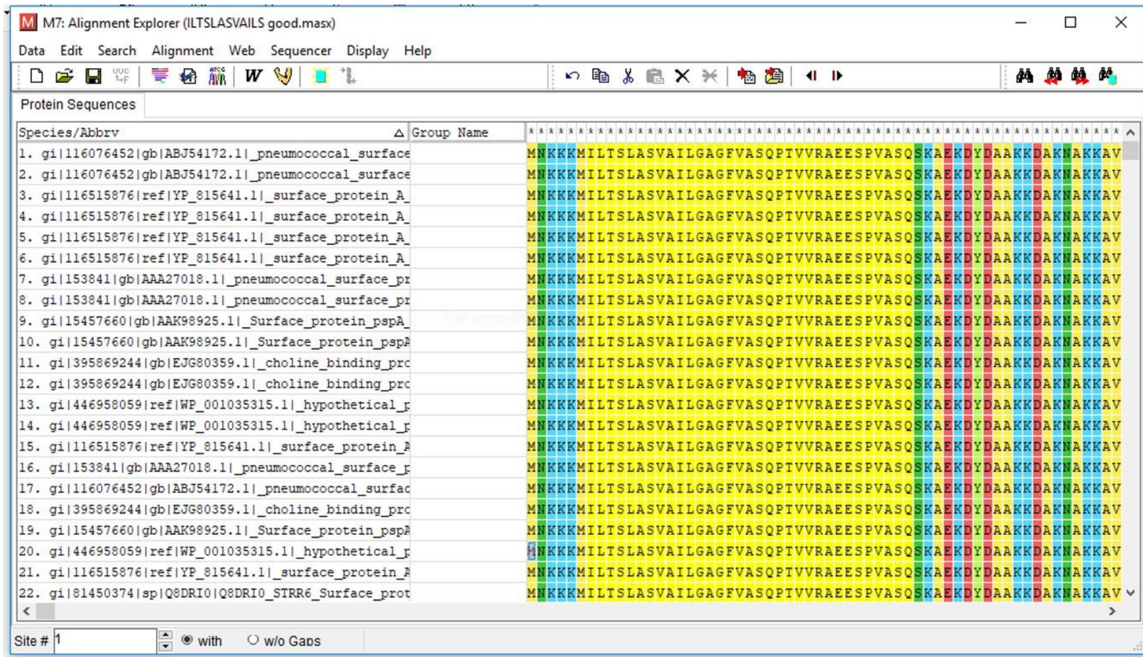

Fig. 3.11: A snapshot of the Multiple Sequence Alignments (MSA) showing conserved region for ILTSLASVAILGAGFVASQPTVVRAEESPVASQ performed by MEGA 7.0.

Fig.3.12: Kolaskar & Tongaonkar Antigenicity Results for ILTSLASVAILGAGFVASQPTVVRAEESPVASQ

3.1.9.10 AQKKYDEDQKKTEE is a highly conserved epitope

The sequences are inspected after BLAST with the sequence of AQKKYDEDQKKTEE. In fig. 3.13 the windows of CLUSTAL W showing the results. In this case also highly conserved region are found that are 100% homolog with the set of protein sequences of PspA. The sequence position starts from 73 and end to 86 (Fig. 3.13).

Fig.3.13: A snapshot of the windows of CLUSTAL W alignment editor in MEGA 7.0 with Multiple Sequence Alignments (MSA) showing conserved region for AQKKYDEDQKKTEE.

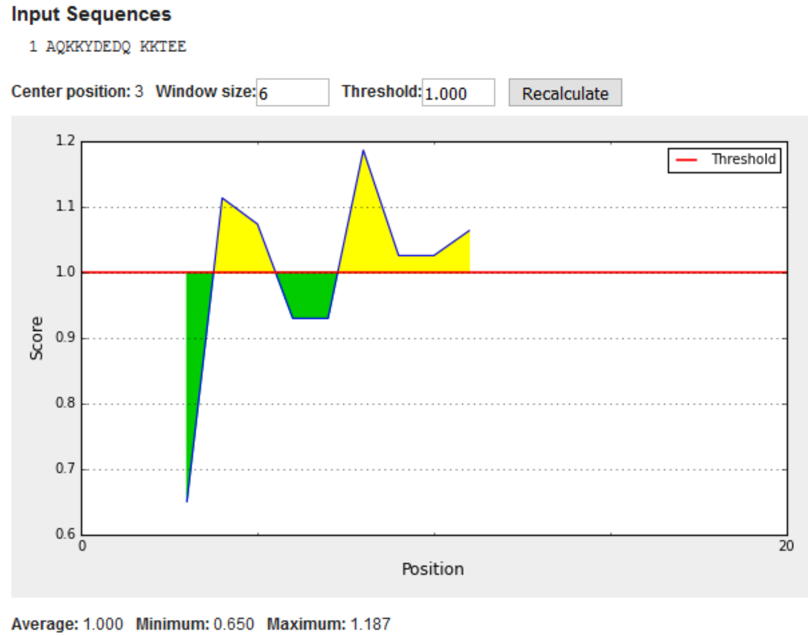

Fig. 3.14: Emini surface accessibility prediction of AQKKYDEDQKKTEE.

3.1.9.11 SKAEKDYDAA is a highly conserved epitope

The sequences are inspected after BLAST with the sequence of SKAEKDYDAA, in fig. 3.15 the windows of CLUSTAL W showing the results. In this case also highly conserved region are found. The sequence position starts from 40 and end to 49 (Fig. 3.15).

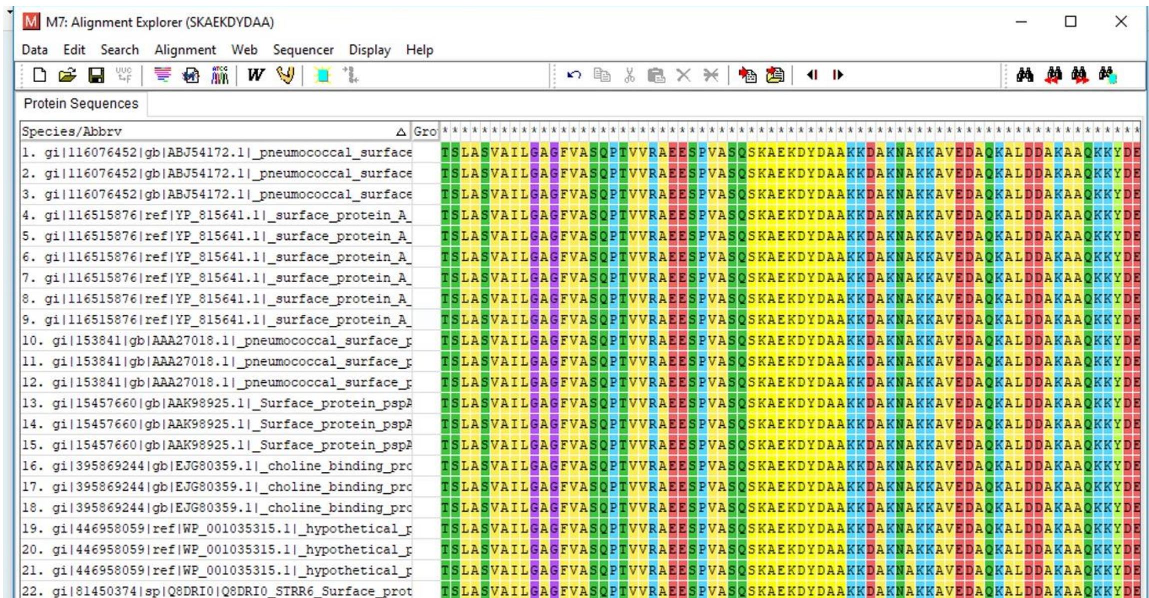

Fig.3.15: A snapshot of the Multiple Sequence Alignments (MSA) showing conserved region for SKAEKDYDAA performed by MEGA 7.0.

Fig. 3.16: Emini surface accessibility prediction of SKAEKDYDAA

3.1.9.12 SESEDYA is a highly conserved epitope

After doing BLAST with the epitope peptide sequence SESEDYA in MEGA 7.0, 100% homology was found. This peptide sequence is matched with all the sequences in a set of PspA. The sequence position starts from 229 and end to 235.After the protein names all the identifiers are denoted with the accession number of NCBI (Fig. 3.17).

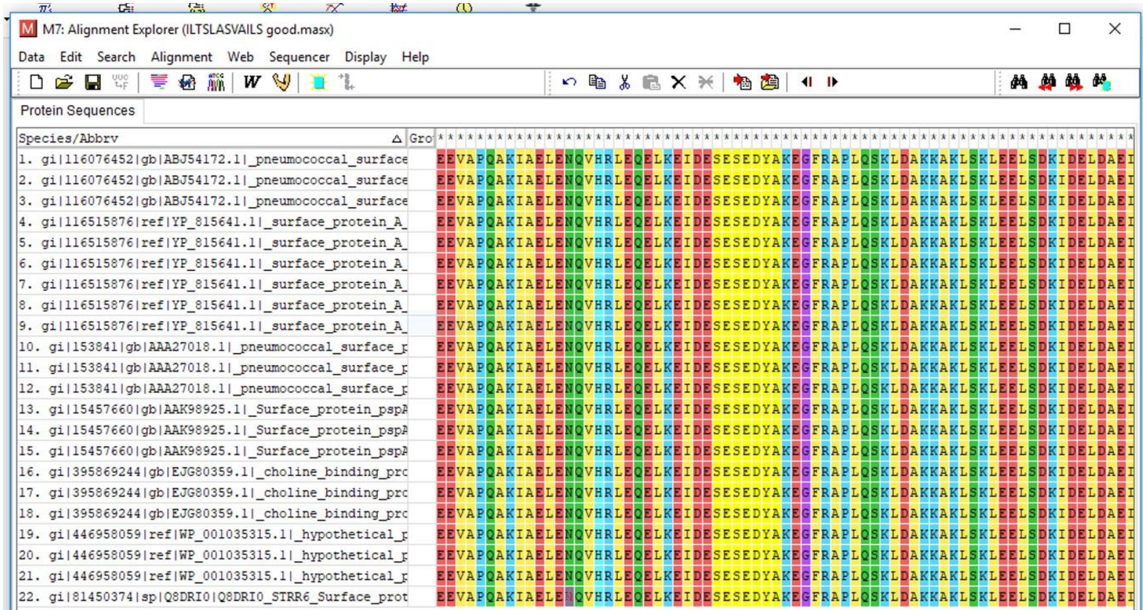

Fig. 3.17: A snapshot of the Multiple Sequence Alignments (MSA) showing conserved region for SESEDYA performed by MEGA 7.0

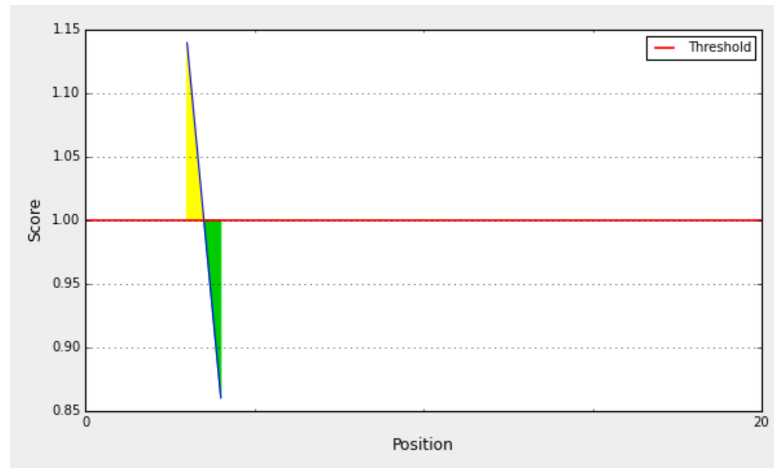

Fig.3.18 – Emini surface accessibility prediction for SESEDYA

3.1.10 Codon optimization of the selected epitope sequences

The present work includes a synthetic polynucleotide sequence that has been optimized for heterologous expression of PspA. The sequences were selected after searching many different serotypes in the Genebank and BLAST. For choosing the sequences, the maximum presence of the same sequence in different serotypes and following IEDB Analysis resource were considered, where we found some predicted peptide sequences.

With this optimization would ensure (in theory) efficient translation of the protein encoded by the transgene. To have an idea on how efficient would be the translation of the original sequence, one can calculate the CAI (Codon Adaptation Index) for the gene of interest according to the codon usage of a particular organism. Redundancy in the genetic code. Synonymous mutations affect protein expression rates up to 1000-fold. Synonymous mutations can also alter protein conformation, PTM, stability, and function.

Codon usage bias refers to differences in the frequency of occurrence of synonymous codons in coding DNA. A codon is a series of three nucleotides (a triplet) that encodes a specific amino acid residue in a polypeptide chain or for the termination of translation (stop codons).

There are 64 different codons (61 codons encoding for amino acids plus 3 stop codons) but only 20 different translated amino acids. The overabundance in the number of codons allows many amino acids to be encoded by more than one codon. Because of such

redundancy it is said that the genetic code is degenerate. The genetic codes of different organisms are often biased towards using one of the several codons that encode the same amino acid over the others—that is, a greater frequency of one will be found than expected by chance. How such biases arise is a much debated area of molecular evolution. Codon usage tables detailing genomic codon usage bias for most organisms in GenBank and RefSeq can be found in the HIVE-Codon Usage Table database.

Codon Optimization:

Introducing synonymous mutations that favor efficient soluble protein expression

```
Optimized  AGTTTTCCAGGTTGAGGTCCGCCCGTT
           || || || || ||||| || || |||
Original   AGCTTCCCGGGATGAGGGCCCCCGGTT
```

The nucleotides of respective peptide sequences which have been obtained after considering codon biasness of *Streptococcus pneumoniae*.

Predicted peptides sequences with back translation

(1) ILTSLASVAILGAGFVASQPTVVRAEESPVASQ

Nucleotide:

ATTTTAACAAGTCTAGCCAGCGTCGCTATCTTAGGGGCTGGTTTTGTTGCG
TCTCAGCCTACTGTTGTA AGAGCAGAAGAATCTCCCGTAGCTAGTCAG

(2) SKAEKDYDAA

Nucleotide:

TCTAAAGCTGAGAAAGACTATGATGCAGCA

(3) AQKKYDEDQKKTEE

Nucleotide:

GCGCAAAAAAAATATGACGAAGATCAAAAGAAAACTGAAGAG

(4) SESEDYA

Nucleotide:

TCTGAATCAGAAGATTATGCT

(Accession No.- NP_357715)

3.1.11 Preparation of constructs

3.1.11.1 Vector Alpha zein fertig

Vector Alpha zein fertig construct contain alpha zein gene. The sequences of *S. pneumoniae* epitopes 1 was placed in fusion with alpha zein. For easy detection, the alpha zein construct (construct alpha) contains a flag- and his-tag. Flag- and His-tags

are important for the localization and determination. FLAG tag having the sequence motif DYKDDDDK (where D=aspartic acid, Y=tyrosine, and K=lysine) which is an artificial antigen (Fig. 3.19). Construct alpha is twice the size of construct gamma (1000bpor36 kD). These constructs were applied to a vector which contained an *E. coli*-origin and a resistance gene for ampicillin. ColE1 is the origin of for E. coli and RK2 is the origin for *A. tumefaciens*. AmpR is the resistance gen against Ampicillin for E. coli and Carbenicilin for *A. tumefaciens*. nptll is the resistance gen against kanamycin (Fig. 3.20, 3.21).

Fig. 3.19: The constructs with alpha zein; "1" is the sequences for the epitops of *S. pneumoniae*. The HIS- and the FLAG-Tag are important for the detection. NcoI, NotI and Xbal are restriction sites.

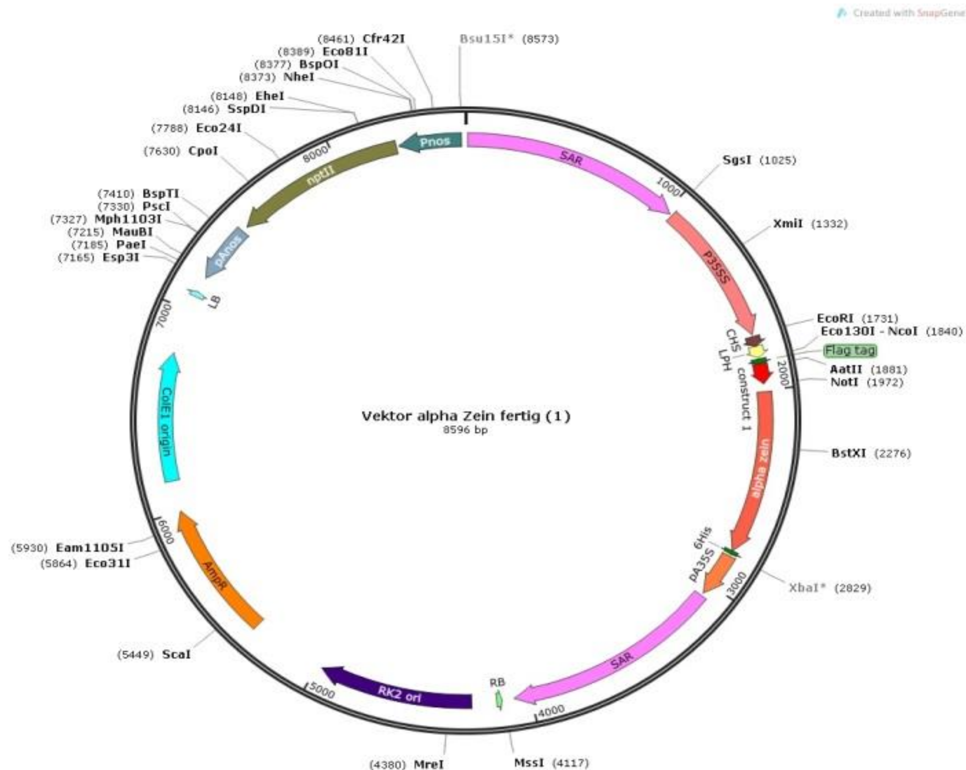

Fig. 3.20: This is an image of the final vector with alpha zein which is transferred into *A. tumefaciens* at the end Ncol, Notl and Xbal are the restriction sites. Construct 1 is the first sequence of *S. pneumoniae*.

18 Features

Feature	Location	Size (bp)	Type
SAR	11 .. 1022	1012	misc_feature
/note = sar			
P35SS	1033 .. 1726	694	misc_feature
/note = 35SS transcription start new			
CHS	1737 .. 1784	48	misc_feature
/note = 5'-UTR from chalcone synthase (petrosinella)			
LPH	1785 .. 1841	57	misc_feature
/note = codon-optimized murine signal peptide of mAb24			
Flag tag	1848 .. 1871	24	CDS
construct 1	1872 .. 1970	99	misc_feature
alpha zein	2010 .. 2807	798	misc_feature
6His	2808 .. 2825	18	CDS
pA35S	2835 .. 3051	217	misc_feature
/note = pA35S			
SAR	3068 .. 4075	1008	misc_feature
/note = sar			
RB	4132 .. 4157	26	misc_feature
/note = right border			
RK2 ori	4272 .. 4980	709	misc_feature
/note = ori for A. tumefaciens			
AmpR	5341 .. 6000	660	CDS
ColE1 origin	6152 .. 6780	629	rep_origin
/direction = RIGHT			
LB	7073 .. 7098	26	misc_feature
/note = left border			
pAnos	7173 .. 7457	285	misc_feature
nptII	7484 .. 8278	795	misc_feature
/note = kanamycin resistance			
Pnos	8291 .. 8577	287	misc_feature

Fig. 3.21: Features of the map of vector Alpha zein fertig which have been performed by SnapGene viewer.

3.1.11.2 Vector gamma zein fertig

Gamma zein fusion are placed in vector gamma zein fertig. The selected sequences of *S. pneumoniae* epitopes 2, 3 and 4 were placed in the N-terminal 90 amino acids of the 27kD gamma zein. The gamma zein construct (construct gamma) contains only the his-tag for easy detection (Fig. 3.22, 23). Construct gamma has about 500 bp which corresponds to 18 kD. All the features are stated in the figure No. 3.24.

Fig. 3.22: The construct with gamma zein; "2, 3, and 4" are the sequences for the epitops of *S. pneumoniae*. The HIS-Tag are important for the detection. NcoI, NotI and Xbal are restriction sites.

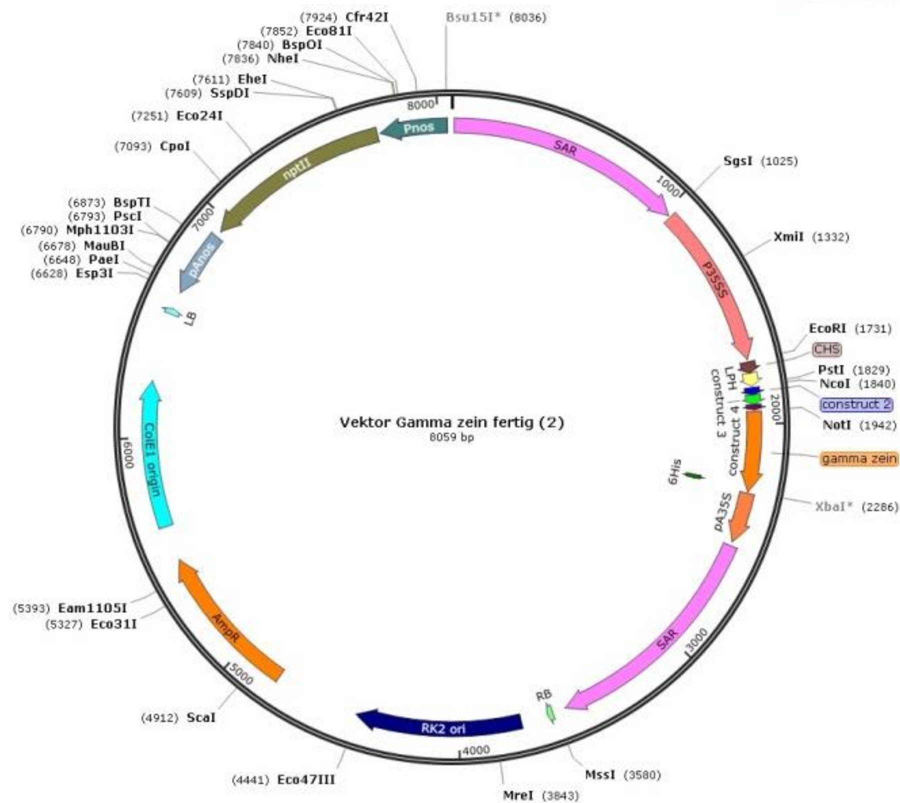

Fig. 3.23: This is an image of the vector with gamma zein. It is quite similar to the vector alpha zein. The differences are: (i) instead of alpha zein there is gamma zein (ii) there are 3 sequences of *S. pneumoniae* and (iii) there is only the his tag available for the localization and determination of the proteins.

19 Features

Feature	Location	Size (bp)	⇄	Type
SAR	11 .. 1022	1012	→	misc_feature
/note = sar				
P35SS	1033 .. 1726	694	→	misc_feature
/note = 35SS transcription start new				
CHS	1737 .. 1784	48	→	misc_feature
/note = 5'-UTR from chalcone synthase (petrosinella)				
LPH	1785 .. 1841	57	→	misc_feature
/note = codon-optimized murine signal peptide of mAb24				
construct 2	1848 .. 1877	30	→	misc_feature
construct 3	1878 .. 1919	42	→	misc_feature
construct 4	1920 .. 1940	21	→	misc_feature
gamma zein	1948 .. 2292	345	→	misc_feature
6His	2262 .. 2279	18	→	CDS
pA35S	2298 .. 2514	217	→	misc_feature
/note = pA35S				
SAR	2531 .. 3538	1008	→	misc_feature
/note = sar				
RB	3595 .. 3620	26	→	misc_feature
/note = right border				
RK2 ori	3735 .. 4443	709	→	misc_feature
/note = ori for A. tumefaciens				
AmpR	4804 .. 5463	660	→	CDS
ColE1 origin	5615 .. 6243	629	→	rep_origin
/direction = RIGHT				
LB	6536 .. 6561	26	→	misc_feature
/note = left border				
pAnos	6636 .. 6920	285	←	misc_feature
nptII	6947 .. 7741	795	←	misc_feature
Pnos	7754 .. 8040	287	←	misc_feature

Fig.3.24. Different features of the map of vector Gamma zein fertig which have been prepared by SnapGene viewer.

3.1.11.3 Vector pTRAkt-glyDs-zen

The cDNA sequence of the red fluorescent protein of *Dictyostelium discoideum* (DsRed) was fused at its N-terminus to the DNA sequence coding the signal peptide and the proline-rich domain (Zera) of γ-zein. The entire chimeric Zera-Ds Red gene was inserted to the plant binary vector pTRA under the control of 35S CaMV promoter and the 35S terminator.

All the selected epitope predicted peptides in both the Alpha zein and Gamma zein have been combinedly introduced into a plant expression vector (pTRA) and

transferred into *Agrobacteria*. This is Ds-red zein gene which also fusion with zein protein. The length of the plasmid is 8655 bps (Fig. 3.25, 3.26).

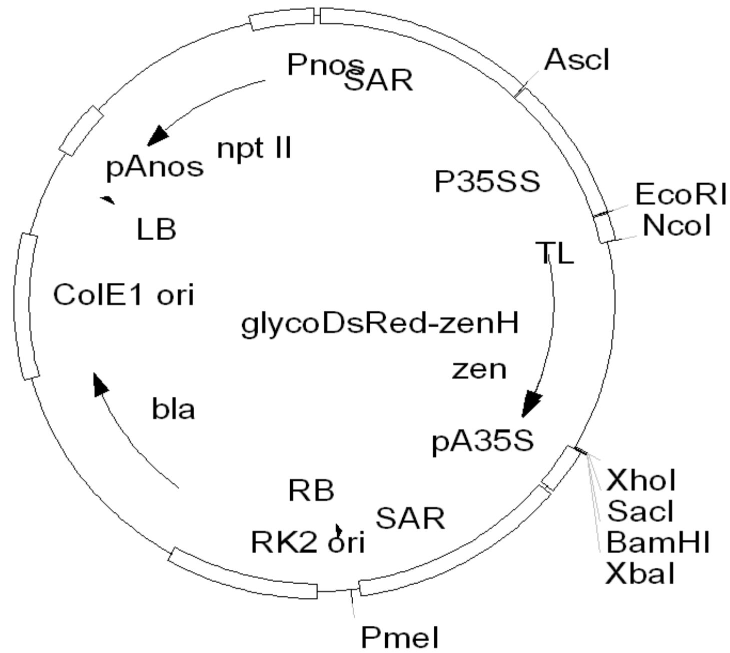

Fig. 3.25: This is an image of the vector pTRAkt-glyDs-zen with Ds-red zein. The size of the circular DNA is 8655bp.

SECentral - [pTRAkt-glyDs-zenH (8655bps)]

File View Clone Map Operations Window Help

Molecule Definition:

Name: pTRAkt-glyDs-zenH Size: 8655 bp circular

Description: Ligation of gdzh into kt

Notes: Vector molecule: kt

Molecule Features:

Name	Start	End		Description
SAR	11	1022		scaffold attachment region of the tobacco RB7 gene (GenBank U67919)
P35SS	1033	1726		CaMV 35S promoter with duplicated transcriptional enhancer
TL	1736	1867		tobacco etch virus 5'UT
glycoDsRed-...	1868	2878		
zen	2546	2848		gamma zein n-terminus
deletion d24	2813	2836		missing in glyDs-zend24-AH
pA35S	2895	3111		CaMV 35S polyadenylation signal
SAR	3128	4135		scaffold attachment region of the tobacco RB7 gene (GenBank U67919)
RB	4217	4192	C	right border
RK2 ori	4332	5040		ori for A.tumefaciens
bla	5203	6063		Ampicillin resistance E.coli / Carbenicillin resistance A.tumefaciens
ColE1 ori	6132	6824		ori for E.coli
LB	7133	7158		left border
pAnos	7251	7504		nopaline synthase gene polyadenylation signal
npt II	8338	7544	C	Kanamycin resistance plant
Pnos	8636	8339	C	nopaline synthase gene promoter

Fig. 3.26: Different features of vector pTRAkt-glyDs-zen

3.1.12 Results of target-specific primers design

In this investigation, a new software tool called SnapGene viewer and Primer-BLAST were used to alleviate the difficulty in designing target-specific primers. These tool combines BLAST with a global alignment algorithm to ensure a full primer-target alignment and is sensitive enough to detect targets that have a significant number of mismatches to primers. Finally Primer-BLAST were used as it allows users to design new target-specific primers in one step as well as to check the specificity of pre-existing primers. Primer-BLAST also supports placing primers based on exon/intron locations and excluding single nucleotide polymorphism (SNP) sites in primers.

The selected Primers:

Finally the primers that have been selected for those of the target gene e.g. Alpha zein, Gamma zein and Ds-red are given bellow-

3.1.12.1 Primers prepared and used in this investigation

Alpha zein:

1. Long Primer

Primer type	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	
Forward primer	ATCCTGGGTGCTGGTTTTGT	Plus	20	139	158	59.81	50.00	3.00	0.00
Reverse primer	TTGCTGTTGCAATTGTGCGA	Minus	20	476	457	59.90	45.00	6.00	3.00
Product length	338 bp								

2. Short primer

Primer type	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACGTCCCTAGCCTCCGTA	Plus	19	6	24	60.15	63.16	6.00	2.00
Reverse primer	GGACTTTCTTCAGCCCGAAC	Minus	20	86	67	58.84	55.00	5.00	3.00
Product length	81 bp								

3. Alpha zein nptll:

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGATATTCGGCAAGCAGGCA	Plus	20	206	225	60.11	50.00	4.00	0.00
Reverse primer	TGCTCGACGTTGTCACTGAA	Minus	20	556	537	59.90	50.00	5.00	3.00
Product length	351 bp								

Gamma zein:

4. Long primer

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGAAAGACTACGACGCTGCC	Plus	20	25	44	60.11	55.00	3.00	1.00
Reverse primer	GAGAGGGATGCGGTTGTTGA	Minus	20	411	392	60.04	55.00	2.00	1.00
Product length	351 bp								

5. Short primer

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCAAGGCAGAGAAAGACTACGA	Plus	22	2	23	59.77	50.00	3.00	0.00
Reverse primer	TCTGATTCGCTTTCCTCTGTTTTC	Minus	24	83	60	59.55	41.67	3.00	0.00
Product length	82 bp								

6. Gamma zein nptll

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATGCGATGTTTCGCTTGGTG	Plus	20	373	392	59.83	50.00	4.00	0.00
Reverse primer	TTGTCACTGAAGCGGGAAGG	Minus	20	547	528	60.25	55.00	5.00	0.00
Product length	175 bp								

Ds-red zen:

7. Ds-red zen

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAGTTCATGAGGTTTAAGGTTCG	Plus	23	7	29	57.47	43.48	6.00	2.00
Reverse primer	CCCAAGCAAATGGCAAAGGT	Minus	20	154	135	59.60	50.00	3.00	0.00
Product length	148 bp								

8. Ds-red zen nptll

	Sequence (5'->3')	Template strand	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCCTGATGCTCTTCGTCCAG	Plus	20	60.18	60.00	3.00	1.00
Reverse primer	TGCTCGACGTTGTCACTGAA	Minus	20	59.90	50.00	5.00	3.00
Product length	253 bp						

3.2 *Agrobacterium*-mediated genetic transformation

An initiative have been taken to develop an edible vaccine through *Agrobacterium* mediated genetic transformation. Pneumococcal Surface Protein Antigen (PspA) were selected in this attempt, as it is the major virulence factor of *S. pneumoniae* causing pneumococcal disease. The sequences of PspA B-cell epitopes were analyzed and placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, and combinedly. Three constructs were produced using three different inserts namely alpha, gamma and Ds-red construct. *Agrobacterium* strain LBA4404 containing plasmid harboring *npt*II (neomycin phosphotransferase) was used for selection in all cases for the integration of gene of interest. Antigens of *S. pneumoniae* fused to either α-zein, γ-zein or Ds-red zein in *N. tabacum* and *L. esculentum* were done through *Agrobacterium*-mediated genetic transformation.

This study was carried out in three phases. In the first phase, several computational studies were conducted to constructs the plasmid vector carrying the sequences of B-cell epitope predicted peptide sequences fusion with zein protein.

In the second phase, a series of experiments were conducted for genetic transformation using three different genetically engineered construct in an *A. tumefaciens* LBA4404. They were namely LBA4404 harboring plasmid Vector Alpha zein fertig (considering as Alpha zein), Vector gamma zein fertig (considering as Gamma zein) and LBA4404 harboring plasmid pTRAkt-glyDs-zen (considered as Ds-red zein).

For successful transformation purpose two plants were selected in this research. One is *N. tabacum*, which were used as an experimental model and another is *L. esculentum* as the main candidate. All these constructs mainly used to integrate the gene of interest into the experimental plants. The constructs contained a marker *npt*II (neomycin phosphotransferase) as selectable marker gene.

For the establishment of an efficient *Agrobacterium*-mediated transformation protocol further experiments were carried out using three constructs for optimization of various parameters required for *Agrobacterium*-mediated genetic transformation including the

optical density of *Agrobacterium* suspension, suitable incubation and co-cultivation periods required for effective transformation. Beside these experiments the concentration of selectable agent (kanamycin) was optimized to obtaining required transformed plantlets. Experiments were also conducted to regenerate plantlets from *Agrobacterium* infected explants. Through proper selection procedures the transformed shoots were selected and rooted properly and then the plantlets were established in soil following optimum hardening. Further molecular characterization of transformed plantlets was carried out through Polymerase chain reaction (PCR) and Western blot analyses. The results of the present study obtained from different experiments are presented in the following sections. And finally in the third phase of this research work, mice model system was used to analyze the immunogenicity and protective efficacy of the predicted peptide sequences. Assessment of immunogenicity as well as appropriate interpretation of immunogenicity data is therefore, of critical importance for assaying of the transgenic plants for the purpose of their effectiveness testing.

3.2.1 *In vitro* regeneration of plantlets

A transformation compatible *in vitro* regeneration system is a prerequisite for the development of successful genetically engineered plants. The success of *in vitro* regeneration is greatly influenced by the age of tissue or organ that is used as initial explant. Explants taken from juvenile plant tissues, particularly from seedlings are highly responsive. In the present study, as the explants immature leaflet, node, cotyledonary leaf, cotyledonary node and hypocotyl were used to develop transformation compatible *in vitro* regeneration system. The explants were obtained from aseptically germinated seeds. Data was recorded by estimating the number of shoots per explants, number of responsive explants, as well as the days required for initiation of shoots from explants. After successful shoot development, the *in vitro* raised shoots were cultured for *in vitro* root induction. Rooted plantlets were consequently acclimatized in soil following proper hardening and allowed to grow under field conditions.

3.2.2 *In vitro* regeneration of shoots

Various explants of *N. tabacum* and *L. esculentum* were used for *in vitro* regeneration of shoots on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of auxins and cytokinins. The results of these experiments are described under the following headings.

3.2.3 Preparation of seed derived explants for *in vitro* regeneration

Seeds were surface sterilized with 70% alcohol and 0.1% $HgCl_2$ solution was used to maintain the aseptic condition and to avoid unwanted contamination of explants. Healthy seeds were dipped into 70% alcohol for one minute and then sterilized with 0.1% $HgCl_2$ solution for 8 - 10 minutes. Afterwards the seeds were rinsed with sterilized distilled water for 4 - 5 times to remove the traces of $HgCl_2$. For obtaining immature leaflet explants surface sterilized seeds were germinated in conical flasks containing autoclaved cotton soaked with sterile distilled water and in MS media in both cases. In both of the cases germination undertaken in water soaked cotton bed whereas, using MS media no or very poor germination takes place (Fig. 3.27 & 3.28). Using water soaked cotton germination of seeds in both plants required 4 - 7 days (Table 3.7). In case of tobacco, leaflet explants were collected from 6 - 8 days old seedlings (Fig. 3.28). In case of tomato cotyledonary leaf, cotyledonary node and hypocotyl were considered as the co-cultivation part.

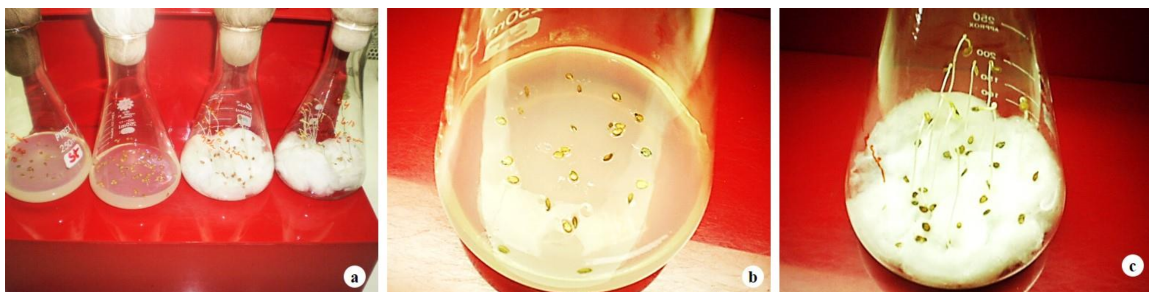

Fig. 3.27 (a-c): Germinating seeds of *L. esculentum* on both MS media and water soaked cotton bed.

Fig. 3.28: Germinating seeds of *N. tabacum* on water soaked cotton bed

Table-3.7: Germination of seeds of *N. tabacum* and *L. esculentum* on sterile cotton bed soaked with sterile distilled water.

Plants	No. of seeds inoculated	No. of seeds germinated	% of germination	Days required for seed germination
N. tabacum	250	150	60%	4 – 7
L. esculentum	50	25	50%	5 – 7

3.2.4 Effect of age of immature leaflet explants towards *in vitro* regeneration

It was observed that the age of the immature leaflet explants played vital role towards *in vitro* regeneration of shoots. In case of regeneration of shoots from leaflet explants, it was found that in most cases shoot regeneration was seriously influenced by the age of leaflet. Both the plant seeds took 4 - 7 days for germination (Table 3.7). It was found that leaflet explants collected from early germinating seeds (4 - 5 days) was not suitable for initiation of shoots (Fig. 3.29). On the other hand it was also noticed that leaflet explants collected from delayed germinated seeds (10-12 days) were also not suitable for *in vitro* regeneration. The leaflet explants collected from longer period of germinated seeds produced more callus rather than initiation of shoots. Leaflets explants collected from 7

days old germinated seed of tobacco showed best result towards shoots regeneration. But in case of Tomato, it took 8 days to collect the most responsive leaflet explants from the germinated seeds. Results of these experiments have been shown in fig. 3.29. It was also noticed that the best responsive young leaflet explants were characterized with light gree or yellowish mid rib. Different stages of germinated seeds of tobacco and tomato in collecting the leaflet explants are presented in fig. 3.29.

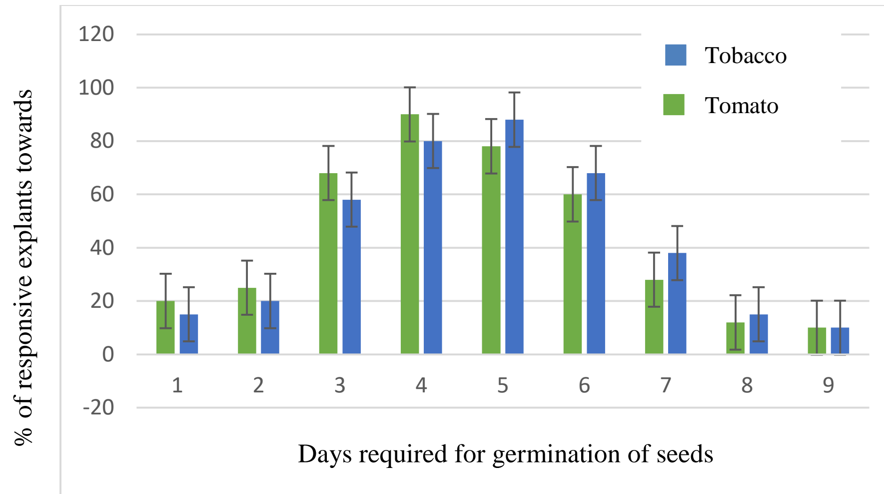

Fig.3.29: Effect of germination period of tomato and tobacco seeds in collecting the immature leaflet explants towards regeneration of shoots.

3.2.5 Effect of culture medium and hormonal supplements towards regeneration of shoots from different explants

Specific hormonal supplements require in each type of explants for the initiation of organogenesis from *in vitro* grown cultures. Composition of culture medium is always considered as an important factor for the successful *in vitro* regeneration from any explant. In the present investigation, MS medium with different concentrations and combinations of hormonal supplements were used for induction and elongation of shoots. For this purpose, MS medium with different hormonal supplements such as BAP, NAA and NAA were used to obtain the proper initiation of shoots from immature leaflet, cotyledonary leaf, cotyledonary node and hypocotyl explants for both tomato and

tobacco. The explants of immature leaflet and cotyledonary leaf, cotyledonary node and hypocotyl produced adequate responses towards the initiation and development of shoots. In the present set of experiments it was noticed that cotyledonary node explants did not show any response towards regeneration on MS medium containing the above mentioned hormonal supplements.

Fig. 3.30: Survived cotyledonary leaf and hypocotyl of *L. esculentum*

Fig. 3.31: Survived leaflets of *N. tabacum* subcultured on regeneration media

3.2.6 Effect of different concentrations of BAP on regeneration of shoots from immature leaflet explants of *N. tabacum*.

MS medium supplemented with different concentrations of BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l) were employed to examine their effects on initiation of regeneration

and development of shoots from immature leaflet explants of *N. tabacum*. The results of experiment have been presented in Table 3.8.

From the immature leaflet explants, regeneration was initiated through the intervention of callus (Fig. 3.32 b). Induction of callus was initiated after 9 - 16 days of inoculation of leaflet explants in MS medium supplemented with BAP. Occasionally leaflet explants produced shoots directly without the formation of callus. The best regeneration response via callus formation was observed on MS medium with 2.0 mg/l BAP. In most of the cases 10 -15 days were required for the induction of callus regeneration. Shoot buds were found to initiate within the developed callus on the same hormonal supplements. Numerous shoot buds as well as elongation of previously formed shoots were found to initiate from the induced calli within 105 days of culture (Fig. 3.32 c and d). Highest mean number of shoots (9.0) was obtained on MS media supplemented with 2.0 mg/l BAP.

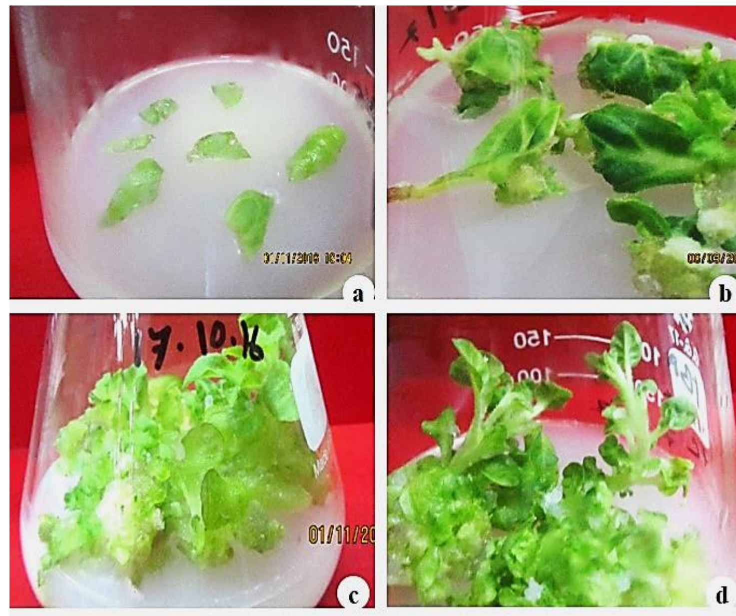

Fig. 3.32 (a-d): Different stages of shoot formation from leaflet explants of *N. tabacum* on MS medium supplemented with different concentrations of BAP; (a) Inoculation of leaflets, (b) Initiation of shoots via callus formation from leaflet explants on MS supplemented with 2.0 mg/l BAP, (c) Multiple shoots formation, (d) Elongated multiple shoots on the same medium.

Table 3.8. Response of leaflet explants towards multiple shoot regeneration using different concentrations of BAP in MS medium in case of *N. tabacum*.

Conc. of BAP (mg/l)	No. of explants inoculated	% of responsive explants towards callus formation	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
1.0	45	86.66	10 - 15	66.66	5.16
1.5	45	88.88	9 - 15	60.00	6.4
2.0	**45**	**91.11**	**10 - 15**	**80.00**	**9.0**
3.0	45	93.33	10-15	72.00	8.6
3.5	45	93.33	10 - 15	75.00	8.5
4.0	45	91.11	11 - 16	60.00	6.0

3.2.7 Effect of different concentrations of BAP towards regeneration of shoots from immature leaflet explants of *L. esculentum*.

MS medium with different concentrations of BAP (0.50, 1.00, 1.50, 2.00 and 2.50 mg/l) were employed to examine their effects on initiation of regeneration and development of shoots from immature leaflet explants obtained from *L. esculentum*. Similar to *N. tabacum* multiple shoot buds initiated from the callus of the leaflet explants. The results of this experiment are presented in Table 3.9. The best regeneration responses were observed on MS medium with 2.0 mg/l BAP where 12 - 16 days were required for initiation of callus. Multiple shoots were found to initiate from the developed callus on the same media. Numerous shoot buds and elongation of previously formed shoots were initiated within 105 days of culture. In this concentration of BAP 73.33% explants were responded towards shoot initiation from the callus and highest mean number of shoots per explants were 8.5 in case of *L. esculentum*. Different stages of shoot regeneration on this hormonal combination of BAP are shown in Fig. 3.33 (a-d). In this concentration of

BAP rate of elongation of shoots was comparatively better than that of *N. tabacum* (Fig. 3.32d).

Fig. 3.33 (a-d): Different stages of shoot regeneration from leaflet explants of *L. esculentum* on MS medium supplemented with various concentrations of BAP. (a) Initiation of multiple shoots on MS medium containing 2.0 mg/l BAP; (b) Development of multiple shoots on the same medium; (c) Elongation of multiple shoots on the same medium; (d) Multiple shoot formation and elongation of shoots on MS medium containing 2.0 mg/l BAP.

Table- 3.9: Effect of different concentrations of BAP containing MS medium for multiple shoot regeneration from leaflet explants in case of *L. esculentum*.

Conc. of BAP (mg/l)	No. of explants inoculated	% of responsive explants towards callus formation	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
0.5	45	93.33	10 - 16	60.00	4.25
1.5	45	86.66	10 - 16	75.00	5.8
2.0	**45**	**88.88**	**12 - 16**	**73.33**	**8.5**
2.5	45	91.11	11 - 16	62.50	8.2
3.0	45	88.88	12 - 17	53.33	5.6

3.2.8 Combined effect of different concentrations and combinations of BAP and NAA towards shoot regeneration from immature leaflet explants of *N. tabacum*.

MS medium supplemented with different concentrations and combinations of BAP (1.0 to 2.0 mg/l) and NAA (0.10 to 0.20 mg/l) were employed to examine their effects on callus induction and shoot development from leaflet explants of *N. tabacum*. Satisfactory growth was found after using BAP and NAA combinedly (Fig. 3.34 a-d). In this experiment, a total of four different combinations of BAP and NAA were tested. Results of these experiment have been presented in Table 3.10.

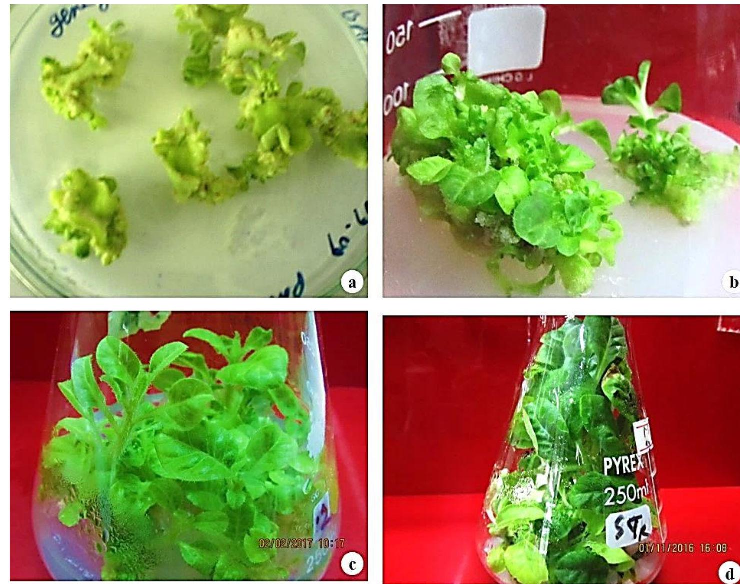

Fig. 3.34 (a-d): Different stages of shoot development from leaflet explants of *N. tabacum* on MS medium supplemented with innumerable concentration of BAP and NAA; (a) Tiny shoot bud formation on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA, (b) Multiple shoots formation via callus formation on same medium, (c) Elongated multiple shoots developed on the same medium, (d) Multiple shoot bud formation along with elongated shoots via callus formation on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA.

Table 3.10. Combined effect of different combination of BAP and NAA towards multiple shoot regeneration using leaflet explants of *N. tabacum*.

Conc. of hormones (mg/l) BAP + NAA	No. of explants inoculated	% of responsive explants towards callus induction	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
1.0 + 0.10	50	94	10 - 16	55.0	6.2
1.5 + 0.10	50	92	14 - 18	66.66	7.4
2.0 + 0.2	50	90	12 - 17	65.5	6.5
2.0 + 0.20	**50**	**96**	**10 - 15**	**90.00**	**9.0**

The best regeneration responses towards callus formation as well as shoot regeneration was observed when leaflet explants were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA. In this hormonal combinations of BAP and NAA, 96% explants produced callus and from the callus producing explants 90.00% was found to initiate shoots within 60 - 75 days of culture. The mean number of shoots/explant was 9.0 on 2.0 mg/l BAP and 0.2 mg/l NAA supplemented media after 3.5 months of culturing of the explants. Different stages of regeneration (shoot bud formation, proliferation and multiplication of shoots) of shoots using the combination of 2.0 mg/l BAP and 0.2 mg/l NAA on MS medium are shown in Fig. 3.34 (a-d). Regenerated shoots were sub-cultured on the same medium containing the same hormonal supplements for their multiplication and elongation at regular intervals of four weeks. In each subculture newly developed shoot buds were observed along with the elongated shoots. MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA also showed good response towards multiple shoots formation (Fig. 3.34d). In this hormonal combination of 2.0 mg/l BAP and 0.2 mg/l NAA 66.66% explants was found to initiate shoots within 60-75 days of culture and the mean number of shoots/explant was 7.4 (Table 3.10).

3.2.9 Effect of various combinations of BAP and NAA on shoot regeneration from immature leaflet explants of *L. esculentum*.

In case of *L. esculentum*, MS medium supplemented with different concentrations and combinations of BAP (2.0, 4.0 and 6.0 mg/l) and NAA (0.1and 1.0 mg/l) were applied to observed their effects towards initiation of shoots using leaflet explants. Six different combinations of above mentioned hormonal supplements were employed to induce callus as well as regeneration of shoots. Results of this experiment has been shown in Table 11. It was observed that in all the hormonal combinations of BAP and NAA, all leaflet explants produced very poor number of shoots. This plant required 6 - 16 days for initiation of callus at various concentrations of BAP and NAA. Among the various combinations maximum shoot regeneration was observed on MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA (Fig. 3.35a and 3.35b). In this combination of BAP and NAA 40% explants showed initiation of shoot and mean number of shoots per explants was recorded to be 4.0. It was observed that when explants were cultured on MS with higher BAP and NAA supplemented medium the explants produced large amount callus instead of producing shoots (Fig. 3.35c and 3.35d).

Fig. 3.35 (a-d): Different stages of shoot development from leaflet explants of *L. esculentum* on MS medium supplemented with innumerable concentration of BAP and NAA; (a) Tiny shoot bud formation on MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA (b) Multiple shoots formation via callus formation on same medium (c) Elongation started on the same medium (d) Multiple shoot bud formed.

Table 3.11. Response of leaflet explants from Tomato towards multiple shoot regeneration using different concentrations of BAP and NAA in MS medium.

Conc. of hormones (mg/l) BAP+NAA	No. of explants inoculated	% of explants formed callus	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
2.0 + 0.1	**45**	**95.55**	**6 - 8**	**40.00**	**3.8**
3.0 + 0.1	45	95.55	7 - 10	40.00	3.5
4.0 + 0.5	45	93.33	8 - 11	33.00	2.0
4.5 + 0.5	45	93.33	10 - 14	30.00	3.0
5.0 + 1.0	45	95.55	11 - 16	25.00	1.75
6.0 + 1.0	45	88.88	10 - 16	20.00	2.25

3.2.10 Combined effect of BAP and Kn towards multiple shoot formation from immature leaflet explants of *L. esculentum*.

In case of *L. esculentum*, MS medium supplemented with different concentrations and combinations of BAP (0.5 to 2.0 mg/l) and Kn (0.1 and 0.2 mg/l) were used to observed their effects on induction of callus as well as initiation of shoot from leaflet explants. Results of this set of experiment have been presented in Table 3.12. More or less 88-94% of cultured leaflet explants showed response towards callus initiation depending on different concentrations of hormonal supplements. Afterwards shoots were initiated from the induced callus depending on the nature of hormonal supplements. Among the various concentrations, the best regeneration response towards shoot formation was observed on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l Kn. In this hormonal combination 75% explants showed shoot initiation and mean number of shoots/explant was recorded as 7.2 after 3.5 months of culture (Fig. 3.36a).

Regenerated shoots were sub-cultured on the same medium with same hormonal combinations for their multiplication and elongation at regular intervals of four weeks (Fig.3.36). Moreover from the table 3.12, it is observed that in the hormonal

combination of 2.0 mg/l BAP and 0.2 mg/l Kn, 62% explants showed shoot initiation within 65 - 75 days of culture and the mean number of shoots/explant was 7.0. MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l Kn as well as MS with 1.5 mg/l BAP and 0.2 mg/l Kn showed 58% and 78% shoot regeneration responses, respectively where mean number of shoots/explant was 6.0 in both the combinations (Fig. 3.36d, table-12).

Fig.3.36 (a-d): Different stages of shoot formation from leaflet explants of *L. esculentum.* on MS medium supplemented with different concentration of BAP and Kn; (a) Shoot formation from the callus on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l Kn, (b) Multiple shoot formation on the same medium (c) Elongated multiple shoots developed on the same medium (d) Fully developed multiple elongated shoots.

Table 3.12. Effect of different concentrations of BAP and Kn in MS medium for multiple shoot regeneration in case of *L. esculentum* from leaflet explants.

Conc.of hormones (mg/l) BAP + Kn	No. of explants inoculated	% of responsive explants	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
0.5 + 0.1	50	90	12 - 18	58	6.0
1.0 + 0.1	50	90	12 - 16	62	7.0
1.5 + 0.2	50	88	12 - 16	78	6.0
2.0 + 0.2	**50**	**94**	**10 - 16**	**75**	**7.2**

3.2.11 Effect of different concentration and combinations of BAP and Kn on regeneration of shoots from immature leaflet explants of *N. tabacum*

To evaluate the combined effect of BAP and Kn towards initiation of shoots from the leaflet explants of *N. tabacum*, six different hormonal combinations were applied. For this purpose MS medium with different concentrations and combinations of BAP (2.0, 4.0 and 6.0 mg/l) and Kn (0.1and 1.0 mg/l) were used to induce callus and consequent development of shoots. Table 3.13 is presented to elaborate the results of this experiment. In the present experiment it was noticed that all leaflet explants showed early response towards callus induction but percentage of shoot formation was low in all the combinations of BAP and Kn compare to BAP alone (Table 2) and in combination of BAP and NAA (Table 4). About 6 - 14 days were required for callus induction at various concentrations of BAP and Kn. Among the various combinations tested maximum response towards shoot regeneration was observed on MS medium supplemented with 4.0 mg/l BAP with 0.5 mg/l Kn (Fig. 3.37a and 3.37b). In this combination 38.88% explants showed shoot initiation response where mean number of shoots per explants were 4.0. The mean number of shoots per explant was found to vary from 2.0 to 4.0 when explants were cultured on the other BAP and Kn supplemented medium (Fig. 3.37c and Table 3.13). It was observed that in many cases when the explants were cultured on MS with various BAP and Kn supplemented medium each explants produced large amount of callus rather than formation of shoots compared to other combination of BAP alone and in combination of BAP and Kn (Fig. 3.37d).

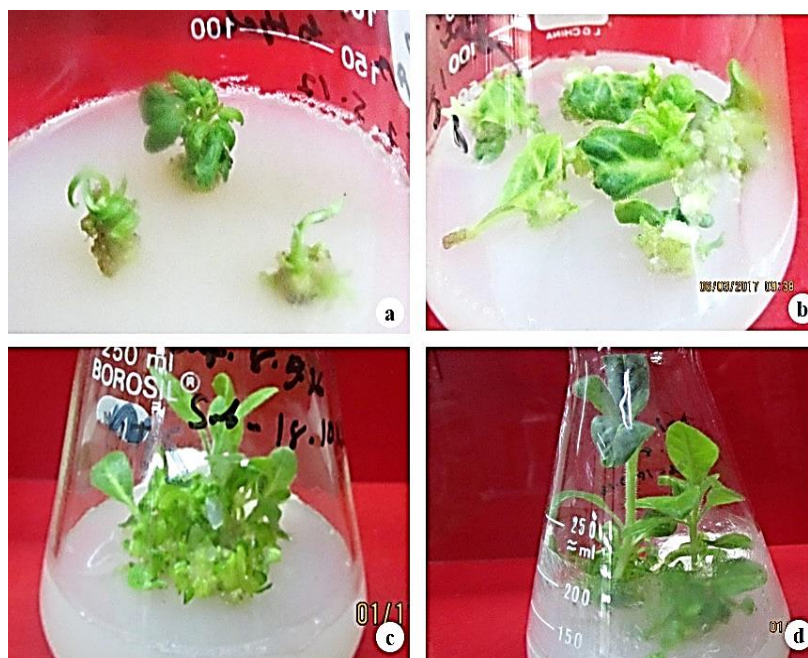

Fig. 3.37 (a-d): Different stages of shoot formation from leaflet explants of *N. tabacum.* on MS medium supplemented with different concentration of BAP and Kn. (a) Shoot formation from the callus (arrow) on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l Kn; (b) Multiple shoot formation on the same medium; (c) Elongated multiple shoots developed on the same medium; (d) Fully developed multiple elongated shoots.

Table 3.13- Response of leaflet explants towards multiple shoot regeneration using different concentrations of BAP and Kn in MS medium in case of *N. tabacum*.

Conc.of hormones (mg/l) BAP+ Kn	No.of explants inoculated	% of callus producing explants	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
2.0 + 0.1	45	97.77	6 – 9	26.66	2.4
3.0 + 0.1	45	95.55	7 – 9	20.00	2.6
4.0 + 0.5	**45**	**88.88**	**8 – 12**	**38.88**	**4.0**
4.5 + 0.5	45	95.55	8 – 13	33.33	3.0
5.0 + 1.0	45	91.11	8 – 14	38.88	2.5
6.0 + 1.0	45	93.33	9 – 14	30.00	2.0

3.2.12 Effect of different growth regulators on induction of roots from regenerated shoots

Induction of root at the base of *in vitro* regenerated shoots is a vital step for the production of plantlets. Generally spontaneous formation of roots was not observed from the *in vitro* regenerated shoots of tobacco whereas in case of tomato that could be found. For the induction of roots various concentrations of three different auxins, namely, IAA, IBA and NAA were used in the present study. Approximately 2.5 - 4.0 cm long *in vitro* derived shoots were excised and cultured on half strength of MS medium containing various concentrations of the above mentioned auxins. Rooted plantlets from all three varieties were transplanted to soil in small plastic pot for their establishment.

3.2.13 Effect of different concentration of IBA on half strength of MS for induction of roots in *N. tabacum and L. esculentum.*

Half strength of MS media with three different concentrations of IBA (0.1, 0.25 and 0.5 mg/l) were used to examine their effects for the induction of roots from the *in vitro* regenerated shoots of *N. tabacum* and *L. esculentum*. Results on root induction using different concentrations of IBA have been shown in Table 3.14. High frequency of root induction in both *N. tabacum* and *L. esculentum* was observed on half strength of MS medium containing 0.5 mg/l IBA (Fig. 3.38 and 3.39). It was recorded that 85% regenerated shoots of tomato and that of 80% of tobacco formed well developed roots on 0.50 mg/l IBA supplemented media. Root initiation was noticed within 8-20 days of culture while it took about 22-30 days were required to get fully developed roots in both the varieties. The number of roots/shoot was 7-14 in case of tomato and that of 6-10 for tobacco (Fig. 3.38). Initiation of roots was started within 7-16 days of culture and maximum numbers of roots were found to form on both 0.25 mg/l and 0.50 mg/l IBA supplemented half strength of MS medium (Fig. 3.38a-d). It was observed that in IBA supplemented media roots were initiated directly at the base of cut end of *in vitro* regenerated shoots without the formation of any callus. Some calli were occasionally

found to form at the cut end after 20 – 35 days of culture maximum in case of tomato (Fig. 3.39).

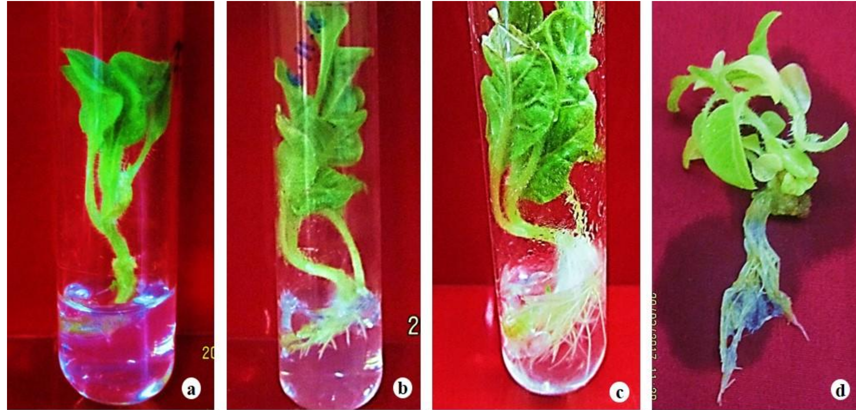

Fig. 3.38 (a-d): Formation on roots from the cut ends of the *in vitro* regenerated shoots on half strength of MS medium containing various concentrations of IBA in case of *N. tabacum*. (a & b) Initiation of roots from the shoots; (c) Fully developed roots; (d) Formation of fully developed roots at the base of cut end.

Fig. 3.39 (a & b): Formation on roots from the cut ends of the *in vitro* regenerated shoots on half strength of MS medium containing IBA *L. esculentum*. (a) Initiation of roots from the shoots; (b) Formation of fully developed roots at the base of cut end.

Table 3.14. Effect of different concentration of IBA on half strength of MS towards formation of roots from regenerated shoots in *N. tabacum* and *L. esculentum*.

Varieties	Conc. of IBA (mg/l)	No. of shoot inoculated for root induction	% of shoots forming roots	Days required to initiate roots	Days required to get well developed roots	No. of roots/ shoot
N. tabacum	0.1	20	60	10 - 22	22 - 30	3 - 7
	0.2	20	80	9 - 20	20 - 28	6 - 11
	0.5	**20**	**80**	**8 - 20**	**22 - 30**	7 - 14
L. esculentum	0.1	20	55	9 - 18	20 - 30	5 - 6
	0.2	20	75	10 - 22	25 - 30	5 - 10
	0.5	20	85	9 - 20	25 - 30	6 - 10

3.2.14 Transplantation of rooted plantlets into the soil

The plantlets with well-developed roots of *N. tabacum* and *L. esculentum* were successfully transplanted into small plastic pots containing autoclaved soil (Fig. 3.40a and 3.40a,b). The transplantation procedure has been described in the materials and methods section. Using this method the survival rate of the transplanted plantlets was found to be about 95% in case of *N. tabacum* and it was 90% in case of *L. esculentum* (Table 3.15). For their further growth and establishment, the survived plantlets were transferred to earthen pots and finally in the field. Fig. 3.40 (a-b) and Fig. 3.41 (a-d) shows the plantlets of both species which transferred to earthen pots and field. The plants were harvested from *in vitro* grown plantlets after 130-140 days finally acclimatized into the soil (Fig. 3.40a-b and Fig. 3.41a-d).

Fig. 3.40 (a&b)- Acclimatization of *N. tabacum* into soil.

Fig. 3.41(a-d): Acclimatization of *L. esculentum* into soil.

Table 3.15- Transplantation of rooted plantlets in soil and their survival rate.

Varieties	No. of plants transplanted in soil	No. of survival plants in soil	% of survival plants in soil
N. tabacum	10	9	95
L. esculentum	10	8	90

3.2.15 Genetic Transformation

In this phase of investigation, a series of experiments were carried out to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation using three constructs e.g. alpha zein, gamma zein and Ds-red zein along with a selectable marker gene *npt*II (Neomycin phosphotransferase). All the vectors were constructed with four selected B-cell epitope predicted peptide sequences of Pneumococcal surface protein antigen (PspA). For this purpose, genetically engineered *Agrobacterium* LBA4404 containing three different binary vector plasmid namely; alpha zein fertig, gamma zein fertig and pTRAkt-glyDs-zen were used for transformation experiments. Transformation experiments were conducted using different explants, namely, immature leaflet, cotyledonary node, hypocotyle and single cotyledon in case of *L. esculentum* and in case of *N. tabacum* leaves were used. During the regeneration experiments (section 3.2.1-3.2.17), it was noticed that the regeneration capability of immature leaf, cotyledonary leaf and hypocotyl explants was found to be comparatively heigher than that of other explants like cotyledonary node in tomato. In case of tobacco leaves were found suitable to work for genetic transformation. Results of these experiments are presented below in different headings.

3.2.15.1 Identification of *A. tumefaciens* using Biolog

Identification of *A. tumefaciens* were done using The Biolog (GEN_lll_v2.7.1.42.15G) utilizes and identification test panel (YT Micro Plate) consisting of a matrix of 8–12. The first three rows contain 35 carbon source oxida-tion tests using tetrazolium violet as an indicator of oxidation. The next five rows contain carbon assimilation tests which are scored turbidometrically against a negative control panel con-taining only water. The last row contains two carbon sources and tests for the co- utilization of various carbon sources with D-xylose. The hardware (Biolog MicroStation Reader) con-sists of an automated plate reader coupled with a computer, which interprets the results and compares them with the resi-dent database which currently includes 267 species.

The biochemical composition of bacterial isolates was determined by standard determinative tests as outlined in the BACTID system, in which pre-selected media were incubated after inoculation with single bacterial colonies.

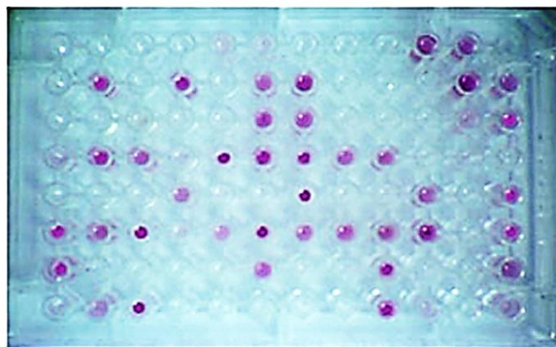

Fig. 3.42: 96-well microliter plate using in Biolog microbial ID system.

This automated identification system uses a 96-well microliter plate with 95 different carbon sources. The microor-ganism of interest is applied to each well. Each microorganism has a unique capacity to oxidize some of the various carbon sources. When these carbon sources are oxidized by the microorganism, a purple dye develops visible patterns of positive (purple) and negative (clear) wells which provide a metabolic signature of the organism. The system's computer examines the pattern signature with its database to determine bacterial species identification.

3.2.15.2 Determination of DNA Concentration of *A. tumefaciens*.

After extraction of DNA by DNA extraction method the determination of the concentration of the DNA had been done. The absorbance was measured at 260 nm wave length by the spectrophotometer.

1. For Alpha zein:

Absorbance of the sample at 260 nm ($OD_{260)}$ = 0.135

Dilution of Sample = 100

So, DNA concentration (μg/ml) = 0.135X100X50 ng/ml

= 675ng/ml

2. For gamma zein:

Absorbance of the sample at 260 nm ($OD_{260)}$ = 0.125

Dilution of Sample = 100

So, DNA concentration (µg/ml) = 0.125X100X50 ng/ml

= 625ng/ml

3. For Ds-red zein:

Absorbance of the sample at 260 nm (OD_{260}) = 0.150

Dilution of Sample = 100

So, DNA concentration (µg/ml) = 0.150X100X50 ng/ml

= 750ng/ml

3.2.15.3 Determination of DNA Concentration of *A. tumefaciens* using Nanodrop photometer.

After genomic DNA isolation from *A. tumefaciens* harboring alpha zein, gamma zein and Ds-red zein, the concentration of DNA also measured using Nanodrop photometer to be sure about the concentration. In table-3.16 it was found that, the average value of bacteria concentration measured 680 ng/ml.

Table 3.16: Determination of DNA Concentration of *A. tumefaciens* using Nanodrop photometer.

Sl. No.	Bacteria containing different constructs	DNA concentration (ng/ml)	Mean value of DNA concentration (ng/ml)
1.	Alpha zein	650	680
2.	Gamma zein	680	
3.	Ds-red zein	710	

3.2.15.4 Confirmation of presence of the target gene into *Agrobacterium* LBA4404 by Plasmid DNA isolation.

After the isolation of plasmid DNA of *Agrobacterium tumefaciens,* the DNA was performed in agarose gel electrophoresis and gave the bands for three different vectors. Fig. 3.43).

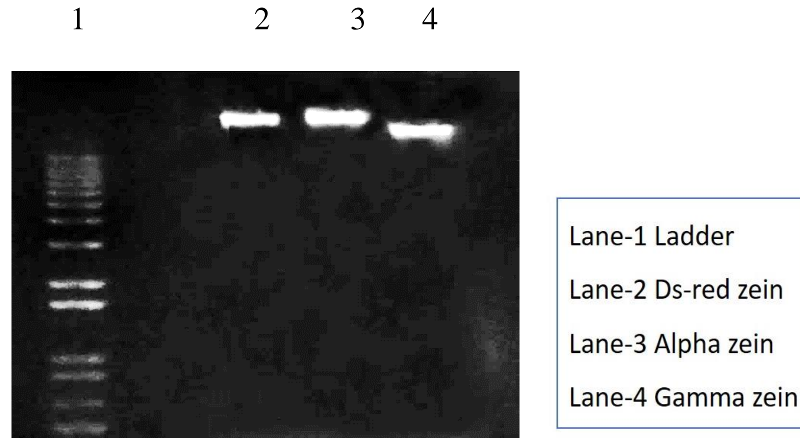

Fig. 3.43: Confirmation of presence of plasmid DNA in *Agrobacterium* LBA4404. Lane-2 Ds-red zein (8655bp), lane-3 Alpha zein (8596bp) and lane-4 Gamma zein (8059bp).

In Fig. 3.43, it was found that three distinct bands were appeared at the upper side of the gel. The band length 8655bp indicated the presence of pTRAkt-glyDs-zen which contain the Ds-red zein gene. The band length 8596bp point to the presence of vector alpha zein fertig that contain alpha zein gene and bend length of 8059bp specified to the presence of vector gamma zein fertig that contain the gamma zein gene in that particular plasmid.

3.2.15.5 PCR confirmation of presence of the target gene into *Agrobacterium* LBA4404 using specific primer.

The genomic DNA from *Agrobacterium* LBA4404 containing three different constructs were isolated and was subjected to PCR analysis for the amplification of alpha zein, gamma zein, Ds-red zein gene and *npt*II genes with the specific primers. Total eight primers were designed. Of them primer number 1, 2 and 3 are for genomic DNA of *Agrobacterium* LBA4404 containing alpha zein gene. Here primer number 1 and 2 designed as long and short sequences of alpha zein gene whereas primer number 3 is for the confirmation of *nptII* gene. Primer number 3, 4 and 5 are for genomic DNA of *Agrobacterium* LBA4404 containing gamma zein gene. In this case, primer number 4 and 5 designed as long and short sequences of gamma zein gene whereas primer number

6 is for the confirmation of *nptII* gene. And primer number 7 and 8 are for genomic DNA of *Agrobacterium* LBA4404 containing Ds-red zein gene, where 8 is for the confirmation of *nptII* gene.

After the performance of PCR amplification it was observed after agarose gel run that, all these primers gave good bands against specific primers. The results were represented in the Fig. 3.44. Here the lane 1 represented the 1kb ladder. All three *npt*II gene corresponding bands were produced after PCR amplification, identical to the band of positive control (Fig. 3.44). Lane 3 and 4 are the two replicates corresponding bands for primer 1 which designed for alpha zein long primer. The sequences for forward and reverse primer are 5'ATCCTGGGTGCTGGTTTTGT3' and 5'TTGCTGTTGCAATTGTGCGA3' respectively. Lane 5 and 6 are the two replicates corresponding bands for primer 2 which designed for alpha zein short primer. The sequences for forward and reverse primer are 5'GACGTCCCTAGCCTCCGTA 3' and 5'GGACTTTCTTCAGCCCGAAC 3' respectively. Lane 7 represented the band for primer 3 that represented the *nptII* gene in alpha zein. The sequences for alpha zein nptII forward and reverse primer are 5'TGATATTCGGCAAGCAGGCA3' and 5'TGCTCGACGTTGTCACTGAA3' respectively.

Lane 8 and 9 corresponding bands for primer 4 and 5 which designed for gamma zein long and short sequence primers. The sequences for forward and reverse for primer 4 are 5'AGAAAGACTACGACGCTGCC3' and 5'GAGAGGGATGCGGTTGTTGA3' respectively. The sequences for forward and reverse for primer 5 are 5' CCAAGGCAGAGAAAGACTACGA 3' and 5'TCTGATTCGCTTTCCTCTGTTTTC 3' respectively. The sequences for gamma zein nptII is the primer 6 and the forward and reverse primer are 5'ATGCGATGTTTCGCTTGGTG3' and 5'TTGTCACTGAAGCGGGAAGG3' respectively which can be found in lane number 10.

Lane 12 to 15 corresponding bands for ds-red zein gene. In lane 12 and 13 the primer 7 for Ds-red zein used. The sequences of forward and reverse primers are 5' GAGTTCATGAGGTTTAAGGTTCG3' and 5'CCCAAGCAAATGGCAAAGGT3' respectively. Lane 14 and 15 are the two replicates of Ds-red zein NptII gene. This

corresponds primer number 8. The sequences of forward and reverse primers for primer 8 are 5'CCCTGATGCTCTTCGTCCAG 3' and 5' TGCTCGACGTTGTCACTGAA 3' respectively (Fig. 44). All the description regarding these primer designed are described in section 3.1.12.1.

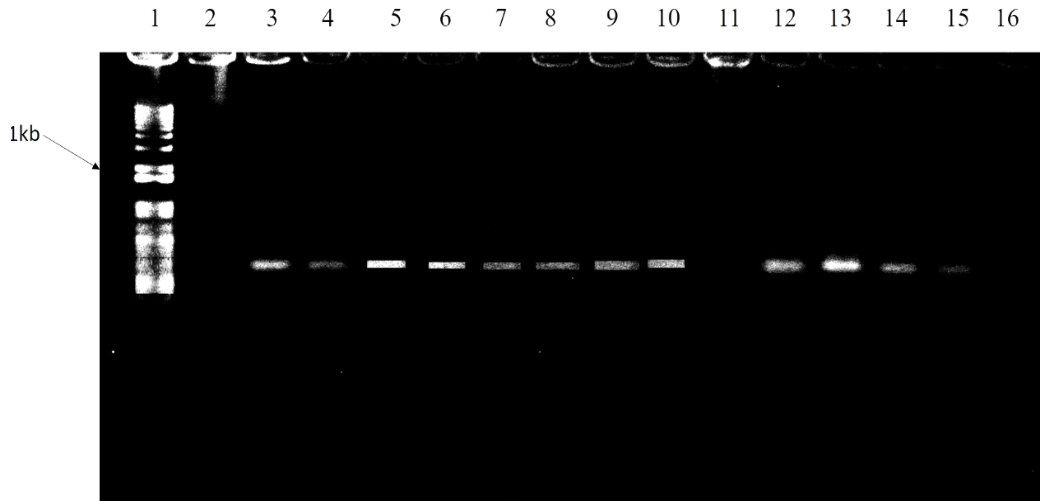

Fig. 3.44: PCR confirmation of the presence of target gene into *Agrobacterium* LBA4404 using specific primers. Lane 3-7 for alpha zein; lane 8 to 10 for gamma zein; and lane 12 to 15 represented primers for Ds-red zein.

3.2.15.6 Confirmation of presence of the target gene into *Agrobacterium* LBA4404 by restriction digestion.

The sequences of *S. pneumoniae* epitopes were placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, respectively. Ds-red zein is the combination of these two constructs (Fig. 3.45).

Fig.3.45: The constructs with alpha and gamma zein. 1,2,3 and 4 are the sequences for the epitops of *Streptococcus pneumoniae*. The HIS- and the FLAG-Tag are important for the detection. NcoI, NotI and XbaI are restriction sites.

The constructs are of different size. Construct gamma has about 500 bp which corresponds to 18 kD. Construct alpha is twice the size of construct gamma (1000bpor36 kD). These constructs were applied to a vector which contained an *E. coli*-origin and a resistance gene for ampicillin. After determining the concentration using NanoDrop, the vectors were digested with a NcoI- and Xbal. The digestion mix was loaded on an agarose gel and separated at 70 V for 40 min.

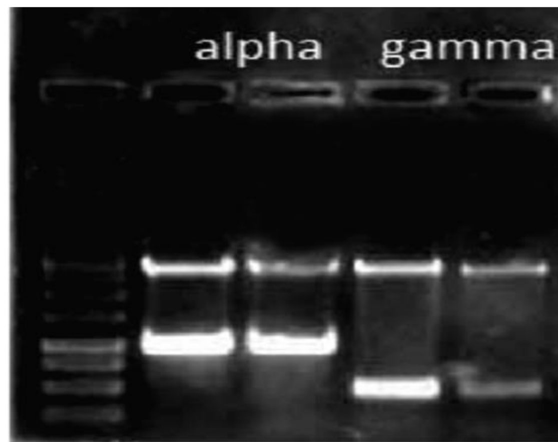

Fig. 3.46: The agarose gel after the electrophoresis of the digestion mix. Lane 1 is the ladder, Lane 2 and 3 are construct alpha and Lane 4 and 5 are construct gamma.

In Fig. 3.46 the gel after the electrophoresis is shown. The first two lanes are the digested vectors of the alpha zein and the following ones are the vectors of the gamma zein. The upper bands with the same size are the vectors and the lower ones are the inserts. Alpha zein insert is about twice the size of the gamma zein insert. After restriction digestion it was confirmed that *Agrobacterium* LBA4404 contained the gene of interests.

3.2.15.7 Genetic transformation with *Agrobacterium* LBA4404 separately containing alpha zein, gamma zein and Ds-red zein along with marker gene in *N. tabacum* and *L. esculentum*

In this investigation, genetic transformation experiments were carried out using *Agrobacterium* LBA4404 with plasmid alpha zein fertig containing the marker gene *npt*II gene as selectable marker conferring kanamycin resistance. Therefore different factors

or parameters which influenced the transformation efficiency were optimized using *N. tabacum*. Generally transient assay of such marker genes were routinely performed as a preliminary step to optimize the conditions required for successful transfer of desired gene/s. Results obtained from the optimization of different parameters for *Agrobacterium*-mediated transformation are presented under the following heads.

3.2.15.8 Optimization of different parameters influencing transformation efficiency

From the previous reports on *Agrobacterium*-mediated genetic transformation it was evident that efficiency of transformation was influenced by several factors, such as optical density (OD) of *Agrobacterium* suspension, duration of incubation period, duration of co-cultivation period, influence of light and dark period during co-cultivation of explants, etc. During this study optimization of these parameters were carried out using *N. tabacum* and *L. esculentum* by selection with the expression of the *nptII* gene.

3.2.15.8.1 Influence of optical density (OD) of *Agrobacterium* suspension on transformation

One of the most important influencing factors of transformation is optical density of *Agrobacterium* suspension. Overnight grown *Agrobacterium* suspension was used to infect the explants for transformation. Optical density of overnight grown bacterial culture ranging from 0.6 – 1.2 was measured at 600 nm. Findings of this set of experiment have been shown in Table 3.17. In case of immature leaflet explants, the maximum percentage (80.0%) of the transformation efficiency of explants was observed at 1.0 optical density of *Agrobacterium* suspension. In case of cotyledonary node explants the maximum transformation efficiency was observed at 0.8 optical density of *Agrobacterium* suspension (Table-3.17). Therefore, bacterial suspension with an optical density of 0.8 was used for all the transformation experiments to avoid bacterial overgrowth in the regeneration medium.

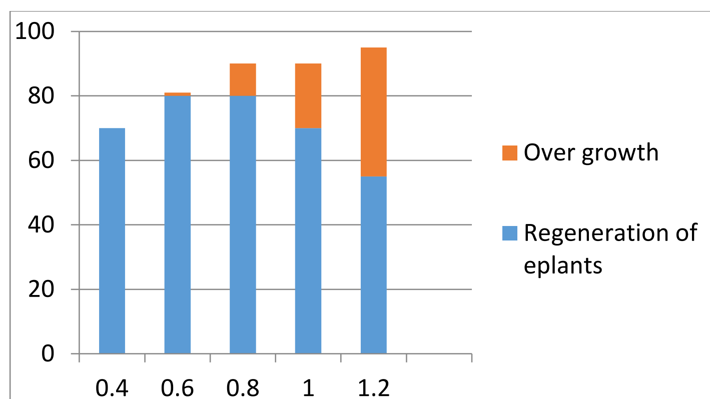

Fig. 3.47: Effect of optical density (measured at 600 nm) infected with *A. tumefaciens.*

Table 3.17: Influence of optical density (measured at 600 nm) of overnight grown *Agrobacterium* LBA4404 suspension on transformation of different explants.

Explants	OD_{600}	No. of explants infected	No. of explants assayed for nptII gene	No. of explants +ve for nptII gene	% of nptII +ve explants
Cotyledonary node	0.4	75	30	21	63.30
	0.8	80	30	22	73.33
	1.0	**80**	**30**	**24**	**80.0**
	1.5	80	30	22	70.33
Cotyledonary leaf	0.6	80	30	22	73.33
	0.8	**80**	**30**	**26**	**86.66**
	1.0	80	30	22	73.33
	1.2	80	30	22	73.33
Immature leaflet	**0.6**	**80**	**30**	**25**	**83.33**
	0.8	80	30	22	73.33
	1.0	80	30	20	66.50
	1.2	80	30	22	73.30

3.2.15.8.2 Influence of incubation period of explants in bacterial suspension on transformation

Incubation period in *Agrobacterium* suspension was another important parameter where the explants were incubated for a definite period to allow the bacteria to infect the plant tissues. In this set of experiment, the effect of different incubation periods was investigated. For this perseverance, different incubation periods, such as 1, 3 and 5 minutes were applied using bacterial suspension with a constant optical density of 0.8 in case of both tobacco and tomato explants. Leaflet explants showed maximum positive expression when the explants were incubated for a period of 5 minutes in *Agrobacterium* suspension. It was observed that bacterial overgrowth was visualized in culture media when explants were incubated more than 3 minutes in *Agrobacterium* suspension. Therefore, explants were incubated for below 3 minutes with *Agrobacterium* suspension. Immature leaflets and single cotyledon explants showed maximum positive activity in3 minutes of incubation with *Agrobacterium* suspension. In many cases the explants showed bacterial over-growth if the explants were incubated for longer period with *Agrobacterium* suspension (Fig. 3.48, 3.49). Therefore, the optimum incubation period was maintained at 3 minutes for both leaflet explants and cotyledon explants at 0.8 OD of the *Agrobacterium* suspension (Table 3.18).

Fig. 3.48 (a-b): Overgrowth and normal condition of *Agrobacterium* in co-culture media.

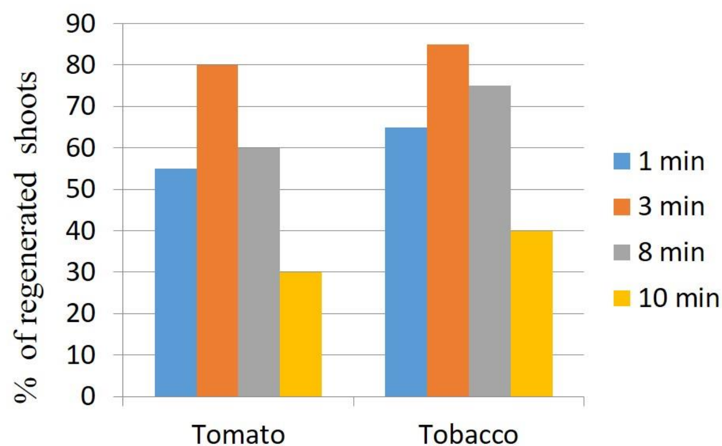

Fig. 3.49: Effect of incubation period infected with *A. tumefaciens*.

Table 3.18: Influence of different incubation periods of overnight grown *Agrobacterium* (LBA4404) suspension on transformation of various explants of *N. tabacum*.

Explants	Incubation Period (mins)	No. of explants infected	No. of explants assayed for kanamycin	No. of explants +ve for kanamycin	% of kanamycin +ve explants
Immature leaflet	1	80	30	18	60.00
	3	**80**	**30**	**24**	**80.00**
	5	**80**	**30**	**26**	**76.60**
Cotyledon	1	80	30	23	76.66
	3	**80**	**30**	**24**	**80.00**
	5	**80**	**30**	**27**	**75.00**

3.2.15.8.3 Influence of co-cultivation period on transformation

Co-cultivation period of explants is another important factor which influences transformation efficiency. In this experiment the transformed explants were co-cultivated for different days such as 2, 3, 4 and 5 days obtain specific time period for sufficient transformation of explants. It was observed that the transformation efficiency was found

to be slightly variable for different periods of co-cultivation of the explants (Table 3.19). In this experiments optical density of *Agrobacterium* suspension was maintained at 0.8. A period of 3 - 4 days co-cultivation period showed the optimum transformation efficiency. However, percentage of transformation could be increased with the increase of co-cultivation period but longer co-cultivation period (more than 4 days) produced bacterial over-growth on the co-culture medium (Fig. 3.50). This type of bacterial over-growth was not suitable for survival of co-cultured explants. Therefore, co-cultivation period of 3 - 4 days was found to be the most suitable when transformation experiment was performed under optimum condition (Table 3.19).

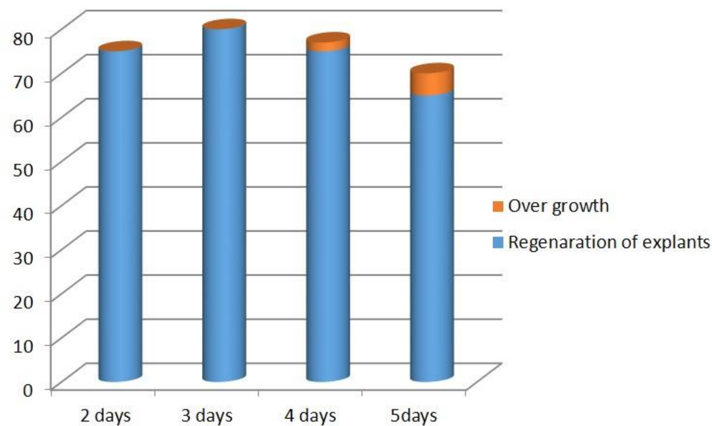

Fig. 3.50: Effect of co-culture period infected with *A. tumefaciens.*

Table-3.19: Impact of different co-cultivation periods on at a constant OD (600 nm) and incubation period of *N. tabacum*.

Co-cultivation period (days)	No. of explants assayed	No. of survived explants	% of survived explants
2	25	19	75
3	**25**	**21**	**78**
4	25	21	73
5	25	22	68

3.2.15.8.4 Effect of co-cultivation under dark and light conditions on transformation

An experiment was carried out to observe the effect of dark (within the incubator) and light (growth room condition) conditions on transformation efficiency during co-cultivation of explants. The result of the experiment was also analyzed by kanamycin and immature leaflet, cotyledon and cotyledonary node explants were used. The two treatments (dark and light) during co-cultivation did not show any significant differences on the transformation efficiency. Results of this experiment have been presented in (Table 3.20). It was observed that the immature leaflet and cotyledon explants showed similar positive response both under light and dark conditions. However, leaflet explants showed slightly better response when the explants were co-cultivated under dark condition.

Table 3.20: Effect of darkness and light (growth room condition) in co-culture period towards transformation at optical density 0.8, 5 mins. of incubation period and 3 days of co-cultivation.

Condition of light intensity	Explants	No. of explants assayed	No. of positive explants	% of positive explants
Dark condition	Leaf	10	7	70
	Cotyledon	**10**	**8**	**80**
	Node	10	7	70
Light (growth room condition)	Leaf	10	7	70
	cotyledon	**10**	**8**	**80**
	Node	10	7	70

3.2.15.9 Regeneration from co-cultured explants

Transformation experiments were performed with immature leaflet, cotyledon and cotyledonary node explants of *N. tabacum* and *L. esculentum*. During these experiments a few selected explants infected with the *Agrobacterium* were subjected for kanamycin selection to monitor the transformation efficiency of the explants. The remaining co-cultivated explants were washed with sterile distilled water containing 300 mg/l carbenicilin for the elimination of *Agrobacterium* attached on the surface of the explants.

Following this step the explants were transferred to MS medium containing 2.0 mg/l BAP for leaflet and cotyledon explants for the regeneration of shoots. Selection pressure was not applied immediately after co-cultivation, instead the explants were allowed to regenerate (minimum for 15 days) in regeneration medium without the selection pressure only containing 100 mg/l carbenicilin. It was observed that if selective agent (kanamycin) was applied immediately after co-cultivation the *Agrobacterium* infected explants did not show further regeneration and in most of the cases the explants failed to survive. When the multiple shoots elongated up to 0.2 - 0.5 cm in length the explants with the newly developed shoots were transferred to the medium with selective agents.

3.2.15.9 Determination of optimum kanamycin concentration for selection of transformed shoots

Agrobacterium strain LBA4404 harbouring alpha zein, gamma zein and Ds-red contained *npt*II gene conferring resistance to kanamycin. Therefore, kanamycin was used as selective agent during the transformation experiments with that *Agrobacterium*. To determine the optimum selection level using kanamycin, shoots derived from non-infected explants (those served as negative control) were subjected to selection pressure. From this experiment it was revealed that with the increase of kanamycin concentration the percentage of survived shoots were found to decrease. For this purpose, the regenerated non-transformed shoots where transferred to suitable regeneration medium containing various concentration of kanamycin. The concentration of kanamycin was increased gradually from 50 mg/l to 300 mg/l in the following manner 50, 100,150, 200, 250 and 300 mg/l kanamycin. Non transformed (control) shoots were transferred to various kanamycin contained selection medium. In case of both tobacco and tomato, transformed shoots did not show albinism in presence of 50 mg/l kanamycin after 30 days of culture, whereas all the non-transformed plant became albino (Fig. 3.51 a&b). Most of the cases non-transformed leaves were found to become albino after 8 - 16 days and then the bleaching extended to the stems. All the non-infected explants (negative control) failed to survive in presence of kanamycin within 30 days (Fig. 3.51b.).

Fig. 3.51 (a&b): Non- transformed plant on medium containing 50 mg/l kanamycin. (a) Plant parts getting albino; (b) Plant parts become albino.

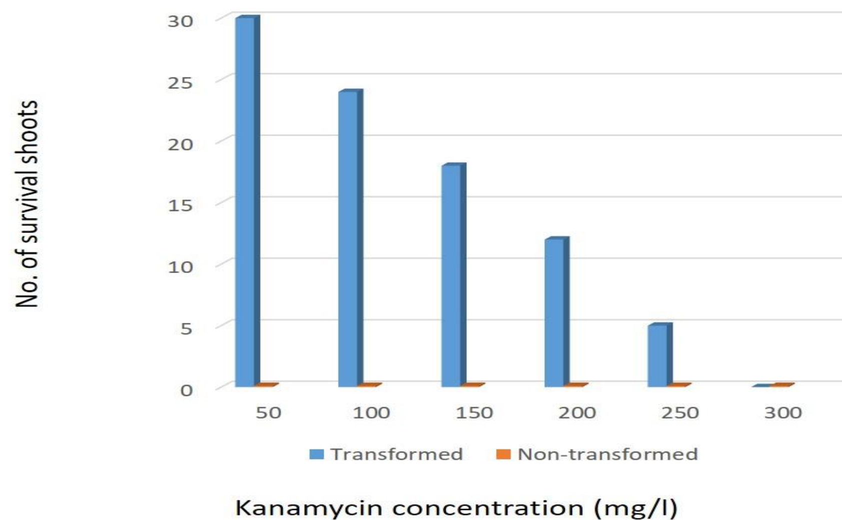

Fig.3.52: Effect of kanamycin concentrations on transformed and non-transformed plant.

For this reason 50 mg/l kanamycin was considered as first selection pressure and 300 mg/l kanamycin was considered as highest selection pressure in the present study. Effect of kanamycin towards the control shoots have been shown in Fig. 3.52.

3.2.15.11 Selection of different transformed shoots of *N. tabacum* using kanamycin

For the development of transformed plantlets it is important to select the transformants from the regenerating shoots. As successfully transformed shoots have the capability to express the gene conferring kanamycin resistance, they were supposed to survive in the selection medium. On the contrary, shoots regenerated from uninfected explants became

albino and could not survive in the selection medium since they did not possess the kanamycin resistant gene. It has been mentioned before that 50 mg/l kanamycin concentration was taken as the first selection pressure then the concentration of kanamycin was increased gradually like 100 mg/l, 150 mg/l, 200 mg/l, 250 mg/l and 300 mg/l.

Fig. 3.53 (a-c): Survived regenerated transformed shoots of *N. tabacum* on medium containing 50 mg/l kanamycin. (a) transformed with alpha zein; (b) transformed with gamma zein; and (c) transformed with Ds-red zein constructs.

Shoots those remained green and healthy and survived on the medium containing optimum selection pressure (100 mg/l) for 30 days were selected as putatively transformed shoots. Different stages of regeneration and selection of transformed shoots gene integration with alpha zein, gamma zein and Ds-red zein in *N. tabacum* have been shown in Fig. 3.53 (a-i). In all these three cases, the survived green colored healthy plants after using kanamycin proved that, the plants are successfully transformed. The results of transformation experiment are shown in Table 3.21.

It was observed that, all the explants showed poor regeneration response if the infected explants were inoculated directly in first selection pressure containing medium (50 mg/l) after co-culture. After co-cultivation, 10-15 days later the explants supposed to transfer on selection medium. Among the three constructs studied Ds-red zein showed better transformation efficiency then the others. In case of alpha zein, out of approximately 850 infected explants, 520 shoots were recovered on final selection medium. Therefore the frequency of recovery of putatively transformed shoots from cotyledon explants was about 61.17%. In case of gamma zein, the frequency of recovery of putatively

transformed shoots was about 76.26%. The highest rate of 83.87% were observed in case of plants transformed with Ds-red zein (Table 3.21).

Table 3.21: Effect of kanamycin on selection of infected shoots of *N. tabacum* transformed with the constructs alpha zein, gamma zein and Ds-red zein.

Sl. No	Constructs	No. of explants infected	No. of survived shoots in regeneration media with 50 mg/l kanamycin	% of survived shoots
1.	Alpha zein	850	520	61.17
2.	Gamma zein	590	450	76.27
3.	Ds-red zein	620	520	**83.87**

3.2.15.12 Selection of different transformed shoots of *L. esculentum* using kanamycin

It has been mentioned before that 50 mg/l kanamycin concentration was taken as the first selection pressure then the concentration of kanamycin was increased gradually like 100 mg/l, 150 mg/l, 200 mg/l, 250 mg/l and 300 mg/l. Shoots those remained green and healthy and survived on the medium containing optimum selection pressure (100 mg/l) for 30 days were selected as putatively transformed shoots. Different stages of regeneration and selection of transformed shoots gene integration with alpha zein, gamma zein and Ds-red zein in *L. esculentum* have been shown in Fig. (3.54 a-c). In all these three cases, the survived green colored healthy plants after using kanamycin proved that, the plants are successfully transformed. The results of transformation experiment are shown in table-3.22.

After co-cultivation, 10-15 days later the explants supposed to transfer on selection medium. It was observed that, among the three constructs studied Ds-red zein showed better transformation efficiency then the others which also found in case of tobacco. In

case of alpha zein, out of approximately 530 infected explants, 220 shoots were recovered on final selection medium. Therefore the frequency of recovery of putatively transformed shoots from cotyledon explants was about 41.50%. In case of gamma zein, the frequency of recovery of putatively transformed shoots was about 51.02%. The highest rate of 58.18% were observed in case of plants transformed with Ds-red zein in tomato. All these percentage range is very less than that results found in case of tobacco (Table 3.22).

Fig. 3.54 (a-c): Survived regenerated transformed shoots of *L. esculentum* on medium containing 50 mg/l kanamycin. (a) transformed and non-transformed plants; (b) transformed with alpha zein; (c) transformed with gamma zein; and (d) transformed with Ds-red zein constructs

Table 3.22: Effect of kanamycin on selection of infected shoots of *L. esculentum* transformed with the constructs alpha zein, gamma zein and Ds-red zein.

Sl. No	Constructs	No. of explants infected	No. of survived shoots in regeneration media with 50 mg/l kanamycin	% of survived shoots
1.	Alpha zein	530	220	41.50
2.	Gamma zein	490	250	51.02
3.	Ds-red zein	550	320	**58.18**

3.2.15.13 *In vitro* root formation from the putatively transformed shoots of *N. tabacum* and transplantation of plantlets to soil

During the transformation experiments the shoots that survived in the kanamycin selection medium were transferred to half strength of MS medium containing 0.5 mg/l IBA with 100 mg/l carbenicilin for the induction of roots. During this study altogether 80 shoots of *N. tabacum* were inoculated in root induction medium which was survived in selection pressure contained medium. It was observed that the shoots that survived in selection pressure containing medium showed slow and lower response towards root formation in all cases (Table 3.23). In this variety 50% shoots showed response towards induction of roots (Fig. 3.55a, b). It was noticed that 90% plantlets were found to survive on soil (Fig. 3.55c, e).

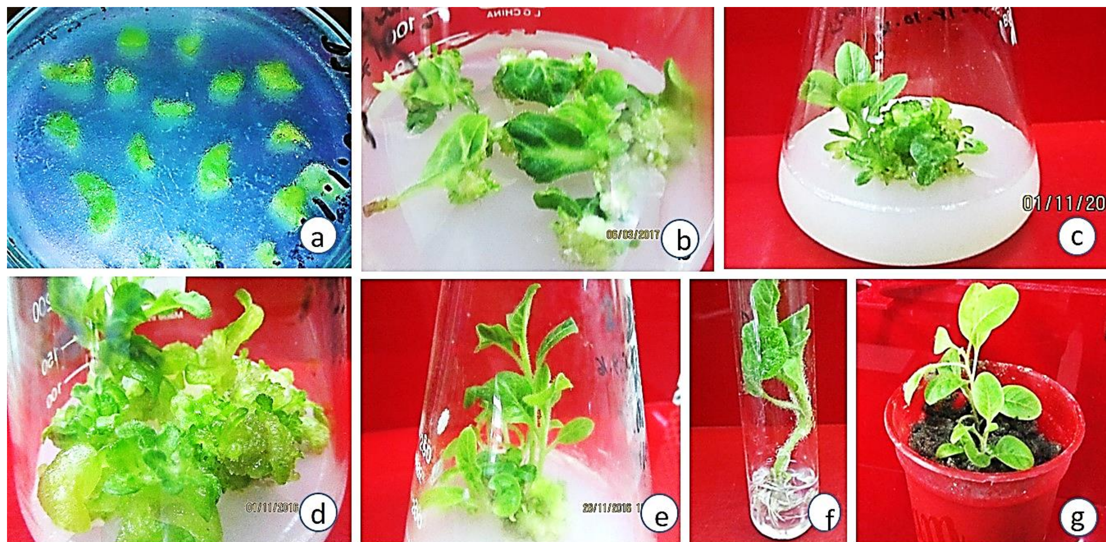

Fig. 3.55 (a-g): Regeneration and selection of putative transformants of *N. tabacum*. (a) Infected leaflet explants in MS medium; (b) Shoots regenerated from infected explants on selection free regeneration medium; (c) Multiple shoot regenerated from infected without selection pressure; (d) Shoot formation from infected leaflet explants after co-cultivation; (e) Development of multiple shoots from infected leaflet explants on 50 mg/l kanamycin supplement; (f) Rooting survived after selection (g) Putative transformed shoots survived on soil.

Table 3.23: Formation of roots and transplantation of plantlets in soil from the *in vitro* regenerated shoots of transformed *N. tabacum* survived in selection pressure.

Plants	No. of transformed shoots inoculated for root induction	Days required for root initiation	No of shoots showed rooting response	% of shoots forming roots	% of survival rate of transplanted plantlets
N. tabacum	80	20 - 45	40	50	90

3.2.15.14 *In vitro* root formation from the putatively transformed shoots of *L. esculentum* and transplantation of plantlets to soil

In the transformation experiments the shoots that survived in the kanamycin selection medium were transferred to half strength of MS medium containing 0.5 mg/l IBA with 100 mg/l carbenicilin for the induction of roots. During this study altogether 60 shoots of *L. esculentus* were inoculated in root induction medium which was survived in selection pressure contained medium. It was observed that the shoots that survived in selection pressure containing medium showed slow and lower response towards root formation in all cases (Table 3.24). In this variety 36.66% shoots showed response towards induction of roots (Fig. 3.56 a, b). It was noticed that 70% plantlets were found to survive on soil (Fig. 3.56 e-g).

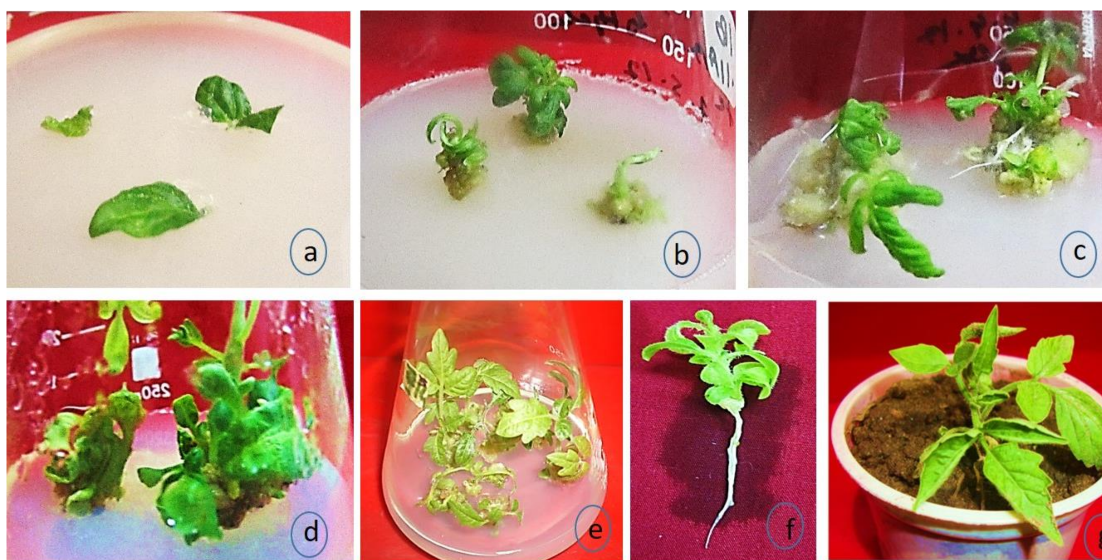

Fig. 3.56 (a-g): Regeneration and selection of putative transformants of *L. esculentum*. (a) Infected leaflet explants in MS medium; (b) Shoots regenerated from infected explants on selection free regeneration medium; (c) Multiple shoot regenerated from infected without selection pressure; (d) Shoot formation from infected leaflet explants after co-cultivation; (e) Development of multiple shoots from infected leaflet explants on 50 mg/l kanamycin supplement; (f) Rooting survived after selection (g) Putative transformed shoots survived on soil.

Table 3.24: Formation of roots and transplantation of plantlets in soil from the *in vitro* regenerated shoots of transformed *L. esculentum* survived in selection pressure.

Plants	No. of transformed shoots inoculated for root induction	Days required for root initiation	No of shoots showed rooting response	% of shoots forming roots	% of survival rate of transplanted plantlets
L. esculentum	60	20 - 45	22	36.66	70

3.2.15.15 PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene along with *npt*II genes in *N. tabacum.*

The genomic DNA isolated from leaves of 25 rooted plantlets of *N. tabacum* was subjected to PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene and *npt*II genes. 20 plantlets of *N. tabacum* were found showing corresponding band for the genes (at 150 bp for alpha zein, 150 bp gamma zein and 180 bp Ds-red zein gene) after PCR amplification, identical to the band of positive control (Fig. 3.56).

In the fig.3.56 it can found that, lane 1 is the 1 kb ladder. Lane 3, 4 and 5 for alpha long primer, short prime and alpha with nptII. In the lane 4 there is no band found where as in third and fifth lane showing band. In case of gamma in lane 6, there is no band found whereas lane 7&8 are showing band. Lane 9 and 10 represent bands for DS-red zein.

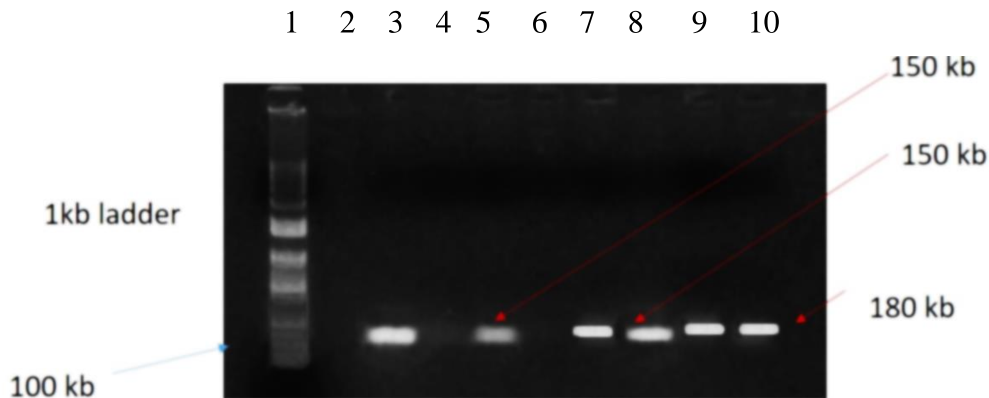

Fig. 3.57: PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene in *N. tabacum.* In this picture lane 1=ladder 1kb. Lane 3, 4 and 5 for alpha, 6, 7 and 8 for gamma and 9 & 10 for Ds-red zein.

3.2.15.16 PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene along with *npt*II genes in *L. esculentum.*

In case of *L. esculentum*, genomic DNA of 8 putatively transformed rooted plantlets were used for PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene along with *npt*II genes. It was observed that in case of alpha zein, gamma zein and

Ds-red zein gene amplification, all maximum plants were found showing band, identical to the band of positive control (Fig. 3.58).

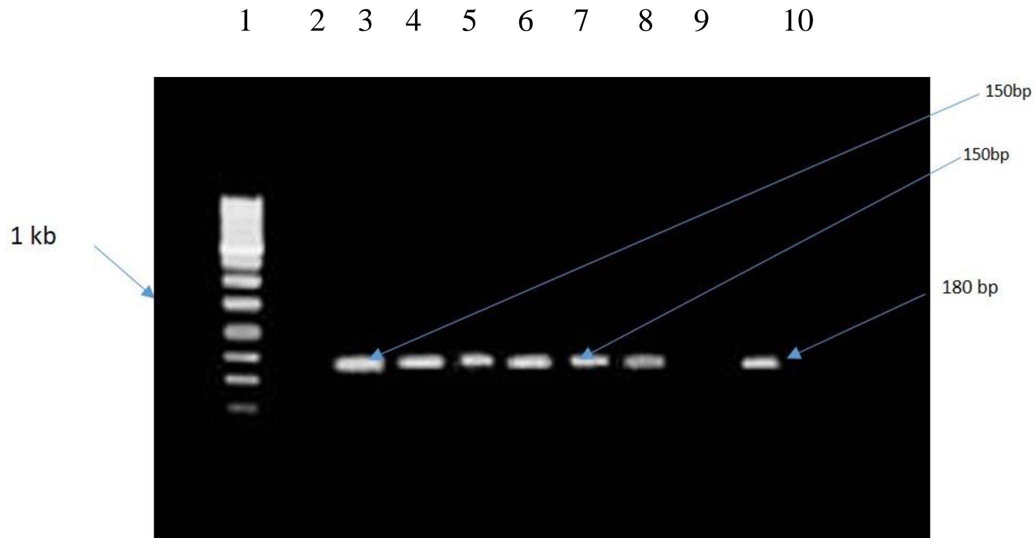

Fig.3.58: PCR analysis for the amplification of alpha zein, gamma zein and Ds-red zein gene in *L. esculentum.* In this picture lane 1=ladder 1kb. Lane 3, 4 and 5 for alpha; lane 6, 7 and 8 for gamma; and 9 & 10 for Ds-red zein.

3.2.15.17 Immunoblot analysis of T_0 plants

Tobacco leaves were transformed with construct alpha, construct gamma and Ds-red. The plant samples were taken from the transformed leaves from tobacco plant and shock frozen in liquid nitrogen to avoid protein degradation. The samples were prepared for the SDS-PAGE which have been described in materials and method section. Protein profiling through the SDS-PAGE was done followed by western blotting.

The membrane was incubated with antibodies against the his6- tag. The construct gamma shows the strongest band than that of alpha zein. Surprisingly, the mass of the gamma fusion protein was much higher than expected. One reason could be the formation of protein dimers. The mass of a dimer would correspond to the result. The construct Ds-

red also shows slight bands. In the Ds-red yielded bands the amount of the protein was much less than the amount obtained with the gamma zein construct only (Fig. 3.59).

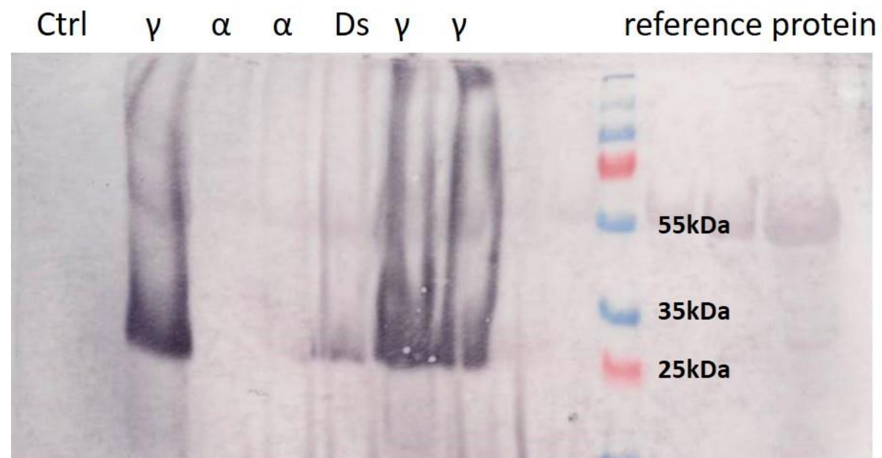

Fig. 3.59: The recombinant proteins were detected using an anti-His-tag antibody (γ = gamma zein fusion, and α = alpha zein fusion, and Ds= Ds-red zein fusion).

3.3 Immunization testing of Mice

After six times (0 week, 1st week, 2nd week, 3rd week, 4th week and 5th week) immunization of mice with transgenic plant tissue they were challenge with live cell to observe the survival of immunized mice.

3.3.1 LD_{50} Dose and challenge of Mice

The mice were grouped into 1, 2, 3 and 4. Group 1 were those mice which had been fed the transgenic plant that was integrated by alpha zein gene. Group 2 represented the mice mice which had been fed the transgenic plant integrated by gamma zein gene. Group 3 included those mice that was fed by the transgenic plant. Different group of mice with the transformed plants (alpha, gamma, Ds-red and control) were each challenged approximate 10^7 live cells of the virulent *S. pneumoniae* serotype 7F. This dose was the 10 time of the 50% of the lethal dose (LD_{50}).

3.3.2 Mice challenge test

All the mice grouped in different type including control were challenged by *S. pneumoniae* 7F. In each mice 0.50 ml bacterial solution was injected in each mice intraperitonially.

Fig. 3.60: Challenging of mice using *S. pneumoniae* serotype 7F.

3.3.2.1. Results of mice challenge after 1st trial of mice challenge test

Much differences were observed in the survival time between the immunized mice and the control mice after first trial of mice challenge. Among 12 control mice 6 mice died within a period of 8-10 days. Among the immunized mice in case of mice immunized with alpha zein 2 mice were died within 16 days but other 10 mice were remain alive within 30 days observation and hence survived the live cell challenge.

In case of mice immunized with gamma zein, among the immunized mice 3 mice were died within 16 days but other 9 mice were remain alive within 30 days observation and hence survived the live cell challenge.

In case of mice immunized with Ds-red zein, out of 12 immunized mice 2 mice were died within 16 days but other 10 mice were remain alive within 30 days observation and hence survived the live cell challenge. After first trial, the survival rate of immunized mice using alpha zein, gamma zein and Ds-red zein are 83.33%, 75% and 83.33% respectively. Whereas in case of control the rate is 50% (Table-3.25).

Table-3.25: Survival rate of mice after first trial of mice challenge test.

Sl. No	Name of the insert	Total No. of Mice challenged	No. of Mice dead	No. of Mice Alive	Percentage of survival (%)
1	Alpha zein	12	2	10	83.33
2	Gamma zein	12	3	09	75.00
3	Ds-red zein	12	2	10	83.33
4	Control (Non-transformed)	12	6	06	50.00

First trial: 13.08.17, Doses: 6 (7 days interval), 0.50 ml/mice, 10^7 cells/ml

3.3.2.2. Results of mice challenge after 2nd trial of mice challenge test

Significant differences were also observe in the survival time between the immunized mice and the control mice after second trial. Among 12 control mice 6 mice died within a period of 8-10 days after challenged with *S. pneumoniae* serotype 7F. So, in this case the survival rate is 50%. Among the immunized mice in case of mice immunized with alpha zein out of 14 mice, 2 mice were died within 16 days but other 12 mice were remain alive within 30 days observation and hence survived the live cell challenge. In this case, from the table- 3.26, we can found that the survival rate is 85.71%. This rate is higher than the first trial.

In case of mice immunized with gamma zein, among the all 12 immunized mice no mice were died. That means all mice were remain alive within 30 days observation and hence survived the live cell challenge. In this case the survival rate is 100%.

In case of mice immunized with Ds-red zein, out of 10 immunized mice all mice were remain alive within 30 days observation and hence survived the live cell challenge. In this case the survival rate also 100%.

In this experiment, it was observed that the mean volume of survived mice was 80.55% in the 1st trial and it was 95.23% after 2nd trial. In case of control (Mice feeding non-transformed leaves) the rate is 50% in both trials.

Table- 3.26: Survival rate of mice after second trial of mice challenge test.

Sl. No	Name of the insert	Total No. of Mice challenged	No. of Mice dead	No. of Mice Alive	Percentage of survival (%)
1	Alpha zein	14	2	12	85.71
2	Gamma zein	12	0	12	100.0
3	Ds-red zein	10	0	10	100.0
4	Control (Non-transformed)	12	6	6	50.00

2nd trial: 18.01.18, Doses: 6 (7 days interval), 0.50 ml/mice, 10^7 cells/ml

3.3.4. Comparative study of Mice challenge experiment between 1st and 2nd trial

In this experiment, after performing two challenges upon mice in different period, more or less similar results were found. In both cases the survival rate for control is 50%. And in case of alpha zein there is no significant differences in survival rate between 1st and 2nd trial. In case of 2nd trial, using gamma zein and Ds-red zein gene 100% survival rate observed, whereas, in 1st trail gamma zein and Ds-red zein showed slight differences which is also acceptable. 50% survival rate in both cases, indicate that this result is significant.

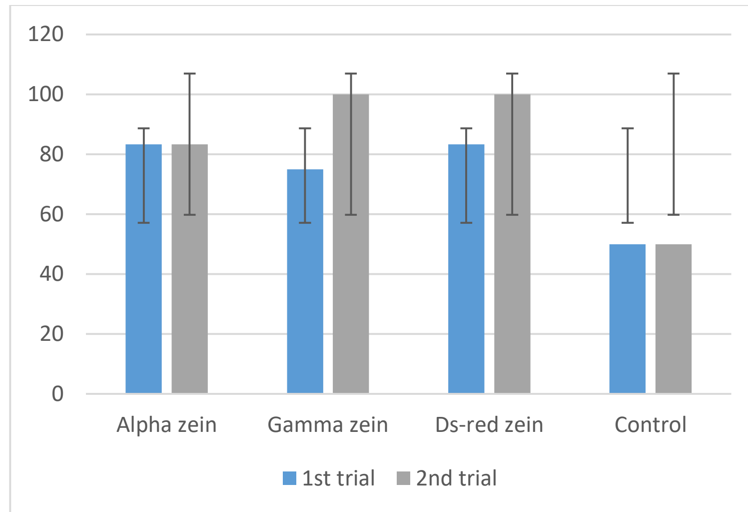

Fig. 3.61: Comparative study after Mice challenge experiment. The results are expressed as mean ± standard deviations.

The variation of 1st and 2nd trial in case of control mice.

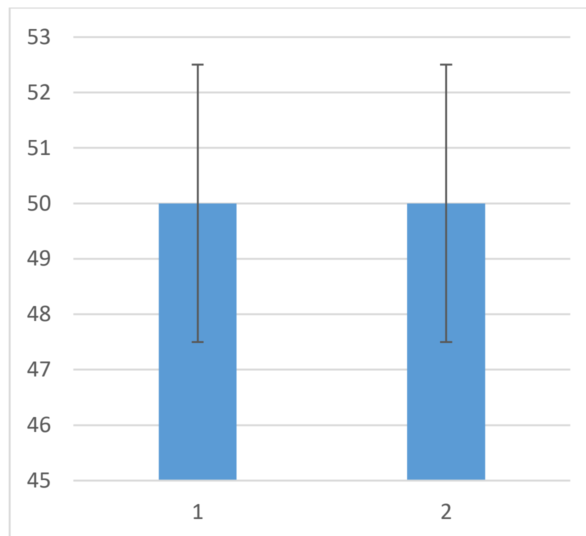

Fig. 3.62: Difference between 1st and 2nd trial of control mice challenge result.

Two cluster groups in this scatterplot showed significant variation between control and immunized mice. The distance between these two cluster indicate that significant variation if present between control and immunized mice on survival rate.

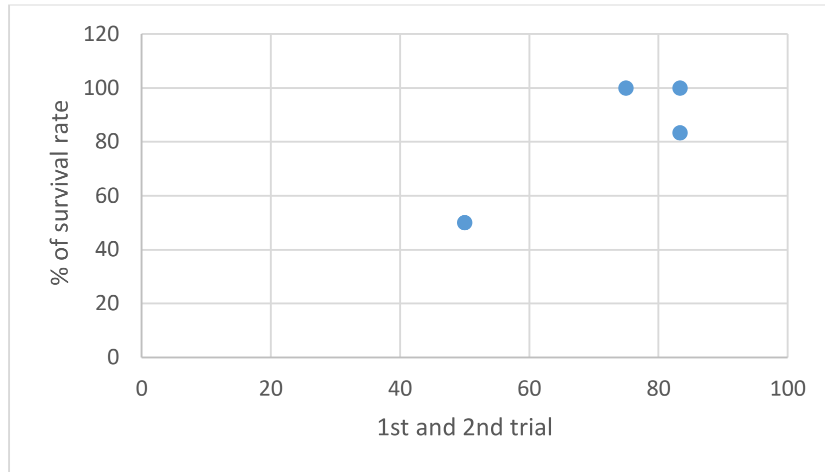

Fig. 3.63: Relationship between control and immunized mice survival rate.

3.3.5 Statistical analysis of mice challenge result

All the tests are performed with SPSS of its version 22.0. The results of statistical analyses of mice challenge test is given bellow:

T-Test

Table-3.26: Comparison of 1st and 2nd trials of mice challenge test.

Group Statistics					
	trial	N	Mean	Std. Deviation	Std. Error Mean
Survival	Trial 1	4	74.1650	17.02243	8.51121
	Trial 2	4	83.9275	23.60016	11.80008

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
Survival	Equal variances assumed	.355	.573	-.671	6	.527	-9.76250	14.54932	-45.36340	25.83840
	Equal variances not assumed			-.671	5.457	.530	-9.76250	14.54932	-46.24072	26.71572

In order to test the equality of average survival number from two trials (1st and 2nd), t-test has been performed. Test results shows that the means are not significantly different ($p>0.05$). That means, the survival rate in both the trials are almost similar.

There is no significant variation in between 1st and 2nd trial of mice challenge experiment.

Oneway

ANOVA					
Survival					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2275.928	3	758.643	6.671	0.049
Within Groups	454.877	4	113.719		
Total	2730.805	7			

To test the equality of survival rate in control group and three other constructs namely Alpha, Gamma and Ds-red, Analysis of Variance (ANOVA) has been done. It can be seen from the test results that average survival rates in the three constructs are different from the control group ($p<0.05$).

Post Hoc Tests

Homogeneous Subsets

Survival			
Duncan[a]			
Inserts	N	Subset for alpha = 0.05	
		1	2
Control	2	50.0000	
Alpha Zein	2		87.0200
Gamma Zein	2		87.5000
Ds-red zein	2		91.6650
Sig.		1.000	.689
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 2.000.			

For further understanding of the ranks of the average number of survival rates in the four groups, Duncan Multiple Ranked Test (DMRT) has been applied. It can be noticed from the findings that all the constructs are in one subset, and they are far higher than control set which belongs to another group at 5% level of significance.

4. DISCUSSION

Despite the presence of antibiotics and vaccines, *S. pneumoniae* is still a major cause of morbidity and mortality, both in the US and worldwide. For the year 1999, statistics collected by the Centers for Disease Control show that *S. pneumoniae* infections resulted in 100,000 to 135,000 hospitalizations for pneumonia, 6 million cases of otitis media, and over 60,000 cases of invasive disease, including 3,300 cases of meningitis in the US [6]. *S. pneumoniae* is the sixth leading cause of death in the US and is a major killer in the developing world.

In these times of emerging antibiotic-resistant strains, preventive vaccines have become more important than ever. A polysaccharide vaccine developed in the 1980s is readily available, but children below the age of 2 years and the elderly have not benefited significantly from the vaccine [330]. This lack of benefit has mainly been attributed to the inability of young children to elicit immune responses effectively against polysaccharide structures and to tolerance in the elderly, leading to the same result. The current strategy for enhancing the efficacy of polysaccharide structures to elicit immunity stems from the successful implication of protein conjugation seen for the vaccine against Hib [16, 306]. Pneumococcal protein conjugate vaccines have been more successful than the polysaccharide vaccine in preventing infection in the main target populations [35, 112, 334] but problems of increasing the number of capsule types to yield complete protection will not be easily solved. Also, the prohibitive cost of these vaccines makes them inaccessible to children in developing countries, where the need is most acute.

Recent research for new vaccines has focused heavily on protein vaccine candidates that can be employed to overcome the lack of immune responsiveness of infants to the polysaccharide vaccine. Of the proteins evaluated so far, PspA, alone or in conjunction with other protein molecules such as PspA and/or pneumolysin, has resulted in promising protection in a variety of model systems [50, 276, 277, 248, 247, 361, 231].

Although PspA is a highly variable molecule based on both serological data and gene sequencing, the structure still contains cross-reactive epitopes. Antibodies reacting with these epitopes have also been shown to have the highest level of cross-protection against strains expressing other PspAs [49, 48]. It has been speculated that these epitopes may be involved in performing the functional aspects of PspA during colonization and infection of the host.

Sequence homology studies of one region of the molecule, the CDR, indicated groupings of sequences within the family structure and resulted in the definition of 5 clades comprising two major families. The PspA sequences within each of these families show greater than 60% sequence identity [162]. This CDR corresponds to the localization of protective and cross-protective epitopes of the family 1, clade 2 PspA from *S. pneumoniae* Rx1 [249].

Despite the advantages of vaccination, limitations restricting the use of vaccines remain. Not all pathogenic agents can be cultivated in an exogenous medium and, due to their highly pathogenic features, the cultivation of some agents demands biosecurity and biosafety infrastructures that are difficult for some countries to afford. Consequently, the production of certain vaccines remains costly and restricted in numerous countries, thus generating an undesirable dependence on hygiene [419]. Another restrictive factor is that, although the attenuation of bacteria or viruses involves very controlled processes, the possibility that these pathogens could revert to their original pathogenic form must be considered [200]. Additionally, the highly specified expiration time and refrigeration requirements inherent to nearly all commercial vaccines demand constant attention to the pathogen contained in such vaccines, thus increasing control, storage, and distribution costs [135]. Vaccine degradation after acid digestion in the stomach is another concern [86].

Recognizing these limitations and exploiting advances in recombinant DNA technology, Mason et al., succeeded in expressing a surface antigen from hepatitis B in tobacco plants [243]. This finding immediately suggested that plants were potentially effective vectors for the production of vaccines to prevent diseases, giving rise to the concept of "edible vaccines," a term coined in 1990 by Charles Arntzen [11]. However, the development of

edible vaccines remains in its infancy, and various medical, legal, ethical, and environmental uncertainties have emerged [135].

Edible vaccines represent a valuable solution to treating certain diseases whose control and prevention is restricted by the inherent limitations of traditional vaccines, such as their production costs, storage requirements, and expensive logistics. Sixteen foods are already producing antigens to counter human and animal diseases globally.

Beyond the pros and cons of edible vaccines, one of the most complex problems to address is the establishment of collaborations for the development of a stable vaccine that can actually be used in human medicine. Advances in the development of transgenic plants and antigen expression for stimulation of the immune system associated with the mucosa have been in the botanical field and not in immunology. It is very difficult to establish a stable antigenic protein concentration in plant tissues, and there is no certainty that the expressed antigen will produce an immune response. Efforts by immunologists and conventional vaccine developers could be of great value to advance this alternative to current vaccines. In addition to their possible benefits, edible vaccines will decrease the costs of vaccination and allow minimally invasive vaccine administration. Furthermore, reiterating the need to increase vaccine performance and stability, developments in the generation of transient vaccines using viruses do not obviate the development of transgenic plants as a long-term and longer-lasting measure. The potential opposing role of oral tolerance might be beneficial for the treatment of autoimmune diseases in which dendritic cells play a fundamental role in regulating and maintaining the balance between immunity and tolerance [76, 2015, 189, 189].

Peptides possess many properties that indicate their potential use as vaccine candidates, and they have been used as tools in immunological research for several years. In order to be an effective subunit or peptide-based vaccine, the constituent sequences should be conserved so that the pathogen cannot overcome the constraint by evolutionary pressure. It is also aimed to evaluate whether the predicted cluster was capable of eliciting an immune response using *in vivo* systems. Bioinformatics analyses revealed that the cluster epitopes would be able to stimulate an immune response in mice. It was determined that

pneumococcal surface protein antigen (PspA) is in fact a highly conserved protein, and as such highly immunogenic peptides of PspA can serve as good vaccine candidates. In this investigation, after all necessary sequence analyses using Gene bank (NCBI), protein analysis (IEDB), Protein Data bank (PDB) and different software use like Mega, SnapGene viewer facilitated to hand-picked the epitope sequences of PspA. Thus, the immune-informatics analysis has predicted some multi-epitope cluster. The results in this experiment, demonstrate the potential of selected four epitope predicted peptide sequences, as the pneumococcal vaccine candidate, as well as the utility of a peptide-based vaccine design approach. With these four sequences vector plasmid were constructed and used for *Agrobacterium*-mediated genetic transformation to get edible vaccine.

In this experiment, the immune-informatics analysis has predicted four epitope clusters those are 33, 10, 17 and 7 amino acids in length, and which is predicted to provide high population coverage.

Now a days plant genetic transformation technology is considered as one of the most useful biotechnological methods which exploring an alternative to conventional methods towards the improvement of this crops. This transformation technology is regarded as a pre-breeding method that can provide a solution to certain constraints that hampered crop production.

The present study was carried out in tobacco (*N. tabacum*) and tomato plant (*L.esculentum*), because these plant can grow quite easily in the sunny, hot and humid climates of our country. The plants can be easily maintained in both *in vivo* and *in vitro* conditions, due to their growth conditions and various factors. It was found that enriching the media with low sugars and low pH were both important factors in achieving efficient transformation in plants. In the present study, the leaf discs of *in vivo* grown tobacco plants and tomato plants were used for co cultivation with *Agrobacterium* and the transformed callus tissue was obtained by applying cefatoxime, kanamycin, and carbenicilin which prevents the growth of other bacterial cells, prevent excessive growth of *Agrobacterium* and allows only the kanamycin resistant ones to grow. Plant transformation vectors and methodologies have been improved to increase the efficiency of plant transformation and

to achieve stable expression of transgenes in plants. Due to simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti Plasmid – based vectors continue to offer the best system for plant transformation. Binary vectors have been improved by the incorporation of super virulent vir genes, matrix attachment regions (MAR) and the insertion of introns in marker genes and reporter genes. With these improvements transformation in plants using *Agrobacterium* has almost become a routine process [294, 381]

In this study, transgenic tomato and tobacco plants expressing the PspA virulent factor of *S. pneumoniae* specific antigen Alpha, Gamma and Ds-red were developed fused to the zein protein. To achieve high expression and accumulation of these fusion proteins in the selected plant, a highly active promoter was used to express the synthetic vaccine sequences. Synthetic vaccine sequences were codon-optimized for expression in plants, as it has been reported that such an approach results in increased protein expression [197]. For instance, optimizing the codon usage of the selected epitope sequences for expression in rice achieved accumulation levels of ~2.1% of total seed protein [272].

Three constructs were produced using three different inserts namely alpha, gamma and Ds-red construct. In alpha, epitope sequence 1 is positioned; in gamma epitope sequence 2, 3 and 4 and Ds-red is the combination of alpha and gamma zein gene. *Agrobacterium* strain LBA4404 containing plasmid harboring *npt*II (neomycin phosphotransferase) was used for selection in all cases for the integration of gene of interest. Vector alpha zein fertig containing alpha zein gene and neomycin phosphotransferase (*nptII*) genes (named as alpha zein); Vector gamma zein fertig containing gamma zein gene and neomycin phosphotransferase (*nptII*) genes (named as gamma zein); and pTRAkt-glyDs-zen containing Ds-red zein gene and neomycin phosphotransferase (*nptII*) genes (named as Ds-red zein) in a *Agrobacterium* strain LBA4404 were used to integrate the desired gene in both tomato and tobacco genome to make edible vaccine. All of these constructs carrying sequences for epitope against PspA of *S. pneumoniae*. The transformants developed through this study were characterized through molecular analysis, like PCR and immunoblot analysis to confirm the introgression of foreign genes in nucleotide and protein levels.

As an integral part of *Agrobacterium*-mediated genetic transformation, experiments were carried out to establish an efficient *in vitro* regeneration protocol using plants, *N. tabacum* and *L. esculentum*, which are commonly cultivated in Bangladesh. Several experiments were carried out to optimize *in vitro* regeneration system using four different explants, namely, immature leaflet, cotyledonary leaf, cotyledonary node and hypocotyl. Establishment of regenerated plants to soil was also carried out following the development of sufficient root system.

An efficient and reproducible *in vitro* regeneration system is required to be developed for the particular plant species to establish a transformation protocol [127]. Earlier studies revealed that most of the legumes, including groundnut, are considered recalcitrant towards regeneration and genetic transformation [124]. However, recent literature survey yielded a number of reports on the transformation and regeneration of different cultivars of tobacco and tomato across the globe.

In case of tomato, it is reported that cotyledonary leaf explants *L. esculentum* can be infected by *A. tumefaciens* strain LBA4404.
The shoot regeneration protocol described here is efficient and reproducible. The selectio n procedure developed during the study has been found to be effective in recovering tran sformed plantlets showing that nptII gene is an efficient selectable marker for tomat oes [124]. It was also reported that transgenic tobacco plants were produced that express an anti-*Salmonella enterica* single chain variable fragment (ScFv) antibody that binds to lipopolysaccharide of *S. enterica* Paratyphi B was used in diagnosis and detection, as a therapeutic agent, and in applications such as water system purification [201].

Throughout the present study *in vitro* plant regeneration was carried out through organogenesis using different types of explants, e.g. immature leaflet, cotyledonary leaf, cotyledonary node, single cotyledon and hypocotyl from tobacco and tomato. Cotton soaked with sterile distilled water was found to be most effective for *in vitro* seed germination in both tomato and tobacco.

The various factors on which *in vitro* callus induction and shoot regeneration depends include the composition of culture medium, proper concentration of growth regulators and

the responses of explants as well as the genotype of the plant material. The results obtained from the study demonstrated that, MS medium with different concentrations and combinations of BAP alone and in combination with BAP and Kn was effective in regenerating multiple shoots directly or via intervention of callus phase from different explants of tomato. This result is in agreement with that of the findings of Sarker et al., (2009) [325]. MS medium with different concentrations and combinations of BAP alone and in combination with BAP and NAA were effective in regenerating multiple shoots directly or via intervention of callus phase from different explants of tobacco. This result is in agreement with that of the findings of [211].

In the present investigation, it has been observed that the immature leaflet explants of *N. tabacum* produced highest number of healthy multiple shoots on MS medium supplemented with 2.0 mg/l BAP. In this case the 73.33 - 80% explants responded towards shoot initiation. A series of experiments were also conducted to investigate the effect of different hormonal supplements such as BAP, Kn and NAA on *in vitro* shoot regeneration from the leaflet explants. In case of BAP and NAA supplemented MS medium 2.0 mg/l BAP and 0.2 mg/l NAA showed best response towards regeneration of multiple shoots, though the mean no of shoots/explant was low compare to the shoots regenerated on MS with 2.0 mg/l BAP. Previously Rahman et al., [299] observed best shoot regeneration using leaflet explants of *N. tabacum* on MS with 2.0 mg /l Kn and 2.0 mg/l IAA which does not corresponds to the concentrations of BAP and Kn used in the present investigation. Dixit et al., [98] got best result after using MS media supplemented with 1.0 mg/l BAP along with 1.0 mg/l NAA in callus formation. As *in vitro* regeneration of tobacco is not that much inflexible to get good regeneration, many combination might be applicable for tobacco multiplication.

Experiments were also carried out to observe the multiple shoot regeneration ability of leaflet explants on MS containing various concentrations of BAP and Kn. It was observed that *N. tabacum* showed positive response but reduced towards regeneration of shoots on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l Kn (38%), whereas *L. esculentum* showed best response on 2 mg/l BAP and 0.2 mg/l Kn (75%) containing MS medium. In this combination of BAP and Kn 95% explants showed responses towards

initiation of shoots. Large amount of callus was produced in the various combinations of BAP and Kn. So, in the present investigation it was noticed that leaflet explants in all combinations of BAP and NAA with MS medium did not show notable response towards shoot initiation in *L. esculentum* whereas, this combination is worthy for *N. tabacum* also.

Induction of healthy root, after successful regeneration of shoots, is an essential part for successful development of plantlets. In the present investigation various concentrations of auxin like 0.1, 0.25 and 0.50 mg/l of IBA, IAA and NAA were used for root induction. The results of the present study demonstrated that although roots were induced more or less in all the three combinations of IBA, IAA and NAA, 0.5 mg/l concentration of IBA was found most effective for root induction in regenerated shoots of *N. tabacum* and *L. esculentum*, whereas 0.1 mg/l gave poor rooting and 0.25 mg/l IBA showed optimum response towards inducing healthy roots. According to Rahman et al., [299], the rooting response of regenerated shoots was observed by using 1/2 MS medium with IBA (0.0, 0.5, and 1.0 mg/L). The highest root formation was found in Motihari (90%) [325] with ½ MS medium supplemented with 0.5 mg/L IBA In tobacco. This results is similar with the present experiment. The maximum root induction from the regenerated shoots were achieved on half the strength of MS medium supplemented with 0.2 mg/l IAA observed by Sarker et al., in *L. esculentum* [325].

After proper hardening, *in vitro* regenerated shoots with well-developed roots of *N. tabacum* and *L. esculentum* were successfully transplanted into soil. Almost 95% transplanted plantlets of *N. tabacum* were survived in soil. And in case of *L. esculentum* 95% acclimatized into the pot containing treated soil. The survived plantlets grew well in large clay pots and afterwards they were transferred in experimental field.

The second phase of the investigation dealt with the genetic transformation of *N. tabacum* and *L. esculentum*. Among the different approaches, *Agrobacterium*-mediated genetic transformation has been considered as the most common and successful method used in various plants such as, tomato [10,325,384] tobacco [351,201]. Reports are also available for using several other methods of genetic transformation of plants including microprojectile bombardment, electroporation, sonication, chemical method of

transformation, etc. [271, 14,226,236,341]. All these methods are plant specific and cost intensive. Generally, *Agrobacterium*-mediated genetic transformation has been considered as the most convenient and cost effective than other techniques. It is the most popular method for genetic transformation because of high co-expression of introduced genes, potentially low copy number and preferential integration into active transcription regions [366,100,328].

In the present transformation study, genetically engineered *Agrobacterium* strain LBA4404 containing three different binary vector plasmid Alpha zein fertig containing alpha zein and *nptII* (Neomycin phosphotransferase) genes (considered as construct alpha zein); LBA4404 containing plasmid Gamma zein fertig containing gamma zein gene and *nptII* (Neomycin phosphotransferase) gene (considered as construct gamma zein) and vector plasmid pTRAkt-glyDs-zen containing Ds-red zein (considered as construct Ds-red) were used as transformation vectors to transform tomato and tobacco plant. As mentioned before, the sequences of *Streptococcus pneumonia* epitopes were placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, respectively. For easy detection, the alpha zein construct (construct alpha) contains a flag- and his-tag, the gamma zein construct (construct gamma) contains only the his-tag. Vector plasmid pTRAkt-glyDs-zen is the combination form of alpha and gamma zein gene. All these three constructs are newly prepared in this experiment. There is no report present with these plasmids. Therefore, required information, statistics, data or any evidence could not be found regarding these vectors. These are confirmedly unique product of the present research work.

It is well known fact that the transformation and regeneration are influenced by various physical and chemical factors such as choice of genotypes, explants, co-cultivation time, virulence inducing agents, hormonal combinations and selectable markers [238]. An ideal transformation protocol should be qualified as the genotype independent and rapid, with minimal presence of chimerism in the regenerated transgenic plants, with high frequency of transformation. Factors like explant type, polarity and orientation of explants, hormonal combinations, addition of various antioxidants, time of infection, co-cultivation period, temperature, pre-culture, bacterial strains, nature of the genes and promoters used, use of

sonication, selective agent, selection pressure and time of application, binary vectors with enhanced virulence, phenolic compounds are reported to increase transformation efficiency in tomato and tobacco as well as many recalcitrant plant species [328, 121,367,313,372,365,104,34,296,4,275].

In the present study different explants, namely, immature leaflet, cotyledon and single cotyledonary node, cotyledon and hypocotyl both plants were used for the evaluation of various factors influencing transformation efficiency. Factors that influence successful transformation efficiency, such as *Agrobacterium* strain-variety (host) compatibility and responsiveness of explants toward *Agrobacterium* infection were evaluated. Several other factors of transformation like optical density (O.D) of *Agrobacterium* suspensions, incubation duration of explants in to bacterial suspension, co-cultivation period, co-cultivation condition (dark/night), etc. were also optimized.

During optimization of the above mentioned factors it was found that maximum transformation efficiency was observed with bacterial suspension having an optical density of 1.0 at 600 nm. Eapen and George, reported to obtain regeneration after 3 to 4 weeks from transformed explants which was incubated for 5 min in the bacterial suspension followed by co-cultivation for three days [105]. Sarker et al., [325] applied 30 mins of incubation period for transformation of tomato and co-cultured for 72 hours using immature leaflet explants and got the transformed plants [325]. Tiwari et al., Tiwari and Tuli, Tiwari et al., reported that late log phase (OD600 1.0 to 1.6) of bacterial growth was optimum for the transformation in peanut [365, 366, 367]. They infected the cotyledon explants with the A*grobacterium* suspension having an optical density of 1.4 - 1.6 with 20 minutes incubation period followed by 5 days of co-cultivation period to get transgenic peanut. In chickpea, Krishnamurthy et al., incubated mature embryo explants for 20 minutes and then co-cultured the explants for 3 days to obtain transgenic plants whereas Tripathi et al., [372] obtained notable transformation efficiency when the infected explants were incubated in 2 days of co-cultivation. In the present study, cotyledon and leaflets explants showed best result towards transformation when *Agrobacterium* suspension had an optical density of 0.6 - 0.8 followed by 3 minutes of incubation period with 3 - 4 days of co-cultivation with all the *Agrobacterium* constructs [372].

In the present study it was found that, longer incubation in the A*grobacterium* suspension leads to the over-growth of bacteria in regeneration culture medium thus hampering the proper growth of infected explants. Overgrowth of *Agrobacterium* was the real problem, when infection time increased to 45 min (data not shown). Similarly, increase of infection time to more than 35 min caused browning of the target tissue and did not allow it to flourish [363]. Experiments were carried out to find the effect of dark (in incubator) and light conditions (growth room condition) during co-cultivation period after infection with *Agrobacterium*. No significant differences was observed towards the transformation efficiency between the dark and light (growth room condition) condition during co-cultivation. Both of the conditions showed almost similar results. Tiwari and Tuli, Tiwari et al., and Chen et al., obtained good transformation efficiency using dark conditions for the incubation of explants whereas Venkatachalam et al., Anuradha et al., Vasudevan et al., Iqbal et al., co-cultivated the explants under light condition instead of dark [367, 366, 66, 380, 4 and 379].

Three of the constructs (alpha zein, gamma zein and Ds-red zein) used in this investigation contained *npt*II gene within its T-DNA region and this gene confers kanamycin resistance to the transformed cells. Therefore, for the selection of transformed tissue kanamycin was applied to the regeneration medium for the selection of transformants. To determine the level of selection agents (kanamycin) in medium, different concentrations of kanamycin were tested. In many reports kanamycin was applied immediately after co-cultivation for the selection of transgenic shoots/cells [70,379,380,347]. However, in the present study it was found that addition of kanamycin immediately after co-culture greatly hampered the growth of the explants. It was observed that even in the presence of lower concentration of kanamycin, co-cultivated explants failed to regenerate. Following the reports of Sharma and Anjaiah [328]; Iqbal et al., [169], Anuradha et al., [4], Beena et al., [30], Bhatnagar-Mathur [34] selection agent was added when the shoot buds were started showing their first signs of emergence. Therefore, a pre-culture period and a delayed selection with kanamycin were followed in obtaining regeneration from explants with high rate of transformation efficiency.

In the present study, it was observed that all control shoots died in the selection medium with 50 mg/l kanamycin. Non-transformed shoots were started to become albino in

presence of 50 mg/l kanamycin. To establish the initial selection pressure 50 mg/l kanamycin was applied and this concentration of kanamycin increased gradually up to 250 mg/l to optimize the proper selection pressure. Shoots that survived on the selection pressure of 250 mg/l for 20 - 25 days were considered as putative transformed shoots. Sharma and Anjaiah [328] reported that 200 μg/ml kanamycin as optimum for the selection of transformed shoots raised from cotyledon explants. However, Sarker and Nahar [324] reported that 300 mg/l kanamycin was optimum to identify the non-transformed explants and they applied higher selection pressure to select the transformed shoots. Eapen and George [105] observed an average of 6.7% of shoot regeneration from leaf tissues on the selection medium containing 50 mg/l kanamycin. Anuradha et al., [4] cultured the completely green shoots on shoot elongation medium with 175 mg/l kanamycin for two selections of two-week duration each. They suggested that 90% elimination of non-transformed shoots was observed when selection pressure was 125 mg/l kanamycin. During the study 100 mg/l carbenicilin was used in all these culture media to check bacterial over growth. However, Venkatachalam et al., [380], Anuradha et al., [4] Beena et al., [30] used 250-300 mg/l cefatoxime to inhibit the growth of *Agrobacterium*. Selection of the transformed shoots was carried out by gradually increas ing the concentration of kanamycin to 200 mg/l since kanamycin resistant gene was used for transformation experiments of tomato by Sarker et al., [325]. Pugalendhi et al., [294] found best result in tobacco transformation using 50 mg/l kanamycin as selectable marker. This result is similar to the present investigation.

Cheng et al., [67] reported that among the different *Agrobacterium* strains (AGLO, LBA4404, C58 and EHA105) the most efficient one was LBA4404 followed by AGLO, EHA105 and C58. This could be due to differences in the functioning of different *vir* genes, which are important factors that influence the infection process by different *Agrobacterium* strains [203]. The rate of infectivity is influenced by the combination and number of virulent genes involved in transcription [147]. Yadav et al., [401]] also reported that among the different *Agrobacterium* strains e.g. LBA4404, EHA105 and GV3101, LBA4404 infected plants showed highest GUS activity in. Rohini and Rao [310] reported the use of *Agrobacterium* strain LBA4404 in groundnut. Similar results were reported in *Vigna*

mungo where transformation frequencies were superior with bacterial stain LBA4404 than those infected by EHA105 [187]. High transformation frequency using *Agrobacterium* strain LBA4404 has been reported in pigeon pea [297], in pea [280], in papaya [18] and in mungbean [400].

Shoots that survived in higher concentration of selection medium were transferred to root induction medium. It was observed that no root induction was initiated when putatively transformed shoots were cultured on root induction medium containing kanamycin. For this reason, only carbenicilin was used in root induction medium to control the overgrowth of *Agrobacterium*. These findings were in agreement with the results of Anuradha et al., [4]. Sharma and Anjaiah [328] also cultured the elongated shoots on root induction medium without any antibiotic.

Detection of integrated gene/s in the targeted host plant is an integral part of developing transgenic plants. Most trusted way to detect the integration of transgene is through polymerase chain reaction (PCR) analysis. For the present experiment primers had been designed using Primer-BLAST, which describes a robust and fully implemented general purpose primer design tool that designs target-specific PCR primers. Primer-BLAST offers flexible options to adjust the specificity threshold and other primer properties. This tool is publicly available at http://www.ncbi.nlm.nih.gov/tools/primer-blast.

In the present investigation, integration of transgene in the transformed plantlets was confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. The DNA isolated from both transformed and non-transformed shoots was subjected to PCR for the amplification of alpha, gamma and Ds-red zein gene and *nptII* gene. PCR amplified DNA was analyzed through agarose gel electrophoresis. 150 bp amplified bands corresponding to alpha and gamma and 180 bp for *nptII*, were observed in each of the transformed plantlets for both tomato and tobacco were identical to the positive control. This result indicated that the respective target genes were inserted in the genomic DNA of transformed plantlets.

For antigen detection total leaves protein extracts of transgenic tobacco leaves, Western Blots were performed. All three constructs contain His-tag, in order to detect this sequence.

In case of alpha zein along with His tag also FLAG-tag inserted for the detection purpose. The construct gamma shows the strongest band than that of alpha zein. Surprisingly, the mass of the gamma fusion protein was much higher than expected. One reason could be the formation of protein dimers. The mass of a dimer would correspond to the result. The construct Ds-red also shows slight bands. In the Ds-red yielded bands the amount of the protein was much less than the amount obtained with the gamma zein construct only. This results indicates that, anti-his polyclonal antibodies were raised in mice and significant amounts of anti-his IgG were detected. Furthermore, published literature on plant based vaccines reports WB and protein studies only from the T2 or T3 generation of seeds, which reinforces the present investigation [272,154].

Oral vaccination using transgenic plants is a viable approach to the development of a novel vaccine. Availability of the vaccine in an edible form as a fruit or vegetable would enhance vaccination coverage by providing an inexpensive, easy to deliver and relatively heat-stable package for distribution. Such a vaccine would have the potential to enable rates of vaccination to reach the targets required for global eradication.

In this study tomato transformed leaves were orally fed to 48 mice divided into four groups. The groups were divided by alpha, gamma, Ds-red and one is control (Non-transformed). The experiments were conducted within two different trials. Leaves of transgenic plant were first made like powder with lyophilizer and prepared a tablet form mixed along with mice feeding pellet in the ration 1:1. For immunogenicity testing mice were orally fed the special tablets. For each feeding, each mouse was given ~1 gm of transgenic leaves containing recombinant protein. 6 doses were performed in each trial with 7 days intervals. They were treated under observation for more than 30 days after challenged with *S. pneumoniae*.

In both of the 1st and 2nd trials, among 36 orally immunized mice, 80.55% mice remain alive after challenge with *S. pneumoniae* of serotype 7F in case of 1st trial and 95.23 in case of 2nd trial. In case of control (Mice feeding non-transformed leaves) the rate is 50% in both trials. Therefore, mice that received 1gm of transgenic leaves over the course developed a better immune response to PspA than those fed in non-transformed leaves.

After statistical analyses using SPSS version 22.0, it has also be proven that there is no significant difference between the first and second trial of the mice immunization test. In order to test the equality of average survival number from two trials (1st and 2nd), t-test has been performed. Test results shows that the means are not significantly different ($p>0.05$). That means, the survival rate in both the trials are almost similar. So, there is no significant variation in between 1st and 2nd trial of mice challenge experiment.

To test the equality of survival rate in control group and three other constructs namely Alpha, Gamma and Ds-red, Analysis of Variance (ANOVA) has been done. It can be seen from the test results that average survival rates in the three constructs are different from the control group ($p<0.05$).
After applying Duncan Multiple Ranked Test (DMRT) , it can be noticed from the findings that all the constructs are in one subset, and they are far higher than control set which belongs to another group at 5% level of significance.

Kong et al., [194] have demonstrated that HBsAg (a subunit antigen of a nonenteric pathogen) expressed in potato tubers and delivered to mice as an edible vaccine can stimulate serum antibodies specific for HBsAg at levels that very significantly exceed the protective level (in humans) of 10 mIUyml. The unique features of bioencapsulation of the antigen within plant cells may be the actual reason why plant-based HBsAg is effective for oral immunization.

Gao et al., [125] reported that a new foreign breed of cherry tomatillo was used as the vector of producing transgenic plant vaccine. Through the research of transforming HBsAg gene into tomatillo, a high efficient transformation system of tomatillo was produced, and the recombinant protein from transgenic plants possessed good immunogenicity. The work demonstrated the feasibility of expressing the oral immunogenic rHBsAg in transgenic tomatillo, and provided some theoretic and experimental directions for the production oral vaccines using transgenic tomatillo in large scale.

Conclusion

From the foregoing discussion, it may be concluded that the preparation of the constructs, the transformation into A. tumefaciens and the expression of gamma zein, alpha zein and Ds-red zein as intact fusion proteins with Pneumococcus antigen in plants were successful. Those transgenic plants have been confirmed through selective agent (antibiotic) as well as polymerase chain reaction (PCR). The immunoblot analysis clearly showed that the fusion proteins were expressed. The gamma zein protein however, had an unexpected mass, perhaps due persisting dimers. Mice challenge experiment clearly proved that transgenic plant can stimulate serum antibodies specific for PspA at levels that very significantly exceed the protective level.

To reduce outbreaks of infectious diseases worldwide, the implementation of control and prevention measures on a massive scale is required. In this scenario, edible vaccines represent a valuable alternative to mitigate and prevent infectious outbreaks in countries where the conventional vaccination is difficult. In addition, in countries where the prevalence of infectious diseases is controlled, edible vaccines may support public health programs to reduce the risk of disease outbreaks, analogous to the use of prebiotics and probiotics as a complement to food. As shown in this work, the current production of edible vaccines is focused on a tomato and tobacco plants, of which tomatoes are consumed globally. However, promoting the genetic transformation of plants with higher impact on the consumption chain in specific countries remains challenging. In addition, increasing the agricultural products of each country must be based on a consideration of country-specific policies with respect to the production or commercialization of genetically modified plants as well as ecological and cultural regulations, especially in those countries considered centers of origin of some important crops.

Moreover, before consideration for releasing these edible vaccine further confirmation and many other immunogenicity testing using proper bioassay for screening of transgenic plants under the laboratory, biosafety net house and field conditions. Food safety assessment as well as Environmental Risk Assessment (ERA) will also be needed to perform as the integral part of biosafety regulations of the country. To the best of our

knowledge, this is the first report on the successful attempt of edible vaccine production using *Agrobacterium*-mediated genetic transformation in Bangladesh.

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APPENDIX – A

Apparatus/Machines

Apparatus	Company
Laminar Air Flow Cabinet	Class-II A1, SAARC, Japan
Nanodrop Photometer	IMPLEN, Netharlands
Autoclave	Model H42 AE, Hirayama manufacturing corp., Japan.
High speed Centrifuge	SORVO
Deionizer	Ultraflow, USA
Disposable micropipette tips	Eppendrof, Ireland
Distilled water plant	Fistreen water purification, UK
Electronic balance	Adventurer, OHAUS corp, USA
Power pack	Biometra, Germany.
Centrifuge tube (1.5 mL)	Eppendorf, Ireland.
Incubator	WTB-binder, Germany
Hot plate stirrer	Gallenkamp, UK
Refrigerated Centrifuge	Hettich, Germany
Centrifuge	Hettich, Germany
SDS-PAGE apparatus	Bio-Rad, USA
Western blot apparatus	Neido, Japan
4°C Centrifuge	Sigma, USA
Sterilizer	NDS-600D, EYELA, Tokyo, Japan
Spectrophotometer	HACH, UV visible, Model no. DR/4000U
Vortex mixer	Whirli Mixer, Fisons, England
Magnetic stirrer	Stuart Scientific, Grate Britain
Orbital shaker incubator	Thermo Forma, USA
Gel documentation	Bio-Rad, USA
Biological safety cabinet	Class-II A1, Thermo Forma, USA

Ultra low Temperature Freezer	Thermofisher
Thermal Cycler (PCR)	BioRed
Gel electrophoresis	BioRed
Water Distillation Plant	SRL Bio Lit
Digital PH meter	Neido, Japan
Digital Analytical Balance	Esco
Orbital Shaker	BioRed
Biolog microbial ID system	GEN_lll_v2.7.1.42.15G

APPENDIX – B

Chemicals

Chemicals	Company
Acetone	Loba chemic, Bombay, India
Acrylamide, monomer	Nacalai Tesque, Inc.,Kyoto, Japan
Ammonium hydroxide	Sigma, USA
Ammonium persulfate (APS)	Serva, Germany
Anti-Chicken IgG (Alkaline phosphatase conjugate)	Abcam, UK
Bile salts	Oxoid, England
β-Mercapto ethanol	Sigma, USA
Bromophenol blue indicator	Loba chemic, Bombay, India
Citric acid	Oxoid, England
Coomassie Brilliant Blue R250	Serva, Germany
Disodium hydrogen phosphate	Sigma,USA
Ethylhydrocupreine, hydrochloride (Optochin disk)	BBL, USA

EGTA	Sigma, USA
Formaldehyde	Sigma,USA
Glacial acetic acid	BDH Laboratory Supply, England
Glycerol	BDH Laboratory Supply, England
Glycine	Nacalai Tesque, Inc., Kyoto, Japan
Hydrochloric acid	BDH Laboratory Supply, England
Methanol	BDH Laboratory Supply, England
Molecular weight marker	Bio-Rad, USA
N, N Methylene- bis-acrylamide	Sigma, USA
Na2HP04	Merck, Germany
Nitrocellulose membrane	Highbond C
PVDF membrane	Bio-Rad, USA
Potassium chloride	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Silver nitrate	Sigma, USA
Sodium azide (NaN3)	Sigma, USA
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate(SDS)	Sigma, USA
Sodium hydroxide	Oxoid, England
TEMED	Serva, Germany
Trizma base	Sigma, USA
Tris-Cl	Sigma, USA
Tween 20	Sigma, USA
FBS (Fetal Bovine Serum)	Sigma, USA
BHI (Brain Heart Infusion)	Sigma, USA

Solutions and Buffers

SDS-PAGE Analysis

1) 30% acrylamide-bis acrylamide solution:

Acrylamide	29.0 gm
Bis-acrylamide	1.0 gm
Distilled water	10 mL

2) 10% ammonium per sulfate

Ammonium per sulfate (APS)	1 gm
Distilled water	10 mL

Store at -20°C

3) 0.1% BMB (Bromophenol blue solution) or Tracking dye

Bromophenol blue	0.1 gm
Distilled water	100 mL

4) Destaining solution (7 % acetic acid)

Glacial acetic acid	7 mL
Distilled water	93 mL

5) Staining solution

Coommassie brilliant blue G-250	0.20 gm
10 % acetic acid	100 mL

6) Sample loading buffer

0.5 M Tris- Cl (pH 6.8)	10 mL
10% SDS	10 mL
2-mercaptoethanol	1 mL
Glycerol	10mL
Distilled water	19 mL

7) Electrophoresis buffer (pH 8.3)

Tris	3.0 gm
Glycine	14.4 gm
10% SDS	10 mL

Distilled water 1000 mL

8) Upper gel buffer (pH 6.8)

Tris-base 6.1 gm

SDS 0.4 gm

Distilled water up to 100 mL

pH adjusted to 6.8 by adding HCl

9) Lower gel buffer (pH 8.8)

Tris base 18.17 gm

SDS 0.4 gm

Distilled water up to 100 mL

pH adjusted to 8.8 by adding HCl.

Western Blot Analysis

1) 0.1% Amido Black

Amido Black 0.1 gm

1% acetic acid 100 mL

2) Blocking solution

Skim milk 2 gm

PBS 100 mL

3) 10% Na-azide solution

Na-azide 1 gm

Distilled water 10 mL

4) 0.1% Tween 20

Tween 20 0.2 mL

Distilled water 200 mL

5) PBS (pH 7.2)

NaCl 8.56 gm

Na_2HPO_4 1.18 gm

KH_2PO_4 0.23 g

KCI	0.20 g
Distilled water	1 L

6) Transfer buffer (Blot buffer)

Deionized water	1L
Methanol	250 mL
Tris base	3.6gm
Glycine	18 gm

7) 50 mM Tris-HCl (pH 9.4)

Tris base	605 mg
Distilled water	200 mL

pH adjusted to 9.4 by adding few drops of HCl

8) Substrate preparation

MgCl2	12 mL
NaCl (0.5 M)	4 mL
Tris base (0.5 M)	4 mL
NBT	88 µL
BCIP	66 µL

Media

1) Brain Heart infusion broth (pH 7.2)

BHI dehydrated media	7.4 gm
Distilled water	200 mL

2) Gentamycin blood agar media

Nutrient agar	100 mL
Sheep Blood	5-7mL
FBS	5 mL

Gentamycin powder (Antibiotic)	0.035g

3) Nutrient agar media (150 mL)

Peptone	0.75 g
Beef extract	0.45 g
NaCl	0.75 g
Agar	2.70 g

Preparation of YMB media

Components	Amount needed for 100 ml medium
Bactopeptone	1.0 g
Bacto Yeast extract	1.0 g
NaCl	0.5 g

Murashige and Skoog (MS) Medium 1962

Components	**Concentration**
Macronutrients	**(mg/l)**
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30

Dhaka University Institutional Repository

H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.

APPENDIX- C

Sequences

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CTGTTGGCTGGCTGGTGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACA
ACTTAATAACACATTGCGGACGTTTTTAATGATCGAATACTAACGTCTCTACCAGATA
TCAGCTTGCATGCCGGTCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTAT
CCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGAC
TCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACA
TGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGA

TTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGATCTGCTTGACTCTAGCTAG
AGTCCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCG
ATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCA
TTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCG
GTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCAC
CATGATATTCGGCAAGCAGGCATCGCCCTGGGTCACGACGAGATCCTCGCCGTCGGG
CATCCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTC
GTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTCCTCGCTCGAT
GCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCG
CCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAG
GAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGAC
AACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCG
CTGCCTCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAA
CCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCT
GTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGT
GCAATCCATCTTGTTCAATCATGCCTCGATCGAGTTGAGAGTGAATATGAGACTCTA
ATTGGATACCGAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTGACAAGAAATAT
TTGCTAGCTGATAGTGACCTTAGGCGACTTTTGAACGCGCAATAATGGTTTCTGACGT
ATGTGCTTAGCTCATTAAACTCCAGAAACCCGCGGCTCAGTGGCTCCTTCAACGTTGC
GGTTCTGTCAGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATA
ACGTGACTCCCTTAATTCTCCGCTCATGATCGATATCCATTGAAGAGCAAGCT

Ds-red zein construct:

AATTCCGCCCCTAGAAATATTTGCGACTCTTCTGGCATGTAATATTTCGTTAAATATGAA
GTGCTCCATTTTTATTAACTTTAAATAATTGGTTGTACGATCACTTTCTTATCAAGCGTT
ACTAAAATGCGTCAATCTCTTTGTTCTTCCATATTCATATGTCAAAATCTATCAAAATTC
TTATATATCTTTTTCGAATTTGAAGTGAAATTTCGATAATTTAAAATTAAATAGAACATA
TCATTATTTAGGTGTCATATTGATTTTTATACTTAATTACTAAATTTGGTTAACTTTGAA
AGTGTACATCAACGAAAAATTAGTCAAACGACTAAAATAAATAAATATCATGTGTTATTA
AGAAAATTCTCCTATAAGAATATTTTAATAGATCATATGTTTGTAAAAAAAATTAATTTT
TACTAACACATATATTTACTTATCAAAAATTTGACAAAGTAAGATTAAAATAATATTCAT
CTAACAAAAAAAAACCAGAAAATGCTGAAAACCCGGCAAAACCGAACCAATCCAAACCGA
TATAGTTGGTTTGGTTTGATTTTGATATAAACCGAACCAACTCGGTCCATTTGCACCCCT
AATCATAATAGCTTTAATATTTCAAGATATTATTAAGTTAACGTTGTCAATATCCTGGAA
ATTTTGCAAAATGAATCAAGCCTATATGGCTGTAATATGAATTTAAAAGCAGCTCGATGT
GGTGGTAATATGTAATTTACTTGATTCTAAAAAAATATCCCAAGTATTAATAATTTCTGC
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TCCTTTGCATGTAACTATTATGCTCCCTTCGTTACAAAAATTTTGGACTACTATTGGGCG
CGGGGCGCGCCCGGTCCAAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATAT
CGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAG
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AGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGAATTCTCAA
CACAACATATACAAAACAAACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCA
ATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTACGAAC
GATAGCCATGGGGAACTCTTCCAAGAATGTTATCAAGGAGTTCATGAGGTTTAAGGTTCG
CATGGAAGGAACGGTCAATGGGCACGAGTTTGAAATAGAAGGCGAAGGAGAGGGGAGGCC
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TTGGGATATTTTGTCACCACAATTTCAGTATGGAAGCAAGGTATATGTCAAGCACCCTGC
CGACATACCAGACTATAAAAAGCTGTCATTTCCTGAAGGATTTAAATGGGAAAGGGTCAT
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CAAACTGGATATAACAAGCCACAACGAAGACTATACAATCGTTGAGCAGTATGAAAGAAC
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CCAACAGCCGCATCCAAGCCCGTGCCAGGCGGCCGCTCATCACCATCACCATCACTAGCT

CGAGCTCGGATCCTCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAATCTATCTCT

CTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTA

TAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAA

AATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTGACCGGGCGGCCG

GCCGCCCCTAGAAATATTTGCGACTCTTCTGGCATGTAATATTTCGTTAAATATGAAGTG

CTCCATTTTTATTAACTTTAAATAATTGGTTGTACGATCACTTTCTTATCAAGCGTTACT

AAAATGCGTCAATCTCTTTGTTCTTCCATATTCATATGTCAAAATCTATCAAAATTCTTA

TATATCTTTTTCGAATTTGAAGTGAAATTTCGATAATTTAAAATTAAATAGAACATATCA

TTATTTAGGTGTCATATTGATTTTTATACTTAATTACTAAATTTGGTTAACTTTGAAAGT

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AAATTCTCCTATAAGAATATTTTAATAGATCATATGTTTGTAAAAAAAATTAATTTTTAC

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CATAATAGCTTTAATATTTCAAGATATTATTAAGTTAACGTTGTCAATATCCTGGAAATT

TTGCAAAATGAATCAAGCCTATATGGCTGTAATATGAATTTAAAAGCAGCTCGATGTGGT

GGTAATATGTAATTTACTTGATTCTAAAAAAATATCCCAAGTATTAATAATTTCTGCTAG

GAAGAAGGTTAGCTACGATTTACAGCAAAGCCAGAATACAAAGAACCATAAAGTGATTGA

AGCTCGAAATATACGAAGGAACAAATATTTTTAAAAAAATACGCAATGACTTGGAACAAA

AGAAAGTGATATATTTTTTGTTCTTAAACAAGCATCCCCTCTAAAGAATGGCAGTTTTCC

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GGCGTGAAAAGGTTTATCCGTTCGTCCATTTGTATGTGTACATCACCGACGAGCAAGGCA

AGACCGAGCGCCTTTCCGACGCTCACCGGGCTGGTTGCCCTCGCCGCTGGGCTGGCGGCC

GTCTATGGCCCTGCAAACGCGCCAGAAACGCCGTCGAAGCCGTGTGCGAGACACCGGCCG

CCGGCGTTGTGGATACCTCGCGGAAAACTTGGCCCTCACTGACAGATGAGGGGCGGACGT

TGACACTTGAGGGGCCGACTCACCCGGCGCGGCGTTGACAGATGAGGGGCAGGCTCGATT

TCGGCCGGCGACGTGGAGCTGGCCAGCCTCGCAAATCGGCGAAAACGCCTGATTTTACGC

GAGTTTCCCACAGATGATGTGGACAAGCCTGGGGATAAGTGCCCTGCGGTATTGACACTT

GAGGGGCGCGACTACTGACAGATGAGGGGCGCGATCCTTGACACTTGAGGGGCAGAGTGC

Dhaka University Institutional Repository

TGACAGATGAGGGGCGCACCTATTGACATTTGAGGGGCTGTCCACAGGCAGAAAATCCAG
CATTTGCAAGGGTTTCCGCCCGTTTTTCGGCCACCGCTAACCTGTCTTTTAACCTGCTTT
TAAACCAATATTTATAAACCTTGTTTTTAACCAGGGCTGCGCCCTGTGCGCGTGACCGCG
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CTCGATCGAGTTGAGAGTGAATATGAGACTCTAATTGGATACCGAGGGGAATTTATGGAA
CGTCAGTGGAGCATTTTTGACAAGAAATATTTGCTAGCTGATAGTGACCTTAGGCGACTT
TTGAACGCGCAATAATGGTTTCTGACGTATGTGCTTAGCTCATTAAACTCCAGAAACCCG
CGGCTGAGTGGCTCCTTCAACGTGCGGTTCTGTCAGTTCCAAACGTAAAACGGCTTGTCC

CGCGTCATCGGCGGGGGTCATAACGTGACTCCCTTAATTCTCCGCTCATGATCGATATCC
ATTGAAGAGCAAGCT

Construct 1 (raw) containing antigen peptide 1 fused to alpha-zein as carrier:

C C A TGG GG GAC TAC AAA GAC GAT GAC GAC AAGATT TTA ACA AGT CTA GCC AGC GTC GCT ATC TTA GGG GCT GGT TTT GTT GCG TCT CAG CCT ACT GTT GTA AGA GCA GAA GAA TCT CCC GTA GCT AGT CAG GCG GCC GCG GGC GGC GGC GGC AGC GGC GGC GGC GGC AGCATG GCC ACC AAG ATC CTG GCC CTG CTG GCC CTG CTG AGC CTG AGC GTG AGC GCC ACC ACC

GCC TTC ATC ATC CCC CAG TGC AGC CTG GCC CCC AAC GCC ATC ATC CCC CAG TTC CTG CCC

AGC GTG ACC AGC ATG GGC ATC GAG CAC CCC ATC GTG CAG GCC TAC AGG CTG CAG CAG GCC

CTG GCC GCC AGC GTG CTG CAG CAG CCC TTC GCC CAG CTG CAG CAG CAG AGC CTG GCC CAC

CTG ACC ATC CAG ACC ATC GCC ACC CAG CTG GAG CAG CAG TTC GTG CCC GCC CTG AGC CAG

CTG GCC GCC GTG AAC CCC GTG AGC TAC CTG CAG CAG CAG ATG CTG GCC AGC AAC CCC CTG

GCC CTG GCC AAC ACC GCC GCC TAC CAG CAG CAG CTG CAG CTG CAG CAG TTC CTG CCC GCC

CTG AGC CAG CTG GCC AGG GTG AAC CCC GCC ACC TAC CTG CAG CAG CAG CAG CTG CTG AGC

AGC AGC CCC CTG GCC GTG GGC AAC GCC GCC ACC TAC CTG CAG CAG CAG CTG CTG CAG CAG

ATC GTG CCC GCC CTG AGC CAG CTG GTG GTG GCC AAC CCC ACC GCC TAC CTG CAG CAG CTG

CTG CCC TTC AAC CAG CTG GAC GTG GCC AAC AGC GCC GCC TAC CTG CAG CAG AGG CAG CAG

CTG CTG AAC CCC CTG GCC GCC GCC AAC CCC CTG GTG GCC GCC TTC CTG CAG CAG CAG CAG

TTC CTG CCC TAC AAC CAG ATC AGC CTG ATG AAC CTG GCC CTG AGC AGG CAG CAG CCC ATC

GTG GGC GGC GCC ATC TTCcat cat cac cat cac cat tga TCT AG AG

M G D Y K D D D D K I L T S L A S V A I L G A G F V A S Q P T V V R A E E S
P V A S Q A A A G G G G S G G G G S **M** A T K I L A L L A L L S L S V S A T T
A F I I P Q C S L A P N A I I P Q F L P S V T S **M** G I E H P I V Q A Y R L Q Q A
L A A S V L Q Q P F A Q L Q Q Q S L A H L T I Q T I A T Q L E Q Q F V P A L S
Q L A A V N P V S Y L Q Q Q **M** L A S N P L A L A N T A A Y Q Q Q L Q L Q Q
F L P A L S Q L A R V N P A T Y L Q Q Q Q L L S S S P L A V G N A A T Y L Q
Q Q L L Q Q I V P A L S Q L V V A N P T A Y L Q Q L L P F N Q L D V A N S A
A Y L Q Q R Q Q L L N P L A A A N P L V A A F L Q Q Q Q F L P Y N Q I S L **M**
N L A L S R Q Q P I V G G A I F H H H H H H **Stop** S R

Construct 2 (raw) containing antigen peptides 2,3 and 4, to be fused to gamma-zein:

CCATGGGG
TCTAAAGCTGAGAAAGACTATGATGCAGCAGCGCAAAAAAAATATGACG
AAGATCAAAAGAAAACTGAAGAGTCTGAATCAGAAGATTATGCTGCGGC
CGC

MG S K A E K D Y D A A A Q K K Y D E D Q K K T E E S E S E D Y A A A

Codon optimized construct 1:

C C A TGG GGGACTATAAGG ATGATGACGA CAAGATATTG ACTAGTCTGG CATCTGTCGC TATCTTGGGC GCTGGTTTTG TGGCCAGCCA ACCAACCGTA GTTAGAGCTG AAGAAAGTCC CGTAGCCAGT CAAGCG GCC GCG GGC GGC GGC GGC AGC GGC GGC GGC GGC AGC**ATG GCC ACC AAG ATC CTG GCC CTG CTG GCC CTG CTG AGC CTG AGC GTG AGC GCC ACC ACC**

GCC TTC ATC ATC CCC CAG TGC AGC CTG GCC CCC AAC GCC ATC ATC CCC CAG TTC CTG CCC

AGC GTG ACC AGC ATG GGC ATC GAG CAC CCC ATC GTG CAG GCC TAC AGG CTG CAG CAG GCC

CTG GCC GCC AGC GTG CTG CAG CAG CCC TTC GCC CAG CTG CAG CAG CAG AGC CTG GCC CAC

CTG ACC ATC CAG ACC ATC GCC ACC CAG CTG GAG CAG CAG TTC GTG CCC GCC CTG AGC CAG

CTG GCC GCC GTG AAC CCC GTG AGC TAC CTG CAG CAG CAG ATG CTG GCC AGC AAC CCC CTG

GCC CTG GCC AAC ACC GCC GCC TAC CAG CAG CAG CTG CAG CTG CAG CAG TTC CTG CCC GCC

CTG AGC CAG CTG GCC AGG GTG AAC CCC GCC ACC TAC CTG CAG CAG CAG CAG CTG CTG AGC

AGC AGC CCC CTG GCC GTG GGC AAC GCC GCC ACC TAC CTG CAG CAG CAG CTG CTG CAG CAG

ATC GTG CCC GCC CTG AGC CAG CTG GTG GTG GCC AAC CCC ACC GCC TAC CTG CAG CAG CTG

CTG CCC TTC AAC CAG CTG GAC GTG GCC AAC AGC GCC GCC TAC CTG CAG CAG AGG CAG CAG

CTG CTG AAC CCC CTG GCC GCC GCC AAC CCC CTG GTG GCC GCC TTC CTG CAG CAG CAG CAG

TTC CTG CCC TAC AAC CAG ATC AGC CTG ATG AAC CTG GCC CTG AGC AGG CAG CAG CCC ATC

GTG GGC GGC GCC ATC TTCcat cat cac cat cac cat tga TCT AG AG

M G D Y K D D D D K I L T S L A S V A I L G A G F V A S Q P T V V R A E E S P V A S Q A A A G G G G S G G G G S **M** A T K I L A L L A L L S L S V S A T T A F I I P Q C S L A P N A I I P Q F L P S V T S **M** G I E H P I V Q A Y R L Q Q A L A A S V L Q Q P F A Q L Q Q Q S L A H L T I Q T I A T Q L E Q Q F V P A L S Q L A A V N P V S Y L Q Q Q **M** L A S N P L A L A N T A A Y Q Q Q L Q L Q Q F L P A L S Q L A R V N P A T Y L Q Q Q Q L L S S S P L A V G N A A T Y L Q Q Q L L Q Q I V P A L S Q L V V A N P T A Y L Q Q L L P F N Q L D V A N S A A Y L Q Q R Q Q L L N P L A A A N P L V A A F L Q Q Q Q F L P Y N Q I S L **M** N L A L S R Q Q P I V G G A I F H H H H H H

Codon optimised construct 2:

CCATGGGGTCCAAGGCC GAAAAAGAT TATGACGCTGCA GCACAAAAGA AATATGATGA GGATCAGAA GAAGACAGA GGAATCTGAATC AGAAGATTA CGCAGCGGCCGC

This has been cloned into a vector containing the partial gamma-zein sequence to result in:

M G S K A E K D Y D A A A Q K K Y D E D Q K K T E E S E S E D Y A A A A A G G G S G G G G S G G C G C Q **P P P P V H L P P P V H L P P P V H L P P P V H L P P P V H L P P P V H L P P P V H V P P P V H L P P P P** C H Y P T Q P P R P Q P H P Q P H P C P C Q Q P H P S P C Q A A A H H H H H H

The 20 Amino Acids and their official codes

No.	1-Letter code	3-Letter code	Name
1	A	Ala	Alanine
2	R	Arg	Arginine
3	N	Asn	Asparagine
4	D	Asp	Aspartic acid
5	C	Csy	Cystein
6	Q	Gln	Glutamine
7	E	Glu	Glutamic acid
8	G	Gly	Glycine
9	H	His	Histidine
10	I	IIe	Isoleucine
11	L	Leu	Leucine
12	K	Lys	Lysine
13	M	Met	Methionine
14	F	Phe	Phenylalanine
15	P	Pro	Proline
16	S	Ser	Serine
17	T	Thr	Threonine
18	W	Trp	Tryptophan
19	Y	Tyr	Tyrosine
20	V	Val	Valine