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Transformation of Salt Tolerant *Lactuca sativa* with Cholera toxin B Gene for Production of Edible Vaccine

A Dissertation

Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of The Requirements for the Degree of Doctorate of Philosophy in Science (Biotechnology)

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Summary

Lettuce (*Lactuca sativa*) is one of the most important edible plant worldwide. At the time that lettuce is the candidate plant to carry the foreign vaccine gene for human. The B subunits of toxin of *Vibrio cholerae* (CTB) are candidate vaccine antigens. This study was conduct to express *CTB* gene in lettuce chloroplast. After surface sterilization of lettuce seeds, they were germinated on Murashige and Skoog (MS) half strength medium. Direct regeneration of lettuce by tissue culture was carried out in the full strength MS medium containing 0.1 mg/l alpha-Naphtaleneacetic acid (NAA) and 0.2 mg/l 6-benzylaminopurine (BAP). Lettuce plant was experimented for tolerance of betaine aldehyde (BA) and sodium chloride (NaCl) by tissue culture technique and it was found that the wild type lettuce tolerated 10 and 75 mM from each substance respectively. Genes required in this study were obtained by polymerase chain reaction (PCR) technique using specific forward and reverse primers, and these genes were cholera toxin B subunit (CTB), betaine aldehyde dehydrogenase (BADH),and *prrn* promoter and many other regulatory genes. Some of these genes were isolated from their hosts(*Vibrio cholera*, Spanish, and lettuce) and some were obtained from previous work available at Daniell laboratory. All these genes beside many techniques for ligation, extension, sequencing, orientation confirmation were used to construct the cassette vector pLS-BADH-LS-CTB which carries the gene of interest. Transformation event was high when gene gun technology was used and it was found that the distance of 6 cm and 900 pound per square inch (psi) were the optimum parameters to get high transformation frequency reaching 10% transformation events per bombarded leaf .In addition, more efficient age of leaves for bombardment was 40 days. The B subunits of toxin of *Vibrio cholerae* (CTB) are the candidate vaccine antigens. In this work the *CTB* gene with *BADH* gene were transferred to the chloroplast of

lettuce plant and selection of transformed plant cell was performed on the MS medium containing BA and NaCl without any antibiotic selectable marker. Integration of an unmodified CTB-coding sequence into chloroplast genomes (up to 1000 copies per cell) resulted in the accumulation of up to 6.2% of total soluble lettuce leaves protein as functional oligomers. PCR and Southern blot analyses confirmed stable integration of the *CTB* gene and *BADH* gene into the chloroplast genome in addition to the integration in the right orientation and in specific region between *trna*I*trn*A.Western blot analysis showed that the chloroplast synthesized CTB assembled into oligomers and were antigenically identical with purified native CTB. Transgenic lettuce plant showed ability to tolerate 150 mM NaCl and 35 mM BA. Introduced genes were stable and inherited in subsequent generations, as confirmed by PCR and Southern blot analyses. Increased production of an efficient transmucosal carrier molecule and delivery system, like CTB, in transgenic chloroplasts makes plant based oral vaccines and fusion proteins with CTB needing oral administration commercially feasible. Successful expression of foreign genes in transgenic chromoplasts and availability of marker-free chloroplast transformation techniques augurs well for development of vaccines in edible parts of transgenic plants. Furthermore, since the quaternary structure of many proteins is essential for their functions, this investigation demonstrates the potential for other foreign multimeric proteins to be properly expressed and assembled in transgenic chloroplasts.

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List of Abbreviations

aadA	Aminoglycoside 3’ adenosyltransferase
Amp	Ampicillin
BA	Betaine aldehyde
BAP	Benzylaminopurine
BADH	Betaine aldehyde dehydrogenase
BME	β-mercaptoethanol
bp	base pair
BSA	Bovine serum albumin
CGS	Cystathionine gamma-Synthase
CTB	Cholera toxin B subunit
CTB-Pins	Cholera toxin B subunit-human proinsulin fusion protein
DMSO	Dimethyl sufloxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ELISA	Enzyme Linked Immunosorbent Assay
GM1	Monosialotetrahexosylganglioside
g10	Translation control region from bacteriophage T7 *gene 10*
GFP	Green fluorescent protein
GSH	Glutathione
GUS	Glucuronidase
IR	Inverted repeat
kb	Kilobases
kDa	KiloDalton
LB	Luria-Bertani broth
LGM	Lettuce germination medium
LRM	Lettuce regeneration medium
LSC	Large single copy region
MS	Murashige and Skoog basal salts
NEP	Nuclear encoded plastid localized RNA polymerase
NAA	Naphthalene acetic acid
NOD	Nonobese diabetic mouse
nptII	Neomycin Phosphotransferase
OD	Optical Density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

PEP	Plastid encoded RNA polymerase
PMSF	phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride
P*rrn*	Plastid ribosomal operon promoter
P*rrn*1	PEP promoter element 1
psbA	Photosystem II core polypeptide D1
Pt-DNA	Plastid DNA
RA	Retinyl acetate
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
RBP	RNA binding proteins
RBS	Ribosome binding site
rps16	Plastid ribosomal protein S16
rrn	Plastid ribosomal operon
RNA	Ribonucleic acid
SOC	Super Optimal Broth with Catabolite repression
SSC	Sodium Chloride and Sodium Citrate solution
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEKDEL	The sequence Ser-Glu-Lys-Asp-Glu-Leu
TAE	Tris-acetate-EDTA
TCR	Translation control region
TLP	Total leaf protein
TSP	Total soluble proteins
WT	Untransformed Plant
UTR	Untranslated region

Chapter One
Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

There is currently much enthusiasm for the potential of genetically engineered plants through production of edible vaccines to help controlling human and animal diseases. Plants expressing bacterial and viral antigens as nuclear transgenes are capable of triggering immune responses when the transgenic tissues are administered orally. Indeed, it has been suggested that plant cells containing an oral vaccine may actually potentiate vaccine activity by protecting against premature digestion of the antigen (Ruhlman *et al.*, 2010).

Despite this promise, transgenes expressed via the nucleus often yield insufficient antigen levels, reported as total soluble protein (TSP) or fresh weight (FW): B subunits of enterotoxigenic *Escherichia coli* (0.01% TSP), hepatitis B virus envelope surface protein (0.01% TSP), human cytomegalovirus glycoprotein B (0.02% TSP), and transmissible gastroenteritis coronavirus glycoprotein S (0.06% TSP).Therefore, one ever-present mission is to increase the level of transgene expression within transgenic plants (Maliga and Bock, 2011).

Cholera toxin B subunit (CTB) is a candidate oral subunit vaccine against cholera, a disease that causes acute watery diarrhea by colonizing the small intestine and producing the enterotoxin, cholera toxin (CT). Cholera toxin is a hexameric AB5 protein consisting of one toxic 27 kDa A subunit having ADP ribosyl transferase activity and a nontoxic pentamer of 22 kDa B subunits that binds to the A subunit and facilitates its entry into the intestinal epithelial cells. When administered orally, CTB is a potent mucosal immunogen; this is believed to be a result of CTB binding to eukaryotic cell surfaces via GM1 ganglioside receptors present on the intestinal epithelial surface, eliciting a mucosal immune response to pathogens and enhancing the immune response when coupled chemically to other antigens (Arakawa *et al*., 1997).

The B subunits of enterotoxigenic *E. coli* (LTB)and cholera toxin of *Vibrio cholerae* genes have been expressed at different levels via the plant nucleus. When the native LTB gene was expressed via the tobacco nuclear genome, it accumulated at levels less than 0.01% of the total soluble leaf protein.To improve LTB expression, a synthetic gene that contained plant-preferred codons was created and eliminated potential mRNA processing signals and destabilizing motifs were found in the native gene. Using the native *CTB* gene for comparison, the synthetic gene led to increase antigen accumulation in the leaves and tubers by 3 to 14 fold (Arakawa *et al*., 1997) .

However, extensive codon modification of genes is laborious and expensive. One of the consequences of these constitutively expressed high CTB levels was the stunted growth of transgenic plants that was eventually overcome by tissue-specific expression in potato tubers. By altering the native *CTB* gene to code for a C-terminal SEKDEL sequence, which targets expression to the endoplasmic reticulum, CTB expression of up to 0.3% TSP was achieved in auxin-induced potato tissues via the nuclear genome (Daniell *et al*., 2001a).

Increased expression levels of several proteins have been attained by expressing foreign proteins in chloroplasts of higher plants. Recently, human somatotropin (7% TSP) and antimicrobial peptides (21% TSP) have been expressed in transgenic chloroplasts. The accumulation levels of the Bt Cry2Aa2 operon in tobacco chloroplasts were as high as 46.1% of the total soluble plant protein (Ruhlman *et al*., 2007).

Besides the ability to express polycistrons, another advantage of chloroplast transformation is the lack of recombinant protein expression in pollen of chloroplast transgenic plants. Absence of chloroplast DNA in the pollen of most crops reduces pollen-mediated outcross of transgenes. Also, stable incorporation of the *CTB* gene into spacer regions between functional genes of the chloroplast genome by homologous recombination eliminates the

"position effect" frequently observed in nuclear transgenic plants. Lack of gene silencing in chloroplasts should allow uniform expression levels in different transgenic lines. Integration of the transgene into chloroplast genomes should result in a high level of *CTB* gene expression, since each plant cell contains up to 10,000 copies of the plastid genome. Similar to the endoplasmic reticulum, the production of CTB in chloroplasts allows formation of disulfide bridges, which are necessary for the correct folding and assembly of the CTB pentamer (Daniell *et al*., 2001b).

Salt stress is a major abiotic stress in plant agriculture. The problem of soil salinity has been compounded by irrigation and excessive use of fertilizers. High salinity causes ion imbalance, toxic levels of cytoplasmic sodium, and drought stress . Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components. One of the metabolic adaptations to salt stress is the accumulation of osmoprotectants. Glycine betaine is quaternary ammonium compound that accumulate in many plant species in response to salt stress Glycine betaine protects the cell from salt stress by maintaining an osmotic balance with the environment and by stabilizing the quaternary structure of complex proteins (Kumar *et al*., 2004).

This substance occurs naturally in some crops, like spinach, sugar beet and cotton, as well as in many highly salt- or drought-tolerant wild plants, including halophytes, However, many stress-susceptible crops do not contain significant amounts of glycine betaine or other osmoprotectants. It was proposed that genetic engineering of osmotolerance in plants could be achieved by producing betaine in nonaccumulators. This has been demonstrated in several reports where transgenic plants accumulating glucine betaine exhibit moderate levels of tolerance to salt stress (Liu *et al*., 2011).

Most transformation technique co-introduce a gene that confer antibiotic resistance , along with gene of interest to impart a desired trait. Regeneration

transformed cells in antibiotic containing media permits selection of only those cells that have incorporated foreign genes. Once transgenic plant are regenerated, antibiotic resistance gene serve no useful purpose but they continue to produce their gene products. One of the primary concerns of genetically modified crops is the presence of clinically important antibiotic resistance gene product in transgenic plant that could inactivate oral dose of the antibiotic. Another concern is that the antibiotic resistance gene could be transferred to pathogenic microbes in the gastrointestinal tract or in soil rendering them resistant to treatment with such antibiotic (Daniell *et al*., 2001 a). However several approaches are currently available to eliminate antibiotic resistance gene from transgenic plant. Use of safe selectable markers to identify transgenic plant cells from other untransformed cell is one of preferred approaches. One of the safe selectable marker used for selection of transgenic cell line is osmoprotectant gene *BADH* or other slat and drought resistance gene, and the selection was done on media containing salt or toxic osmoprotectant.

Objectives of the study:

- Cloning of cholera toxin B gene in leafy edible plant (lettuce).
- Using of organelle genetic engineering (chloroplast genetic engineering) to produce foreign protein in plant.
- Exploring salt tolerant gene *BADH* for production of salt tolerant lettuce plant.
- Using salt tolerant gene as a selectable marker for transgenic plant instead of the antibiotic selection technique.
- Constructing new cassette vector for lettuce chloroplast transformation with *CTB-BADH* genes.
- Determination of optimum conditions to achieve high rate of transformation by gene gun technology.

1.2 Literature Review

1.2.1 Lettuce (*Lactuca sativa*)

Lettuce (*Lactuca sativa* NC_007578) is a member of the Asteraceae family. Thousands of species are included in this large family. The family was originally referred to as compositae because the species seemed to share a compact structure often sprouting in the shape of a head . *Lactuca* means 'milk forming', *sativa* means 'common'. Lettuce is considered 'milk forming' because of the creamy substance that is often found when stem are snap (Kiple and Ornelas, 2000).

The plant is an important commercial vegetable crop cultivated worldwide in a diverse range of environments, with USA, Spain, Italy, Japan and France as the main producer countries (Contreras, 2007).

Lettuce leaves contain small amounts of opiate-like substance, lactucarium "lettuce opium", which is a mild sedative. Our ancestors used lettuce to treat anxiety, insomnia, and neurosis. Lettuce is a rich source of antioxidants such as quercetin, caffeic acid, vitamins A and C. It was shown that ethanol extract of lettuce injected subcutaneously, significantly decreased accumulation of lipofuscin pigment granules "age granules" in brain of mice under accelerated ageing regimen (mice were administered with D-galactose) (Deshmukh *et al*., 2007).

Molecular markers have previously been used in lettuce genomic studies, including construction of genetic maps, investigation of the diversity and origin of cultivated lettuce, examination of the genetic relationships among *Lactuca* species, and assessment of allelic diversity of selectively amplified microsatellite polymorphic loci markers (Jinguo *et al*., 2005).

The common cultivated species of lettuce has its origin traced back to the Eastern Mediterranean basin. It is a cultivated crop lettuce can be found growing all over the world. It is considered as an annual and polymorphic plant. An annual plant has a life span of only one year; it must be replanted

every year if desired for cultivation. As a polymorphic plant, lettuce can take on a wide variety of appearances in its final stage of growth. Although all types can be found in a typical salad today, each group appears fairly different and is used more specifically for different purposes. For example, romaine is often the base of a Caesar salad while iceberg lettuce is often found in a "tossed garden salad" as it is often named on a restaurant's menu (Weaver, 1997).

1.2.2 *Vibrio cholerae*

Vibrio cholerae, is a bacterium of the family Vibrionaceae, the bacterium's name originates from the Greek words meaning "flow of bile". It is a facultatively anaerobic, Gram-negative, non-sporeforming curved rod, about 1.4–2.6 mm long, capable of respiratory and fermentative metabolism; it is well defined on the basis of biochemical tests and DNA homology studies (Baumann *et al*., 1984). The bacterium is oxidase-positive, it reduces nitrate, and is motile by means of a single, sheathed, polar flagellum. Growth of *V. cholerae* is stimulated by addition of 1% sodium chloride (NaCl). However, an important distinction from other *Vibrio* species is the ability of *V. cholerae* to grow in nutrient broth without added NaCl.

Differences in the sugar composition of the heat-stable surface somatic O antigen are the basis of the serological classification of *V. cholerae* first described by Gardner and Venkatraman (1935), currently the organism is classified into 206 O serogroups (Shimada *et al*., 1994; Yamai *et al*., 1997). Until recently, epidemic cholera was exclusively associated with *V. cholerae* strains of the O1 serogroup. All strains that were identified as *V. cholerae* on the basis of biochemical tests but that did not agglutinate with O antiserum were collectively referred to as non-O1 *V. cholerae*. The non-O1 strains are occasionally isolated from cases of diarrhea (Ramamurthy *et al*., 1993a) and from a variety of extra intestinal infections, from wounds, ear, sputum, urine,

and cerebrospinal fluid (Morris and Black, 1985). They are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin. The O1 serogroup exists as two biotypes, classical and El Tor; antigenic factors allow further differentiation into two major serotypes- Ogawa and Inaba. Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. A third serotype (Hikojima) expresses all three antigens but is rare and unstable (Morris, 1990).

Between 1817 and 1961, six pandemics of cholera were recorded. The classical biotype was responsible for the fifth and sixth pandemics and is believed to have been associated with the earlier pandemics as well, although there is no hard evidence. The causative agent of the seventh and current cholera pandemic,which began in 1961, is the El Tor biotype. The classical biotype has been completely displaced worldwide, except in Bangladesh where it reappeared in epidemic proportions in 1982 (Samadi *et al*., 1983), remained prominent there for a few years, and seems to have become extinct again (Siddique *et al*., 1991).

The simple distinction between *V. cholerae* O1 and *V. cholerae* non-O1 became obsolete in early 1993 with the first reports of a new epidemic of severe,cholera-like disease in Bangladesh (Albert *et al*., 1993) and India (Ramamurthy *et al*., 1993b). At first, the responsible organism was referred to as non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum. However, further investigations revealed that the organism did not belong to any of the O serogroups previously described for *V. cholerae* but to a new serogroup, which was given the designation O139 Bengal after the area where the strains were first isolated (Shimada *et al*., 1993). Since recognition of the O139 serogroup, the designation non-O1 non-O139 *V. cholerae* has been used to include all the other recognized serogroups of *V. cholerae* except O1 and O139 (Nair *et al*., 1994).

The emergence of *V. cholerae* O139 as the new serogroup associated with cholera, and its probable evolution as a result of horizontal gene transfer between O1 and non-O1 strains (Bik *et al*., 1995), has led to a heightened interest in the *V. cholerae* non-O1 non-O139 serogroups. There is evidence for horizontal transfer of O antigen among *V. cholerae* serogroups; Karaolis, Lan and Reeves (1995) reported that isolates of nearly identical *asd* gene (chromosomal housekeeping gene, which encodes aspartate semialdehyde dehydrogenase, sequences had different O antigens and that isolates with the O1 antigen did not cluster together but were found in different lineages. There has been elevated activity of the non- O1 non-O139 serogroups in the recent past, and localized outbreaks of acute diarrhoea caused by *V. cholerae* serogroups such as O10 and O12 have been reported (Dalsgaard *et al*., 1995; Rudra *et al*., 1996).

1.2.2.1 Cholera Disease and Epidemiology

The major features of the pathogenesis of cholera are well established. Infection due to *V. cholerae* begins with the ingestion of contaminated water or food. Usually, in healthy individuals cholera is asymptomatic or mild; in the latter, diarrhea may be the only symptom.Following a usual incubation period of 6-72 hours, severe illness is heralded by a sudden onset of profuse, watery diarrhea accompanied by nausea and vomiting. Up to 20 litres of diarrhoea can be passed in a 24 hour period, which if left untreated rapidly leads to serious dehydration and circulatory collapse (Taylor *et al*., 1987).

After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of the toxin co-regulated pili and possibly other colonization factors such as the different haemagglutinins, accessory colonization factor, and core-encoded pilus, all of which are thought to play a role. To cause the severe diarrhea in cholera, *V. cholerae* releases CT toxin to obstruct the phospholipids of erythrocytes and

causes the intestinal capillaries to be porous. The CT toxin attaches itself to the intestinal epithelium and releases an enzyme activated subunit, which dramatically increases the amount of cyclic Adenine monophosphste production in the body, causing abundant release of water and electrolytes into the intestines. The intestines are irritated by *V. cholerae*'s toxins to the point that it starts to release profuse amounts of fluids, while the epithelial cells of the intestines are slowly being eroded. The affected epithelial cells die, and eventually form scaly mounds that peel off from their original bases. This is followed by the opening of the capillaries and the release of fluids from the vessels and into the neighboring tissues. The destruction of the intestinal walls and capillary leakage cause fluid accumulation in the body, hence the severe diarrheal symptom of cholera. Cholera is a disease that occurs in regions of the world where sanitation, food and water hygiene are inadequate or lacking. In circumstances where there is no clean water or adequate sewage disposal (as may occur for example, after natural disasters or in displaced populations in war zones) , cholera can spread very quickly. The main areas of the world where cholera is currently prevalent are in Africa, Asia, the Middle East, Peru and some countries of Central America. Imported cases are reported sporadically around the world from travelers to endemic countries (Sach *et al*., 2004).

Cholera usually occurs in large epidemics or pandemics and in the 19th century pandemics frequently originated from the Ganges delta in India and up to the mid 20th century, were largely confined to Asia (except for a large epidemic in Egypt in 1947). The current, seventh pandemic caused by *V. cholerae* O1 El Tor originated in Indonesia in 1961 and spread rapidly through most of Asia into eastern Europe. In 1970, this biotype was introduced into West Africa, where it spread rapidly and is endemic in many African countries, and in 1991, it was re-introduced into Peru, where it had been absent for over 100 years. Another serogroup, *V. cholerae* O139, was

discovered as being the cause of cholera epidemics in India and Bangladesh in 1992 and has since spread to eleven other countries in south east Asia. A part from a few imported cases, this serogroup is not known to have occurred outside of these countries (Colwell, 1996**).**

Cholera is considered to be an endemic disease in many parts of Iraq since 1966 when the first cholera cases appeared (227 cases including 20 deaths, case fatality rate 8.8%).Trends of cholera in the last 40 years show that each epidemic lasted several months followed by a second wave in the next year. Data reflecting the seasonality is not available, but traditionally cholera started in the north and spread to the centre and south.In 1998, the largest cholera outbreak was reported with 2560 cases.In 2003, a total of 187 cholera cases with no death were reported from three governorates in the Lower South. The recent report of Iraq Ministry of Health indicates that 574 laboratory confirmed Cholera cases have been detected and registered in Iraq, up to 26 October 2008. 56% of the cases are found in children below 5 years of age.There have been 8 deaths (6 males and 2 females). 50% of the deaths have been in below 5 year old children. Epidemiological findings have indicated that contaminated water is the most probable source of the outbreak. 573 cases have been identified as Inaba and 1 case as Ogawa serotype (W.H.O., 2008).

In 2002, 142,311 cases of cholera including 4564 deaths were officially reported to the World Health Organization , although this is almost certainly an under estimate. Ninety-seven percent of reported cases were reported from Africa. Large outbreaks occurring in Malawi, Mozambique and Zambia accounted for 44% of the African cases. *Vibrio cholerae* O1 El Tor is responsible for the majority of reported cases. *V. cholerae* O139, which emerged in the Bay of Bengal in 1992, is confined to south east Asia and is responsible for approximately 15% of laboratory-confirmed cases of cholera reported from cholera-endemic countries in Asia. Five cases were reported in

Australia in 2002, two of which were imported and three were locally acquired (Faruque *et al*.,1998).

1.2.2.2 Cholera Toxins

The existence of cholera enterotoxin (CT) was first suggested by Robert Koch in 1884 and demonstrated 75 years later by De (1959) and Dutta, Pause and Kulkarni (1959) working independently. Subsequent purification and structural analysis of the toxin showed it to consist of an A subunit and 5 smaller identical B subunits(49 kDa) (Finkelstein and LoSpalluto, 1969).

The CTA consist of two subunits(22 and 5kDa) possesses a specific enzymatic function and acts intracellularly, raising the cellular level of cyclic Adenine monophosphste and thereby changing the net absorptive tendency of the small intestine to one of net secretion. The B subunit pentamers(22 kDa) serves to bind the toxin to the eukaryotic cell receptor, ganglioside GM1. The binding of CT to epithelial cells is enhanced by neuraminidase (Mekalanos , 1985)

Apart from the obvious significance of CT in the disease process, it is clear that the production of CT by *V. cholerae* is important from the perspective of a serogroup acquiring the potential to cause epidemics. This has become particularly evident since the emergence of *V. cholerae* O139. A dynamic 4.5-kb core region, termed the virulence cassette, has been identified in toxigenic *V. cholerae* O1 and O139 but is not found in non-toxigenic strains. It is known to carry at least six genes, including *ctxAB* (encoding the A and B subunits of CT), *zot* (encoding zonula occludens toxin) *cep* (encoding core-encoded pilin), *ace* (encoding accessory cholera enterotoxin), and *orfU* (encoding a product of unknown function) (Trucksis *et a*l., 1993; Fasano *et al*., 1991; Pearson *et al*., 1993). In the El Tor biotype of *V. cholerae*, many strains have repetitive sequence insertion elements on both sides of the core region; these are thought to direct site-specific integration of

the virulence cassette DNA into the *V. cholerae* chromosome The core region, together with the flanking RS sequences, makes up the cholera toxin genetic element CTX (Mekalanos, 1983).

Studies have shown that the entire *CTX* element constitutes the genome of a filamentous bacteriophage (CTXf). The phage could be propagated in recipient *V. cholerae* strains in which the CTXf genome either integrated chromosomally at a specific site, forming stable lysogens, or was maintained extrachromosomally as a replicative form of the phage DNA (Waldor and Mekalanos, 1996). Extensive characterization of the CTXf genome has revealed a modular structure composed of two functionally distinct genomes, the core and RS2 regions. The core region encodes CT and the genes involved in phage morphogenesis, while the RS2 region encodes genes required for replication, integration, and regulation of CTXf (Waldor *et al*., 1997). Generally, CTXf DNA is integrated site-specifically at either one (El Tor) or two (classical) loci within the *V. cholerae* genome (Mekalanos, 1985). In El Tor strains, the prophage DNA is usually found in tandem arrays that also include a related genetic element known as RS1. The RS1 element contains the genes that enable phage DNA replication and integration, plus an additional gene (*rstC*) whose function is unknown but that does not contain *ctxAB* or the other genes of the phage core region that are thought to produce proteins needed for virion assembly and secretion (Davis *et al*., 2000). CTXf gains entry to the *V. cholerae* cell by way of the toxin-regulated pili—the surface organelles required for intestinal colonization, genes are then incorporated into host chromosome, inducing the cell to secrete CT.

The *zot* gene increases the permeability of the small intestinal mucosa by an effect on the structure of the intestinal tight junctions (Fasano *et al*., 1991), while *ace* affects ion transport in the intestinal epithelium. Another factor whose gene resides outside the CTX genetic element and which is thought to contribute to the disease process is haemolysin/cytolysin .In contrast to the

watery fluid produced by CT, the haemolysin can cause accumulation in ligated rabbit ilea loops of fluid that is bloody with mucous (Honda and Finkelstein, 1979).

Although not fully characterized, other toxins produced by *V. cholerae* include the shiga-like toxin , a heat-stable enterotoxin (Takeda *et al*., 1991), sodium channel inhibitor, thermostable direct haemolysin-like toxin , and a cell-rounding cytotoxic enterotoxin known as the non- embrane-damaging cytotoxin (Saha *et al*., 1997).

1.2.2.3 Cholera Vaccine

Studies to-date on patients with cholera suggest that different components of the immune system, both humoral and cell mediated, innate as well as adaptive, are activated in response to natural infection . The best studied responses are the humoral immune responses and both mucosal and systemic antibody responses have been found to be related to protection . The serological responses such as the complement mediated vibriocidal antibody response, antibody responses to lipopolysaccharide and CT as well as to protein antigens have been found to be significantly increased in response to clinical cholera (Qardi *et al*., 1995) .

The antibacterial responses include, in addition to lipopolysaccharide, responses to the toxin-coregulated pilus, which is a colonization factor and potentially protective antigen, as well as to the mannose sensitive haemagglutinin, a type IV pilus antigen which is also immunogenic and gives rise to antibody secreting cell responses and fecal as well as plasma antibodies in patients. SIgA antibodies to the major protective antigens have been detected in mucosal secretions of patients, e.g. in intestinal lavages, feces as well as in breast milk and saliva specimens. Of these, fecal extracts have been found useful due to the ease of collection, and relatively satisfactory mucosal responses have been estimated in patients and vaccinees using these samples .

There is however a need for more sensitive analytical methods and appropriate clinical specimens to better gauge the mucosal response (Sanchez and Holmgren, 2005).

Vaccines which reduce the rates of cholera will provide an overall health benefit for children and adults who are at risk of disease. There are currently three oral cholera vaccines that are licensed in different parts of the world. The first, Dukoral, has been developed at the University of Gothenburg and is commercially produced by SBL Vaccine, Stockholm, Sweden. This vaccine contains recombinant CTB plus heat and this cholera vaccine should be given as two doses to individuals up to 6 years, and as three doses to children aged 2-6 year, at 1–6 week intervals between doses, with a buffer to protect CTB against stomach acidity. Before being licensed, this vaccine was extensively tested in both adults and children in large field trials in cholera endemic areas and it is licensed in over 50 countries of the world. The vaccine provided a very high degree of short term protection in all agegroups, 85-90% , but a more lasting protection in adults (~60% during 3 years) than in children in a field trial carried out in Matlab in Bangladesh . Subsequent analyses of data from the field trial in Bangladesh showed that a greater than 90% reduction in cholera disease burden could be achieved by this vaccine through herd protection, even when the level of coverage was only moderate (~50% - 60%) **(**Savarino *et al.*, 1999).

The vaccine gives rise to intestinal SIgA responses directed against CTB as well as against *V. cholerae* LPS, which are thought to synergistically contribute to the protection afforded by the vaccine .The vaccine enhances serum vibriocidal antibody responses, which is known to be the best available indirect correlate of protection after oral immunization or infection; it also induces systemic antibody responses against CTB and LPS . However, less is known about the T-cell responses induced after immunization with this cholera vaccine. In mice, T-cell responses to CT are strictly dependent on the

presence of CD4+ T cells. The cholera vaccine has mostly been tested in adults and children >2 years, but the disease is also seen in infants and under 2 year old children . Therefore, it is important to test the vaccine in younger children down to 6 months of age where the disease is prevalent, especially when maternal antibody protection wanes and weaning from breast feeding is generally initiated (Savarino *et al*., 2002).

The second licensed oral cholera vaccine, CVD 103HgR or Orochol that was previously produced by Berna/Crucell, is a single-dose, live attenuated vaccine. It was derived from the classical Inaba 569B strain with 94% deletion of the enzymatically active A-subunit of the cholera toxin leaving only the immunologically active B-subunit . This vaccine was shown to be safe and immunogenic in various trials in North America , Switzerland , Peru ,Indonesia. However, a large field trial with more than 67,000 subjects in Indonesia failed to show protective efficacy . Production of this vaccine was stopped several years ago (Simanjuntak *et al*.,1993).

Another killed oral whole cell cholera vaccine is available which is produced in Vietnam by the local manufacturer Vabiotech following technology transfer from Sweden. This vaccine consists of killed *V. cholerae* O1/O139 whole cells and has been shown to be safe and immunogenic in subjects aged 1 year and older and to have 50% long term effectiveness in Vietnam . This vaccine was initially only licensed in Vietnam but has also been licensed in India. In order to expand the use of this vaccine globally, the vaccine has been reformulated, and is currently under trial in Kolkata, India ;production is being conducted by a WHO-prequalified vaccine manufacturer in India (Shanta Biotech, India).

Cholera toxin B subunit has been expressed in transgenic tobacco and potato plants. The CTB protein, purified from transgenic tobacco plants was found to be antigenically similar to authentic protein. Efficacy of potato-based choera toxin B subunit to elicit immune response in mice has also been

established. However, tobacco is not palatable because of high level of toxic compounds and potato needs cooking,which can denature antigens (Jani *et al*., 2002).

The integration of *CTB* gene to tobacco chloroplast genome was firstly done by Daniell lab, this eliminates the results in high levels of CTB accumulation and assembly of functional oligomers in chloroplasts which overcome the need to modify the *CTB* gene for optimal expression in plants (Daniell *et al*., 2001).

1.2.3 Abiotic Stress in Plant

In the natural environment, plants often grow under unfavorable conditions, such as drought, salinity, chilling, freezing, high temperature, flooding, or strong light. These conditions are known collectively as abiotic stresses, and any of them can delay growth and development, reduce productivity and, in extreme cases, cause the plant death. Abiotic stresses represent the most limiting environmental factors affecting agricultural productivity. To overcome these limitations and to improve production, to feed the ever-increasing population, it is imperative to develop crop cultivars that are stress tolerant. When crop plants are subjected to environmental stress conditions, they fail to express their full genetic potential for production (Ghosh *et al*., 2001).

The effect of stress depends on the developmental stage, genotype of plant species as well as duration and intensity of the stress. Generally, plants respond to these stresses under low or moderate levels, but when the stress levels exceed a certain critical level (which varies from crop to crop), the physiological mechanisms imparting tolerance to plants start breaking down causing ultimately plant death. Consequently, the abiotic stress factors cause a massive loss to the productivity of crop plants. Biotic and abiotic stress factors lead to a loss of US 15.74$ billion in five most important crops of

semi arid tropics, sorghum, pear millet, pigeonpea, chick pea and groundnut. These crops are the main food source for poor people of the developing countries. Amongst the various stresses affecting crop plants, loss due to abiotic stresses is much more significant as compared to the losses that occur due to insect, pests, weeds and diseases (Kesseru *et al*., 2002)**.**

Classical plant breeding methods involving inter-specific or inter-generic hybridization and *in vitro* induced variation have been applied to improve the abiotic stress tolerance of various crop plants but without much success. The conventional breeding strategies are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress condition and lack of efficient selection criteria. It is important, therefore, to look for alternative strategies to develop stress tolerant crops. Recently, marker assisted selection of specific traits that are linked to yield, e.g. osmotic adjustment, membrane stability or physiological tolerance indices, has been recommended. However, quantative trait loci QTL that are linked to tolerance at one stage in plant development can differ from those linked to tolerance at other stages. Furthermore, desirable QTLs can require extensive breeding to restore suitable traits along with the introgressed tolerance trait. The best alternative, therefore, is the direct introduction of genes by genetic engineering to incorporate tolerance traits in target crops (Bohnert *et al*.,2005).

Research over the past two decades has provided a better understanding of the molecular biology of stress responses in plants. Many genes and gene products have been identified which get induced upon exposure of plants to various abiotic stresses drought, salinity, low and high temperature stress, etc. Consequently, biotechnological tools have been applied to transfer some of these useful genes implicated in stress tolerance to plants. In addition to these stress-induced proteins, genes encoding enzymes of the biosynthetic pathways of different osmolytes such as proline, glycine betaine, trehalosc, sorbitol,

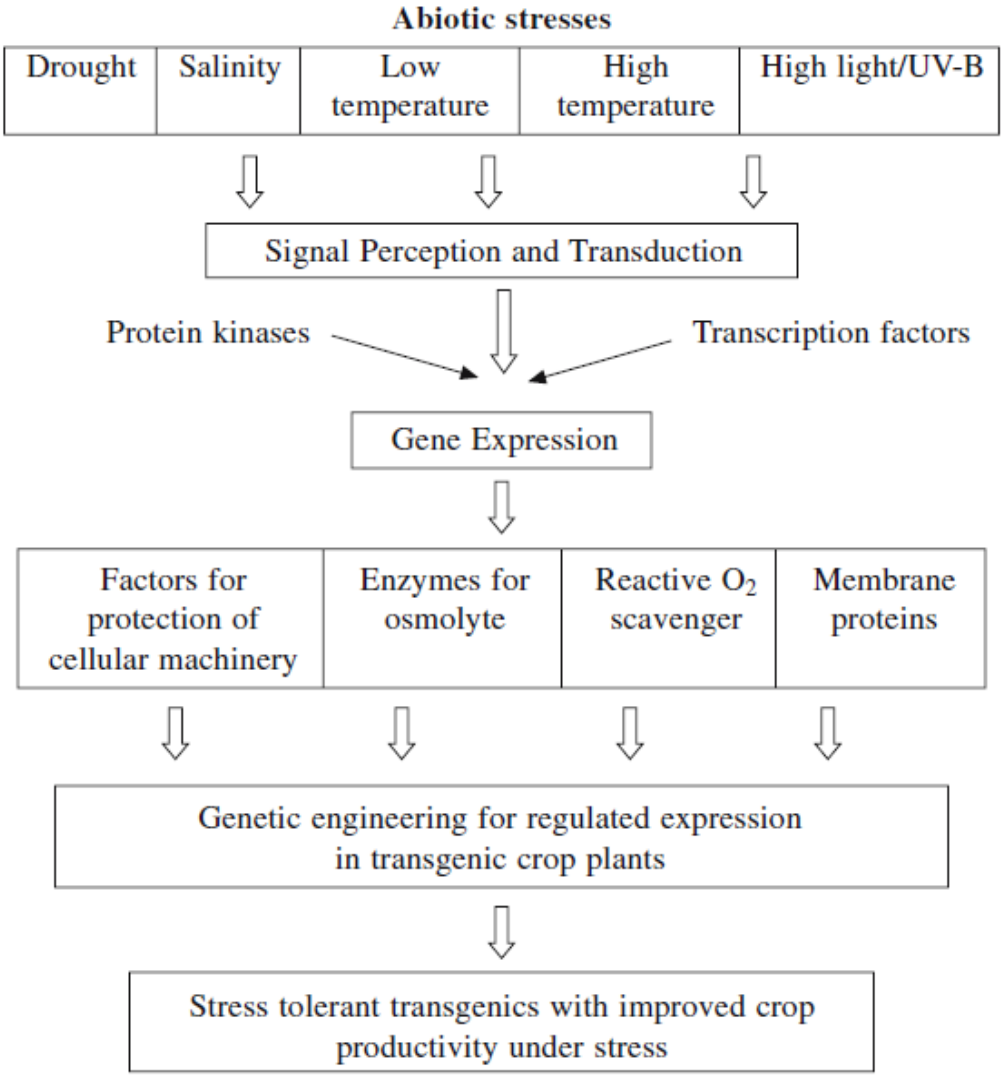
pinitol, etc. have been cloned and exploited in improving abiotic stress-tolerance in plants through genetic engineering (Cramer *et al*.,2007).

Under different abiotic stress conditions, a large number of genes show elevated transcript levels in plants . Up-regulation of these genes does not always confirm their role in stress tolerance. Changes in gene expression may be due to disruption of physiological and metabolic processes of the cell. However, precise physiological function of any such gene can be studied by its altered expression (overexpression or suppression) in transgenic plants. Indeed, transgenic approach has emerged as a valuable tool in determining or confirming the precise function of the stress-induced genes and to develop stress-tolerant transgenic crop plants. Normally, genes isolated under stress conditions are first tested in model species such as tobacco and *Arabidopsis* for their role in stress tolerance before transferring them to economically important crop species (Zhu, 2000).

There are four categories of stress induced genes/proteins with known function which have been exploited for generating stress tolerant transgenic plants (Figure 1-1).

1.2.3.1 Role of Betaine aldehyde dehydrogenase in Resistance of Abiotic Stress(Salt and Drought)

Salt stress and drought are major abiotic stresses in agriculture. The problem of soil salinity has been compounded by irrigation and excessive use of fertilizers. About 20% of the world's irrigated lands are affected by salinity (Zhu, 2007). High salinity causes ion imbalance, toxic levels of cytoplasmic sodium, and drought stress. Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components (Hanson *et al*., 1991). One of the metabolic adaptations to salt stress is the accumulation of osmoprotectants. Glycine betaine and *b*-Ala betaine are quaternary ammonium compounds that accumulate in many plant



Figure(1-1) Schematic representation of stress perception,transduction, stress-induced gene expression, and genetic engineering utilizing the candidate genes for developing stress tolerant transgenic crop plants (Zhu, 2007).

species in response to salt stress (Rhodes and Hanson, 1993). Glycine betaine protects the cell from salt stress by maintaining an osmotic balance with the environment and by stabilizing the quaternary structure of complex proteins (Papageorgiou and Murata, 1995).

Betaine aldehyde dehydrogenase (BADH) enzymes are classified as substrate-specific oxidoreductases (EC 1.2.1.8) and belong to family 10 of the large superfamily of aldehyde dehydrogenases (Sophos and Vasiliou, 2003).

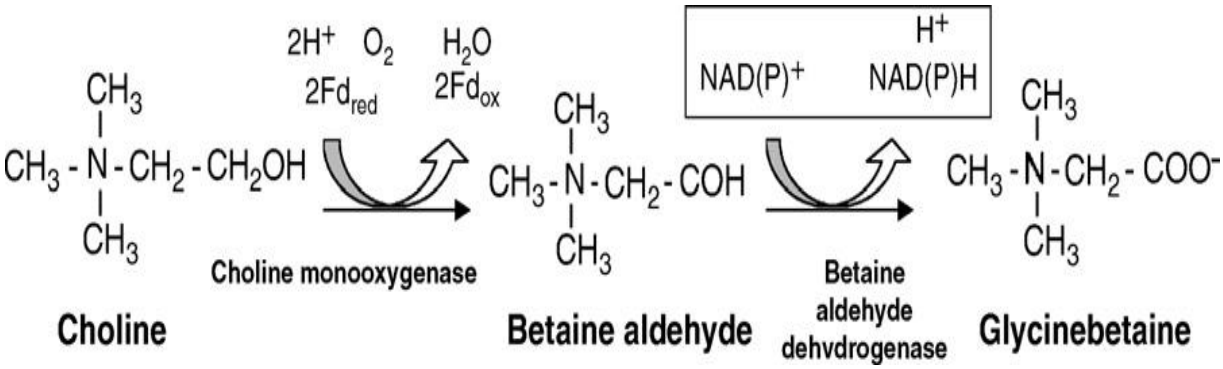
BADH catalyse the oxidation of betaine aldehyde to betaine. Trimethylglycine was the first betaine to be characterised and is by far the most extensively studied; this compound is now referred to as 'glycine betaine (GB) to distinguish it from other betaines. In plants, the study of betaine has almost exclusively focused on GB which is known to be particularly effective in conferring protection against abiotic stresses such as salt, water deficit, heat and chilling (Le Rudulier *et al*., 1984).

In general terms, a plant BADH refers to an enzyme that converts BA to GB, using an oxidising co-factor (Fig. 1-2). The reaction can be either NAD+ or NADP+ dependent, however, plant BADHs show higher activity using NAD+ (Nakamura *et al*., 1997). Choline monooxgenase (CMO) and BADH work together in the pathway to GB production in plants, with CMO initially converting choline, a derivative of proline, to BA (Figure 1-2).

Many plants possess more than one putative *BADH*-encoding gene homologue, most commonly two have been reported, and these are generally referred to as *BADH*1 and *BADH*2 .

Glycine betaine is widely distributed in bacteria, algae, higher plants (e.g., sugar beet and cotton) and animals, and is frequently detected in those plant species that are exposed to drought and salinity stresses (Rhodes and Hanson 1993; Reda *et al*., 2004).

However, not all plants accumulate GB, and it has been suggested that this is due to the lack of a functional CMO (Nuccio *et al*., 1998). Initial enzyme activity studies and molecular cloning of a plant *BADH* were performed in spinach (Weretilnyk and Hanson, 1990). Subsequently, numerous putative *BADH*s have been isolated based on the homology of these genes to the spinach *BADH* gene. This has led to the classification of many putative *BADH*-encoding genes without substrate specificity and enzyme activity data for the enzyme that they encode.



Figure(1-2) Biochemical pathway to GB from choline via CMO and BADH (Sakamoto and Murata, 2001).

Betaine aldehyde dehydrogenase was first implicated in plant salt stress response in spinach leaves (Weigel *et al*., 1986), with the finding that BADH activity in spinach increased threefold in plants grown with 300 mM NaCl relative to control plants. Weretilnyk and Hanson (1990) also found that both salt shock and chronic salt stress caused a several fold increase in the level of spinach *BADH*-encoding mRNA. The response of BADH to salt in a major crop species, barley, was reported in 1990; it was found that the level of BADH enzyme activity increased threefold in leaves hydroponically grown with 200 mM NaCl (Arakawa *et al*., 1990).

Glycine betaine is known to protect protein structure and enzyme activities and also stabilize membranes during osmotic and ionic stresses (Rhodes and Hanson 1993). Moreover, it functions to stabilize both PSII complex and Rubisco during photosynthesis under stress conditions (Holmstrom *et al*., 2000).

So far, *BADH* has already been cloned from spinach (*Spinacia oleracea* L.) (Weretilnyk and Hanson, 1990), sugar beet (*Beta vulgaris* L.), *Atriplex hortensis*, L. barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor*), rice (*Oryza sativa* L.), *Amaranthus hypochondriacus* L., mangrove [*Avicennia marina* (Forsk.) Vierh., *A. centralasiatica* (Ahmad *et al*., 2008). This gene was also expressed in transgenic tobacco *(Nictiana tabacum*), which grows normally in a medium containing 1.2% (205 mM) NaCl.

Glycine betaine may also enhance tolerance of plants to high temperature stress. Several *in vitro* studies have indicated that GB protects some enzymes and protein complexes against heat-induced inactivation . GB is in particular effective in protecting highly complex proteins, such as the PSII complex, against heat-induced inactivation An *in vivo* study has further shown that transformed Arabidopsis (*Arabidopsis thaliana*) with accumulation of GB exhibits enhanced tolerance to high temperatures during the growth of young seedlings (Alia *et al*., 1998). However, it is not clear what is the physiological basis of GB *in vivo* for such an enhanced tolerance of growth to high temperature stress.

1.2.3.2 Safe Selectable Marker for Transgenic Plants

The rapid development of plant genetic engineering has led to the creation of transgenic crops, with genes transferred from organisms across their sexual boundaries, which is otherwise impossible through conventional breeding. Since the first transgenic plant was developed in tobacco in 1984 , transgenics in several economically important plants resistant to herbicides, insects, diseases, and also with superior nutritional and post-harvest quality were developed, some of which are already in use. The dramatic increase in area of cultivation of transgenic crops is evident from a virtually ground zero in 1994 to around 125 million acres of land occupied the world over at the end of 2001 (Hasegawa *et al*., 2000).

However, the public awarness against the commercialization of transgenics, due to possible environment and health hazards, demands thorough assessment on this aspect. One such concern is with genes that code for antibiotic and herbicide resistance. Co-transferred into the host genome as selectable markers, they confer selective advantage to the transformed cell/tissue to grow in the presence of the antibiotic/ herbicide. The products of these genes may not be necessarily harmful, albeit their presence in transgenic

plants increases the chances of their escape through pollen or seed dispersal to the wild and weed relatives of the crops, resulting in genetic pollution (Daniell *et al*., 2001a).

Antibiotic resistance genes may get passed onto pathogenic microorganisms in the gastrointestinal tract or soil, making them resistant to treatment with such antibiotics. Similarly, resultant gene products in the transgenic plant may cause unknown health hazards to the consumers. Since a limited number of selectable marker genes are available for plant transformation, the combination of multiple transgenic traits through crosses among different transgenic lines will frequently produce plants that contain multiple copies of the same selectable marker linked to different effectors genes. The presence of such multiple homologous sequences in plants enhances the likelihood for homology dependent gene silencing, which could severely limit the reliable long-term use of transgenic crops. Many new strategies have been developed to eliminate the selectable marker genes from the transgenic plants for the improved safety of both the environment and the consumer .Again, it is possible to re-transform a transgenic plant using the same original marker for multigene transfer, thereby repeatedly stacking the transgene in a stepwise process. The ability to re-use a selectable marker could be particularly helpful for re-transforming species, which are otherwise difficult to transform or regenerate and for which a few markers work well (Fischer *et al*., 1996).

Besides marker removal systems, several other strategies where removal of marker genes is not needed, e.g. development of environmentally safe selectable markers or transplastomic plants, modulating and spatial/temporal expression of transgenes have been developed to ease the public concerns about health and environment.

Recently, Daniell lab. engineered chloroplast genome without the use of antibiotic selection. The betaine aldehyde dehydrogenase (*BADH*) gene from

spinach was used as the selectable marker. This enzyme is present only in the chloroplast of a few plant species (members of Chenopodiaceae, Poaceae, etc.). Rapid regeneration of chloroplast transgenic plants was obtained under betaine aldehyde (BA). The selection process involves the conversion of the toxic BA by the chloroplast BADH enzyme to a nontoxic glycine betaine, which also serves as an osmoprotectant for enhancing drought and salt stress tolerance in plants. Transgenic plants were morphologically indistinguishable from untransformed plants and the induced trait was stably inherited in the subsequent generations. Chloroplast transformation efficiency was 25-fold higher in BA selection than spectinomycin, which is widely used for chloroplast transformation,but the *BADH* gene was also transferred to tobacco and carrot with *aadA* gene which confer the resistance to spectinomycin therefore the transgenic plants still contain antibiotic resistance gene (Kumar *et al*., 2004a).

1.2.4 Transgenic Plants to Produce Therapeutic Proteins

Research in the past few decades has revolutionized the use of therapeutically valuable proteins in a variety of clinical treatments. Because most genes can be expressed in many different systems, it is essential to determine which system offers the most advantages for the production of the recombinant protein. The ideal expression system would be the one that produces the most safe, biologically active material at the lowest cost. The use of modified mammalian cells with recombinant DNA techniques has the advantage of resulting in products that are identical to those of natural origin; however, culturing these cells is expensive and can only be carried out on a limited scale. The use of microorganisms such as bacteria permits manufacture on a larger scale, but introduces the disadvantage of producing products that differ appreciably from the products of natural origin. For example, proteins that are usually glycosylated in humans are not

glycosylated by bacteria. Furthermore, human proteins that are expressed at high levels in *E. coli* frequently acquire an unnatural conformation accompanied by intracellular precipitation, owing to lack of proper folding and disulfide bridges (Nemchinov *et al.*, 2000).

The production of recombinant proteins in plants has many potential advantages for generating biopharmaceuticals relevant to clinical medicine. First, plant systems are more economical than industrial facilities using fermentation or bioreactor systems. Second, the technology is already available for harvesting and processing plants and plant products on a large scale. Third, the purification requirement can be eliminated when the plant tissue containing the recombinant protein is used as a food (edible vaccines). Fourth, plants can be directed to target proteins into intracellular compartments in which they are more stable, or even to express them directly in certain compartments (chloroplasts). Fifth, the amount of recombinant product that can be produced approaches industrial-scale levels. Last, health risks arising from contamination with potential human pathogens or toxins are minimized (Daniell *et al.*, 2001b).

Generally, levels of pharmaceutical proteins produced in transgenic plants have been less than the 1% of total soluble protein that is needed for commercial feasibility if the protein must be purified. Plant derived recombinant hepatitis-B surface antigen induced only a low level serum antibody response in a small human study, probably reflecting the low level of expression (1–5 ng\g fresh weight) in transgenic lettuce. In spite of recent improvements in expression levels in potato with a view to clinical trials, expression levels should be increased further for practical purposes.Also, even though Norwalk virus capsid protein expressed in potatoes caused oral immunization when consumed as food, expression levels are too low for large-scale oral administration (0.37% of total soluble protein).

Expression of genes encoding other human proteins in transgenic plants has been disappointingly low: human serum albumin, 0.020% total soluble protein, human protein C, 0.001% total soluble protein; erythropoietin, ~0.003% total soluble protein; and human interferon-β, <0.001% fresh weight. A synthetic gene coding for the human epidermal growth factor was expressed only up to 0.001% of total soluble protein in transgenic tobacco (Maliga and Bock, 2011)

In spite of several successful reports of high-level expression of non-human proteins (e.g. phytase, glucanase) via the nuclear genome, there is a great need to increase expression levels of human blood proteins to enable the commercial production of pharmacologically important proteins in plants. One alternative approach is to express foreign proteins in chloroplasts of higher plants. Foreign genes have been integrated into the tobacco chloroplast genome, giving up to 10 000 copies per cell and resulting in the accumulation of recombinant proteins at up to 47% of the total soluble protein (Ma *et al*., 2003).

1.2.4.1 Chloroplast Genetic Engineering

The chloroplast is one of the organelles known as plastids in plant cells and eukaryotic algae. It is the site of photosynthesis, which provides the primary source of the world's food productivity. Other important activities that occur in plastids include evolution of oxygen, sequestration of carbon, production of starch, synthesis of amino acids, fatty acids, and pigments, and key aspects of sulfur and nitrogen metabolism (Verma and Daniell, 2007).Plastid is the general organelle category encompassing proplastids, the progenitors of all plastid types and chloroplasts (green plastids), chromoplasts (yellow or red, in some fruits and flowers), and different types of white plastids such as the amyloplasts (starch containing) and elaioplasts (oil containing plastids). Plastids are plant cellular organelles with their own

genome and transcription- translation machinery. The plastid genome (plastome or ptDNA) is a highly polyploid, circular double-stranded DNA 120 kb to 180 kb in size,its present in 1,000–10,000 copies per cell , and maternally inherited in most angiosperm plant species .A salient feature of the plastid genome in most higher plant species is duplication of a large (25 kb) region in an inverted orientation (Hagemann, 2004).

The concept of chloroplast genetic engineering was first conceived in the mid 1980's with the introduction of isolated intact chloroplasts into protoplasts (Daniell and Dhingra, 2005). Later focus was laid on the development of chloroplast systems capable of efficient, prolonged protein synthesis and the expression of foreign genes .Biolistics method of transformation has made it feasible to transform plastids without the need to isolate them. The first successful chloroplast genome complementation was reported in 1988 for the unicellular green alga having single chloroplast, *Chlamydomonas reinhardtii*. For this, the photosynthetically incompetent mutants that lacked *atp*B gene and chloroplast ATP synthase activity were used. The wild-type *atp*B gene was introduced into the cells using tungsten microprojectiles coated with the *atp* gene .The single large chloroplast provided an ideal target for DNA delivery. Restoration of photoautotrophic growth upon selection in the light demonstrated that the deletion mutant phenotype was corrected with the introduction of wild type *atp*-B gene into the cells (De Marchis *et al.*, 2008).

After the successful transformation of the unicellular algae *C. reinhardtii* through chloroplast genome, efforts were made to transform the higher plants through chloroplast genome. Initially in higher plants, foreign genes were introduced and expressed only in isolated but intact plastids. The first expression of a foreign gene in plastids of cultured tobacco cells used autonomously replicating chloroplast vectors .This

became a major breakthrough because the majority of higher plants genetically transformed via the chloroplast genome now use *aad*A gene as selectable marker. Stable integration of the *aad*A gene into the tobacco chloroplast genome was then demonstrated (Svab and Maliga, 1993).

Initially, when the transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions of the chloroplast genome. However, Daniell lab. advanced forward the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes (Daniell and Dhingra, 2002). The *trn*I-*trn*A intergenic region is transcriptionally active because of the read-through transcription of the upstream 16S rRNA promoter capable of transcribing six native genes downstream (fig 1-3)(Dhingra *et al.*, 2004).

Tobacco, a non-food/ feed crop is proven to be ideal for transformation because of its ease for genetic manipulation. Tobacco is an excellent biomass producer (in excess of 40 tons fresh leaf weight/acre based on multiple mowings per season) and a prolific seed producer (up to one million seeds produced per plant), which is ideal for the large-scale commercial production. It has been extensively used for the large-scale production of therapeutic proteins. It has been estimated that the cost of production of recombinant proteins in tobacco leaves will be 50-fold lower than that of *Escherichia coli* fermentation systems .Using the chloroplast transformation, tobacco has been used for hyper-expression of vaccine antigens and production of valuable therapeutic proteins like human elastin-derived polymers for various biomedical applications (Guda *et al*., 2000), vaccines antigens for cholera, anthrax, plague and tetanus , monoclonal antibody Guy's 13, a monoclonal antibody against *Streptococcus* mutans and which protects against dental

carries has also been expressed in the chloroplast (Daniell *et al.*, 2001).

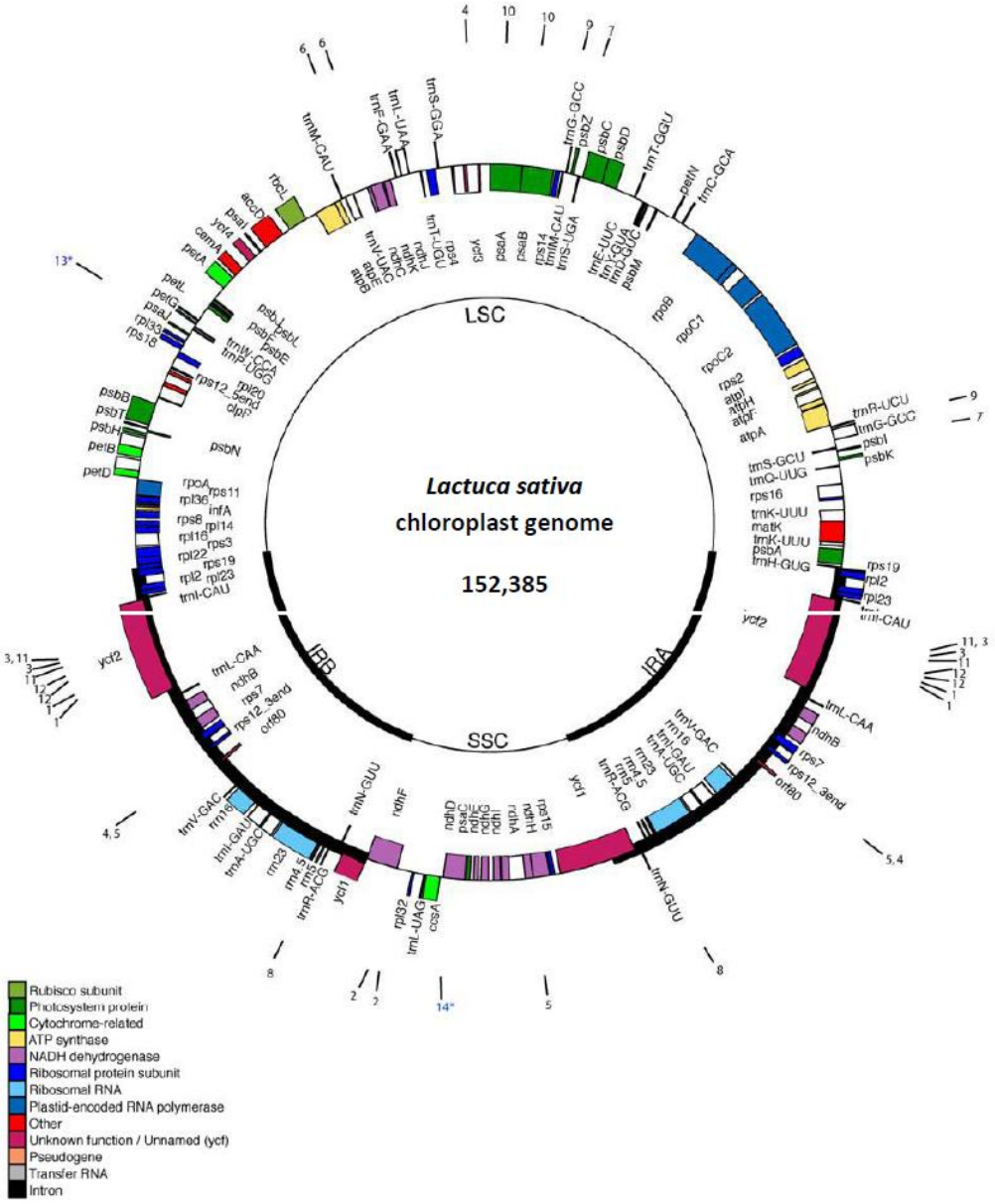


Figure 1-3:- Complete genetic map of *Lactuca Sativa* (Verma and Daniell 2008)

Human therapeutic proteins, including human serum albumin, magainin, a broad spectrum topical agent, systemic antibiotic, wound healing stimulant and a potential anticancer agent , interferon and insulin- like growth factor (Davoodi-Semiromi *et al.*, 2009) have been expressed. Several other laboratories have expressed other therapeutic proteins, including human

somatotropin, and interferon-GUS fusion proteins in transgenic chloroplasts(Tacket, 2007).

The chloroplast transformation technology has been used to introduce various agronomic traits. Insect resistance has been achieved by expressing insecticidal proteins from *Bacillus thuringiensis* (Bt); the Cry2Aa2 protein had accumulated up to 46.1% tsp and this is by far the highest expressed foreign protein in transgenic plants to date (DeCosa *et al.*, 2001). Similarly herbicide resistance against Glyphosate, a broad spectrum herbicide that non-selectively kills the weeds by inhibiting the 5-enolpyruvylshikimate- 3-phosphate synthase, a nuclear-encoded chloroplast localized enzyme in the shikimic acid pathway of plants and microorganisms that is required for the biosynthesis of aromatic amino acids has been achieved. The plastid that was engineered with PSPS gene in tobacco plants developed resistance to glyphosate over the wild type plants .The antimicrobial peptide MSI-99, an analog of magainin has been expressed in the chloroplast genome of transgenic tobacco up to 21.5% TSP . MSI-99, offers protection against prokaryotic organisms due to its high specificity for negatively charged phospholipids found mostly in bacteria. Extracts from MSI-99 transformed plants inhibited growth of *Pseudomonas aeruginosa*, a multi-drug resistant bacterium, which acts as an opportunistic pathogen in plants, animals, and humans. It is also biologically active against *Pseudomonas syringae*, a major plant pathogen. The yeast trehalose phosphate synthase gene expressed in the chloroplast showed a high degree of drought tolerance (Lee *et al*., 2006). Different biomaterials, enzymes and amino acids also were expressed in the transgenic chloroplasts. For example, xylanase which is an industrially important enzyme when expressed through the nuclear transgenic plants showed cell wall degradation and affected plant growth. But when was expressed via the chloroplast, there were no such effects seen as in nuclear transgenics (Agrawal *et al*., 2011).

Chloroplast genetic engineering technology is currently applied to other useful crops such as potato, tomato, carrot, cotton and soybean by transforming different plastid genomes (Kumar *et al.*, 2004a). The limitations in extending the plastid transformation technology to major crops that regenerate via somatic embryogenesis include inadequate tissue culture protocols, lack of selectable markers and also the inability of expressing the transgenes in non-green tissues (Daniell *et al*., 2005).For the first time carrot plastid genome has been transformed using the non-green tissue as explants and regenerated via somatic embryogenesis (Kumar *et al.*, 2004).

A useful plant trait (salt tolerance) has been expressed for the first time in a non-solanaceous crop via the chloroplast genome. The toxic betaine aldehyde (BA) is converted to non-toxic glycine betaine by the chloroplast BADH enzyme. This glycine betaine also serves as an osmoprotectant and confers salt tolerance. The transgenic calli obtained from cultured cells expressing BADH were green in color in the absence of selection and the untransformed cells were yellow. Somatic embryos of carrot are single cell derived and multiply through recurrent embryogenesis, which provides uniform source of cell culture and homogeneous single source of origin (Matsui *et al*., 2011).

Furthermore, Kumar *et al*. (2004) have transformed the cotton plastid genome. The transgenic seeds obtained were resistant to kanamycin selection whereas the untransformed seeds were not (Kumar *et al*., 2004a). Similarly, the first successful development of transgenic soybean plants was achieved by Dufourmantel *et al.* (2004). Therefore, the successful plastid transformation of the above said crop plants was suggested to be due to the 100% homologous plastid DNA sequences used in the species-specific vectors. Even though the concept of universal vector was proposed several years ago, the use of species-specific vectors has demonstrated successful plastid transformation (Lee *et al.*, 2006).

Plastid transformation has been developed for edible leafy crops. One such crop, lettuce, has been used to produce, via nuclear transformation and chloroplast engineering, a hepatitis B virus subunit vaccine for clinical trials. Lettuce grows quickly and can be harvested within few months after planting. The movement of plastid integrated transgenes to the nucleus has been reported. However, the frequency of pollen derived from transplastomic plants carried the transgene that was integrated in the plastid genome is rather low .Furthermore, lettuce is suitable for indoor cultivation by hydroculture systems. Thus, the horizontal propagation of transgenes can be prevented by fail-safe (Kanamoto *et al.*, 2006).

1.2.4.2 Expression of Foreign Protein in Chloroplast Genome

Several environmental stresses such as disease, drought, insect pests, salinity and freezing, can severely limit plant growth and development. In order to improve the plant traits, many researchers had done a series attempts. Many important agronomic traits have already been engineered via the plastid genome, such as herbicide resistance, insect resistance, and tolerance to drought and salt. The insect resistance genes were investigated for high-level expression from the chloroplast genome (De Cosa *et al*., 2001).

Plant diseases have affected global crop production. Transgenic chloroplasts conferred resistance to the fungal pathogen *Colletotrichum destructive* in tobacco. The chloroplasts were estimated to express MSI-99 at 21.5% to 43% of total soluble protein (TSP). MSI-99 was expressed at high levels to provide inhibition of growth against *Pseudomonas syringae* pv *tabaci*, a major plant pathogen. This data suggests that MSI-99 expressed in tobacco chloroplasts can offer significant protection from both bacterial and fungal pathogens (DeGray *et al*., 2001).

Chloroplast engineering had been successfully applied for the development of plants with tolerance to salt, drought and low temperature.

Previous research has shown that over-expression of enzymes for Glycine betaine biosynthesis in transgenic plants improved tolerance to various abiotic stresses. Choline monooxygenase from beet (*Beta vulgaris*), the enzyme that catalyzes the conversion of choline into betaine aldehyde, has been transferred into the plastid genome of tobacco. Transplastomic plants demonstrated that higher photosynthetic rate and apparent quantum yield of photosynthesis in the presence of 150 mmol/L NaCl (Rhodes and Hanson, 1993).

A therapeutic protein, human serum albumin was firstly expressed in transgenic chloroplasts of tobacco at an expression level up to 11.1% of TSP, which is 500-fold greater than the nuclear expression.So far, most efforts have been focused on the high-level production of antigens for use as vaccines and their tests for immunological efficacy in animal studies(Richter *et al*., 2000).

1.2.4.3 Vaccine Antigens Expressed Via the Chloroplast Genome

Expressing vaccine antigens via the chloroplast genome has proven to be advantageous: subunit vaccines are not toxic even when expressed at high levels, bacterial genes have high AT content allowing for high expression in the chloroplast; and oral delivery of vaccines yields high mucosal IgA titers along with high systemic IgG titers, enabling the immune system to fight against germs at their port also entry (yang *et al*., 2008).

Vaccines that have already been expressed in the chloroplast include the Cholera toxin B- subunit (CTB), which does not contain the toxic component that is in CTA (Daniell *et al*., 2001), the F1~V fusion antigen for plague (Singleton 2003), the 2L21 peptide from the Canine Parvovirus (Molina *et al*., 2004), Anthrax Protective antigen (Watson *et al*., 2004), C terminus of *Clostridium tetani* (Maliga, 2003).

CTB was expressed at 4 to 31% of Total Soluble Protein (TSP) and was effective in the GM1-ganglioside binding assay which indicates proper

folding and formation of disulfide bonds to form pentamers (Daniell *et al.*, 2001, Molina *et al.*, 2004). The 2L21 peptide from the Canine Parvovirus fused to green fluorescent protein expressed 22% TSP and CPV fused to CTB 31% TSP (Molina *et al.*, 2004). When mice were immunized intraperitoneally with the leaf extracts from CTB-2L21, the developed anti-2L21 antibodies were able to recognize VP2 protein from CPV. Anthrax PA83 was expressed at 14.7% TSP in transgenic tobacco chloroplasts and elicited immunogenic response in the mice proving that plant derived PA is biologically similar to PA derived from *Bacillus anthracis* (Koya, 2004). The C terminus of *Clostridium tetani* was expressed at 25% TSP for AT rich and 10% TSP for GC rich sequences which shows that chloroplasts favor prokaryotic-AT rich sequences. TetC when administered intranasally produced both IgG and IgA and was immunoprotective against the toxin (Tregoning *et al*., 2003). To date, only few vaccine candidates derived from chloroplast genetic engineering have been tested for immunogenecity in mice and only one vaccine candidate has been tested for immunoprotective property. High expression is not only economically important, but for oral vaccines it is essential for the immune response**.**

1.2.4.4 Advantages of Chloroplast Transformation over Nuclear Transformation

Cross Pollination with pollen from genetically modified crops with the wild type crop is a major concern. However, by genetically engineering the chloroplasts, the possibility of cross-pollination with pollen carrying transgenes is eliminated because chloroplast DNA is maternally inherited. Although pollen from plants was shown to exhibit maternal plastid inheritance through metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation and hence is not transmitted to the next generation. During fertilization, the paternal chloroplasts from pollen are

disintegrated in a synergic cell and only the sperm nucleus enters the egg cell and fuses with the egg to form a zygote. Maternal inheritance thus offers the advantage containment of chloroplast transgenes due to lack of gene flow through pollen (Daniell, 2002). The transgene inheritance thus occurs only via seeds.This environmental eco-friendly feature should eliminate all the concerns of cross contamination with wild type and relative crops (Daniell and Parkinson, 2003).

In order to make plant production of proteins commercially feasible, expression levels greater than 1 % of the total soluble protein must be achieved in plants (Kusnadi *et al*., 1997). Nuclear transformation of plants has usually produced lower expression levels of antigens (Daniell *et al*., 2001; Richter *et al*., 2000; Tacket *et al*., 2000; Daniell *et al*., 2005). For example, plant derived recombinant hepatitis B surface antigen was as effective as a commercial recombinant vaccine, but the levels of expression in transgenic tobacco were low (0.0066% of total soluble protein).For an oral delivery vaccine, it may prove to be extremely important to have high expression levels in order to elicit the immune response.

An alternative approach to nuclear transformation is to integrate foreign genes into the chloroplast genome, which is a powerful technique because of the number of copies of chloroplast genomes per cell (up to 1,000). This high polyploidy leads to high transcript levels and finally accumulation of abundant translated product resulting in high expression levels up to 47% of TSP (De Cosa *et al*., 2001).

Chloroplast transformation occurs exclusively through site-specific homologous recombination. In contrast, nuclear transformation experiments frequently suffer from gene-silencing mechanisms resulting in unstable and inconsistent gene expression or complete loss of transgenic activity. The nuclear genome has mechanisms that may inactivate genes when regulatory sequences are inserted in a repetitive pattern. This occurs because integration

of transgenes into the nuclear genome is random and not via homologous recombination (Daniell and Dhingra, 2002).

This random integration of transgenes may allow for insertion of the transgene into a region of the nuclear genome that is not highly transcribed. Due to the random position of the transgene in the nuclear genome, expression levels vary in different transgenic lines. However, neither gene silencing nor position effects have been observed in genetically engineered chloroplasts(Daniell and Dhingra, 2002).Chloroplast transgenic lines with the accumulation of transcripts 169-fold higher than nuclear transgenic lines have shown no gene silencing (Lee *et al*., 2003; Dhingra *et al*., 2004). Likewise, chloroplast transgenic lines showed no transgene silencing at the translational level regardless of accumulation of foreign protein up to 47% TSP (DeCosa *et al*., 2001).

Besides, the chloroplast genetic engineering offers attractive advantages of introducing multigenes in a single transformation step because of its ability to transcribe the operon with multigenes into polycistronic mRNA and translate the polycistronic mRNA (Ruiz *et al*., 2003). This saves a lot of time to create a transgenic plant expressing multigenes as opposed to the nuclear transformation where several independent transgenic lines have to be created followed by the laborious repetitive breeding (DeCosa *et al*., 2001; Daniell and Dhingra, 2002; Lossl *et al*., 2003).

1.2.4.5 Advantages of Chloroplast Transformation over *E.coli* Transformation

Chloroplast expression system has several advantages over *E. coli* expression system. Production of recombinant proteins in microorganisms is expensive, requires stringent purification protocols, and scale-up requires building costly fermenters. Vaccine production in plants can circumvent these problems. First, farming of plants is straightforward, fairly inexpensive, and

can be scaled-up at low cost in one season. The cost of production of recombinant proteins in tobacco leaves will be 10 to 50 times cheaper than that of *E. coli* fermentation with 20% expression levels in *E. coli*. Second, plants provide a heat-stable environment for proteins, and the technology already exists for harvesting, storage, and purification of transgenic plant proteins.In addition, each transgenic plant generated can produce up to a million seeds per plant. Third, chloroplasts are capable of folding proteins and maintaining their natural conformation (Kusnadi *et al*., 1997).

Folding, assembly and production of disulfide bridges for CTB have already been demonstrated in chloroplasts (Daniell, 2002). Binding assays proved that chloroplast synthesized CTB binds to the GM-1 ganglioside receptor. Many antigens for vaccines that have been expressed in chloroplasts have been proven to be immunogenic (Tregoning *et al.*, 2003; Molina *et al.*, 2004).

Finally, the process of purification of a recombinant protein produced in *E. coli* is costly and time consuming. For example, for insulin production, chromatography accounts for 30% of the production cost and 70% of the set-up cost. A transformed tobacco plant would still need purification. But an edible vaccine such as in the lettuce , carrot or tomato would eliminate this cost. Expenses for delivery by injection would also be eliminated. Therefore, expression of CTB in the lettuce and other edible plant plastids for oral vaccination would be beneficial (Petrides *et al*., 1995).

1.2.5 Molecular Farming (Pharming) and Edible Vaccine

The recombinant production of pharmaceuticals, functional proteins, industrial enzymes and functional secondary metabolites in plants is referred to as plant molecular farming. The terms molecular farming, biofarming, molecular pharming, phytomanufacturing, recombinant or plant-made industrials, plantapharma, plant bioreactors, plant biofactories, pharmaceutical

gardening, and phytomanufacturing are used interchangeably (Basaran and Rodriguez-Cerezo, 2008).

Molecular farming in plants is expected to challenge already established production technologies for pharmaceuticals that currently use bacteria, yeast, and cultured mammalian cells because plants lack human pathogens, oncogenic DNA sequences, prions, and endotoxins (Cabanes-Macheteau *et al.*, 1999). As research in using plants as manufacturing platforms becomes more widespread, the commercial success will rest on the efficiency of the technology, solving current drawbacks in the existing plant expression and production systems, safety of final products, health and environmental testing, economic considerations, the readiness of the regulatory environment, intellectual property regimes, ethical issues, public acceptance, and overcoming of related social and policy challenges (Drossard, 2004).

Delivery and expression of heterologous genes in plants may involve several strategies such as nuclear transformation, plastid (e.g., chloroplast) transformation, transient expression, viral transfection, and agroinfilitration as show in the figure 1-4 (Gleba *et al*., 2005).

Oral vaccines are more affordable and accessible to the inhabitants of developing countries, who needlessly die, in thousands, from diseases, which can easily be prevented by vaccination. Food vaccines are like subunit preparations in that they are engineered to contain antigens but bear no genes that would enable whole pathogens to form. These vaccines basically work in the same way as the injected DNA vaccine, since a peptide sequence similar to an infectious part of a pathogen is synthesized, by itself, and is used to prime T and B cells in the body. The big difference in this case is that the protein sequences are encoded in a plant to form the desired protein. This protein is then ingested, as the plant or its fruit is eaten. One becomes immune against the ingested protein, as T and B cells become stimulated to proliferate and differentiate (Castle *et al.*, 2006).

Edible plant-made vaccines have the potential to provide more benefits compared to injected vaccines. Edible plant-made vaccines are produced in molecular farms, where specialized plants are processed through transformation, and the plant material delivers antigens through oral delivery. Specifically, by oral delivery, transformed plants can be delivered into the body by processing it into pills or integrating transformed plants into foods. Successful expression of antigens in plants was achieved for Rabies virus G-protein in tomato, Norwalk virus capsid protein in tobacco and potato (Mason *et al*., 1996), Hepatitis B virus surface antigen in tobacco and potato , *E. coli* heat-labile enterotoxin B subunit (LT-B) in tobacco and potato Cholera toxin B subunit (CT-B) in potato (Arakawa *et al.*, 1998). Food vaccines are also used to suppress autoimmune disorders like type-1 diabetes, multiple sclerosis, rheumatoid arthritis. Foods under study include potatoes, bananas, lettuce, rice, wheat, soybean, corn and legumes (Prakash, 2001).

Edible vaccines reduce the need for skilled personnel to administer injections and negate the concerns regarding the reuse of needles. A measles vaccine that can be directly consumed would significantly increase the availability in places where maintenance of a cold-chain during storage and transport is difficult(Webster *et al*., 2002).

A concern with oral vaccines is the degradation of protein components in the stomach (due to low pH and gastric enzymes) and gut before they can elicit immune responses (Daniell *et al*., 2001) but the rigid plant cell walls could provide protection from intestinal degradation (Webster *et al*., 2002). The degradation can be compensated by repeating the exposure of the antigen until immunological tolerance is accomplished .The M cells lining the small intestine take in the components that have entered the small intestine (including pathogens) and pass them to other cells of the immune system, such as antigen presenting cells and macrophages. These cells chop up their acquisitions and display the resulting protein fragments on the cell surface.

Helper T lymphocytes recognize the displayed fragments as foreign, induce B lymphocytes to secrete neutralizing antibodies and also help to initiate a broader attack on the perceived enemy. Mucosal immune responses represent a first line of defense against most pathogens (Mason *et al*., 2002).

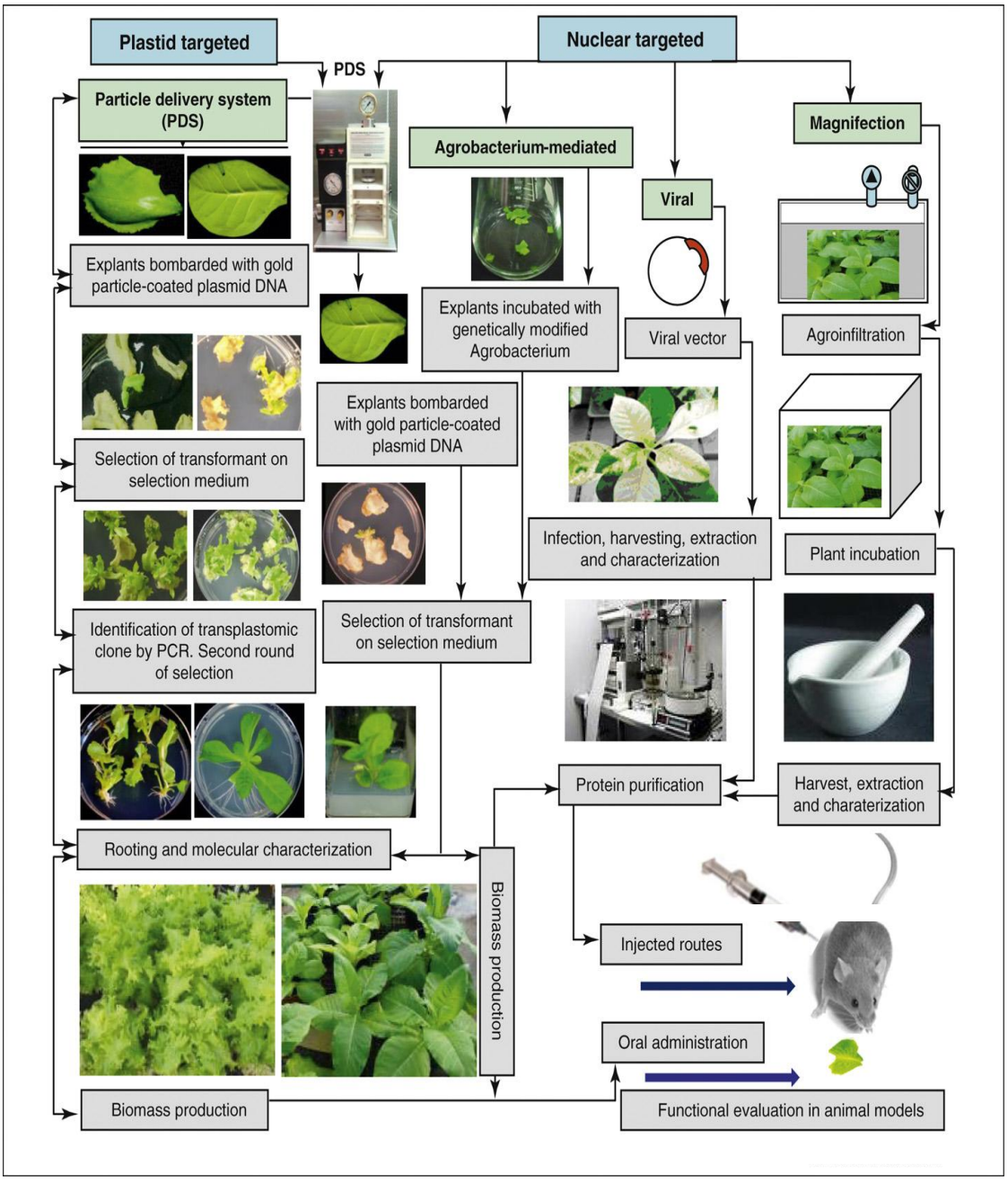
Second generation edible vaccines are also called as multicomponent vaccines that provide protection against several pathogens. An elegant approach to achieve this goal, based on epitope fusion to both subunits of the cholera toxin (CT), was demonstrated by Yu and Langridge (2001). CT provides a scaffold for presentation of protective epitopes of rotavirus and ETEC (Enterotoxigenic *E.coli*), acts as a vaccine candidate by its own right and as a mucosal adjuvant devoid of toxicity.

The trivalent edible vaccine elicited significant humoral responses, as well as immune memory B cells and T helper cell responses, important hallmarks of successful immunization. In the clinical trials described 100 g of raw potato tubers expressing LT-B of ETEC in three doses had to be consumed in order to overcome digestive losses of the antigen and to elicit a significant immune response (Tacket *et al*., 2000).

1.2.5.1 History of Edible Vaccine

From 60,000 BC to the 19th century, plants were the main source for human drugs. There was a great turn in medication history, starting at the beginning of the 19th century until the early 1970s, when pharmacy turned to be dominated by scientific chemistry with both the development of more and more sophisticated processes for extraction, purification and the synthesis of active pharmaceutical compounds. The 20th century became a triumph for drugs produced at an industrial level by chemical synthesis. This evolution probably started with the production of aspirin, a synthetic analogue of salicylic acid previously extracted from willow bark. In parallel, more and more sophisticated extraction and purification procedures were developed

resulting, for example, with the first extraction of morphine from poppy in 1815 or extraction of insulin from pig pancreas in 1922 (Paccalet *et al*., 2007).



Figure(1.4) Schematic representation of vaccine antigens and biopharmaceuticals production in plants and their functional evaluation in animal models. Explants are tissues that have the potential for regeneration into mature plants.(Daniell *et al*., 2010).

As a complement of synthesis and extraction chemistry, modern biology enters the world of pharmaceutical industry with the development of genetic

engineering in the early 1970s, allowing biosynthesis of complex molecules, and are too difficult to extract and purify from living material and inaccessible to synthesis chemistry. In the last decades, genetic engineering has offered an alternative to chemical synthesis and extraction procedures with the production of therapeutic molecules in transgenic bacteria, yeast and animal cells. After a temporary decrease in interest, plants are rapidly moving back into human pharmacopoeia, with the recent development of plant-based recombinant protein production systems offering a safe and extremely cost-effective alternative to microbial and mammalian cell culture (Agrawal *et al*., 2011).

Molecular farming activity has existed since the first higher plant was successfully transformed , because any protein has the potential of being a protein product. One of the earliest marker genes that scientists have used in developing transformation systems in plants, , is a molecular farming product (Zhong *et al.*, 1999).

The first report of human antibodies produced in plants was by During (1988) and was expanded to include secretory antibodies by Hiatt and co worker(Hiatt *et al*., 1989). The first report of a protein being produced in plants for the specific purpose of extraction, purification, and sale of that protein was by Hood and Jilka, who detailed the production of avidin, an egg protein with several important properties (Hood and Jilka, 1999).

Aprotinin, one of the first molecularly farmed pharmaceutical proteins to be produced in plants, may soon be used on medical patients for wound closure and to suppress the systemic inflammatory response during surgery (Zhong *et al.*, 1999).

1.2.5.2 Edible Vaccine vs. Classical Vaccine

Edible vaccines have many advantages over traditional injected vaccines. Edible vaccines are cost-effective, have various routing methods,

low risk of contamination, and eliminate the cost of transportation. Transgenic plants can be cost efficient in many categories, like storage, preparation, production and transportation. Unlike pharmaceutical companies, transgenic plants do not necessitate cold chain storages, which cost 200-300 million dollars yearly to preserve vaccines .The seeds of transgenic plant tissues can be dried out due to the low degree of moisture in the seeds, and plants with oil or water extracts have more storage opportunities (Hood *et al.*, 2003).

Edible vaccines are made in molecular farms, and not multimillion dollar machines. Since most countries have soil-rich land, it is convenient in obtaining edible vaccines, and long distance transportation is not required. The cost of materials needed for field grown plants is lower compared to cell culture grown in bioreactors .Edible vaccines have a low cost for medical equipment as well, because needles and syringes are not needed for delivery . Another reason for the low cost is the lack of administration required, as medical professionals are not needed for oral delivery (Streatfield and Howard, 2003).

Contamination risks are low in transgenic plants as compared to injected vaccines. Needles and syringes used in vaccinations cause environmental contamination and contain the risk of spreading second-hand diseases . There are risks in spreading second hand diseases because there is not an assurance if the needles have been reused. There are various regions in the world where clinics may use outdated techniques or lack knowledge of properly identifying reused needles (Schillberg *et al*., 2002).

Oral delivery is emphasized by many authors to be more efficient compared to injected vaccines. A reason for the efficiency is the increased chance of provoking a mucosal immune response, which produces cell mediated responses. Another crucial advantage to edible vaccines is the multi-component ability that is possible due to the crossing of two plant lines. These vaccines with multi-component abilities are known as second-generation

edible vaccines as they allow for several antigens to approach M cells (micro fold cells) simultaneously. A multi-component edible vaccine can be trivalent in that it can act to prevent multiple diseases from occurring within a specimen - for example, ETEC, cholera, and rotavirus. Injected vaccines lack this feature and so are less effective than edible vaccines (Yu and Langridge, 2001).

1.2.5.3 Advantages of Producing Cholera Vaccine Antigen in Plant Plastids

There are no known human or animal pathogens that affect plants therefore, the production of CTB protein in plants would yield a vaccine free of human pathogens (Streatfield *et al*., 2001).

- The CTB gene has an A/T content of 59%, which is ideal for chloroplast expression of the protein. Because chloroplasts are prokaryotic in nature they tend to exhibit higher expression with higher AT content genes.
- Bacterial proteins have been expressed at extraordinarily high levels in transgenic chloroplasts. This includes AT rich proteins such as Cry2a (67% AT) at 47% Total Soluble Protein (De Cosa *et al*., 2001), CTB (59% AT) at 33% TSP (Daniell *et al*., 2001), and Human Serum Albumin (66% AT) up to 11.1% TSP, (Fernandez-San Millan *et al*., 2003). Disulfide bonds in the above examples were properly formed.
- The CTB antigen is not glycosylated, which is good for our particular system because plastids do not glycosylate proteins.
- The technology to sow, harvest, store, and transport crops already exists.
- Plants have up to 100 chloroplasts per cell, each containing about 100 chloroplast genomes. This provides up to 10,000 genomes per cell to efficiently produce the antigens.

- Using the chloroplasts eliminates cross-pollination of the transgene by pollen because chloroplast's DNA is maternally inherited in most of the plants (Daniell, 2002; Daniell and Parkinson, 2003).
- Transgene integration is specifically targeted to intergenic spacer sites in the chloroplast genome; this eliminates gene silencing and the position effect that is seen in nuclear transformation (Daniell *et al*., 2002; Daniell and Dhingra, 2002).
- AT rich *CTB* has been produced in transgenic chloroplasts and has been shown to have native conformation via the GM1-ganglioside binding assay. It was produced 410 and 3100-fold higher in transgenic chloroplasts than nuclear transgenic plants at 4.1 and 31% total soluble protein (Daniell *et al*., 2001 ;Monila *et al*., 2004).
- Vaccine antigens have already been expressed in plants and have shown to be protected in the stomach through bioencapsulation. Also, they have been proven immunogenic when administered orally in clinical trials (Tacket *et al*., 2003; Tacket *et al*., 2004).
- Using plastid transformation technology can help produce of large quantities of vaccine.
- Proving that this vaccine is immunogenic when produced in tobacco chloroplasts could justify the expense to engineer oral vaccines by transforming plastids of lettuce. Delivery of plant-derived vaccine to mucosal tissues has been proven to induce both a mucosal and a systemic immune response (Haq *et al*., 1995).

1.2.6 Polymerase Chain Reaction(PCR) Technology

PCR is the cardinal laboratory technology of molecular biology. Arguably one of the most powerful laboratory techniques ever discovered, PCR combines the unique attributes of being very sensitive and specific with a great degree of flexibility. With the PCR it is possible to specifically address

a particular DNA sequence and to amplify this sequence to extremely high copy numbers. Kary Mullis invented the polymerase chain reaction (PCR) in 1985. One of the major applications of this outstanding invention is to know the presence of a transgene. The polymerase chain reaction results in the selective amplification of a transgene, if an appropriate primer is provided. Any region of transgene can be chosen, so long as the sequences at the borders of the region are known. The border sequences must be known because in order to carry out a PCR, two short oligonucleotides, must hybridize to the DNA molecule, one to each strand of the double helix. These oligonucleotides, which act as primers for the DNA synthesis reactions, delimit the region that will be amplified (Spoth and Strauss, 2000).

Amplification is usually carried out by the DNA polymerase-I enzyme from *Thermus aquaticus*, which lives in hot springs, and many of its enzymes, including Taq polymerase, are thermo stable, meaning that they are resistant to denaturation by heat treatment. Thermo stability of *Taq* polymerase is an essential requirement in PCR methodology along with dNTPs, $MgCl_2$, reaction buffer, sterile water.

To start a PCR amplification, the enzyme is added to the primed template DNA and incubated so that it synthesizes new complimentary strands. The reaction mixture is then heated to 94° C so that the newly synthesized strands detach from the template, and then cooled, enabling more primers to hybridize at their respective positions, including positions on the newly synthesized strands. *Taq* polymerase, which unlike most types of DNA polymerase is not inactivated by the heat treatment, now carries out a second round of DNA synthesis. The cycle of denaturation hybridization – synthesis is repeated, usually 25-30 times, resulting in eventual synthesis of several hundred million copies of the amplified DNA fragment. At the end of a PCR, a sample of the reaction mixture is usually analyzed by agarose gel electrophoresis, sufficient DNA having been produced for the amplified

fragment to be visible as a discrete band after staining with ethidium bromide. This may by itself provide useful information about the presence of transgene that has been amplified (Landridge, 2000).

1.2.7 Gene Gun Technology

Biolistics was created in part as a response to the limitations of the *Agrobacterium tumefaciens* system, which transforms plants through the creation of crown galls (tumors) that proliferate to form a cancerous growth. Sanford *et al*. (1987) reported the first stable transformants produced by biolistic bombardment in 1987. Their early experiments with onion epidermal cells as well as Christou's studies with soybeans in 1988 and 1989, showed that it was possible to cultivate transgenic roots. These studies were significant because they made up the first wave of concrete evidence that fully active and functional DNA could be delivered by particle bombardment (Sanford *et al*., 1987; Yamagishi *et al.*, 2006).

A proposed dynamic model for the biolistic particle bombardment device separates the gene transfer process into three discrete stages (Figures 1-5 and 1-6). The particle cluster acceleration stage involves the acceleration of the particles already coated with DNA to be transferred. The first stage ends just before the particles reach the end of the conical barrel. In the separation stage, the particle clusters are sped up as they approach the tissue to be transformed and break down into smaller micro-carrier particles. This stage can be further divided into two sections, inside and outside of the conical barrel.The third and final stage is the particle deceleration process. This stage takes place after the particles have entered the tissue to be transformed. Once the micro-carrier particles have entered the tissue, they move with a decreasing speed toward the target cells (Zhang *et al.*, 2007).

The calibration and selection of parameters for the biolistic process is an integral part of ensuring the success of an experiment. The parameters that

determine the efficiency of an experiment may be broken down into three categories: physical parameters, chemical parameters, and biological parameters .The precipitation of DNA onto the metal particles (gold, tungsten or platinum) involves the use of inorganic salts, calcium chloride, magnesium chloride and calcium nitrite, and organic reagents, polyethylene glycol, glycerin, ethanol and spermidine. Calcium chloride was found to be essential to the precipitation of DNA, with efficiency greatest at concentrations of 0.2 – 1.5 M. An increase in the concentration to 2.0 M or higher reduces transformation due to negative effects caused by high salt concentrations.

The most efficient concentration of spermidine was found to be 8-16 mM. However, it is still unclear as to whether it is required for DNA precipitation or not. Biolistic particle bombardment has become a useful tool in plant research, as it has allowed for the direction of specific genes to a predetermined site in the nucleus of a given plant (Rasco-Gaunt *et al*., 1999).

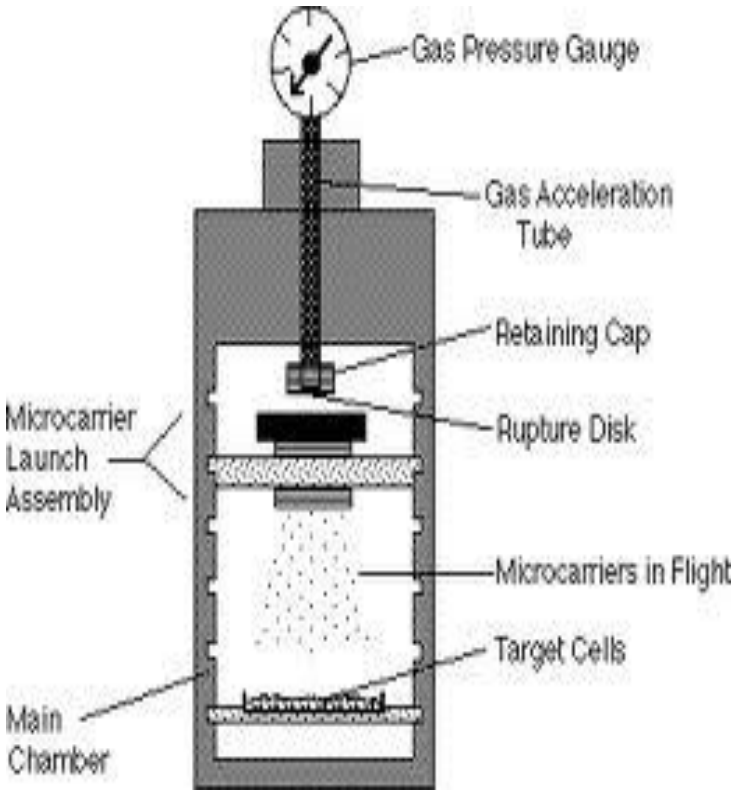


Figure (1.5) Gene gun apparatus (Zhang *et al*., 2007).

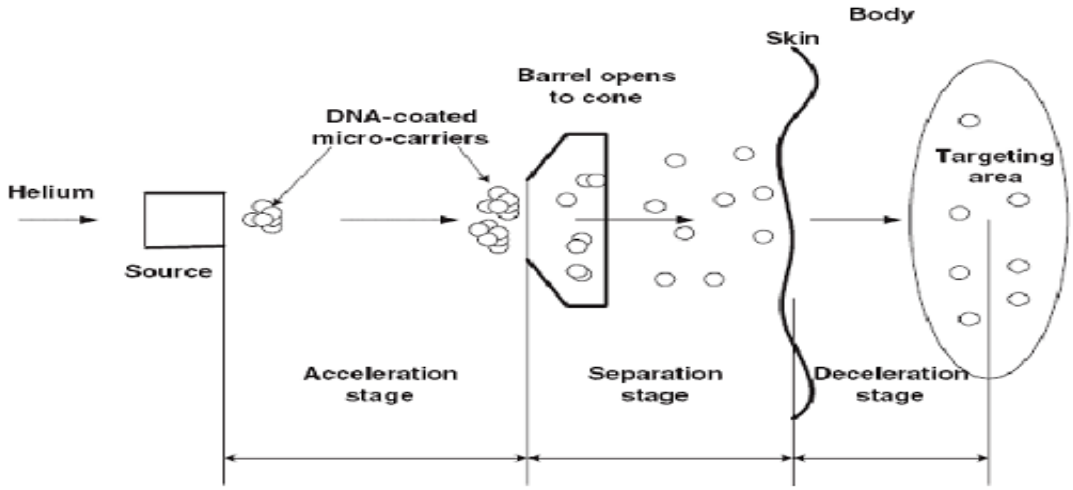


Figure (1.6) The three stages of the gene transfer process using a biolistic gene gun. The DNA-coated micro-carriers are represented by the circles (Zhang *et al*., 2007).

Another important use of the DNA gun involves the transformation of organelles. For the first time, researchers have transformed yeast mitochondria and the chloroplasts of *Chlamydomonas* (algae) using this technology. The ability to transform organelles is significant because it enables researchers to engineer organelle-encoded herbicide resistances in crop plants , to study photosynthetic processes,and to introduce gene encoding for pharmaceutical protein to economic plants (Chawla *et al*., 2006).

Chapter Two
Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments and apparatus were used throughout this study (table 2-1):

Table (2-1) Equipments and apparatus

Equipment	Manufacturer(USA)	Catalogue No.
Gold micro carriers (0.6 µm)	Bio-Rad	165-2262
1,100 psi Rupture disks	Bio-Rad	165-2329
Microcentrifuge tubes	Midwest Scientific	AVSS1700
Five Gallon pots (12 in diameter and 11 in height)	Local market	
Autoradiography cassette	Fisher	FBXC810
Costar 96-well enzymatic immunoassay (EIA) plates	Corning	3590
Film developer mini-medical series	AFP Imaging Corp	9992305300
Greenhouse		
Culture room fitted with fluorescent lights on a controlled timer (16 hrs light/8 hrs dark photoperiod)		
GS GeneLinker UV Chamber	Stratagene	400 071
Hand-operated homogenizer	Sigma	Z359971
Helium gas	Local market	
Hybridization incubator	Fisher	H6743
Laminar air flow	Nuaire	NU-201-630
Macrocarrier	Bio-Rad	165-2335
Macrocarrier holder	Bio-Rad	165-2322
Microtiter plate reader equipped with	BioTek Instruments	EL403

450-, 570- and 595-nm filters		
Nitrocellulose membrane	Bio-Rad	162-0146
Nylon membrane	Bio-Rad	162-0174
Helium Biolistic particle delivery system PDS-1000	Bio-Rad	165-2257
Polypropylene pestle adapters	Sigma	Z359947
Polypropylene tube	Falcon	2059
Porcelain mortars and pestles (50 ml capacity)	Coors	Z247464
Peltier thermal cycler PTC-100	Bio-Rad	PTC-1196
Qiagen-tips(200µl) and (1 ml)	Qiagen	12 123 12 143
Refrigerated microcentrifuge	Eppendorf Fisher	5415R 05-401-05
Shaker incubator	Jencons	397-018
Sterile Petri dishes(100 -25 mm^2)	Midwest Scientific	TPP 93100
Stopping screen	Bio-Rad	165-2336
Surgical blade #21	Henry Schein	100-3535
Syringe-operated ultra filtration unit 0.22 µm pore size	Sigma	Z359904
Thin wall PCR tubes(0.2-ml)	Midwest Scientific	AVTW2
Ultraviolet transilluminators (115V)	VWR	21 475-468
UV Silica Cuvette (10 mm Pathlength)	VWR	BK580012
Vacuum pump	Fisher	01-257-8c
Water bath	Polyscience	510-117
Whatman filter paper, 70 mm	Whatman	1001-070
X-ray film		

2.1.2 Reagents

The reagents listed below were used throughout this study (table 2-2), others were stated within the related methods.

Table(2-2) Reagents, kits, and enzymes used in this study

reagent	Manufacturer (USA)	Catalogue No.
Chloroplast targeting vector, containing flanking sequences specific to the genome	Daniell lab. at Burrnett school for Biomedical science, University of Central Florida, FL, USA	
Chloroplast expression cassette, containing the necessary genome-specific regulatory sequences, each fragment is verified by DNA sequencing	All published chloroplast vectors were kindly supplied from the Daniell Laboratory	
DNeasy Plant Mini Kit	Qiagen	69106
dNTPs	Invitrogen	10297018
Pfu DNA polymerase. high-fidelity DNA polymerase to minimize errors	Promega	M7748
Set of primers to isolate gene and evaluate the transgene	Carefully design using available software such as Primer select module of DNASTAR lasergene program.	
Sterile molecular biology-grade water	Eppendorf	955155033

PCR cloning vector	Promega	A1360
Restriction endonucleases, Keep at-20°C and avoid any cross-contamination	New England Biolabs	
T4 DNA polymerase	Invitrogen	18005017
T4 DNA ligase	Invitrogen	15224017
Calf intestinal alkaline phosphatase	CIAP; Promega	M1821
QIAquick Gel Extraction Kit	Qiagen	28704
QIAquick PCR Purification Kit	Qiagen	28104
QIAprep Spin Miniprep Kit	Qiagen	27104
Chlorox bleach	Local market	
Antibiotics	Invitrogen	11593-019
Plasmid Midiprep Kit	Bio-Rad	732-6120
Ethanol 190 proof for molecular biology	Sigma	E7148-1GA
Murashige and Skoog salts 4.33 g /1 liter	MS; Caisson	MSP 001
6-Benzylamino purine (BAP)	Sigma	B3408
1-Naphthaleneacetic acid (NAA)	Sigma	N0640
Sucrose	Sigma	S0389-5KG
Normal salt (NaCl)	Sigma	S0245-10KG
Betaine aldehyde	Sigma	B1874
Phytoblend	Caisson	PTC 001
1 M phenyl methyl sulfonyl fluoride (PMSF)	Sigma	P7626
Tween-20	Sigma	P1379

Nonfat powdered milk	Carnation	
Electrochemiluminescent (ECL) substrate for western blot	Pierce	32109
Bradford reagent	Bio-Rad	500-0006
Sample buffer (SDS reducing buffer)	Freshly prepared	Detailed in preparation of buffers
10X Electrode buffer (EB)		
Transfer buffer		
Binding buffer		
Wash buffer		
Elution buffer		
EDTA	Sigma	46081
Tris–HCl	Fisher	BP152-5
SDS	Fisher	BP166-500
B-mercaptoethanol	Sigma	M 3148
Sodium carbonate Na2CO3	Sigma	S7795
Sodium bicarbonate NaHCO3	Sigma	S6297
Glycerol	Shelton Scientific	IB15762
Bromophenol blue	Sigma	B8026
Glycine	Bio-Rad	161-0724
Methanol	Fischer	A411
Taq DNA polymerase and buffer and $MgCl_2$	Stratagene	600 131
QuickHyb solution	Stratagene	
Salmon sperm DNA, stored at –20°C	Invitrogen	15 632-011
Acryamide/Bis (ready-made	ICN Biomedical	IC814 320

mixture) stored at 4°C.		
Bovine serum albumin	Sigma	9048-46-8
Calcium chloride $CaCl_2$	EM Science	3000
Potassium chloride KCl	EM Science	4484
Pipes-NaOH	EM Science	6910
Magnesium chloride $MgCl_2$	EM Science	8504
Dimethyl sulfooxide DMSO	Aldrich	47 157-7
Luria media	Sigma	L7602
Ficoll	Voigt Global Distribution	341 691CB
Malic acid	Qiagen	133042
DIG-11 dUTP	Roche	1 093 088
DNA labeling and chemiluminescent detection (CDP-star)	Biolabs	N7001S
Sodium lauroyl sarcosinate (sarkosyl)	Chemieliva	137-16-6
Phenol	EM Science	6610
Chloroform	EM Science	3150
Agarose	EM Science	2090
Ammonium persulfate	Aldrich	21 558-9
Coomassie brilliant blue G-250	EM Science	3300
Dithiotherotol (DDT)	Roche	100 032
Ethidium bromide	Aldrich	16 053-9
TEMED	Roche	100 139
SOC medium	Sigma	S1797

2.1.3 Antibiotics, Buffers, Enzymes, Strains, and Vectors

Many buffers and antibiotics were used in molecular biology experiments. In this study, most of them were prepared and used in southern and western blot analyses, including:

2.1.3.1 Antibiotics :-Ampicillin stock solution (50 mg/ml) was prepared and sterilized by using 0.22 µm Millipore filter, then stored at -20°C up to 2 months.

2.1.3.2 Inoue Transformation Buffer (Inoue *et al*., 1990)

a- Piperazine-1,2-bis-2-ethanesulfonic acid (PIPES) at 0.5 M was prepared by dissolving 15.1g in 80 ml of D.W, the pH of the solution was adjusted to 6.7 with 5 M KOH, and then the final volume (100 ml) was completed with distilled water. The solution was then sterilized by filtration, divided into aliquots and kept frozen at -20°C .

b- Inoue transformation buffer was prepared by dissolving all the solutes listed below (table 2-3) in 800 ml of D.W and 20 ml of 0.5M PIPES was added, then the volume was completed by D.W to one liter before sterilization by ultra filtration and divided into aliquots then stored at -20°C. It was chilled to 0°C before used to prepare competent *E. coli*.

Table (2.3) Reagents used for preparation of transformation buffer

Reagent	Amount per liter	Final concentration (mM)
$MnCl_2.4H_2O$	10.88g	55
$CaCl_2.2H_2O$	2.20g	15
KCl	18.65g	250
PIPES(0.5M)	20ml	10
D.W	to 1 liter	

2.1.3.3 Southern Blot Buffers (Verma and Daniell, 2007)

The following buffers were prepared for southern blot analysis:

a-Washing buffer 1 with a final volume of 100 ml
10ml of 20×SSC (2×SSC)

1 ml of 10% SDS (0.1% SDS)

Brought to volume with D.W

Note: Washed solutions were heated to the desired temperature (65°C) before use.

b- Washing buffer 2 with a final volume of 100 ml

500 µl of 20X SSC (0.1XSSC)

1ml of 10 % SDS (0.1% SDS)

Brought to volume with D.W

c-Depurination solution (0.25 N HCl)

10.4 ml 12 N HCl, the volume was completed to 500 ml with D.W.

d-Transfer Buffer (0.4 N NaOH, 1M NaCl)

16 g NaOH

58.4 g NaCl

Volume was completed to 1 L with D.W.

e-20X SSC (3M NaCl, 0.3M Sodium Citrate)

175.3 g NaCl

88.2 g Na_3 Citrate·$2H_2O$

pH was adjusted to 7.0 using 1N HCl.

Volume was completed to to 1 L with D.W.

It was autoclaved

f-2×SSC

20ml of 20X SSC

180 ml of D.W

2.1.3.4 Western Blot/SDS-PAGE Buffers and Solutions (Verma *et al*., 2008)

Western blot/SDS-PAGE analyses were conducted using different buffers.

a-Acrylamide/bis:- bought from supplier and stored at 4°C.

b-10% SDS

A quantity of 10g of SDS was dissolved in 90ml water and then brought up to 100 ml, stored at room temperature.

c-Resolving gel buffer (1.5M Tris-HCl pH8.8).

A quantity of 27.23 g Tris base was dissolved in 80 ml D.W, the pH was adjusted to 8.8 with 6 N HCl and then the volume was brought to 150 ml, autoclaved and store at 4°C**.**

d- Stacking gel buffer (0.5M Tris-HCl pH 6.8)

A quantity of 6 g of Tris base was dissolved in 60 ml D.W

pH was adjusted to 6.8 with 6N HCl ,the volume was completed to 100ml with D.W and autoclaved then stored at 4°C.

e-Sample buffer (SDS reducing buffer)

Aliquot of 3.55 ml D.W

1.25 ml 0.5 M Tris-HCl pH 6.8

2.5 ml glycerol

2 ml 10% SDS

0.2 ml 0.5% bromophenol blue

All these materials were mixed together, stored at room temperature

*50µl B-mercapto ethanol(BME) was added to 950µl of sample buffer prior to use.

f- 10X Electrode (running) buffer pH8.3

A quantity of 30.3g Tris base

144g glycine

10g SDS

All these materials were dissolved in 700ml water, pH was adjusted to 8.3 then the volume brought to 1 liter and stored at 4°C.

*Adjusting pH by addition of acid and base was avoided one of them used only.

g- 20% APS

A quantity of ammonium persulfate (200 mg) was dissolved in 1 ml of D.W and stored at 4°C

h-10X PBS

80g NaCl

2g KCl

26.8 g $Na_2HPO_4*7H_2O$ (or 14.4 g Na_2HPO_4)

2.4g KH_2PO_4

All these materials were dissolved in 800 ml of D.W and the pH was adjusted with HCl, then the volume was completed to one liter, autoclaved and stored at room temperature.

i-Transfer buffer

300 ml 10X EB

300 ml methanol

900 ml water

0.15 g SDS

The above materials were mixed and stored at room temperature.

j- phenylmethyl sulfonyl fluoride (PMSF)

A quantity of 17.4mg of powdered PMSF was dissolved in 1 ml of methanol then mixed well and stored at -20°C up to one month.

k-Di thiotherotol(DDT)

Solution of sodium acetate (0.01 M) was prepared firstly and then 3.09 g of DDT was dissolved in 20ml of solution ,the mixture was sterilized by filtration, divided to aliquots (1 ml) and stored at -20°C.

l-Plant extraction buffer

All reagents and materials used for extraction were ready before starting the work, extraction buffer was prepared as detailed in table 2-4.

m- Destaining solution

Acetic acid 10%, 50 ml

Methanol 10%, 50 ml

Two solutions were mixed well and stored at room temperature up to three months.

Table (2.4) Reagents for preparation of extraction buffer

Stock solution concentration	Used concentration	Final volume (11 ml)
5 M NaCl	100 mM	180 µl
0.5M EDTA	10 mM	180 µl
1M Tris-HCl(pH 8)	100 mM	1.8 ml
Tween -20	0.05%	6 µl
10% SDS	0.1%	90 µl
BME	14 mM	9 µl
1M sucrose	400 mM	3.6 ml
D.W.		3 ml
100 mM PMSF	2 mM	180 µl
PIC	1 tablet in 1 ml H_2O	900 µl
1M DDT	100 mM	900 µl

2.1.3.5 Enzymes

Restriction enzymes were used throughout this study, they listed in table 2.5

Table 2-5 Restriction enzymes and the recognition site for each one

Enzyme name	Recognition site	Supplier
*Sma*I	CCC/GGG	New England Biolabs and Roche Molecular Biochemicals
*Sna*BI	TAC/GTA	
*Sac*I	GAGCT/C	
*Sal*I	G/TCGAC	
*Not*I	GC/GGCCGC	
*Hin*dIII	A/AGCTT	
*Pvu*II	CAG/CTG	

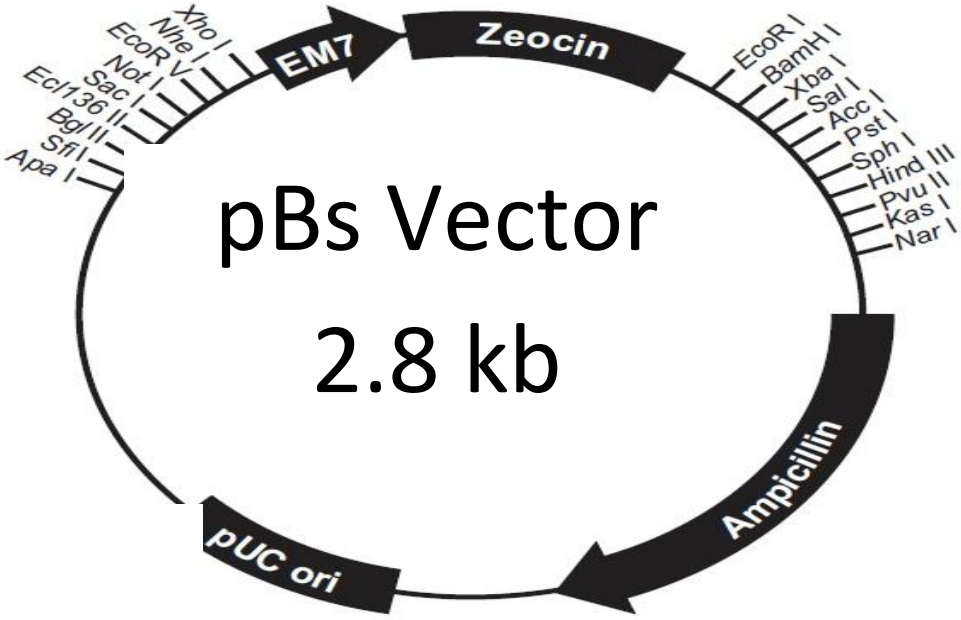
2.1.3.6 Strains

Vibrio cholera ATCC 39315 with accession number of AE003852 was kindly supply by infectious disease laboratory at Burnett School of Biomedical Science in Florida, USA. *E. coli* ATCC 35691with accession number of C2925H/I was propagated from Professor Daniell strain store.

2.1.3.7 Vectors

All vectors used in this study were kindly supplied from Professor Daniell laboratory store and these vectors were:-

a-pBluescript (pBs) vector which used as shuttle vector for *prrn* and *BADH* gene and propagated in *E. coli* , the genetic map of this vector and main features are detailed in the figure 2-1



Comments for pEM7/Zeo
2829 nucleotides

bla promoter (-10 region): bases 160-166
Ribosome binding site: bases 189-193
Beta-lactamase ORF: bases 201-1061
pUC origin: bases 1206-1879
5´ Polylinker: bases 1887-1937
EM7 promoter: bases 1942-2008
Sh ble ORF: bases 2009-2383
3´ Polylinker: bases 2384-2593

Figure 2-1:- Genetic map of pBs shuttle vector with restriction site (Ruhlman., *et al* 2007)

b-Cassette vector pLs-LF which was used to integrate and propagate the whole operon contain CTB, BADH, prrn, and regulatory gene. The genetic map of this vector is detailed in the following figure 2-2

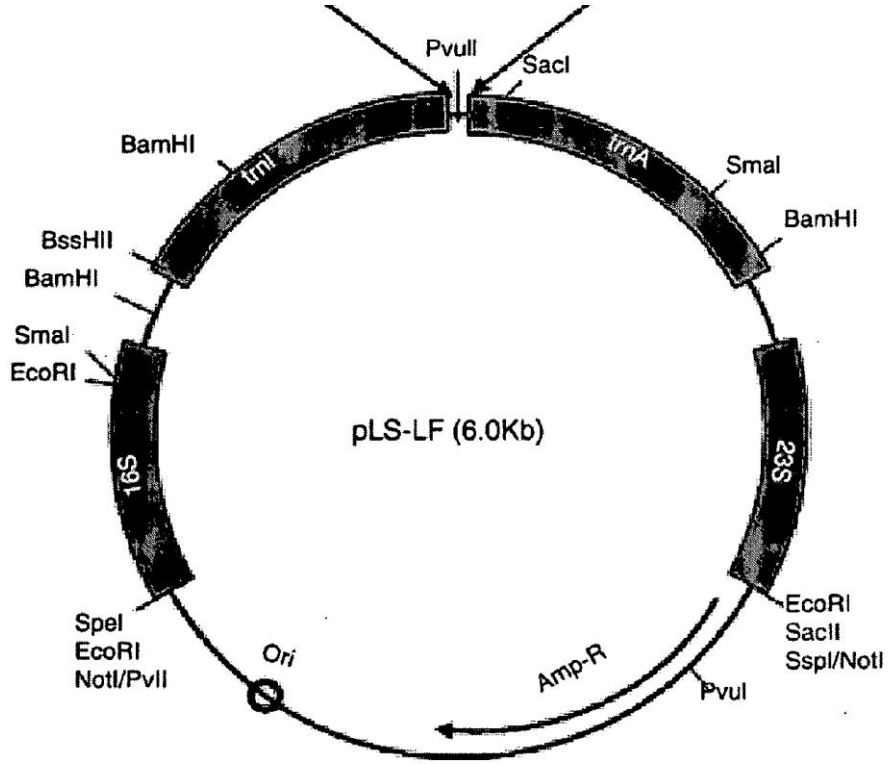


Figure2-2:- Genetic map of pLS-LF cassette vector used to transform lettuce leaf (Ruhlman., *et al* 2007)

2.1.4 Media

2.1.4.1 Seed Germination Medium (Ruhlman *et al.*, 2010)

Lettuce seeds were germinated in the lettuce germination medium (LGM), the following components were used for preparation of one liter.

MS salt (1/2X)(macro and micro)	2.16 g
Thiamine-HCL	1 mg
Myo-inositol	100 mg
Sucrose	30 g
pH	5.8
Phytoblend	6.2 g

All contents were mixed together and sterilized then poured to 50 ml plates.

2.1.4.2 Lettuce regeneration medium (LRM) (Ruhlman *et al.*, 2010)

The regeneration medium for lettuce was prepared as follows:

MS salt (macro and micro)	full strength 4.32g
Thiamine	10 mg/l
Pyridoxine-HCl	1 mg/l
Nicotinic acid	1 mg/l
Myo-inositol	100 mg/l
NAA	0.1 mg/l
BAP	0.2 mg/l
PVP	500 mg/l
Sucrose	30 g/l
Phytoblend	6.2 g/l

All materials above were added to 800 ml of D.W and the pH was adjusted to 5.8, then the volume was completed to one liter using distilled water, the phytoblend was added after pH adjustment, then medium was sterilized by autoclaving and poured into 50 ml plate.

2.1.4.3 Selection medium

Selection of the transgenic plant was performed in the selection medium which was prepared using the LRM with NaCl in concentrations of (25, 50, 75, 100, 125 and 150) mM and betaine aldehyde in concentrations of (1, 5, 10, 25 and 50) mM .

2.1.4.4 Luria broth and Luria agar

Luria medium was prepared by adding the following ingredients to one liter of D.W.

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g

NaCl	5 g

It was used to check the transfromant *Escherichia coli* and to propagate the clones.

2.1.4.5 SOC medium (Inoue *et al*., 1990)

The medium was used to increase the transformation of competent *E.coli* and this medium was prepared as follows:-

Tryptone (pancreatic digest of casein)	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	8.6 mM
KCl	2.5 mM
$MgSO_4$	20 mM
Glucose	20 mM

The prepared medium was sterilized by filtration through 0.2 μm Millipore filter and stored at room temperature until use.

2.1.5 Primers

Various primers were used throughout this study (table 2-5) to amplify the genes of interest and to investigate the transgenic plant. All primers were designed depending on the sequence available on NCBI web site and the primers were carefully designed using available software (primer select module of DNASTAR lasergene program.

All these primers have melting temperature (T_m) ranging between (60-65°C), The annealing temperature (T_a) for each primer pair was generally calculated as 5°C lower than the estimated melting temperature.

Table (2-6) List of primers used in the study

No.	Gene of interset	Forward and reverse primers sequence 5'→3'	Primer properties
1	16SF	CAGCAGCCGCGGTAATACAGAGGA	24 mer
2	CTB	F TATGGATCCATGACACCTCAAAATATTACT R GGCGAATTCATATCTTAATTTGCCATAC	30 mer 28 mer
3	BADH	F CACTCTGCTGGGCCGACACTGACAC R CACTAGCCGACCTTGACCCCTGTT	25 mer 24 mer
4	trnI-trnA	F ACGGGCGAGGTCTCTGGTTCA R GCACGTTTCGGTCCTCTTCC	21 mer 20 mer
5	rbcL	F CCGCCTCATGGGATCCAA R CTTATCCAAAACGTCCAC	18 mer 18 mer
6	psbA	F GATATCGTCGACGTAGAGAAGTCCG R CATATGAAAATCTTGGTTTATTTAA	25 mer 24 mer
7	prrn	F ATCGATGAGCCTGATTATCCTAAG R CAGCAGGTAGACAAAGCGGATTC	24 mer 23 mer

2.2 Methods

2.2.1 Lettuce seeds Surface Sterilization and Germination (Ruhlman *et al.*, 2010)**.**

a-A number ranging between 50-100 seeds was taken in Ependorf tube and treated with 70% alcohol with Tween20 for 45-60 sec(1-2 drop).

b- Seeds were washed once with sterile distilled water.

c- Seeds were treated with 1/3 (v/v) of commercial bleach (chlorax) dissolved in sterile water with one drop of Tween20 for 5-7 min.

d-Seeds were washed five times with sterile water to ensure removing of bleach.

e-Seeds were carefully distributed on filter paper to dry.

f-Ten seeds were cultured on seed germination medium which was poured in tissue culture pertidishs before sealing with parafilm, seeds were grown under a 16:8 hrs (light:dark) photoperiod at 25°C .

2.2.2 Tissue Culture and Regeneration of Lettuce Leaves

For tissue culture of lettuce leaves, LRM was prepared as described above and poured in plates, after seed germination for 20 days. Young and fully expanded leaves (~4 cm^2) which grown aseptically were taken and spliced to small pieces (0.5x0.5) cm and cultured adaxial side down in tissue culture plates and incubated for 16:8 hrs photoperiod (light:dark) at 25°C.

2.2.3 Determination of Lettuce Tolerance to NaCl and Betaine Aldehyde

Different concentrations of NaCl were prepared, (25, 50, 75, 100, 125, and 150 mM) mixed with LRM with plant hormone in concentration efficient for direct regeneration and the explants of lettuce were cultured to examine the tolerance to salt.

Also the same procedure was applied to betaine aldehyde but the concentration used were (1, 5, 10, 25, and 50 mM) .

All plates with different concentration of NaCl and BA were cultured with explants of healthy lettuce leaves 0.5 cm^2 and incubated at 25°C.

2.2.4 Construction of Vector (Verma and Daniell 2007;Verma *et al.*, 2008; Ruhlman *et al.*, 2010).

The pUC-based *L. sativa* long flanking plasmid (pLS-LF) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome inverted repeat region (Ruhlman *et al*., 2007). A transformation cassette for the generation of transplastomic *L. sativa* plants that express CTB-BADH from the T7 gene translational control region

was transferred to pLS-LF from pZERO (Invitrogen, Carlsbad, CA). The cassette included the following published *N. tabacum* plastid regulatory sequence elements: ribosomal operon promoter (P*rrn*), *psbA* 3' UTRs. The *BADH* gene was included conferring salt tolerance and was expressed via a GGAGG ribosome binding site. All digest products (vectors and inserts) were separated by electrophoresis through 0.8% agarose-TAE (400 mM Tris-acetate, 10 mM EDTA) gels containing 1ug mL^{-1} ethidium bromide. Gel fragments were isolated using a sterile blade under UV illumination and DNA was eluted from gel fragments using the QIAquick gel extraction kit .

Plasmid products of T4 ligase mediated reactions were transformed into *E. coli* according to standard protocols (Sambrook *et al.*, 1989). The expression cassette was digested with *Sna*BI. pLS-LF was digested with *Pvu*II and treated with alkaline phosphatase prior to ligation with the *Sna*B1 digested cassette. Recovered plasmids were digested with restriction enzymes to determine correct orientation of the inserted cassette in pLS-LF. Nucleotide sequence of the intermediate plasmid was confirmed. The complete detailed of construction of vector and isolation of genes will be mentioned in the result chapter.

2.2.5 Competent *E. coli* (Inoue *et al*., 1990)

2.2.5. Competent Cells Preparation

The frozen competent *E. coli* cells were prepared according to the standard protocol (Sambrook *et al.,* 1989) the following steps were followed.

a-Aliqout of five ml falcon of LB with 20mM $MgSO_4$ was inoculated with *E.coli* and incubated overnight at 37°C.

b-Next morning, 250ml of LB with 20mM $MgSO_4$ in 1 liter flask was inoculated with 2ml of overnight culture, incubated at 23°C with good aeration (250 rpm) until the OD was reached 0.4-0.6 at A600.

c-Culture was then placed on ice for 10 min and transferred to bottles and spined at 3900 rpm at 4°C for 10 min.

d-The medium was poured off and the open centrifuge bottles were stored on a stack of paper towels for 2 min, the vacuum aspirator was used to remove any drops of remaining medium.

e-Cells were resuspended in 80 ml of ice-cold Inoue transformation buffer, the cells were resuspended by swirling (avoid pipeting or vortexing).

f-Cells were harvested by centrifugation 3900 rpm at 4°C for 10 min.

g- Medium was removed and the open centrifuge bottles were stored on a stack of paper towels for 2 min and the vacuum aspirator used to remove any drops of remaining medium.

2.2.5.2 Freezing of Competent Cells

a-The cells were resuspended gently in 20ml of cold TB.

b-A portion of 1.5 ml of DMSO was added and mixed with the bacterial suspension by swirling, then stored in ice for 10 min.

c-The mixture was divided quickly into chilled, sterile microfuge tubes, and then immediately the cells were snap-freezed by immersing in a bath of liquid nitrogen, the tubes stored at -70°C until use.

d- When needed, a tube was removed from -70°C freezer and thawed by holding in the palm of the hand. Just as the cells were thawed, they were transferred to ice and stored for 10 min.

e-Chilled sterile pipette was used to transfer the competent cells to chilled, sterile polypropylene tubes, stored on ice.

2.2.5.3 Transformation of Competent Cells

a-Transformed DNA was added (up to 25 ng per 50 µl of competent cells) and the volume was less than 5% of that of competent cells. Tubes were swirled gently several times to mix the contents, and stored on ice for 30 min.

b- Tubes were transferred to a rack placed in preheated (42°C) circulating water bath, stored in the rack for exactly 90 seconds. Tubes must be not shaken .

c- Rapidly the tubes were transferred to ice bath and allowed to cooled for 1-2min.

d-SOC medium 800µl was added to each tube, then the culture was warmed to 37°C in a water bath, and then incubated in shaker incubator at 37°C, for 45 min.

e-Apropriate volume (up to 200µl per 90mm plate) of transformed cells was transferred to plates containing $MgSO_4$ (20mM).

f-Plates were stored at room temperature until the liquid has been absorbed.

g- All plates were incubated invertly at 37°C, to detect the transformed colonies which appear after 12-16 hrs.

2.2.6 Extraction of DNA from Bacteria

2.2.6.1 Plasmid Extraction from *E. coli*

The method for plasmid extraction used in this study depended on QIAGEN protocol (QIAprep for purification of molecular biology grade DNA). Buffers and column supplied by manufacturing company and the protocol was as follow:-

a-*E. coli* were cultured in LB for 18 hrs at 37°C in 50ml tube.

b-The grown cells were collected by centrifuge 8000 rpm for 10 min.

c-The supernatant was discarded and the cells were resuspended in the cell resuspension solution.

d-Aliquot of 3 ml cell lysis buffer was added and mixed gently then left for 3 min

e-Neutrilization solution 5ml was added, mixed for 20 times then centrifuged for 10 min at 10000 rpm

f- The supernatant which contain plasmids was taken and put in clearing column (blue) , the plasmids pass through this column.

g-Flow liquid was put in binding column (white), plasmids here were bind to membrane

h-After washing with PE buffer it was centrifuged for 2 min at 5000 rpm

i-Washed with endotoxin buffer (5ml) and allowed to vacuum to suck the solution.

j-Another wash was carried out with PE buffer and the solution was pulled by vacuum allowing it to dry the membrane for 30 sec. until the ethanol odor was removed.

k-The column was removed from vacuum and tap it on paper to more drying and then put in a new tube.

l-Aliqout of 600 µl of nuclease free water was added and left for 5 min before centrifuging for 5 min at 2000 rpm without closing the tubes.

2.2.6.2 Genomic DNA extraction from *Vibrio cholerae* (Dalsgaard *et al.*, 1996)

Vibrio cholerae was obtained from the infectious disease lab. at Burnett College of Biomedical Science, University of Central Florida, USA.

Bacterial strains were grown aerobically in tryptocasein soy broth at 37° C for 72 hrs . Two ml of culture were centrifuged at 4000 rpm for 20 min. The pellet were resuspended in 620 ul of lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, pH 8) containing 1% SDS and 0.4 mg/ml of proteinase K.

The mixture was incubated for 1 hr at 56°C and then at 100° C for 1 hr. An equal volume of phenol/chloroform/isoamylalcohol was then added to the mixture and centrifuged at 10000 rpm for 10 min. The supernatant was added to an equal volume of chloroform and centrifuged at 10000 rpm, the top layer was collected and DNA was precipitated with two volumes of cold

isopropanol at -20°C for 10 min. The pellets were obtained by centrifugation for 20 min and washed with 1.5 ml of 70% cold ethanol.

Finally the pellets were resuspended in 100 ml of TE 1X buffer (10 mM Tris-Cl, 1 mM Na2 EDTA, pH 8).

2.2.7 Conformation of orientation and sequence

Restriction enzymes were used to confirm the orientation of ligated genes(CTB, BADH, prrn) in plasmid. Each restriction enzyme has a specific buffer and a specific time to achieve its work properly. The enzymes used were *Sma*1, *Sna*B1, *Sac*1, *Sal*1 ,*Not*1 *and Hin*dш, the orientation was confirmed depending on the size of the bands on gel.

The right orientation plasmid was sent for sequencing to GENEWIZ company (http://www.genewiz.com/), The plasmids were spotted with two different primers, each overlapping the other so all sequence were covered.

Digestion protocol in all experiments was done in 100 µl final volume which consisted of:

78 µl molecular grade water

2 µl restriction enzyme

10 µl buffer

10 µl DNA were diluted to appropriate concentration

The time for digestion differed depending on the type of restriction enzyme and the aim of the experiment.After each digestion the reaction was stopped as follows:

a- Aliquot of 500 µl of PBI buffer was added and mixed by pipette.

b-The mixture was added to binding column and spinned at 14000 rpm.

c-Washing was carried out with PE buffer (750 µl) and spinned again at 14000 rpm.

d-Ethanol was evaporated then eluted with 70 µl of distilled water.

2.2.8 Protocol to generate genes blunt end and preventing self ligation (Deen *et al.*, 1983)

The T4 DNA polymerase was used to generate blunt end after digestion with enzyme and after PCR amplification of some products. The Invitrogen kit was used for this purpose as follows:

a-The following components were added to a sterile microfuge tube on ice

Component	Amount
5X T4 DNA polymerase buffer	20 µl
0.5mM dNTP mix	20µl
DNA	0.5-2.5 µg
T4 DNA polymerase(5U/µl)	2 µl
Molecular grade water	to 100 µl

b-After the components were gently mixed and incubated at 11°C for 15 min the reaction components were placed on ice.

c-To terminate the reaction, phenol extraction, chloroform extraction and the ethanol precipitation were used.

To prevent self ligation of PCR product and digested DNA, treatment with alkaline phosphatase was carried out using 1 ul(1U) for 6 min.

2.2.9 PCR Protocol

Polymerase chain reaction (PCR) was used during the period of study to isolate and ligate genes of interest as well as for confirmation of transgenic plantlets. Two enzymes were used, *Taq* polymerase and Platinum Pfx polymerase. The protocol for PCR was performed as in the standard protocol

a-For single reaction, the following components were added to sterile PCR tube, on ice.

Components	Volume	Final concentration
5X enzyme buffer	5µl	1X
10mM dNTP mixture	1.5µl	0.3 mM each
50mM $MgCl_2$	1µl	1 mM
Primer mix(10µM)each	1.5µl	0.3 µM each
Template DNA	≥1µl	As required
Enzyme Pfx or Taq	0.4µl	1 unit
Molecular grade water	to 50µl	

b- Tube contents were mixed, then centrifuged briefly at 3000 rpm to collect the contents

c- The PCR amplification was performed (25-35) cycles as follows:

Denaturation	94°C	30 sec
Annealing	55-58°C	30 sec
Extension	68-72°C	1min for 1 kb

Final extension time was 7-10 min at 68-72°C

d- The reaction was stored at 4°C after cycling for short time use, while it is stored at -20 for a long time storage.

e- PCR product was analyzed using agarose gel electrophoresis.

2.2.10 Agarose Gel Electrophoresis

PCR products, the ligation and restriction products were analyzes by agarose gel stained with ethidium bromide. To prepare 0.8% agarose gel, 0.8g of agarose was dissolved in 100 ml of distilled water, boiled using a microwave where agarose was completly dissolved, then cooled down to 58-60°C at room temperature before adding 10 µl from stock solution 0.1µg/ml of ethidium bromide. The mixture was poured into jar containing comb. After solidification the DNA samples were loaded into wells, (Ficoll 6X was added if the mixture didn't contained). The DNA bands visualized under UV transilluminator at 356nm and results were documented using gel

documentation system and printed directly using thermal printer. The desired DNA fragment was eluted from gel for further analyses

2.2.11 Elution of Desired DNA from The Gel

a-DNA fragment was excised from agarose gel by a clean, sharp scalpel, with trying to minimize the gel slice by removing the extra agarose.

b-The gel slice was weighed in a colorless tube, then three volumes of buffer QG were added to each volume of gel (100mg~100µl).i.e 300 µl of QG buffer were added to 100 mg of gel.

c-Tube was incubated at 50°C for 10 min (or until the slice completely dissolved), to assist dissolving gel vortex was done each (2-3) min during incubation.

d-After complete dissolving, the color was checked, if the color was yellow and had the same color of QG buffer means the reaction is good, this was done because the adsorption of DNA to QIA quick membrane is efficient only with pH7.5. Buffer QG contain a pH indicator which is yellow at this pH.

e-One gel volume of isopropanol was added to the sample and mixed, to increase the DNA yield. The centrifugation was avoided at this stage.

g-QIA quick column was placed in 2 ml collection tube.

h-The sample was applied to QIA quick column and centrifuged for 1min .

i-Flow was discarded, and QIA quick column placed back into same collection tube to minimize the plastic waste.

j- A volume of 0.5ml of QG buffer was added to the QIA quick column to remove any trace of agarose then centrifuged for 1 min.

k-Washing was carried out by adding 0.75 ml of buffer PE to QIA quick column and the column was let for 2-5 min before centrifuged for 1 min.

l-The flow was discarded and the QIA quick column was centrifuged for additional 1min at 13000 rpm.

m-The tube was placed at room temperature until the ethanol odor was completely removed.

n- Column was placed in new tube. For elution, 50 µl of buffer EB was added (10 mM tris-Cl, pH8.5) or elution was done using molecular grade water. The addition of elution solution was in center of QIA quick membrane. left stand for 5 min for diffusion and then, centrifuged for one min.

The eluted DNA was used as template for PCR amplification or for ligation to construct vectors after digestion of product with restriction enzyme.

2.5.11.1 Concentration of DNA by Ethanol

When the concentration of DNA resulting from the elution is not enough to progress toward another experiments, the DNA was concentrated by ethanol as follows:

a. Two volumes of absolute ethanol were added to DNA in the tube.

b. The tube was kept at -80°C for 30 min.

3. Centrifugation was done for 1 min at 8000 rpm and the supernatant was discarded.

4.The pellets were washed once with 70% ethanol.

5.A volume of 150 µl of molecular grade water was added.

2.2.12 Ligation of Genes to Vector

BADH gene was ligated with *prrn* promoter by PCR extension, while the *CTB* gene was ligated to shuttle vector and the shuttle vector was ligated to cassette vector using ligase enzyme. T4 DNA Ligase catalyzes the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. The concentration of vector to insert depends on the original concentration of each and the size of insert, the ratio used were 1:3,1:1or 3:1 vector to insert and the end concentration of insert was determined as follows:

Ng. of vector x kb size of insert x molar ratio of insert/vector=ng. of insert
kb size of vector

(Verma and Daniell 2008)

In current vector construction, the ratio used was 3 insert to one vector.

a. The following reaction were assembled in a sterile micro centrifuge tube:

vector DNA

insert DNA

Ligase 5X Buffer 4µl

T4 DNA Ligase (1U/ul) 1µl

Nuclease-Free Water to final volume of 20µl

b. Incubation at:

room temperature for 3 hrs, or

4°C overnight, or

15°C for 4–18 hrs.

After incubation period, the products were transformed with *E.coli* for propagation of plasmids and then the clones were investigated by PCR to confirm the result. The investigation by PCR was done directly to each clone grown after incubation period on selective medium, each colony was replicated on another plate and put in 200 µl PCR tube using sterile sticks, classical PCR reaction was carried out by a set of primers previously used to isolate of inserts.

When the right plasmid was confirmed, the orientation and sequence were tested as mentioned previously and then the transformed *E. coli* were propagated in LB and harvested for plasmid isolation. The isolated plasmid were used to transform lettuce leaves using gene gun.

2.2.13 Transformation of lettuce leaves (Ruhlman *et al*., 2007)

2.2.13.1 Preparation of gold particles

a. A quantity of 50 mg of gold particles (0.6 mm) was suspended in 1 ml of absolute alcohol in a 1.5 ml siliconized tube and vortexing for 2 min.

b. Spine at 13000 rpm for 3 min and the supernatant was discarded.

c. Resuspended the gold pellet in 1 ml of 70% ethanol by vortexing for 1 min.

d. Incubating the tube at room temperature for 15 min. Mixed intermittently during the incubation.

e. Spin for 2 min at 8000rpm, and then the supernatant was discarded.

f. The pellet was resuspended in 1 ml of sterile distilled water by vortexing for 1 min.

g. The particles were allowed to settle down for 1 min at room temperature and then centrifuged for 2 min.

h. Washing steps f and g were repeated for 3 additional times.

i. The gold particle pellet was resuspened in 1 ml of 50% (v/v) glycerol and stored at –20 ° C until use .

2.2.13.2 Loading of Plasmid on Gold Particles

After the fresh lettuce leaves were prepared for bombardment, the plasmids were loaded on gold particles as follows:

a. A portion of 50µl of completely resuspended gold particle were put into a 1.5 ml siliconized tube.

b. While vortexing, 5 ul of plasmid were added (1 µg/ µl) followed by 50 ul of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine-free base. The vortexing was performed for 20 min at 4°C.

c. The DNA-coated gold particles were collected by centrifugation for 1 min at 5000 rpm

d. The pellets were washed with 200 µl of 70% ethanol followed by absolute ethanol at room temperature.

e. Finally, pellets were resuspended in 50 µl of 100% ethanol. DNA-coated particles were kept on ice until use.

f. The sterilized macrocarrier was placed inside the macrocarrier holder by using insertion cap.

g. Vortexing the DNA-coated gold particles were performed to eliminate any clumps. 10 µl of the DNA-coated gold particles were spreaded onto center of each macrocarrier and let dry in a laminar airflow hood

2.2.13.3 Bombardment

a. Five fully grown aseptically propagated lettuce leaves were harvested.

b. The adaxial side of the leaves was placed up on a circular sterile Whatman filter paper (70 mm) and soaked in agar-solidified LRM thin plate without selectable agent.

c. The power of the PDS and vacuum pump were switched on.

d. The knob on the top of the helium gas cylinder was opened anticlockwise.

e. The screw bar was adjusted by tightening slowly clockwise to bring the helium gas approximately to 200–250 psi above the desired pressure of the rupture disc(900 psi).

f. Rupture disk was placed in its holder by using a sterile forceps and screw tightly to the gas acceleration tube .

g. Stopping screen was placed in the macrocarrier holder and placed the DNA-coated macrocarrier was facing downward toward the stopping screen. The assembly was screwed with macrocarrier cover lid and placed into its position in the gene gun chamber.

h. Uncovered Petri dish containing sample was placed to be bombarded on the target plate holder and inserting it into the second slot (6 cm) in the gene gun chamber from the top. The chamber door was closed .

i. Vacuum pressure was created inside the chamber by pressing the vacuum button toward up position (VAC) at least up to 28 in. Hg in the vacuum gauge display. Flipping the switch swiftly to the third position (HOLD) to hold the vacuum and keep pressing the FIRE button up until the rupture disk bursts.

j. FIRE button was free and flip the vacuum button to VENT postposition

to release the vacuum. The bombarded sample was removed.

k. Steps f–j were repeated for additional samples.

l. The system was shut down by rotating the knob of the gas cylinder clockwise. Create, vacuum as in step i to release the gas pressure remaining inside the helium tube. Repeatedly keep pressing and releasing the FIRE button until both the pressure falls to zero on the meter guage of the gas cylinder and loosen the screw bar turning counterclockwise. Release the vacuum and turn off the vacuum pump and PDS.

m. Each Petri dish containing the bombarded leaf was wrapped with parafilm and kept in the dark for 2 days inside the culture room.

2.2.14 Plant Regeneration and Selection of Transgenic Plant

After 2 days of incubation in the dark culture room, the bombarded leaves were transferred to hood and cultured on selective medium.

a. Each of the bombarded leaf was cut into small (5 mm^2) pieces and place the adaxial side (bombarded side) touching LRM selection medium containing 25mM betaine aldehyde . Each Petri dish was wrapped with parafilm and kept in the culture room under appropriate growth conditions. Within 3–5 weeks of culture, putative transformants will appear.

b. Again the leaves of the primary regenerated PCR-positive putative transplastomic shoot were cut into small pieces (2 mm^2) and subject to a second round of selection on fresh LRM selection medium to achieve homoplasmy.

c. The regenerated shoots after second round of selection were separated and transferred to LRM selection medium containing 25mM betaine aldehyde for third round of selection .

2.2.15 Extraction of DNA from Regenerated Leaves (Verma *et al.*, 2008)

The Qiagen DNeasy Kit was used to isolate plant genomic DNA as described in the Qiagen manual. More than 100mg of tissue sample was

obtained from the plant using a septic techniques, placed into a micro centrifuge tube, and grinded by using a micro pestle in liquid nitrogen, then the fine powder weighed to be 100 mg and transfer to fresh tube without thawed, followed directly by addition of 400 µl of buffer AP1 and 4 µl of RNase A (stock solution 100mg/ml).The mixture was incubated for 10 min at 65°C and mixed 2-3 times during incubation by inverting the tube.

Buffer AP2 (130 µl) was added to the lysate, vortexed and incubated for 5 min on ice. Following, a centrifugation was done at 13000 rpm for 5 min and the supernatant was transferred to a Qiashredder spin column (lilac) sitting in a 2ml collection tube. The centrifugation was performed at full speed for 2 min.

The flow through was transferred to a new tube and 1.5 volumes of buffer AP3/E were added to the lysate and mixed immediately. 650 µl of the mixture was applied to a DNeasy mini spin column (clear) and centrifuged for 1 min at 8000 rpm. The flow through was discarded and the collection tube reused to repeat the previous step with the rest of the sample. The tube with the flow through was discarded and the column was placed in 2 ml tube.

Buffer AW (500 µl) was added to the column and centrifuged for 1min at 8,000 rpm. The flow through was discarded, and the tube was reused. The DNeasy column was washed once again by using 500 µl of AW buffer and by centrifuging it for 2 min at maximum speed.

The Column was transferred to a clean 1.5ml tube and 100 µl of preheated (65°C) buffer AE were directly delivered into the DNeasy membrane. The membrane was incubated for 5 min at room temperature and then centrifuged at 8000 rpm for 1 min to elute the DNA. The DNA was kept at -20°C.

2.2.16 Molecular Characterization of Transgenic Plants

PCR technique was used to screen transgenic plants and to distinguish transplastomic plants from the mutants. The integration of transgene into site-

specific chloroplast genome is determined by using sets of primers, one of which anneals to the native chloroplast genome beyond the flanking sequence and the other anneals to the transgene cassette .

No PCR amplification will be observed in mutants and nuclear transgenic plants with these primers. To confirm the site-specific integration of transgene cassette into chloroplast genome, perform the PCR using primer pairs 16SF and BADH reverse primer(table 2-5), which anneals to the native chloroplast genome and *BADH* genes, respectively . Whereas to confirm the integration of gene of interest, perform PCR with primer pairs, tRNI-tRNA reverse primer with *CTB* forward primer, which anneal to tRNA and *CTB* respectively.

a. DNA was extracted from the leaf tissue of wild-type and putative transformants using the Qiagen's DNeasy Plant mini kit following manufacturer's protocol.
b. PCR reaction mixture was prepared for expected transformed plant and wild type along with other PCR component to serve as control, the enzyme used was *Taq* polymerase following the standard protocol.
c. PCR reaction was performed with initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58–60°C for 1 min and extension at 72°C for 1 min/kb followed by final elongation of 10 min at 72°C.
d. The PCR-amplified products were analyzed in agarose gel by electrophoresis and visualized by staining with ethidium bromide under U.V transilluminetor.

2.2.17 Southern Blot Analysis to Confirm Homoplasmy (Singh *et al.*,2009)

Initially, few copies of the chloroplast genomes receive transgene cassette and after several rounds of selection, untransformed genome copies are replaced by transgenic copies, leading to homoplasmy. This can be

ascertained by Southern blot analysis of the total DNA isolated from the plants after third round of selection.

Total plant DNA extracted from transplastomic leaves was digested with *Sma*1 and probed with DIG chloroplast flanking sequences. The following steps were performed carefully.

2.2.17.1 Transfer of DNA

a.Total cellular DNA was extracted from transplastomic as well as from wild-type plants using Qiagen's DNeasy Plant mini kit as described in product manual.

b. Total cellular DNA (1–3 μg) was digested with *Sma*1 (42.5 μl of template ,5 ul of 10X enzyme buffer,2.5 μl of enzyme) and incubated at the prescribed temperature for 3 hrs. wild-type DNA was included as control.

c. Run the digested DNA on 0.8% agarose gel until completely separated, the excess gel was removed, the gel was transferred to appropriate container.

d. The DNA was depurinated by soaking in 0.25 N HCl for 10 min and rinsed twice with distilled water for 5 min each. This will facilitates efficient transfer of DNA fragments from the gel to membrane.

e. Denaturation of the DNA was done by soaking the gel in transfer buffer for 20 min.

f.The gel was turned off upside down so that the smooth surface faces the nylon membrane. Prewet the nylon membrane in water then in transfer buffer for 5 min. The membrane was placed on top of the gel and apply gentle pressure by placing a stack of paper towels and a weight of 300 g on top to ensure even contact between gel and membrane. Allow the DNA to transfer overnight.

h. Remove the membrane with transferred DNA, rinse twice with 2x SSC buffer for 5 min each and air-dry on filter paper.

2.2.17.2 Preparation of Probe

DNA was labeled using DIG-11-dUTB as a substrate for *Taq* DNA polymerase during PCR .For standard PCR setting, the ratio of dTTP to DIG-11-dUTB used was 3:1.The concentration of the labeled probe was estimated by gel electrophoresis .Labeled unused probe was saved at -20C for a couple of months.

2.2.17.3 DNA Fixation on Membrane

UV-cross-linking at 80 °C for 2-3 hrs fixed DNA on positively charged nylon membrane, after brief rinsing of membrane in distill water, the membrane was placed on Whatman 3 mm paper presoaked with 5X SSC. UV-cross-linking was carried out in cross-linker at standard setting (C3).
Prehybridization step was done immediately or the fixed membrane stored at 4°C .

2.2.17.4 Prehybridization and Hybridization

DIG easy Hyb-buffer (20 ml) was heated and the membrane was incubated in it for 1 hr with gentle agitation at 40°C. About 50 ng of DIG labeled DNA probe was prepared by boiling for 5 min and rapidly cooled in ice .

Denatured DIG labeled DNA probe was added to preheated 10ml DIG-Easy Hyb buffer and mixed well but foaming was avoided(bubbles may lead to dark background) .Prehybridization solution was poured off and probe hybridization mixture was added to membrane and incubated at 43°C for 5 hrs or at 4°C overnight.

2.2.17.5 Stringency Washing

a-The membrane was washed twice in sufficient amount of 2X SSC,0.1%SDS with shacking for 5 min each.

b-The membrane was again washed in 0.5X SSC ,0.1%SDS (preheated to 60°C) at 60 °C for 15 min with shacking.

2.2.17.6 Immunological Detection

a-Membrane was incubated in sufficient amount of malic acid buffer with shacking for 2 min.

b-Malic acid buffer was poured off and blocking buffer was added to cover membrane and incubating with shacking for 2 hrs.

c-DIG-antibody was spin for 5 min at 12000rpm ,5 ul of antibody was added to 5 ml of blocking buffer ,mixing well .Blocking buffer was replaced with antibody-DIG solution and incubated with shacking for 30 min.

d-Antibody solution was discarded and washing buffer was added ,shacking was done for 15 min and repeated once.

e-Washing buffer was replaced with detection buffer and membrane was incubated with shacking for 3 min.

f-Blot was placed in plastic film with DNA side up and about 15-20 drops of CDP-star ready to use were applied.Excess liquid was squeezed and waited for 5 min.

g-Blot was removed from plastic sheet and wrapped in saran wrap film.

h-The blot was exposed to X-Ray film for 5-10 min depending on the intensity of signal. The signal were analyzed for the transgenic status of the plant

i-Develop the exposed X-ray film in an automated X-ray film processor to visualize the pattern of hybridization on the film.

j-The confirmed homoplasmic plants were transferred to the pots containing sterilized soil and initially kept under high humidity for acclimatization and then moved them to the greenhouse.

2.2.18 Western Blot and SDS-PAGE (Verma *et al.*, 2008)

Western blot analysis facilitates detection of expression of a specific protein within a tissue or mixture of protein. It can also be used to determine the quantity and the molecular size of the protein.

This technique relies on specificity and quality of antibody used to probe the desired protein. The following steps were performed carefully to minimize error in the experiment.

2.2.18.1 Extraction of Total Protein from Plant Leaves

a-Plant leaves were collected and grinded by liquid nitrogen (any dry from liquid nitrogen was avoided) , any thing used in this step must be in liquid nitrogen(i.e. tubes, spatula … etc)

b- powder was weighed (100mg) and placed in tube cooled by liquid nitrogen, the powder saved in liquid N_2 until the weighing process is complete. c-Extraction buffer was added ,5 volume of EB was added ,mixed and grinded by micro mechanical pestle for 2 min ,the tube was kept at vortex for 20 min

d-Centrifugation was done 13000rpm at 4°C for 5 min ,the supernatant was transferred to new tube and the leftover supernatant was stored at -80 °C

e-The extract was aliquot to new tube,in amount determined by the purpose of experiment

f-25 µl of BME was added to 475 µl of sample loading buffer, and the same amount of loading buffer was added to each aliquot.

Bradford assay was done to know the concentration of protein.

2.2.18.2 Bradford assay protocol (Bradford 1976)

a-Bradford dye solution was made by diluting 5 ml of Bio-Rad protein dye concentrate in 20 ml of dH_2O and filtered using filter paper.

b-BSA standard curve was made in 1.5 ml Eppendorf tubes.8µl of BSA at 10mg/ml were added to 92 µl dH2O to get concentration of 0.8 mg/ml.Four tubes were filled with 50ul dH_2O for 0.4,0.2,0.1,and 0.05 mg/ml

respectively ,then serially diluted by taking 50µl of 0.8 mg/ml BSA into next tube ,the process was continued until all dilutions were made.

c-Dilutions of plant extract were made at 1:5, 1:10, and 1:20 by adding 10 µl of plant extract to 40 µl D.W to prepare 1:5 dilution. Two Eppendorf tubes were filled with 25 µl of dH_2O and serially diluted by taking 25 µl from 1:5 into the 1:10 and so on.

d- Once all dilutions were made, 10 µl of each sample was used per well in duplicate.200 µl of Bradford assay from step one was added to well, waiting for five min and then absorbance was read on plat reader at 595nm.

After the checking the protein in extract the SDS-PAGE and western blot were made .

2.2.18.3 Preparation of The Gel

a- Glass plates and combs were cleaned thoroughly with D.W.

b-Plates were slide into green frame (smaller plate in front), sliding was done against table top.Wings were pushed out to lock the glass plates.

c-The plates were pressed down to another container (grey foam strip) on the bottom.

d-Distilled water was added to plates, and left while the gel solution was prepared.

e- APS solution 20% was prepared in Eppendrof tube and stored in a fridge

f-Resolving and stacking gel were prepared in 15 ml tube, all ingredients were added except APS and TEMED.

g-Water was poured off from plates and 50 µl of 20% APS and 10 µl of TEMED were added to the resolving gel. The solution was added to plates using pipette.

h-Water was added to level out the top of resolving gel, solidification was noticed in about 10 min.

i-Water was poured off and stacking gel was added to plates (after addition of ABS and TEMED) and the comb was placed carefully.

Table(2-7) Gel percentages and its contents

Gel %	D .W ml	30% degassed acrylamide/bis(ml)	Gel buffer	10% w/v SDS (ml)
4	6.1	1.3	2.5	0.1
5	5.7	1.7	2.5	0.1
6	5.4	2	2.5	0.1
7	5.1	2.3	2.5	0.1
8	4.7	2.7	2.5	0.1
9	4.4	3	2.5	0.1
10	4.1	3.3	2.5	0.1
11	3.7	3.7	2.5	0.1
12	3.4	4	2.5	0.1
13	3.1	4.3	2.5	0.1
14	2.7	4.7	2.5	0.1
15	2.4	5	2.5	0.1
16	2.1	5.3	2.5	0.1

*Resolving gel buffer : 1.5M Tris-HCl, pH 8.8

*Stacking gel buffer:-0.5M Tris-HCl, pH 6.8

2.5.18.4 Loading and Running The Gel

Two gels were prepared one for Coomassie stain and one for western blot, for purified extract. A quantity of 15 µg of protein for Coomassie and 5 µg of protein for western were loaded .

a-The box for electrophoresis was filled with 1X electrode buffer (EB), then left until the sample is ready.

b-Sample loading buffer 2X was made by adding 5 µl of BME to 95 µl 2X buffer (BME was added under radiation hood).

c-The sample loading buffer was added to tubes, and then the amount of protein was added in appropriate concentration. CTB standard was made in

the same manner ,which was 20 ng/µl, thus when added to 2X sample buffer it became 10 ng/µl.

d-Tubes were closed tightly and boiled for5 min.

e-The tubes were left to cool for a couple of min, and then centrifuged for few second.

f-Samples and marker were loaded.

g-Running of gel was done at 85 volts until the dye front passed into the resolving gel, then the voltage was turned up to 125 volts.

h-Runnig has been stopped when the dye reached the end of gel.

2.5.18.5 Staining the Gel with Coomassie Brilliant Blue

a-The stacking gel was cut off.

b-Gel was placed in small dish and covered with 0.1% Coomassie blue stain.

c-Dish containing the gel and stain was shaked at room temperature for 10-30 min.

d-Stain was poured back into container.

e-Destaining solution was added, discarded, more destaining was added with some absorbent tissue pool.

f-Shaking at room temperature was conducted for 30-60 min.

g-The solution was discarded and water was added to wash the gel, then visualized by normal light stage.

2.5.18.6 Transfer of Protein to Nitrocellulose Membrane

a-All requirements for transfer were collected before the dye reach the end, these were Pyrex tray, box, sponges, sandwich cassettes, stir bar, and green scraper thingy.

b-Transfer buffer was prepared as described previously

c-Little amount of transfer buffer was poured to Pyrex dish and a sandwich black side down was put in it.

d-The transfer cassette order was as follows:

*-Sponge

*-2 pieces of filter paper

*-Gel**,** the top part where the comb was cut

*-Nitrocellulose membrane ,previously cut from the roll

*-Two pieces of filter paper

*-sponge

Everything was wetted with transfer buffer before adding them to stack, also bubbles were also avoided as much as possible.

e-Cassette was closed and placed into Mini Transfer Blot Module.

f-Ice was added back, stir bar and transfer buffer to the top.

g-Ice was added to Pyrex dish after putting the whole setup in it.

h-Running was done at 85 volts for 1 hour.

2.5.18.7 Probing The Membrane

a-After transfer, membrane was rinsed with water

b-Milk was prepared for blocking and other steps.(PTM) 100 ml 1X PBS-Tween+3 g of dry milk.

c-Membrane was incubated in PTM for at least 30 min at room temperature and then PTM was poured off.

d-Primery antibody was added in correct amount, 5 µl of CTB antibody were added to 20 ml PTM 1:4000.15 ml of antibody solution was added to membrane and incubated in shaker at room temperature for 2 hrs or at 4°C overnight.

e-The membrane was rinsed briefly for two times with water

f-Secondary antibody was added in concentration 1:4000, also 5 µl of goat anti rabbit was used for CTB, this 5 µl was added to 20 ml of PTM.15 ml of secondary antibody solution was added to membrane and incubated at room temperature for 90 min.

g-PBS-T was prepared (1000 ml 1X PBS+1ml Tween 20)

h-Membrane was washed three times with PBS-T for 9 min.

i-Film processor rocker was turned on during the last wash

j-Last wash was done with 1X PBS for 9 min

k- Chemiluminescent solution was added (750 µl) of each, to the membrane after pouring off PBS and pipetting over membrane 50 times.

l-Lightly the membrane was wrapped in plastic wrap

m-In dark room X-Ray film was placed on the membrane in developing cassette, the exposition was for 2 min to judge appropriate time for exposition.

Chapter Three
Results and Discussion

3. Result and Discussion

3.1 Lettuce Seeds Germination and Tissue Culture

Germination occurred after 10 days growth on 1/2 strength MS medium (fig 3-1 A). Lettuce seeds were obtained from the store of Burnett School of Biomedical Science, University of Central Florida, USA. Results indicated that most of seeds were geminated in 1/2 strength medium containing thymine and myo-inositol at pH 5.8 after one week of incubation at 25 °C under 16:8 hrs (dark :light) photoperiod(figure 3-1B), plantlets then were successfully transferred to light box bottle and the resulted leaves were used for tissue culture and transformation experiments (figure 3-1 C).

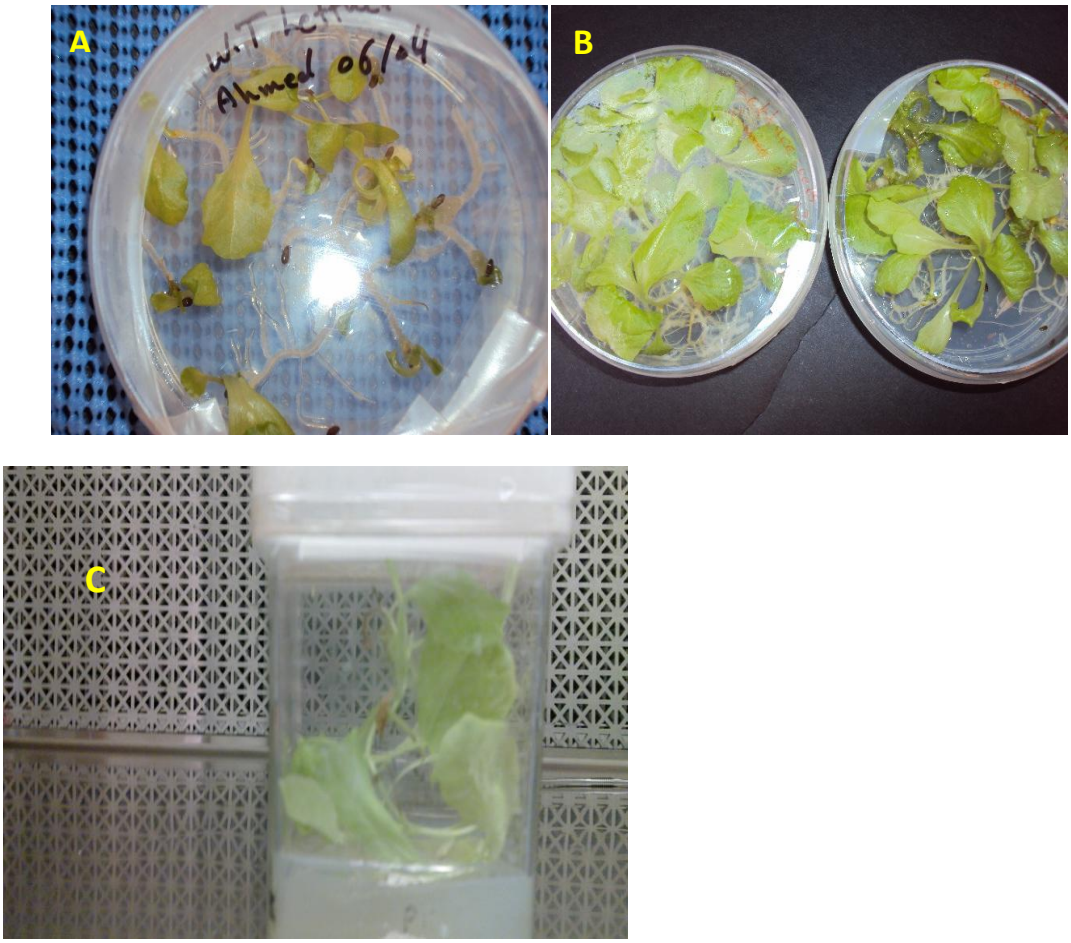


Figure (3-1) Wild type lettuce seeds germination on 1/2 strength MS medium **(A)** after 10 days of germination **(B)** after 21 days of germination **(C)** lettuce planlets germinated in a light box.

Leaves formed on seedling were dissected (figure 3-2 A) to small pieces of (5x5 mm), regeneration ability of lettuce explants to form complete plant was tested on MS medium supplemented with NAA and BAP, (Figure 3-2 B) indicates regeneration occurred with shoot formation after 10 days of culture. Shoots were transferred to another petridish for rooting and plantlets were obtained after 30 days, and thus plants were ready to be transferred to a green house .

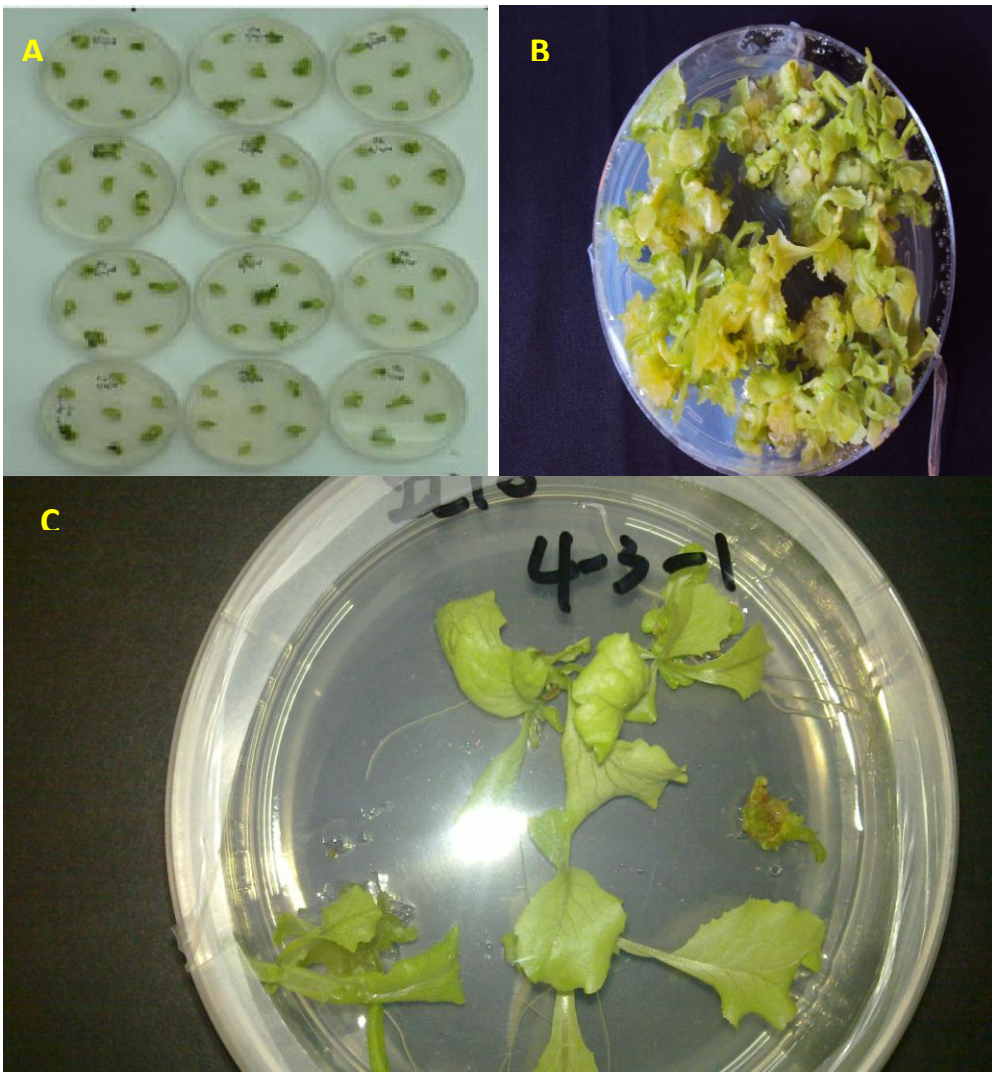


Figure (3-2): **(A)** Tissue culture of lettuce leaf explants on MS medium containing NAA (0.1 mg/l) and BAP (0.2 mg/l) **(B)** Direct regeneration on MS medium **(C)** Root formation.

NAA:- 1-Naphthaleneacetic acid BAP:- 6 benzylaminopurine MS:- Murashige and Skoog

Results showed that low concentrations of NAA and BAP (0.1 and 0.2 mg/l) respectively were needed for regeneration (figure 3-2). However, Regeneration were from leaf explants grown in such concentration of NAA and BAP occurred during the first 10 days, and the number of formed shoots increased at 20th day of culture. The frequency of adventitious shoots dying increased as the period of culture proceeded, ranging from 5 % (day 20) to 33% (day 40).

Seed germination is known to be controlled by a variety of internal and external factors, and some seeds have specific requirements for germination**.** Organ-forming potential is influenced by a variety of factors including genotype, age and physiological status of the donor plant, culture medium, culture environment including light, temperature, and atmosphere, as well as the phytohormones used (Hunter and Burritt, 2002).

It was reported that 72% shoots regeneration was obtained when MS medium supplemented with 0.13 mg/l NAA, 0.25 mg/l BAP. In this regard, Mohebodini *et al*. (2011) found that different combinations of plant growth regulators gave different responses. Higher concentrations of NAA and BAP did not increase the number shoots regenerated from explants. Hunter and Burritt (2002), produced the greatest number of shoots when 0.54 mg/l of NAA in combination with 0.44 mg/l of BAP was used. Similarly, Kanamoto *et al*. (2006) obtained high shoot regeneration from leaf explants cultured on a medium supplemented with 0.1 mg/l NAA and 0.1 mg/l BAP.

3.2 Examination of Salt Tolerance in *Lactuca sativa*

Lettuce explants grown on MS medium containing NAA and BAP were tested using different concentrations of NaCl (25,50,75,100,125, or 150) mM under optimum growth conditions (figure 3-3). Results obtained from this experiment indicated that explants are able to grow in a medium containing 75 mM of NaCl but failed to grow in a medium supplemented with 100 mM

of NaCl. It was also noticed that, growth efficiency in the presence of 50 mM of NaCl was more than those grown on 25 mM. Some explants tended to form callus in presence of NaCl rather than direct regeneration despite the presence of appropriate concentrations of plant growth regulators. Such finding was used in further experiments concerning the transgenic plant ability to tolerate NaCl.

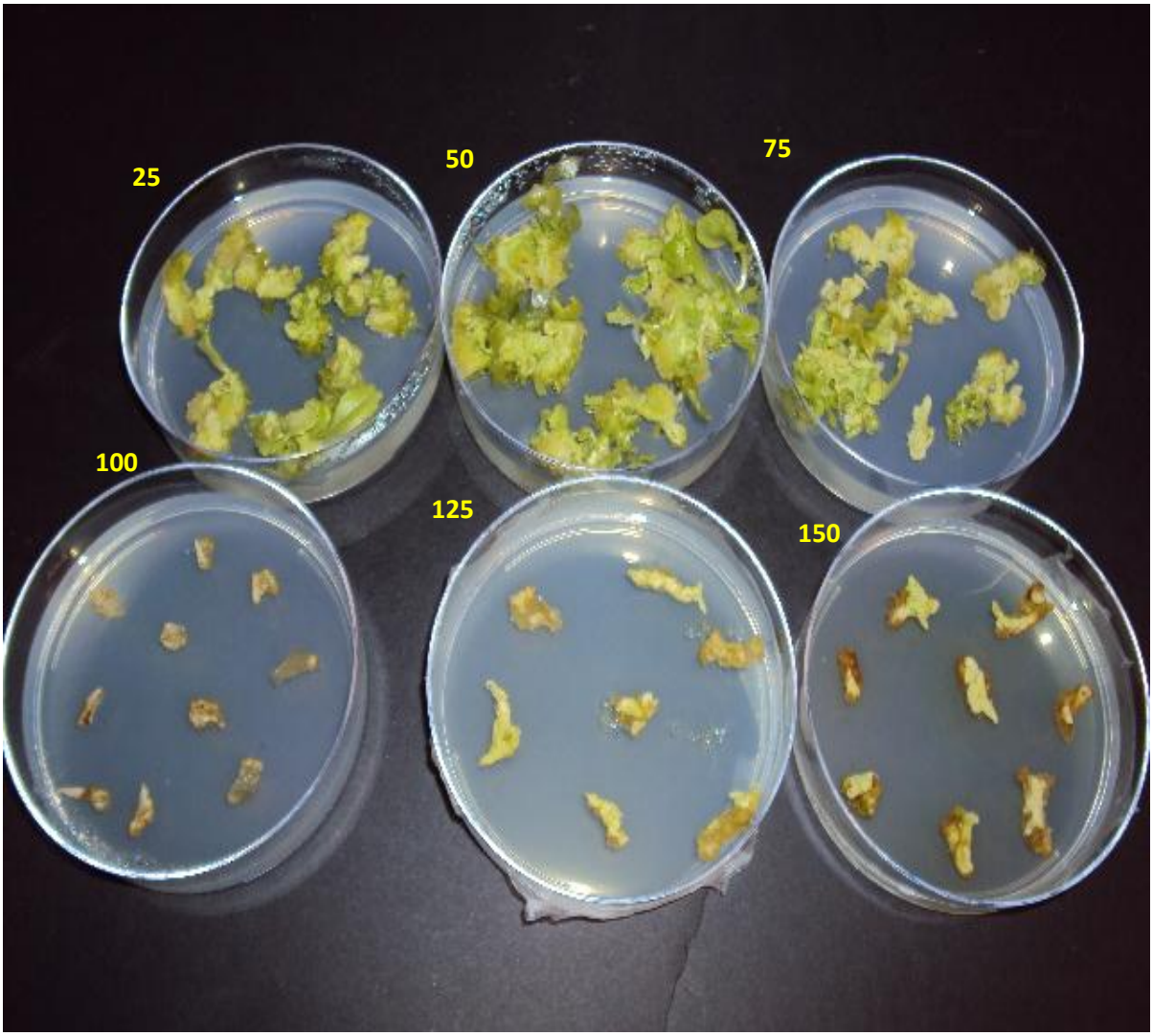


Figure (3-3) Lettuce explants grown on MS medium containing NaCl concentrations (25, 50, 75, 100, 125, 150) mM.

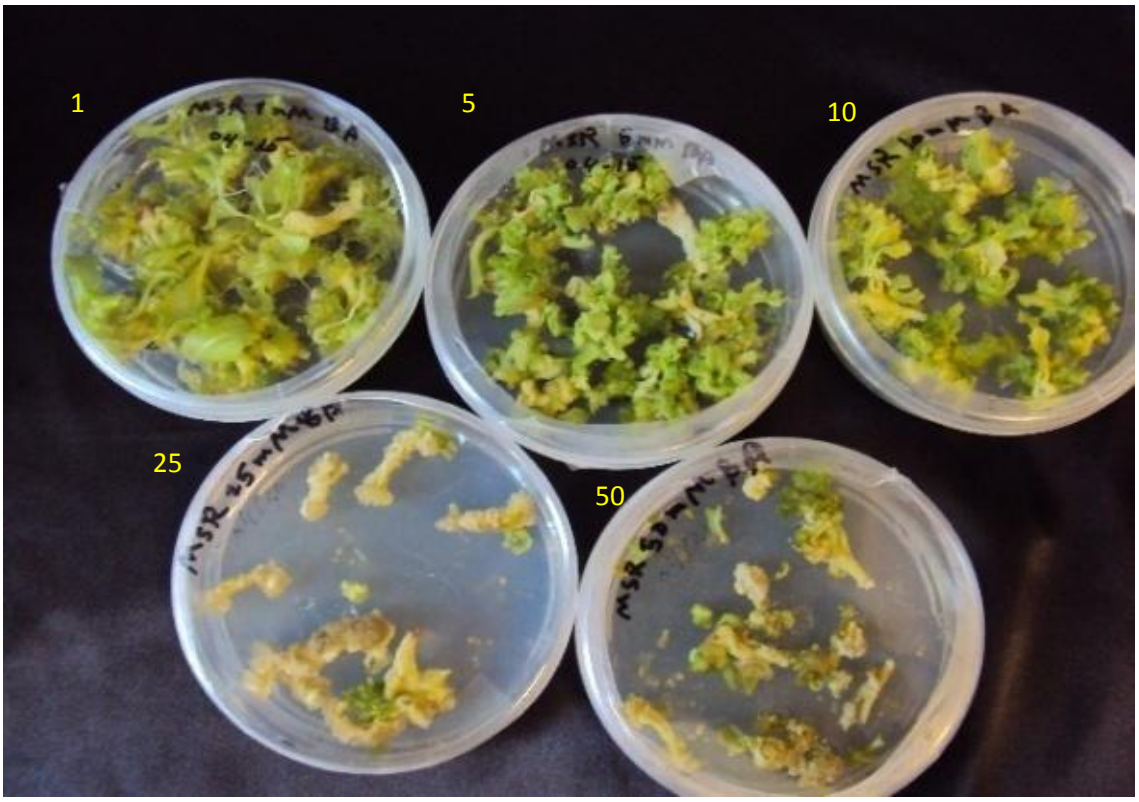
Salinity is an increasingly important environmental constraint to crop production worldwide. Regardless of the cause (ion toxicity, water deficit,

and/or nutritional imbalance), high salinity in the root zone severely impedes normal plant growth and development, resulting in reduced crop productivity or crop failure. The initial effect of salinity at cellular level is due to its osmotic effects. Shoot and root growth rate is reduced, resulting in smaller and fewer leaves and shorter plant stature. when salinity is due to NaCl, its effects are associated with accumulation of Na_+ and Cl_- in cells and/or to ionic imbalance leading to physiological disturbances. The effects of salinity depends on interactions with environmental variables such as water vapour deficit, temperature and solar radiation. Lettuce has been considered as a moderately salt sensitive crop, with a threshold of electrical conductivity (EC) of 1.3 dS/m, and a negative slope of 13.0 for each unit of added salinity above this threshold value.

Similar effect of NaCl was reported on lettuce and other plant by Zhang *et al*. (2008) when they found that salinity delayed lettuce plant growth due to reduction of photosynthesis. It is led to close of stomata and reduction of water entrance into the plant causing duplicate reduction in the plant weight. Luo *et al.* (2007) stated that such reduction in the dry weight of plumule and radicle which was a result of enhancing the salinity concentration is a normal phenomenon and probably may be due to the low water absorbance.

3.3 Effect of Betaine Aldehyde on Lettuce Growth

When five concentrations of betaine aldehyde were added to the growth medium to evaluate the ability of lettuce leaf explants to resist this compound, results showed that the plant was able to resist betaine aldehyde up to 10 mM and died at 25 mM (figure 3-4). In contrast, the 1mM concentration had no effect on the explants growth, while concentrations up to 5 and 10 mM led to callus growth rather than direct shoot regeneration.



Figure(3-4) Leaf explants grown on MS medium containing NAA 0.1 mg/l and BAP 0.2 mg/l and supplemented with different concentrations of betaine aldehyde after 20 days.

NAA:- 1-Naphthaleneacetic acid BAP:- 6 benzylaminopurine MS:- Murashige and Skoog

One common metabolic adaptation to salinity stress is the accumulation of osmoprotectants. Of these osmoprotectants, glycinebetaine, is a bipolar quaternary ammonium compound accumulated in many plant species as a result of stress. This compound protects the cell from salt stress by maintaining an osmotic balance with the environment, and by stabilizing the quaternary structure of complex proteins. In photosynthetic systems, glycine betaine stabilizes the oxygen-evolving photosystem II complex and Rubisco enzymes at elevated salt concentrations (Kumar *et al*., 2004b)

Park *et al*. (2007) stated that salt tolerance in transgenic tobacco, wheat, tomatoes and other crops with *BADH* gene was increased to varying degrees.

The levels of both BADH transcripts and enzymatic activity were found to increase almost 2 folds in leaves of salt stressed spinach plants, and almost 3

and 4 folds in sugar-beet taproots and leaves, respectively. Betaine has previously been shown to accumulate in both roots and shoots of sugar beet as a function of salinity, and to reach higher levels than in spinach. Betaine is compatible with metabolic function and can, therefore, acts as a non-toxic osmolyte in the cytoplasm; betaine may also protect enzymes against the inhibitory effects of elevated salt concentrations. As might be expected for a solute involved in osmotic adjustment of the cytoplasm, the accumulation of betaine is precisely regulated at the biosynthetic level and is proportional to the severity of salinity or water deficit.

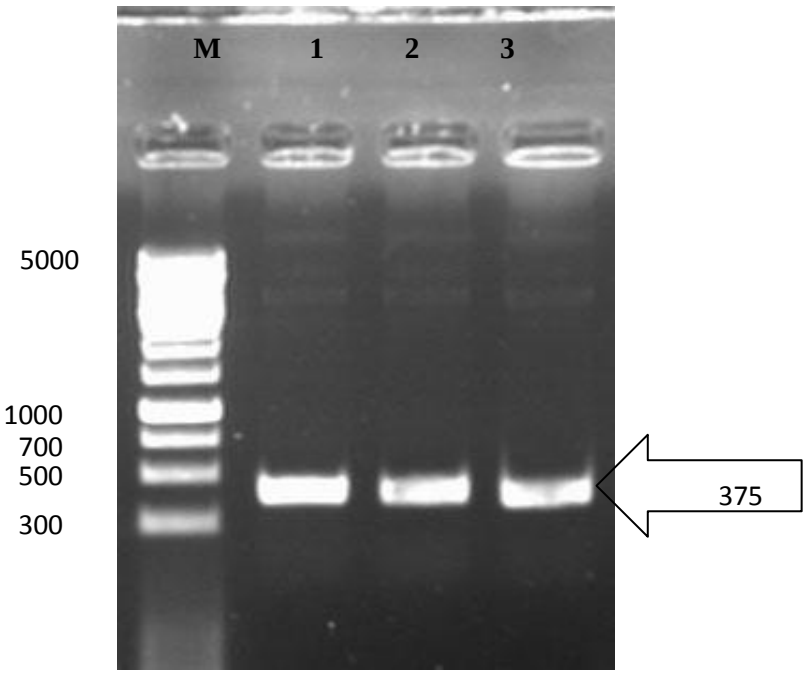
3.4 Isolation of Genes

3.4.1 Isolation of Cholera Toxin B Gene

One of the aims of this study is to produce an edible vaccine against cholera pathogen. To achieve this aim, a method to isolate the *CTB* gene from *Vibrio cholerae* obtained from the infectious disease laboratory at Burnett School for Biomedical Science was applied. Primers for amplification of *CTB* gene were designed according to primer-3 program available at NCBI web site, and a molecular weight of *CTB* gene of about 375 bp was obtained(fig 3-5), while the resulted bands were eluted using standard protocol .

Cholera toxin B subunit (CTB) is the pentameric non-toxic portion of cholera toxin (CT), responsible for the holotoxin binding to the GM1 ganglioside receptor present on most nucleated cells . When conjugated to autoantigens, the CTB dramatically increases their tolerogenic potential after oral administration (Jani *et al*., 2002).

The symptoms of cholera are caused by the cholera toxin (CT), an 85 KDa protein composed of A (CTA) and B (CTB) subunits combined in AB_5 holotoxin. CT functions as a mucosal adjuvant but its use in human is not possible due to its toxicity.



Figure(3-5) Electrophoresis of PCR product on agarose gel (1% w/v). Lane M, 5 kb DNA size marker, lane 2, 3 and 4, single expected band of *CTB* (approximately 375 bp).

Arakawa *et al*. (1997) used nontoxic B subunit to resolve this problem. CTB has been reported as a potent immunogen in the intestinal and nasal mucosal site. A mucosal adjuvant for oral and nasal vaccines and a transmucosal carrier delivery system for induction of oral tolerance when conjugated to auto antigens and allergens (Sergio *et al*., 2010).

3.4.2 Isolation of *BADH* Gene and *prrn* Promoter

Betaine aldehyde dehydrogenase gene was provided from a previous work of Kumar *et al.* (2004) in Professor Dniell laboratory at University of Central Florida using *BADH* forward and reverse primers according to the sequence of *BADH* gene isolated from spinach. Results of electrophoresis showed that the gene could be isolated in a single band with a molecular weight of about 1470 bp (fig 3-6). The gene then was eluted for further experiments to construct the vector.

The *prrn* promoter also amplified from vectors obtained from Professor Daniell laboratory using primers listed in chapter two (table 2-6). Results indicated that this promoter was about 200 pb (figure 3-6) which was eluted by the standard protocol.

Higher plants from several families (e.g., Chenopodiaceae, Poaceae, Asteraceae) accumulate the quaternary ammonium compound betaine in response to salt stress or water deficit. Much evidence indicates that in plants and in other organisms, betaine acts as a nontoxic or protective cytoplasmic osmolyte, allowing normal metabolic function to continue in cells at low solute potential (Foolad, 2004).

The most abundant transcripts in plastids are the rRNAs. The biosynthesis of plastid rRNA is highly regulated during development at both the transcriptional and post-transcriptional levels. Rates of *rrn* transcription vary by 50-fold and rates of rRNA stability vary by 35 fold in response to developmental and environmental status. Rates of *rrn* transcription were induced 10-fold in pea and tobacco chloroplasts in response to light (Ruhlman *et al*., 2010).

Transcription of the plastid rRNA operon (*rrn*) in higher plants is from diverse promoters. The *rrn* operon in tobacco was transcribed by the multisubunit, plastid-encoded RNA polymerase (PEP) from a σ70-type promoter (*Prrn*), as in most higher plants, including maize, pea, carrot, rice, barley, and *Arabidopsis*. In tobacco, in addition to the *Prrn* P1 PEP promoter, *rrn* was transcribed from a second promoter, *Prrn*2, which is recognized by the nucleus-encoded plastid RNA polymerase (Kumar *et al*., 2004).

In plants that naturally accumulate osmoprotectants, the levels are typically 5-50 μmol/g fresh weight (~6-60 mM on a plant water basis) and are highest during exposure to osmotic stress since accumulation is to some extent, stress-induced.

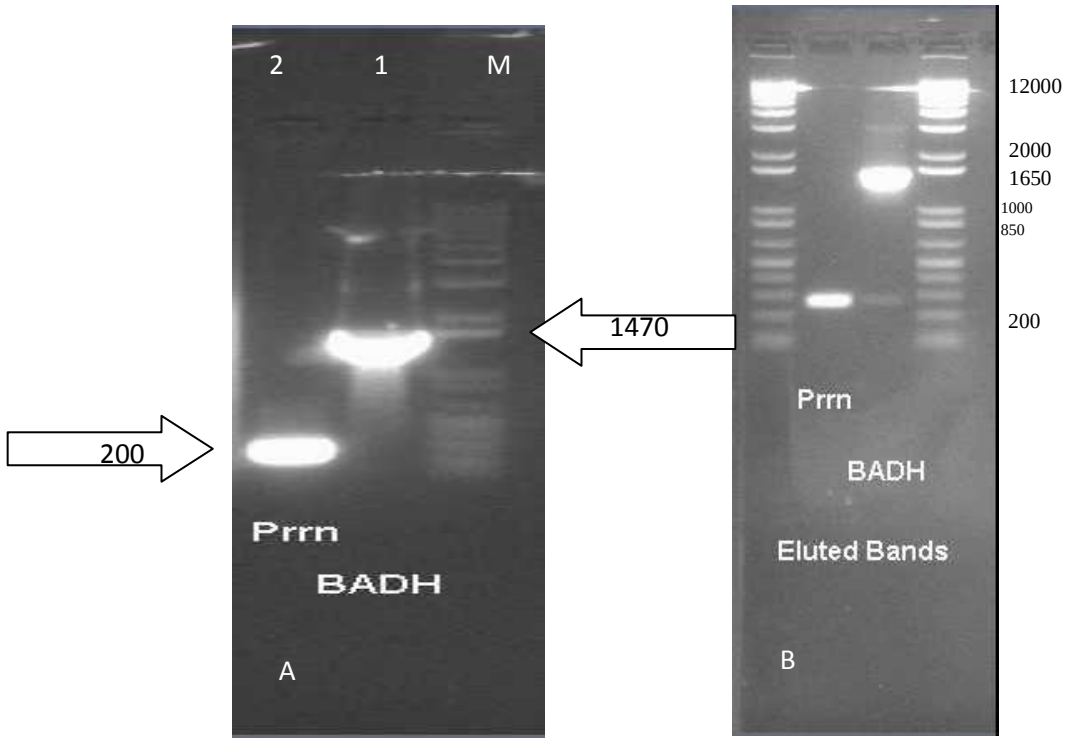


Figure (3-6) Electrophoresis of PCR product on agarose gel (1% w/v).
(A) Lane M, 1 kb DNA size marker, lane 1 *BADH* gene (1470bp) lane 2 *prrn* promoter (200 bp) **(B)** *BADH* and *prrn* after elution from gel.

In plant cells, osmoprotectants are typically confined to the cytosol, chloroplasts, and other cytoplasmic compartments that together occupy 20% or less of the volume of mature cell (the other 80% is the large central vacuole). Natural osmoprotectant concentrations in cytoplasmic compartments can therefore reach or exceed 200 mM. Such concentrations are osmotically significant, and so have vital role in maintaining cell turgor and the driving gradient for water uptake under stress (Rhodes and Hanson, 1993).

The fact that many important crops such as rice, potato and tomato are betaine deficient has inevitably led to the proposal that it might be possible to increase drought and salinity tolerance by genetically engineering GB synthesis.Transgenic potato plants with enhanced tolerance of oxidation, salt, and drought stresses was obtained by synthesizing GB via the introduction of the bacterial choline oxidase (codA) gene (Ahmad *et al*., 2008).

3.5 Construction of Vector

After the required genes were isolated, other steps of the project were started such as the construction of shuttle vector and cassette vector containing *CTB* and *BADH, prrn* promoter, all regulatory genes, and the specific restriction site. To achieve such steps, precise work regarding many PCR processes, restriction, ligation, sequencing, and transformation in *E. coli* were performed. Vector construction started with the ligation of *BADH* gene with *prrn* promoter previously isolated using *BADH* forward and *prrn* reserve primers formally designed in their isolation. The product was about 1700 bp DNA segment eluted and used to complete the shuttle vector (fig 3-7). *Trps*16(*rbcl*) 150 bp was amplified from genomic DNA of lettuce and used as transcription termination and enhancing the translation, and was ligated with *prrn, BADH*. All these products were ligated in pBs vector available at Professor Daniell laboratory at Burnett School of Biomedical Science in USA. This vector contains ampicillin resistance gene, and therefore, it was able to transform *E. coli* with this vector to propagate new vector and to ensure the right work .

Figure (3-8) which displays the result of transformation of competent *E. coli* with pBs vector containing *BADH, prrn, rbcl*, indicated that two colonies were transformed with the pBs vector and the colonies were checked by direct colony PCR with two primers from *BADH* to Trps16.

The resulted plasmids were propagated from replica plating *E. coli* and isolated using plasmid mini preparation Kit. Orientation and sequence of genes were established at this stage.

Orientation of the gene in the plasmid was carried out using restriction enzymes, the resulting bands gave indication of the orientation and arrangement of genes. The plasmid isolated from transformed *E. coli* was cut with *Hin*dɯ and *Noc*1 enzymes (figure 3-9).

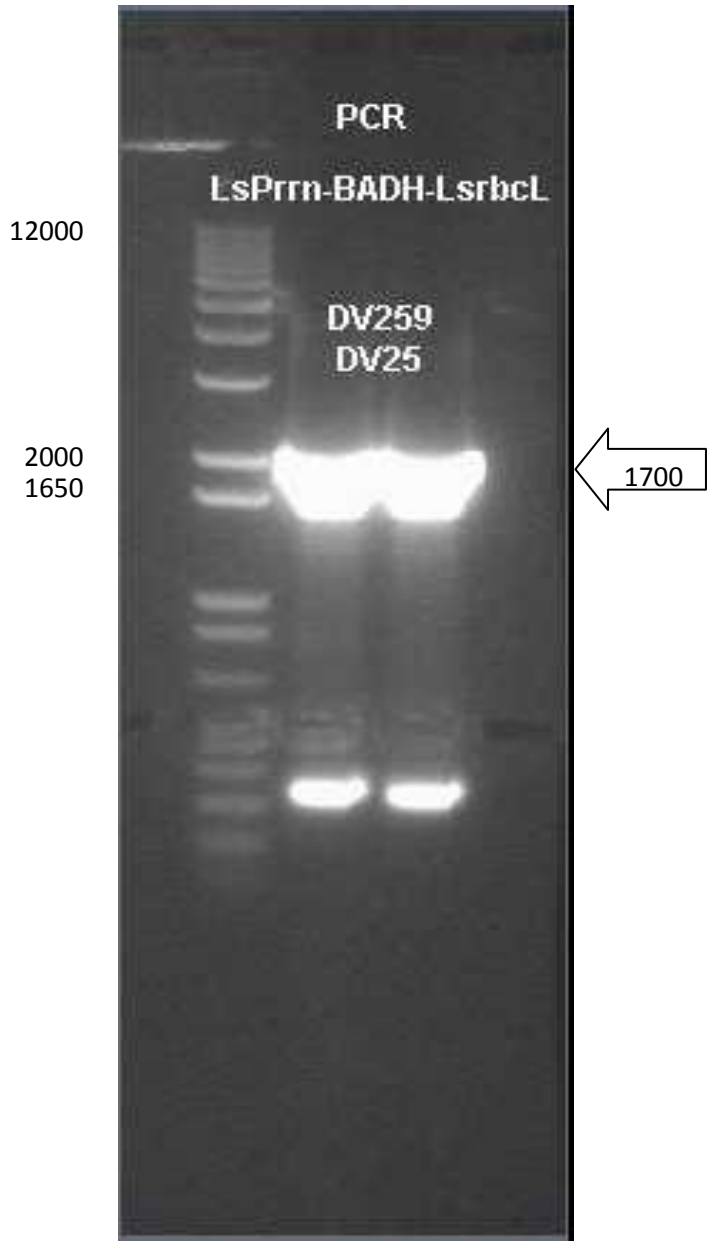
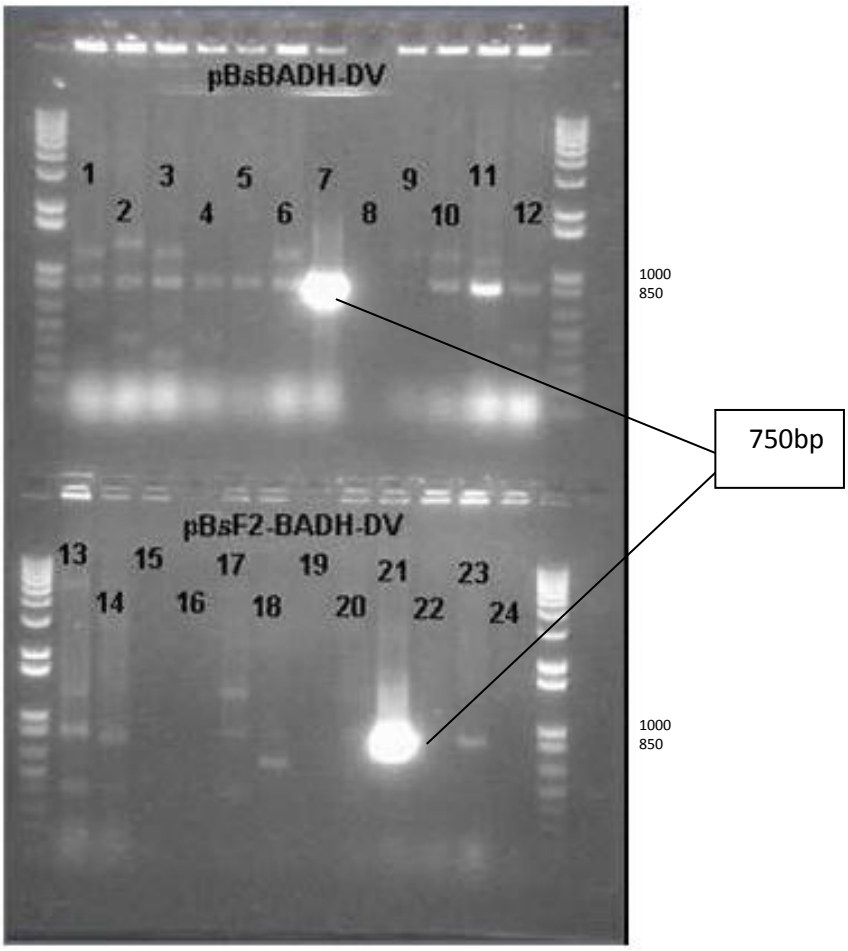


Figure (3-7) Electrophoresis of PCR product on agarose gel (1% w/v).
PCR product for *BADH, prrn* fusion and Trps16 about 250 bp.

Results showed that the genes were in right arrangement depending on the resulted band. Digestion with *Hind*ɯ showed about 1250 bp and the remaining vector and with *Noc*1 showed about 1100 pb and remaining of the vector. When genes in opposite orientation, the result was different, and in this case the digestion with *Hind*ɯ produced about 350 bp band.



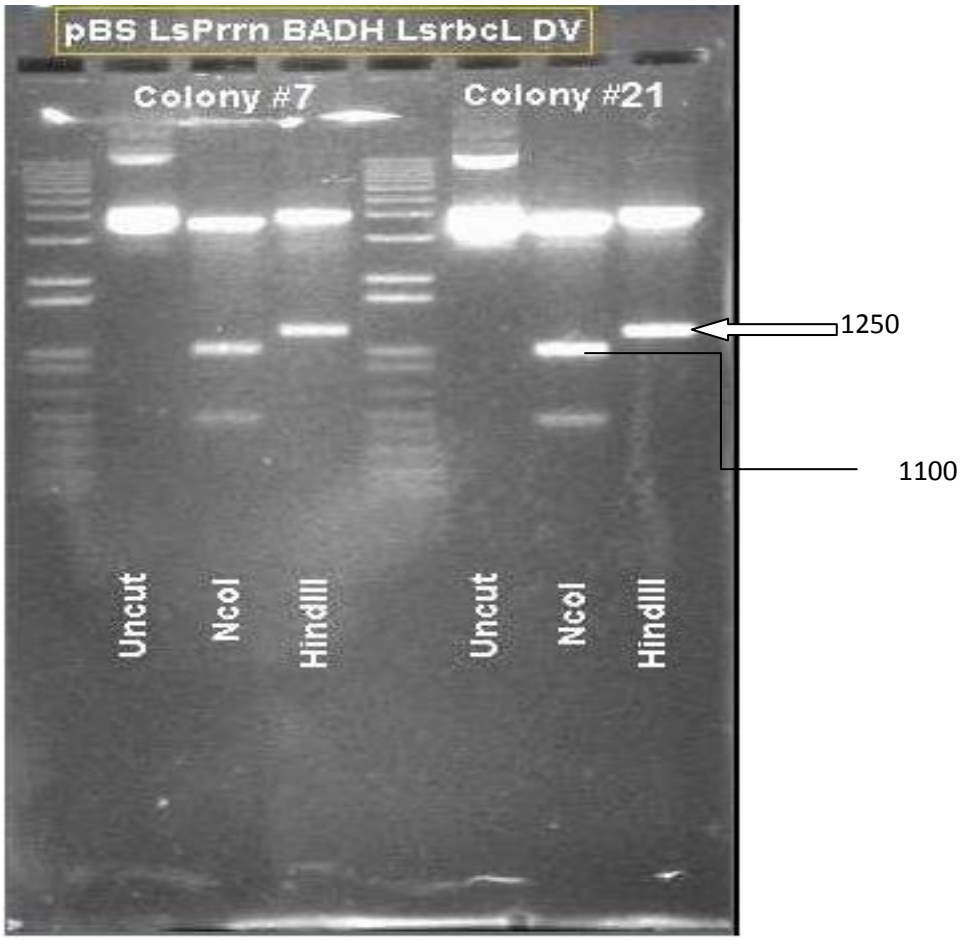
Figure(3-8) Electrophoresis of direct colony PCR from luria agar ampicillin plate culture with *E. coli* transformed with *BADH* gene using primers amplify the region from prrn to mid of BADH gene, 1-24 represent the numbers of colonies that had been checked by direct PCR technique .

Right orientation plasmids were sent to GENEWIZ company to ensure the fidelity of amplification process. Results obtained from above source confirmed the right nucleotide sequence for all plasmids.

Another promoter and terminator P*psb*A and T*psb*A for *CTB* gene were isolated from a previous work conducted by Ruhlman *et al*. (2010) using the restriction enzymes *Eco*R1, *Sma*1, *Not*1 and *Sac*1(figure 3-9).

The resulted promoter and terminator were ligated to the shuttle vector. Work with restriction enzymes was carried out according to the manufacturer protocol using specific buffer and time to achieve restriction and to obtaining

precise results. In addition, blunt ends were conducted using T4 DNA polymerase according to standard protocol mentioned in chapter two 2.2.8.



Figure(3-9) Gel electrophoresis of pBs-Ls prrn plasmid digestion with *Hind*ɯ and *Noc*1 restriction enzymes in compared with uncut plasmid.

The shuttle vector now ready to ligate the gene of interest *CTB* which was previously isolated in this study. *CTB* gene was introduced between the P*psb*A and T*psb*A using the restriction enzymes *Xba*1 and *Nde*1 as shown in figure 3-10. Specific restriction sites were introduced to selected genes and regulatory element by primary designing of primers (Verma and Daniell, 2007). All details of vector construction are shown in figure 3-10**.**

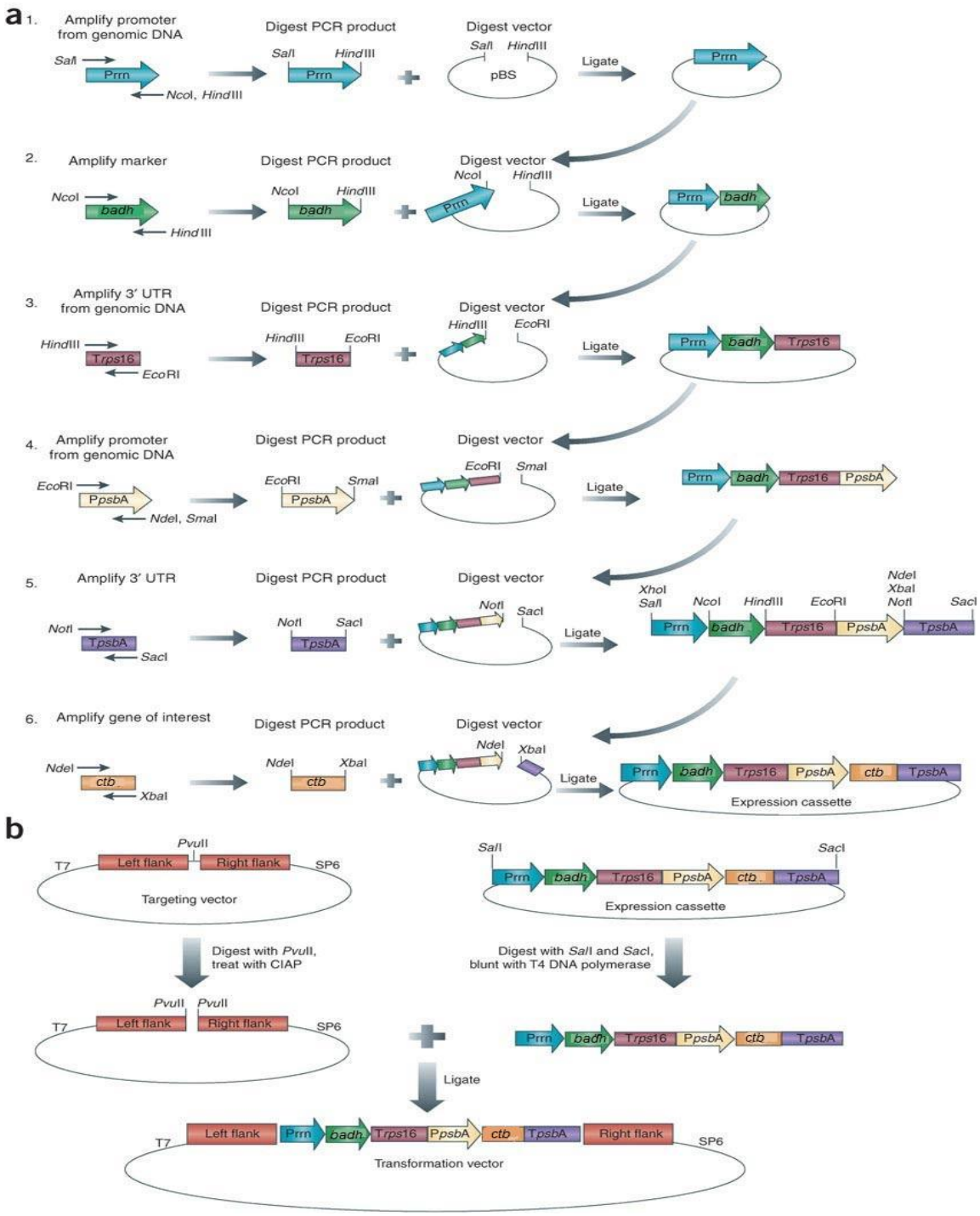


Figure (3-10) Vector construction **(a)** Steps involved in the creation of gene expression cassette with suitable chloroplast-specific promoters and regulatory elements, amplified from the total cellular DNA using primers designed on the basis of the chloroplast genome sequence information available in the GenBank. The ribosome-binding site in reverse primer of the *Prrn* promoter. P*psb*A represents promoter and 5'-UTR. **(b)** Steps involved in the construction of final transformation vector containing species-specific chloroplast flanking sequence and transgene expression cassette.

Figure (3-11b) shows that the pUC-based *L. sativa* long flanking plasmid (pLS-LS) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome (Ruhlman *et al*., 2007). The pLS-LS vector was modified to include the *BADH* gene for selection of transplastomic lines. Expression of BADH is driven by the *L. sativa* endogenous P*rrn* and a GGAGG ribosome binding site. The BADH transcript is stabilized by inclusion of the endogenous 3' UTR of *rbcL* (Verma *et al*., 2008)**.**

The *CTB* gene construct was inserted into the pLSLS vector as previously established by Professor Daniell laboratory (Verma and Daniell, 2007; Ruhlman *et al*., 2010) under the control of the light regulated *psb*A region located within the 5' UTR. All previous vectors contained the *aadA* gene which confers resistance to spectinomycin and were driven by the upstream *prrn* promoter but in the present study *BADH* gene was used instead of *aadA* as a selectable marker to establish transformed plants. Chloroplast vectors included native lettuce DNA flanking regions (*trnI/trnA*) in order to facilitate homologous recombination (Ruhlman *et al*., 2010).

E. coli then was transformed with the cassette vector for propagation of plasmid and also to test the orientation and sequencing to ensure the right vector used to transform the lettuce plant for the production of edible vaccine. Results of *E. coli* transformation showed many clones some of them tested by PCR using P*psbA* forward primer and T*psbA* reverse primer and the product size was about 700 bp as shown in figure 3-12.

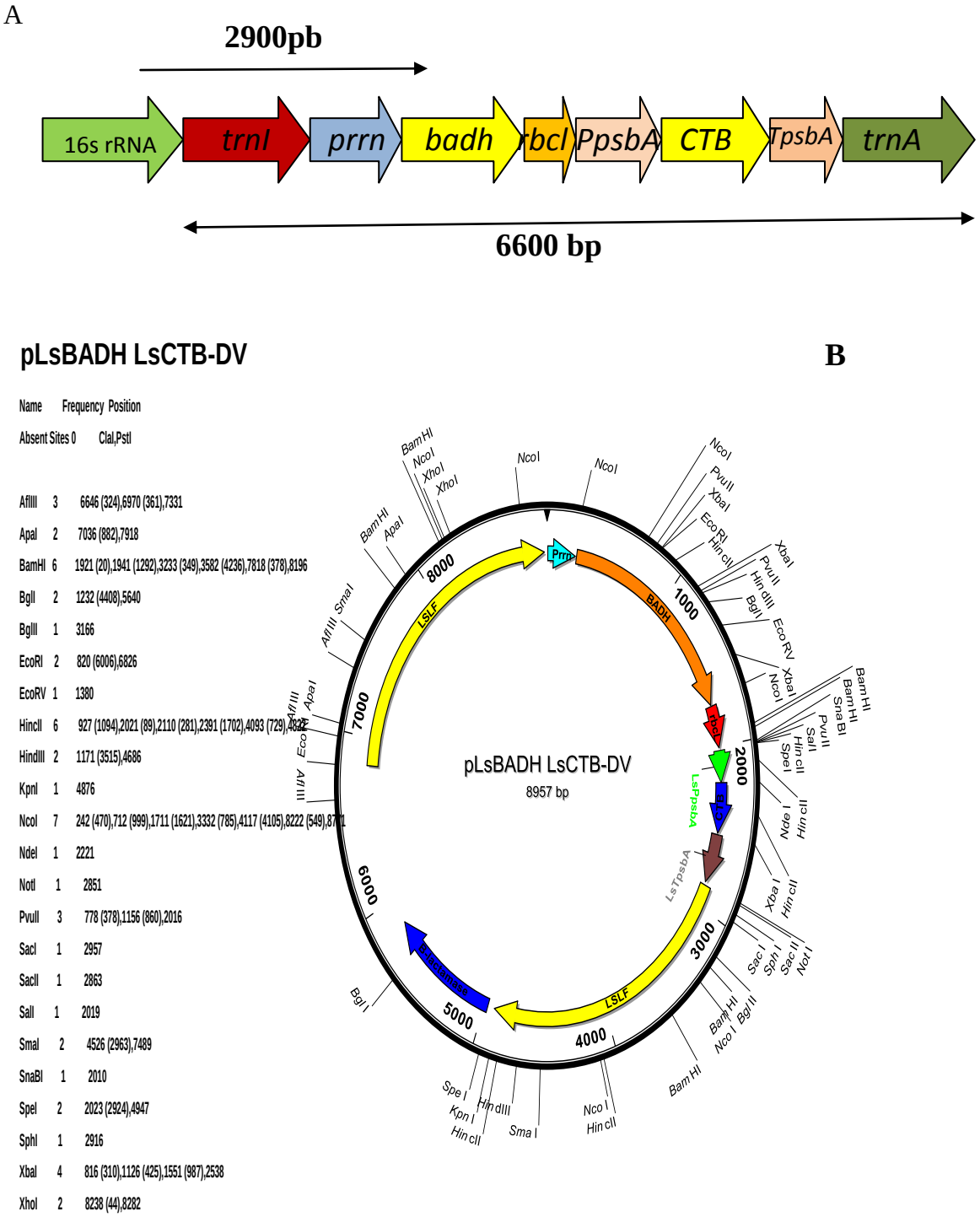


Figure (3-11) **(A)** Schematic representation of the transgene cassette, 16s rRNA gene form rRNA in native chloroplast genome, trnI represent transfer RNA gene for leucine, trnA represent transfer RNA gene for alanine (**B**) Complete map with restriction sites for transformation vector to transform lettuce plant with *CTB* gene using *BADH* as a selectable marker.

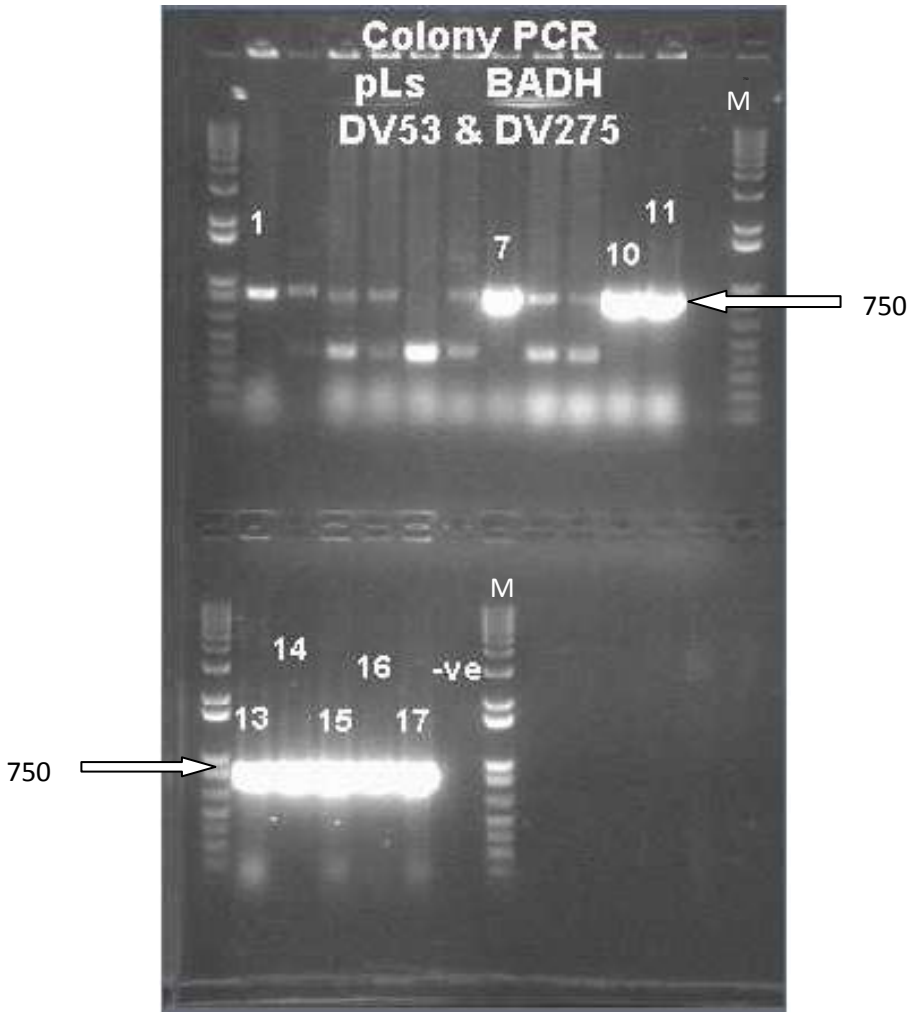
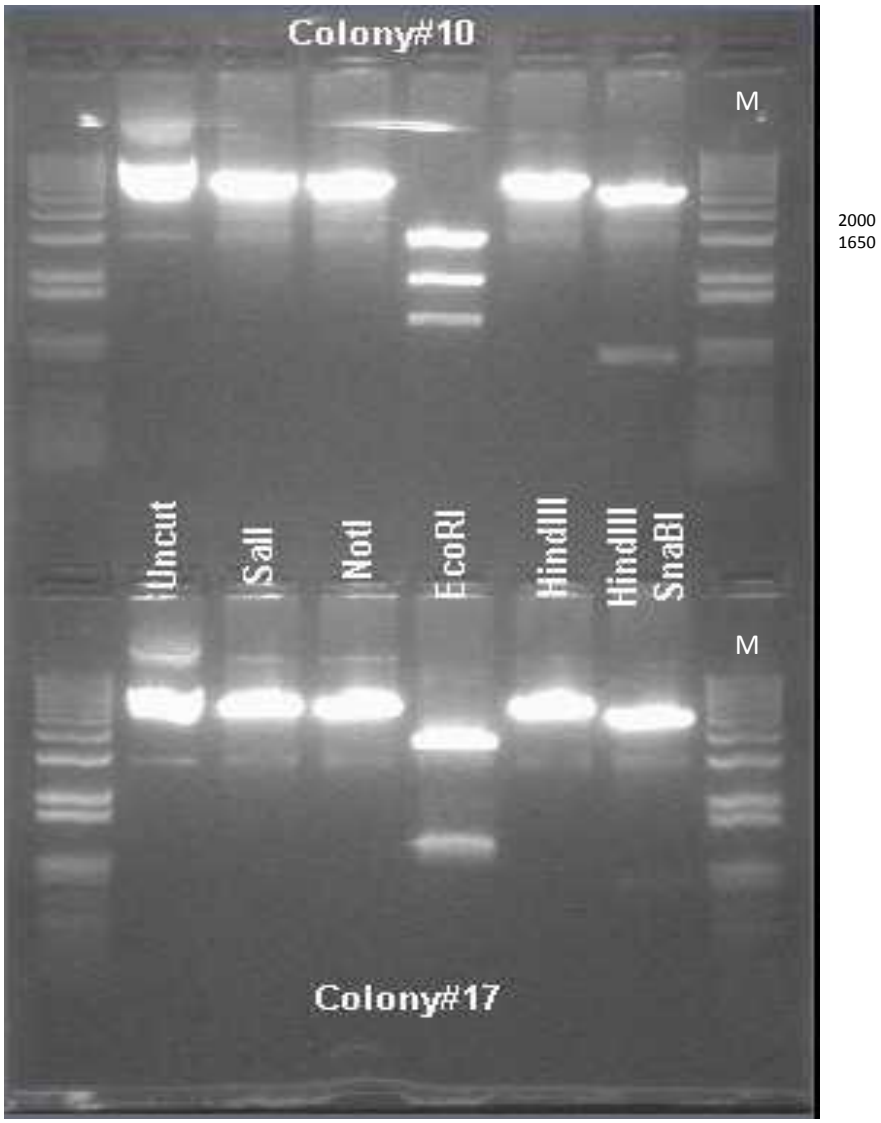


Figure (3-12) Electrophoresis of direct colony PCR from luria agar ampicillin plate culture with *E. coli* transformed with *BADH* gene using primers amplify with 1kb ladder the region from prrn to mid of BADH gene, M 1kb molecular marker 1-17 represent the numbers of clones that had been checked by direct PCR technique .

Clones 10 and 17 were randomly chosen, their orientation and nucleotide sequence were tested. Five enzymes were used to test the orientation. As shown in the physical map the *Eco*R1 should give two bands one about 1600 bp and the other occupies the rest of plasmid and this was present in colony 17 (fig 3-13), while in clone 10 three bands were formed (unknown reason). *Hin*dɯ gave a low quality digestion, this may be due to the time required for digestion with *Hin*dɯ which is more than other enzymes. The confirmed

orientation plasmid was sent to GENEWIZ company to confirm the nucleotide sequence and the result showed that all amplification processes took place without any error.



Figure(3-13) Gel electrophoresis for plasmid digestion with different restriction enzymes *Sal*I, *Not*I, *Eco*RI, *Hin*dIII, *Sna*BI to confirm the orientation of gene and regulatory element, M 1kb molecular marker.

Plasmid at this stage was ready for propagation in *E. coli* cultured in luria broth containing ampicillin. The propagated plasmids were isolated from the bacteria and used to shoot the plant tissue with foreign genes.

Many previous vectors were constructed in Professor Daniell laboratory and others worldwide laboratories to transform various plants with different genes. In the field of edible vaccine, construction of most vectors depends on *aad*A gene which confers spectinomycin resistance as a selectable marker (Barone *et al*., 2009).

As known that the delivery of antibiotic resistance gene to human has many obstacls and may cause health problem, many reports are directed in their recommendations to produce edible vaccine without antibiotic as aselectable marker. This project may be the first one in such field designed for the production of cholera vaccine in plant using a safe selectable marker *BADH* which confers salt and drought resistance. Kumar *et al*. (2004) transformed carrot with *BADH* conferring salt tolerance using *aad*A as a selectable marker.

Many projects were conducted for the production of cholera vaccine in plants using different selectable markers. Arakawa *et al*. (1997) achieved the first attempt to transform potato with *CTB* gene using *Agrobacterium tumefaciens* as a carrier for gene and two antibiotic resistance gene kanamycin and claforan as selectable marker. Li *et al*. (2011) worked on transformation of tobacco plant with *CTB* using *Agrobacterium tumefaciens* and the chloramphenicol resistance gene. Production of cholera vaccine in lettuce was firstly achieved by Kim and his colleagues in 2006 but also by *Agrobacterium tumefaciens* and cefotaxim resistance gene as a selectable marker. Toxin B production in the above work was 0.24% from the total soluble protein.

3.6 Bombardment of Leaves with Cassette Vector

Transformation plasmids were loaded on gold particles as described in the materials and methods(2.2.13.2). Different parameters were used to achieve the bombardment of lettuce leaves.

Figure (3-14) displays the loaded gold particles with cassette vector, the gold particles with DNA showed very high molecular weight in electrophoresis and indicating the presence of DNA loaded on gold particles.

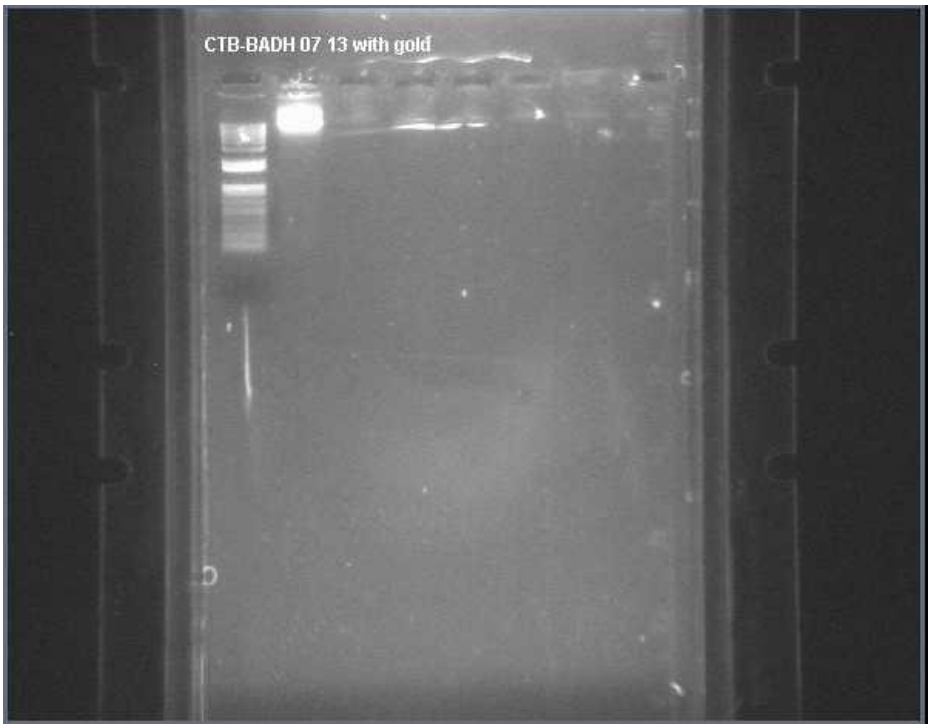


Figure (3-14) Gel electrophoresis on agarose gel for gold particles loaded with cassette vector contains *BADH* and *CTB* genes.

The first parameter used for bombardment of lettuce leaves was the distance between the rapture disk containing the macrocarrier and the leaves. In Bio-Rad helium apparatus used throughout this study, there are three distance which are 6, 9, and 12 cm. These three distances were experimented. Results indicated that the best transformation efficiency was at 6 cm distance (table 3-1). In addition, different gas pressures were tested for best efficiency of transformation. The tested pressures were 650, 900, and 1100 psi and the result showed that 900 psi with a distance of 6 cm more effective in the transformation of lettuce leaves (10%).

Leaf age was reported as one of the parameters that affect transformation efficiency (Xia *et al.*, 2011), so leaves at different ages were examined. Table

3-2 shows that leaves aged 40 days cultured aseptically in light bottle on MS medium were more efficient, the transformed explants were 10% compared

Table (3-1) parameters used to transform lettuce leaves and their relation with transformation efficiency, the transgenic cell lines selected on lettuce regeneration medium containing 150 mM NaCl and 35 mM betaine aldehyde after bombardments were confirmed by PCR for site-specific transgene integration.

No.of plates	Rapture disk* psi**	Distance*** cm	Event per plate	Efficiency**** %
25	650	6	0	0
25	650	9	1\25	4
25	650	12	0	0
28	900	6	3\28	10
28	900	9	1\28	3.5
28	900	12	0.5\28	1.7
20	1100	6	0.3\20	1.5
20	1100	9	0	0
20	1100	12	0	0

*Helium gas pressure used

**pound per square inch

***Distance between rapture disk and leaf

****Percentage of transformation efficiency was calculated from total number of independent transformation events obtained from total number of plates.

Table (3-2) Effect of leaf age on transformation efficiency when optimum bombardment conditions were used 900 psi and 9 cm distance

Leaf age (days)	Total No. of plate	Event per plate	Efficiency %
30	10	0.4	4
40	10	1	10
50	10	0.2	2
60	10	0.2	2

with other leaves aged 30, 50, or 60 days. The relation between the helium gas pressure and distance required for best transformation efficiency is represented in figure 3-15. The best helium gas pressure for lettuce transformation and the best distance was recorded. Many papers indicate wide parameters for bombardment, for example, tobacco plant needs 12 cm distance and 1100 psi to obtain efficient transformation frequency, carrot needs 12 cm and 900 psi (Ginsberg *et al*., 2010). Taha (2008) found that 900 psi with 9 cm gave more transformed *Impatiens balsamina* with GUS gene.

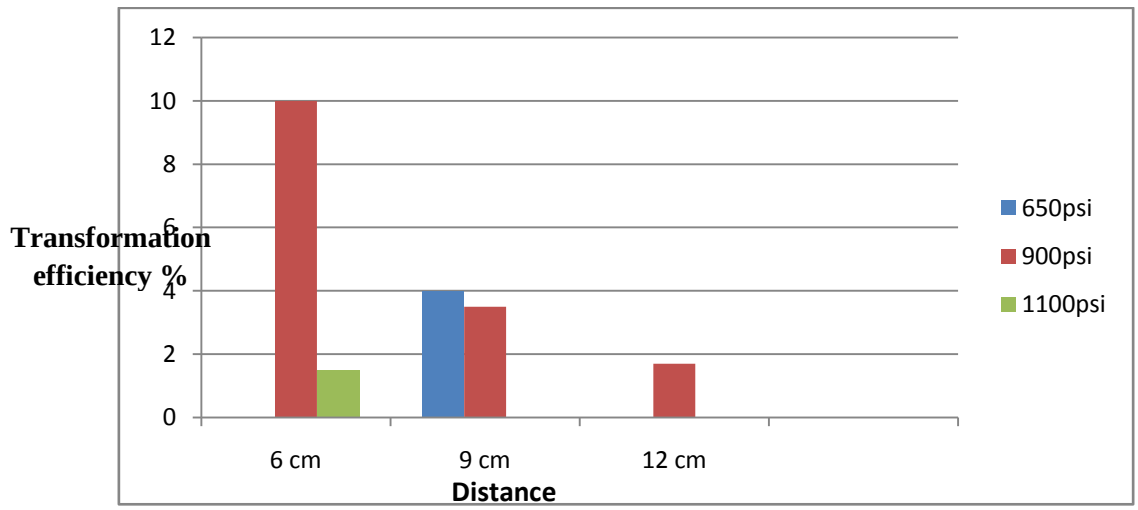


Figure (3-15) Transformation efficiency in relation with leaf distance from rapture disk and the helium gas pressure.

Leaves prepared for bombardment were obtained from aseptic light bottle inside a laminar hood and placed at apxial side in LRM without selectable agent. After bombardment, leaves were incubated in dark incubator for wounds healing occurred during bombardment process. Leaves were then spliced to small pieces 5x5 mm as shown in the fig 3-16, and cultured adxial side on LRM containing selectable agent (BA at concentration of 35mM and NaCl at a concentration of 150 mM). Each bombarded leaf was divided into 7 -12 splices and spreaded equally to each selectable agent plate and incubated in culture room at 25°C, 16:8 hrs light:dark photoperiod.

More expected transformed plants on BA plates than NaCl plates (as a selectable marker) were detected, this might be as a result of BA direct contact with the medium so some explants able to grow even they were not transformed. Therefore, more analyses were conducted to examine the transformed explants.

DNA transfer by particle bombardment makes use of physical processes to achieve the transformation of crop plants. The independence on bacteria such as *Agrobacterium tumefaciens* as a carrier of DNA minimize the limitation present in bacterial method.

The absence of biological constraints, at least until DNA has entered the plant cell, means that particle bombardment is a versatile and effective transformation method, not limited by cell type, species or genotype. There are no intrinsic vector requirements so transgenes of any size and arrangement can be introduced, and multiple gene co-transformation is straight forward (Xia *et al*., 2011).

Biolistic DNA delivery is used when the targets are plastids in intact tissue. Polyethylene glycol treatment is used for DNA introduction into protoplasts. The most commonly used selective marker gene is *aad*A, encoding spectinomycin resistance (Svab and Maliga, 1993). Kanamycin (Carrer *et al*., 1993), chloramphenicol (Li *et al*., 2011), and the amino acid

analogs 4-methylindole and 7-methyl-DL-Trp (Barone *et al*., 2009) have also been successfully employed as selective agents.

Extension of the technology of plastid transformation to new crops has been more challenging than the nuclear gene transformation. Although there are reports of partial success in many species, reproducible protocols for plastid transformation have been described in tobacco (Svab and Maliga, 1993), tomato (*Solanum lycopersicum*), petunia (*Petunia hybrida*) potato (*Solanum tuberosum*) (Valkov *et al*., 2011), soybean (*Gycine max*) (Dufourmantel *et al*., 2004), lettuce (*Lactuca sativa*) (Kanamoto *et al*., 2006), and cabbage (*Brassica oleracea*) (Liu *et al*., 2011). Monocots as a group appear to be the most recalcitrant species.

A major advantage of particle bombardment is that the delivered DNA can be manipulated to influence the quality and structure of the resultant transgene loci. This has been demonstrated in recently reported strategies that favor the recovery of transgenic plants containing intact, single copy integration events, and demonstrating high-level transgene expression. At the current time, particle bombardment is the most efficient way to achieve plastid transformation in plants and is the only method so far used to achieve mitochondrial transformation (Liu *et al*., 2011)

3.7 Screening for Transgenic Plants

Many plantlets appeared after various incubation period on MS medium containing selectable agents. These plantlets should be tested to ensure transformation. PCR technique was used to confirm the transgenic plant using the same primer used for *BADH* isolation, and to confirm plastid integration, 16SF primer which anneal only to native plastid DNA with other primer inside the cassette vector.

Figure 3-17 shows different plantlets that appeared after 14-25 days of incubation on a medium containing NaCl and BA as selectable agents.

DNA was extracted from small leaves that appeared after incubation period (figure 3-18). Very small pieces were cut carefully from leaves to determine if they were transgenic. The leaves sample were cut aseptically and this enabled passing the rest of leaf to another round of selection. DNA was extracted according to standard protocol mentioned in the materials and methods chapter(2.2.15). The extracted DNA was used as template for PCR analysis to detect the transformed plant contain *BADH* gene which then passed to another round of selection.

Results showed that most of regenerated plants were transgenic and contain *BADH* gene (figure 3-19). Testing of *BADH* was carried out by the same primer to isolate *BADH*.

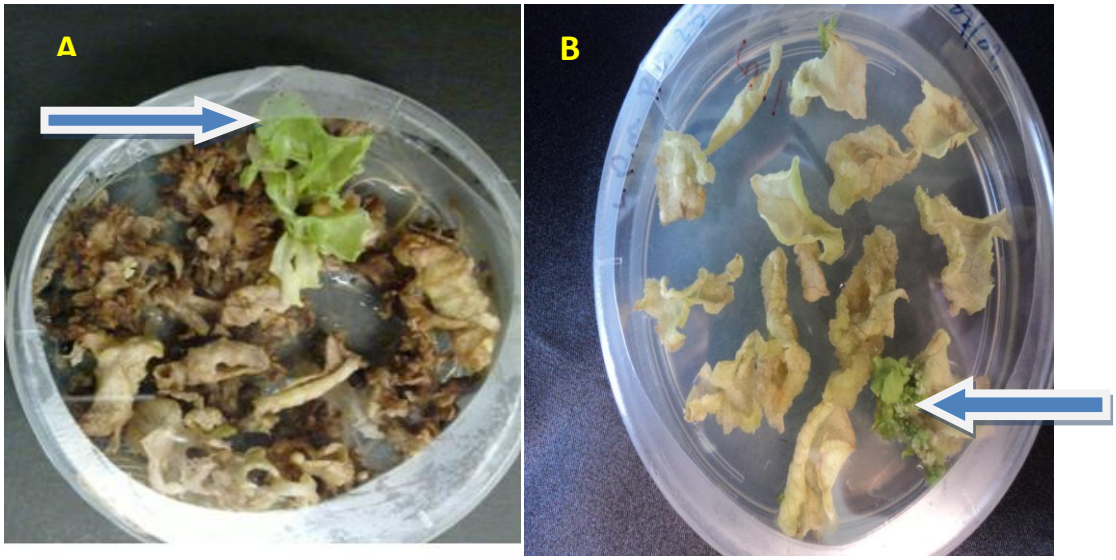


Figure (3-16) Bombarded lettuce explants on a selective medium **(A)**Transgenic plantlets grown on MS medium containing 150 mM NaCl **(B)** transgenic plantlets grown on MS medium containing 35 mM betaine aldehyde, arrow indicate expected transgenic shoots.

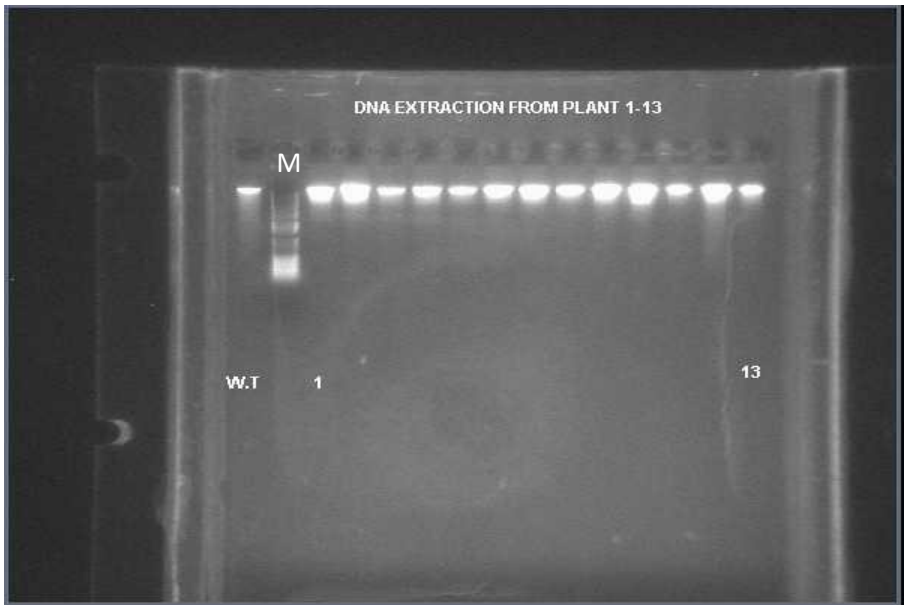


Figure (3-17) Gel electrophoresis of the extracted DNA from expected transgenic plantlets using Qiagen DNeasy Kit lane WT wild type, lane M molecular marker 1 kb, lane 1-13 represent expected transgenic explants DNA.

It seems from the figure 3-19 that different bands appeared by electrophoresis of PCR product using *BADH* primers. This may belong to a gene copy of *BADH* gene and may be the primers anneal to nuclear DNA which has one copy of *BADH* gene in the lettuce plant (Verma *et al*., 2008).

Therefore, only plantlets which showed a high intensity were selected for further analyses to confirm chloroplast integration.

Confirmation of chloroplast integration of cassette vector was conducted by PCR but with primers annealed only to chloroplast native genome which was 16SF primer as shown in chapter two(table 2-6). This primer annealed to the region out of flanking sequence and gave a product more than 2000 pb since the flanking sequence was 2000 bp from each side.

Primers used to confirm chloroplast integration of cassette vector were 16SF and primer inside *BADH* gene, according the primers used the fragment resulted was 2900 bp (figure 3-20).

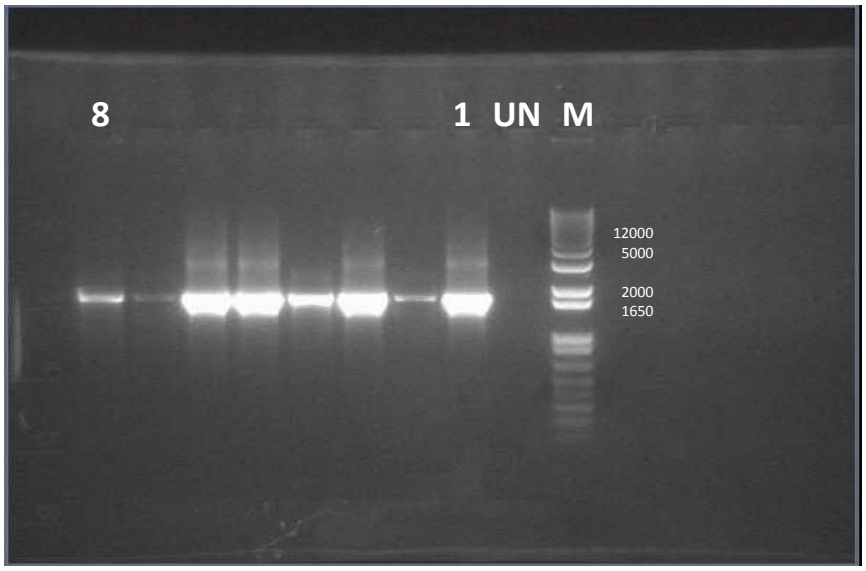


Figure (3-18) Gel electrophoresis of PCR product using *BADH* primers. Lane M 1kb molecular marker, UN untransformed plant cultured on MS medium, lane 1-8 bombarded plantlets with cassette vector on MS medium with selectable agent.

Results showed many regenerated plants with chloroplast integration. Regenerated plants which exhibited chloroplast integration were passed into second and third rounds of selection to become homoplasmy (figure 3-21), and also to ensured that the trait becomes genetically inhereted in the plants. All rounds of selection were carried out under the stress of BA at a concentration of 35 mM.

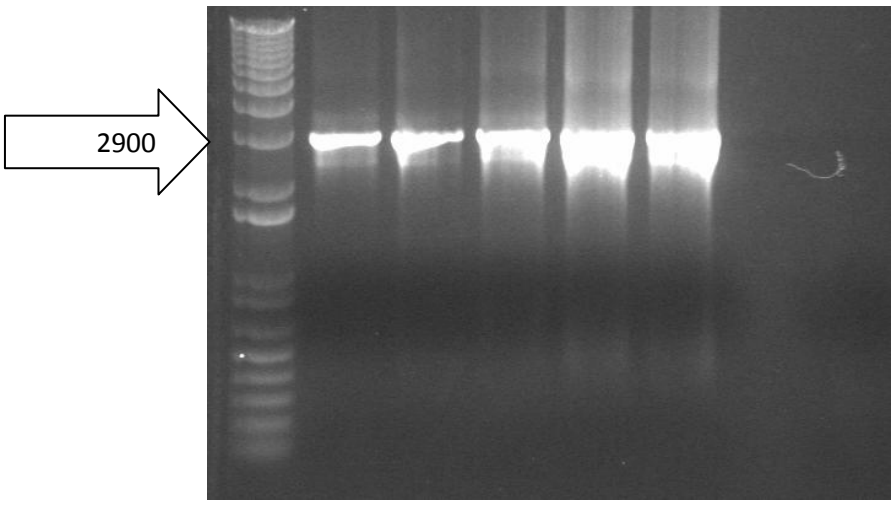


Figure (3-19) agarose gel electrophoresis(0.8%) of PCR product of transgenic plantlets using 16SF and *BADH* internal primers .

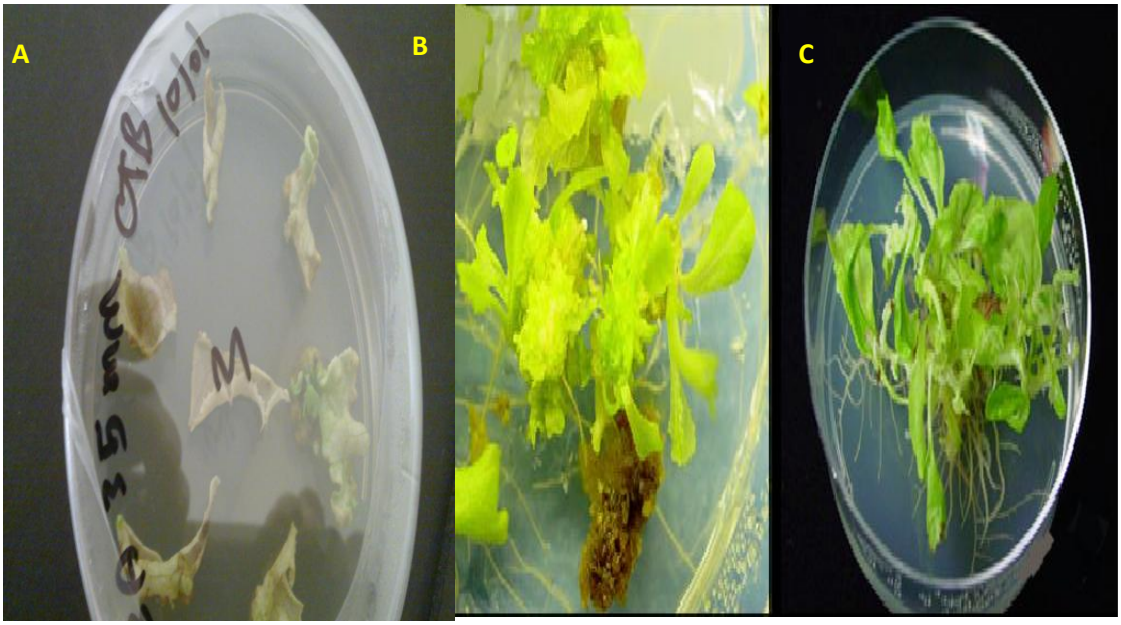


Figure (3-20) The three rounds of selection on MS medium supplemented with 35 mM betaine aldehyde **(A)** first **(B)** second **(C)** third .

Chloroplast transformation strategies have utilized both endogenous and heterologous regulatory elements to facilitate high levels of foreign gene expression. Hybrid systems comprising a modified tobacco (*Nicotiana tabacum*) ribosomal operon promoter (*Prrn*) in conjunction with a translational control region derived from the tobacco plastid-encoded rbcL gene or from bacteriophage T7 gene 10 (g10) to express foreign genes have been utilized in numerous species (Ruhlman *et al*., 2007). Incorporation of foreign DNA is based on homologous recombination between the targeting region of the vector and the ptDNA. The transformation vectors are *E. coli* plasmids that do not replicate in plastids. The marker gene encoded in the vector will be stably expressed only if incorporated in the plastid genome by homologous recombination (Verma and Daniell, 2007).

The choice of the insertion site in the plastome may have a profound effect on the level of protein accumulation. Inserting a transgene in the repeated region of the ptDNA doubles the number of transgene copies per genome, as

compared with insertions in unique regions. Insertion of transgenes between genes of a heavily transcribed operon will further increase the level of translatable mRNA, typically yielding higher protein levels (Daniell *et al*., 2011).

3.8 Homoplasmy Confirmation by Southern Blot

For further confirmation of the transgene integration into the chloroplast genome, and for the determination of homoplasmy, Southern blot analysis was performed. Total DNA extracted from plants in the third round of selection was digested with enzyme *Sma1* (figure 3-22A).

As described in methods, the digested DNA on agarose gel was hybridized with a chloroplast flanking sequence probe (0.8kb). As shown in the figure 3-22B, Wild type plants generated 4.2 kb fragment and transgenic plants generated 4.5 and 2.2 kb fragments.

All transgenic lines appeared homoplasmic (within the levels of detection) which means that all chloroplasts in the plant contained the transgene *BADH-CTB.*

Total plant DNA digested with *Sma1* was also hybridized with a gene specific probe (*CTB*) as shown in figure 3-23. A fragment with 4.5 kb was detected in the transgenic samples confirming correct integration of the entire transgene in the correct spacer region of the chloroplast genome.

No fragment was detected in the wild type plants when gene specific probe was used. When the transformed DNA is introduced on the surface of microscopic particles, only one or a few chloroplasts in a leaf cell may be damaged by the impact, and only a few of the approximately 100 ptDNA copies incorporate the transforming DNA.

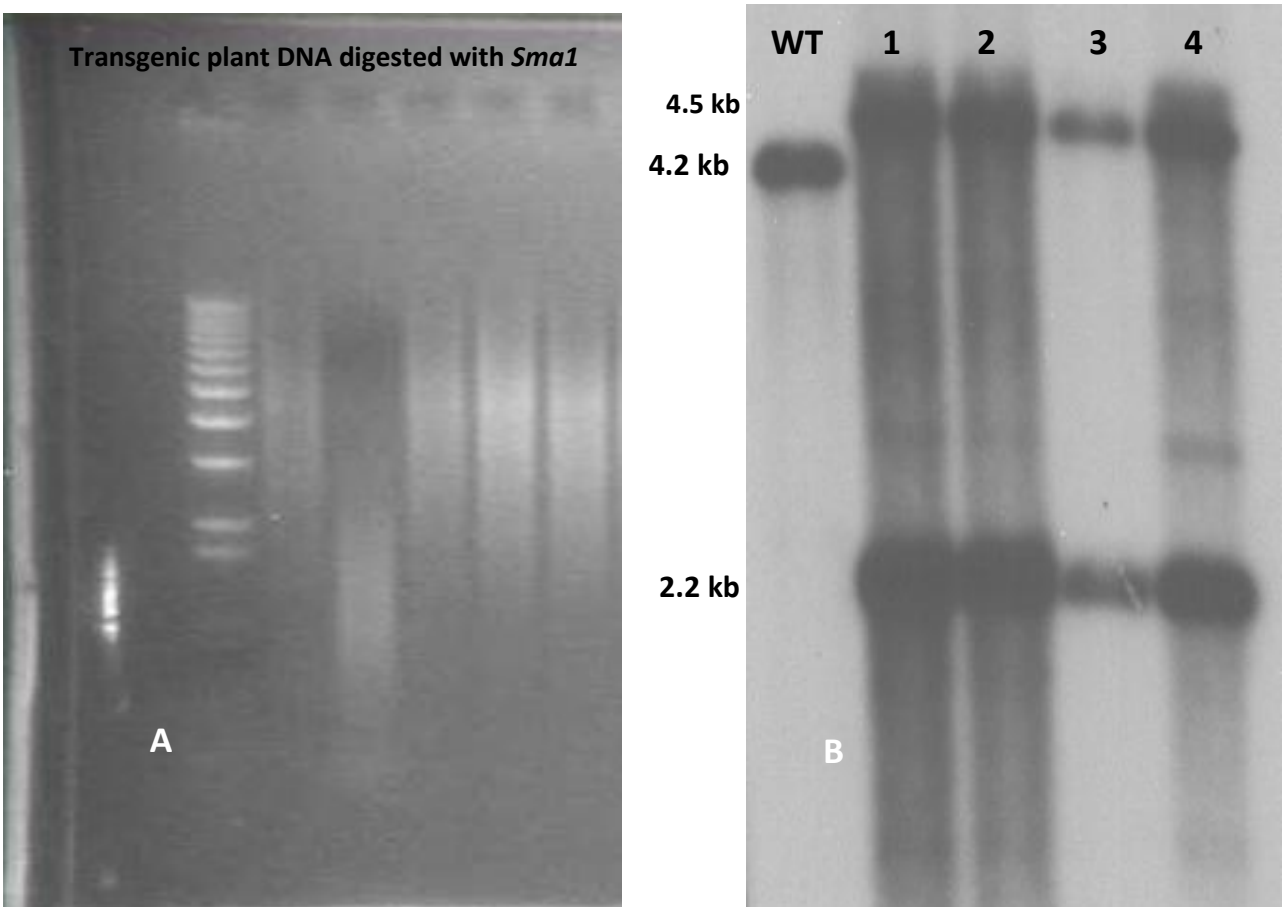


Figure (3-21) Analysis of homoplasmic transformation of lettuce plant **(A)** Gel electrophoresis of total DNA digested with *Sma1* **(B)** Southern blot analysis with flanking sequence probe. Lane WT Wild-type showing 4.2 kb fragment, Lane 1 - 4 Transgenic plants showing 4.5 and 2.2 kb hybridizing fragments.

However, the transforming DNA carries antibiotic detoxifying genes or stress tolerant genes conferring a selective advantage to plastids that carry the T-ptDNA. Because the selective agent can most conveniently be administered in the tissue culture environment, selective enrichment for the T-ptDNA is carried out in tissue culture cells.

The tissue culture medium triggers cell division, yielding meristematic cells with 10 to 14 proplastids, each of which carries only one or two nucleoids. Reduction of plastid number from 100 to 10 to 14 greatly

accelerates plastid sorting during cell division, during which plastids carrying the T-ptDNA are dividing at a faster rate. Plastids carrying only the wild-type ptDNA are ultimately lost by dilution during cell division (De Cosa *et al*., 2001**)**

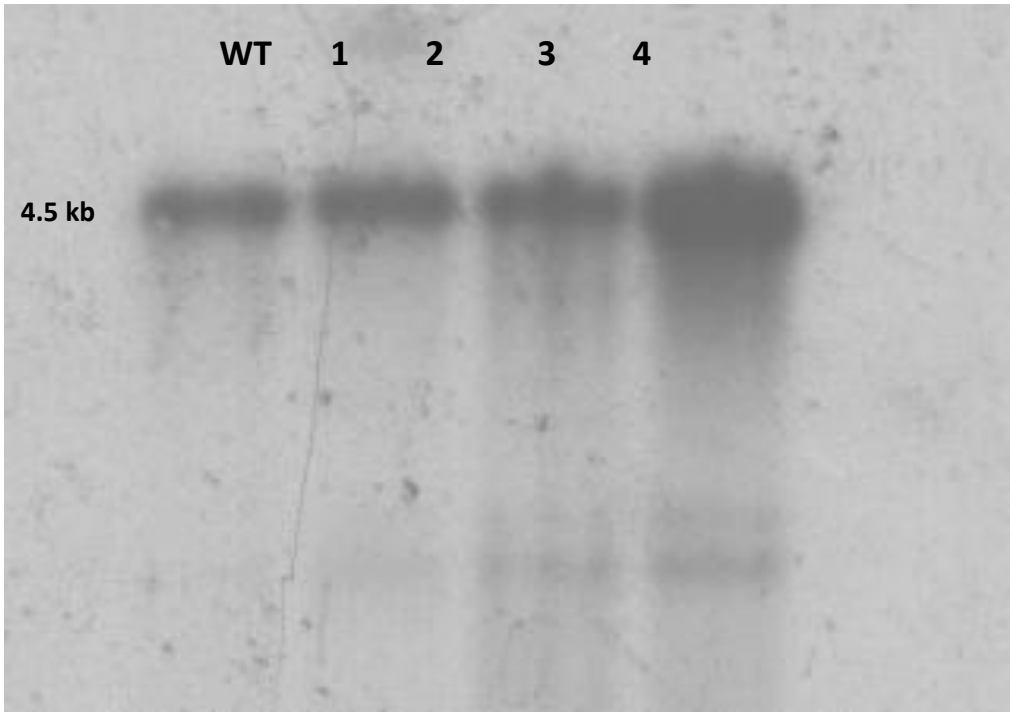


Figure (3-22) Southern blot analysis with gene specific *CTB* probe.
Lane 1 – 4: Transgenic plants showing 4.5 kb fragment, Lane WT: Wild type plant

It is known for all plant biotechnologists that each cell of plant contains about 100 plastids and each plastid contains 10 copies of its own DNA. Biologists explored this feature to make a huge number of copies of foreign genes in chloroplasts. In the case of lettuce gene copy, it can reach up to 2000 copies if the gene is integrated in space between *trn*A*trn*I due to the presence of two homologous sites in each DNA molecule inside plastid and homoplasmy was ensured. Many previous works were done on tobacco and lettuce to produce foreign protein in chloroplast, and homoplasmy was done by the effect of antibiotic stress while in the present work the homoplasmy

was established by salt stress. High copy numbers of a new gene in chloroplast under strong promoter ensures high level of expression of a new proteins by chloroplast genetic engineering (Ruhlman *et al*., 2007).

3.9 Western Blot to Confirm the Gene Expression

Western blot analysis was performed to investigate the expression of the fusion protein CTB-BADH in transgenic lettuce chloroplasts, using anti-CTB antibody. The protein was extracted from the lettuce leaves as described in the materials and methods chapter(2.2.18), its concentration was calculated using standard curve made by bovine serum albumin (BSA) using Bradford method. Results showed that all extracted samples contain protein in amount vary between 0.3-0.8 mg/ml in first dilution of crude extract as detailed in table 3-3. These amounts were estimated according to a standard curve of BSA using absorbance at 595nm (figure 3-24).

Different amounts of total protein were loaded on 12% polyacrylamide gel to show the protein bands, and the resulted bands indicated a new protein band in transformant plant with about 22 KDa (figure 3-25). In comparison with wild type plant, this is an indicator for expression on monomer of CTB protein which has the corresponding molecular mass.

Another gel was prepared to confirm the right expression of inserted genes in crude extracts by western immunoblot with anti CTB antibody as mentioned in the western blot protocol in the materials and methods chapter(2.2.18).

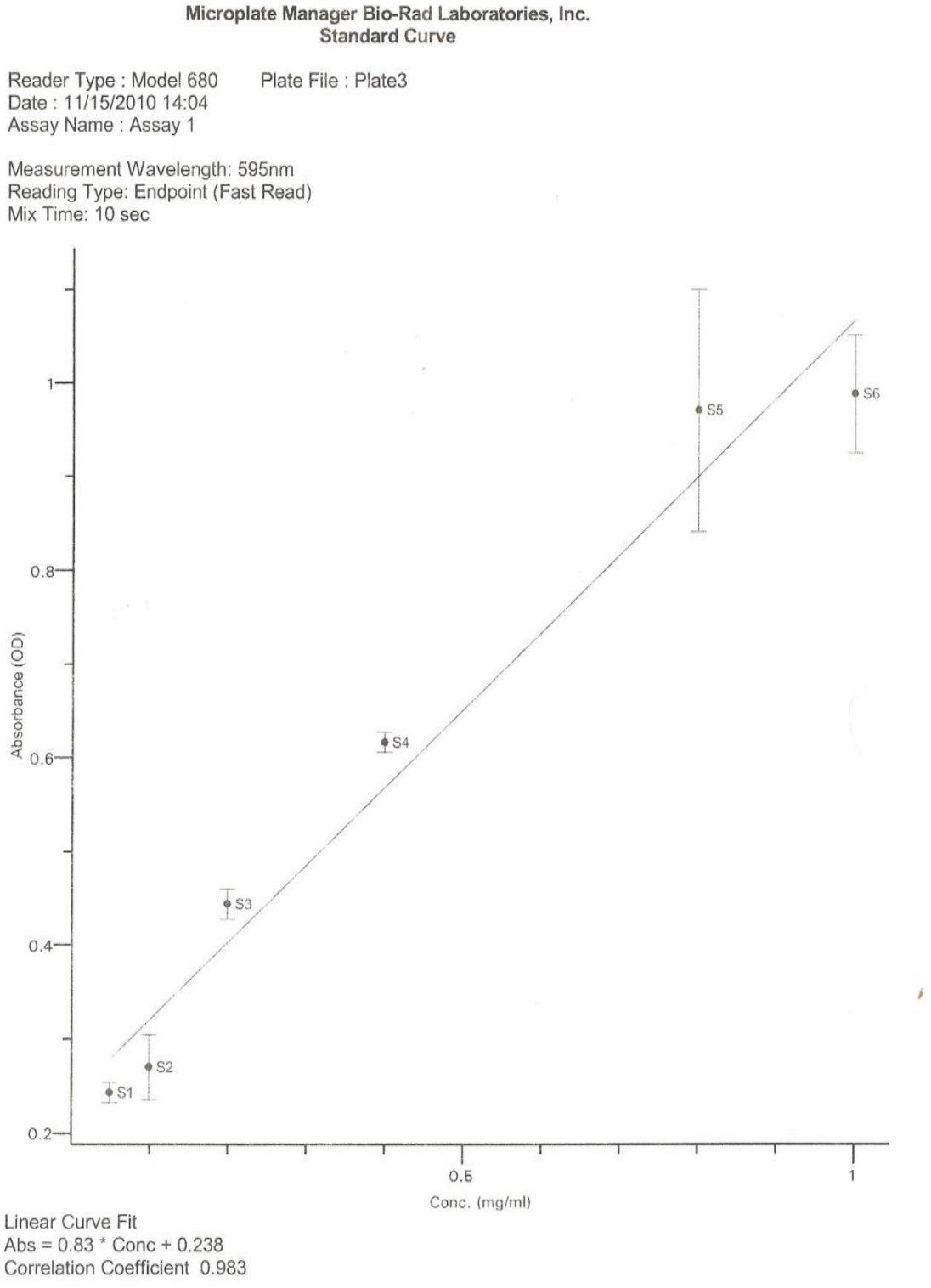
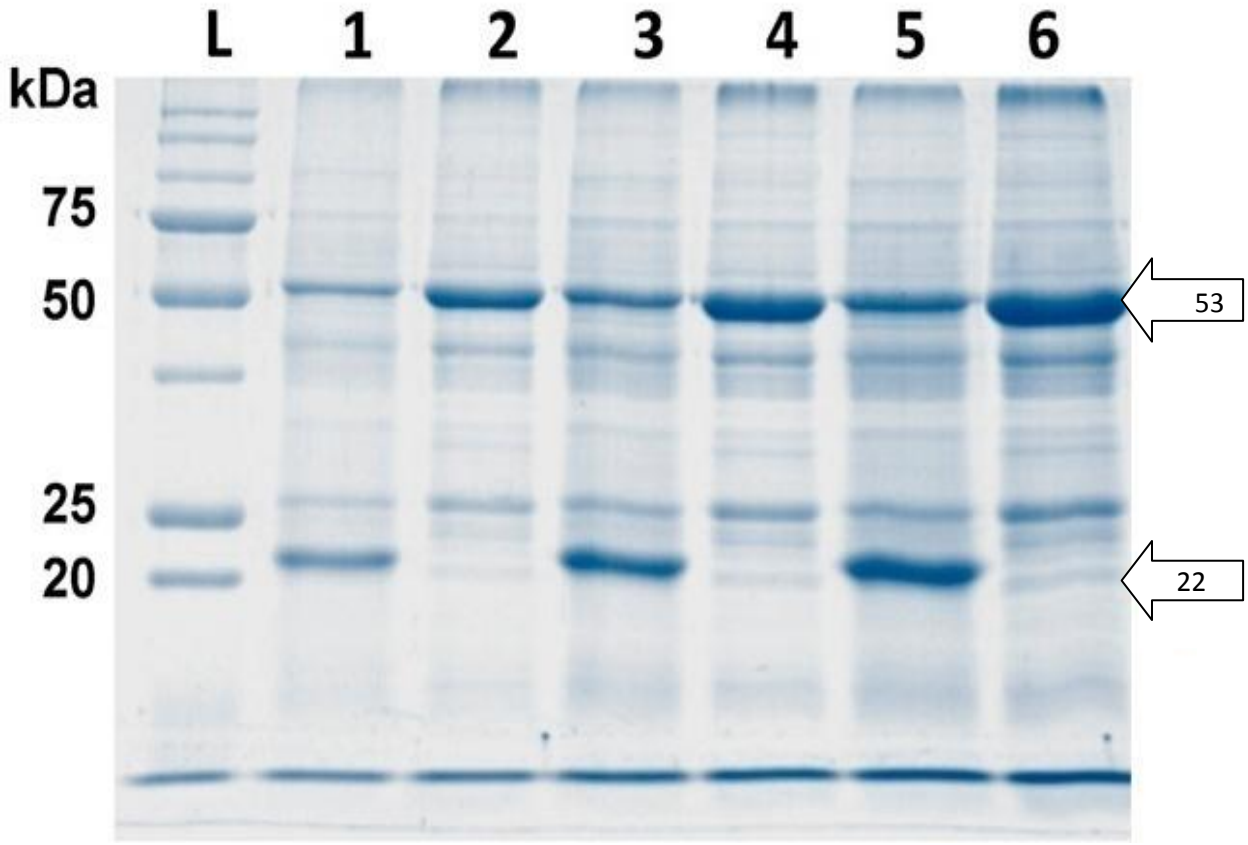


Figure (3-23) Standard curve of bovine serum albumin

Table (3-3) Dilutions and absorbance with concentrations of bovine serum albumin (BSA) and extracted protein

label	OD	Std dev.	Concentration mg/ml	Dilution
S1	0.244	0.011	0.05	
S2	0.271	0.035	0.1	
S3	0.445	0.016	0.2	
S4	0.618	0.011	0.4	
S5	0.972	0.029	0.8	
S6	0.989	0.043	1	
X1	0.295	0.032	0.325	5
X2	0.266	0.011	0.281	10
X3	0.230	0.023	0.118	20
X4	0.345	0.019	0.458	5
X5	0.254	0.014	0.352	10
X6	0.167	0.012	0.322	20
X7	0.483	0.027	0.872	5
X8	0.688	0.016	1.342	10
X9	0.319	0.011	0.639	20
X10	0.465	0.016	0.691	5
X11	0.279	0.022	0.412	10
X12	0.324	0.014	0.553	20
X13	0.472	0.018	0.843	5
X14	0.338	0.011	0.501	10
X15	0.250	0.017	0.281	20
Blank	0.014	0.00		

S1-S6 bovine serum albumin concentration, X1-X15 Dilutions of protein extracts



Figure(3-24) SDS-PAGE stained with Coomassie Brilliant Blue. Lanes 1, 3, and 5 laoded with 10, 20, and 30 ug respectively, of total soluble protein from transgenic leaf, lanes 2, 4, and 6, corresponding amounts of wild type protein extract, lane L, molecular mass standards. Arrow heads indicate positions of CTB (22 kD) and Rubisco (53 kD).

Two different crude extracts were used, boiled and without boiling to explain the presence of CTB-BADH proteins as new proteins appear in the transgenic lettuce. Non-boiled samples showed bands with about 49 kDa which represent primary fusion between CTB and BADH (figure 3-26), while the boiled samples showed about 22 kDa representing CTB protein as explained by the purified CTB on polyacrylamide gel.

After confirmation of the ability of transgenic lettuce to produce CTB and tolerate increased concentrations of salt stress, the plantlets were transferred to green house under the stress of salt. Lettuce was grown in soil

in a green house. The plant performed at 150mM NaCL stress as shown in figure 3-27.

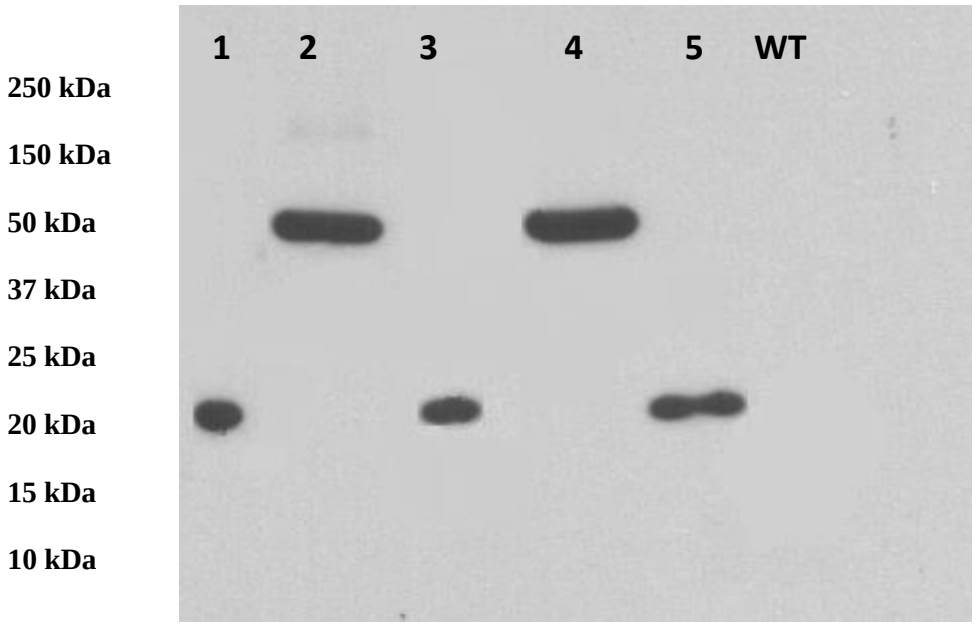


Figure (3-25) Immunoblot analysis of CTB-BADH using anti-CTB antibodies. Lane 1,3 boiled extract showing CTB band, lane 2,4 non-boiled extracts showing CTB-BADH, lane 5 purified CTB standard 200ng, lane WT wild type extract.

Figure (3-26) Transgenic lettuce grown in a green house under salt stress.

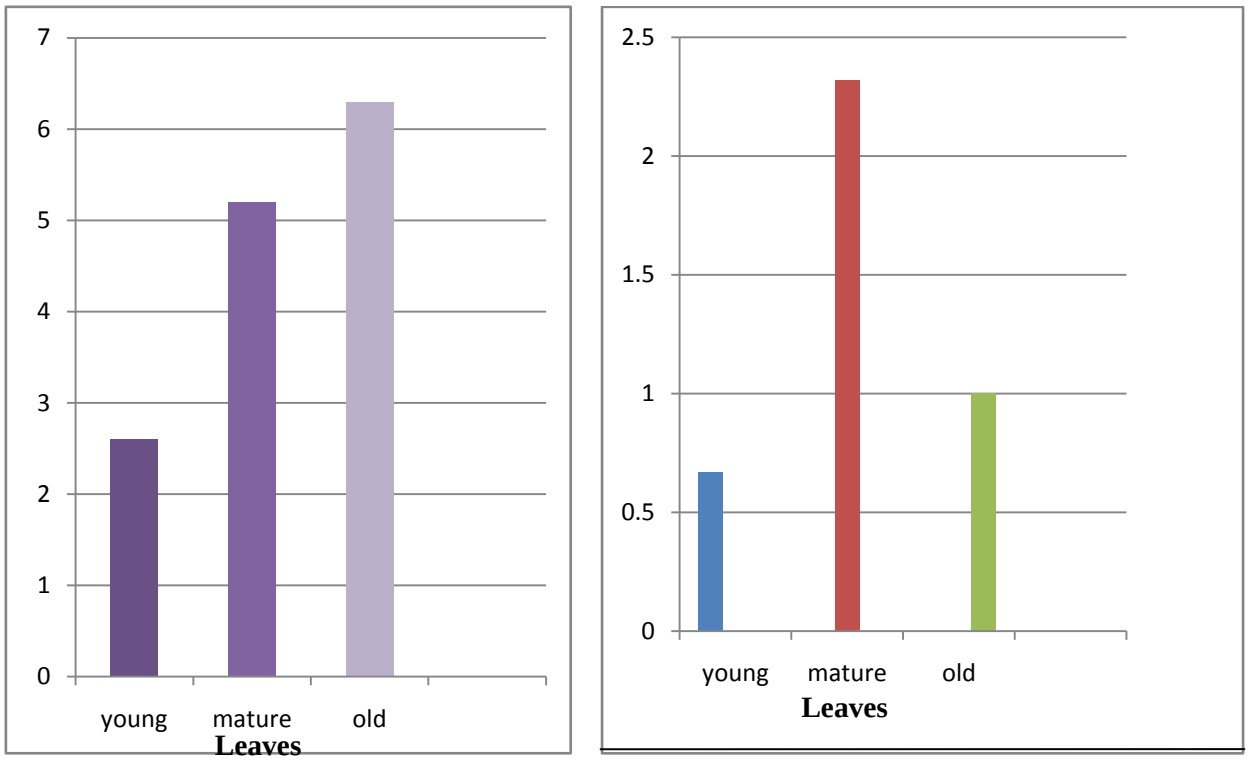
3.10 CTB Quantification

A standard curve was obtained using different dilutions (dilutions were carried out in coating buffer) of purified CTB. Primary antibody used was CTB polyclonal antibodies against CTB, and secondary antibodies were rabbit anti-CTB IgG peroxidase conjugated. The percentage of CTB expressed was as a percent of total soluble protein calculated using the Bradford assay i.e. the CTB percent is inversely proportional to the TSP values. The CTB expression levels reached a maximum of 6.3% of the total soluble protein in the old leaves when compared to 2.6% TSP in young leaves, and 5.2% TSP in mature leaves (figure 3-28A). Maximum CTB expression was observed in the old leaves when compared to the young and mature leaves.

Based on the fresh weight calculations, the amount of CTB obtained from young, mature and old leaves was 0.67mg, 2.32 mg and 1mg per leaf respectively (fig 3-28B). Difference in the CTB expression levels observed when calculating percentages of TSP and based on fresh weight is due to the consideration of total soluble protein. The total soluble protein in old leaves was less when compared to the mature leaves, and this could be due to degradation of the other soluble proteins in comparison to CTB as the leaves grew old.

The expression levels of CTB were higher in mature leaves when compared to old ones. When calculation based on fresh weight, the trend clearly shows higher expression of CTB in the mature leaves, which may be due to more number of chloroplasts and high number of chloroplast genomes (up to 1000 copies/cell) in the mature leaves. Also, the large size and more mature leaves per plant may contributed to the higher levels of CTB in mature leaves. Increased expression levels of several proteins have been attained by expressing foreign proteins in chloroplasts of higher plants. Recently, human somatotropin (7% TSP) and antimicrobial peptides (21% TSP) have been

expressed in transgenic chloroplasts. The accumulation levels of the Bt Cry2Aa2 operon in tobacco chloroplasts were as high as 46.1% of the total soluble plant protein (Singh *et al*., 2009).



(A) CTB from TSB % (B) CTB in mg per leaf

Figure (3-27) Quantification of CTB expression levels in transgenic plants **(A)** Expression levels in %TSP of CTB expressed in Young, Mature and Old leaves under regular conditions **(B)** Amount CTB (in mg) obtained from each of the young, mature and old leaves based on the fresh weight.

Besides the ability to express polycistrons, another advantage of chloroplast transformation is the lack of recombinant protein expression in pollen of chloroplast transgenic plants. Absence of chloroplast DNA in the pollen of most crops reduces pollen-mediated outcross of transgenes. Also, stable incorporation of the *CTB* gene into spacer regions between functional genes of the chloroplast genome by homologous recombination eliminates the (position effect) frequently observed in nuclear transgenic plants. Lack of gene silencing in chloroplasts should allow uniform expression levels in

different transgenic lines. Integration of the transgene into chloroplast genomes should result in a high level of *CTB* gene expression, since each plant cell contains up to 1000 copies of the plastid genomes (Daniell *et al*., 2011).

Similar to the endoplasmic reticulum, the production of CTB in chloroplasts allows formation of disulfide bridges, which are necessary for the correct folding and assembly of the CTB pentamer (DeGray *et al*., 2001).

High levels of constitutive expression of CTB in transgenic tobacco did not affect growth rates, flowering or seed setting, unlike previous reports for the synthetic LTB gene, constitutively expressed via the nuclear genome. Therefore, there is no need to regulate CTB expression in transgenic chloroplasts or express it in specific tissues to overcome pleiotropic effects.

The potential use of the chloroplast technology developed in many studies is threefold, it can be used for (a) large-scale production of purified CTB, (b) as an edible vaccine expressed in an edible plant or (c) as a fusion transmucosal carrier peptide, to either enhance mucosal immunity or induce oral tolerance to the products of these peptides (Agrawal *et al*., 2011)

Large-scale production of purified CTB in bacteria involves the use of expensive fermentation techniques and stringent purification protocols, making this a prohibitively expensive technology for developing countries. The cost of producing 1 kg of recombinant protein in transgenic crops has been estimated to be 50 times lower than the cost of producing the same amount by *E. coli* fermentation. If 5% of the alfalfa soluble protein is the target protein, one could harvest about 15 kg of the transgenic-expressed protein/acre per year. While existing expression levels of CTB via the chloroplast genome are adequate for commercial exploitation, levels could be increased further (up to 50% total soluble protein) by insertion of a putative chaperonin, which may also aid in the subsequent purification of recombinant CTB due to crystallization (Daniell *et al*., 2011).

If used as an edible vaccine, a selection scheme eliminating the use of antibiotic-resistance genes should be developed. One such scheme uses the betaine aldehyde dehydogenase (*BADH*) gene, which converts toxic betaine aldehyde to non-toxic glycine betaine, an osmoprotectant. Other strategies have been developed to eliminate antibiotic-resistance genes from transgenic plants. Development of edible vaccines in transgenic leaves are ideal for animal vaccines. Future development of edible vaccines for human consumption would be more appealing if the vaccines are expressed in edible parts of transgenic plants. Expression of a transgene in chromoplasts has been demonstrated, and the efficiency of translation of foreign genes in chromoplasts is similar to that of chloroplasts. Therefore, it is anticipated that elevated edible vaccine expression in chromoplasts of transgenic fruits is both feasible and forthcoming (Davoodi-Semiromi *et al*., 2009).

Previous studies expressing the CTB protein in plants have been performed in potato (Arakawa *et al*., 1998). The expression level in nuclear transgenic potato tubers was 0.1% of TSP. This low expression level required the feeding of mice with large amounts of fresh potatoes. Ruhlman *et al. (*2007) were expressed the CTB protein in transplastomic tobacco to up to 4% of TSP, 40-fold greater than that achieved in nuclear transgenic potatoes. Mice were given 8 mg of CTB tobacco leaf tissue (containing 14 μg of the protein) per week, this represents a 175-fold decrease in the amount of plant tissue administered compared with the 3 g per week used previously (Arakawa *et al*., 1998). The use of these small concentrated doses reduces the possibility of the potential confounding effects of leaf tissue, and eliminates the need to process or purify large quantities of plant material. Hyperexpression of CTB in plant plastids should make this fusion protein abundantly available for animal studies or human clinical trials.

The levels of CTB accumulation in transplastomic lettuce in the present study were observed to be higher than previous studies of Ruhlman *et al*.

(2010), the average value determined for lettuce (6.2% of TSP) represents a level of protein sufficient to proceed with animal or preclinical studies

The difference observed between tobacco and lettuce may be attributed, in part, to the 5′ regulatory elements used in our study. Lettuce expression of CTB is driven by the endogenous *psbA* 5′ UTR, whereas tobacco expression is regulated by the inclusion of the translational control region of bacteriophage T7 gene 10. Previous studies have demonstrated that the level of foreign protein accumulation is lower when these translation elements are used to drive expression of the same gene, with *psbA* 5′ UTR being more efficient (Dhingra *et al*., 2004). In addition, intrinsic variation in the nature of the leaves from tobacco and lettuce may influence the accumulation of foreign protein expressed in chloroplasts(Verma and Daniell , 2008). We here developed new transformation constructs for CTB expression which employed lettuce endogenous translation elements, *psbA* 5′ UTR, to further increase the level of expression.

Chapter Four
Conclusions and Recommendations

4.1 Conclusions

1.A pioneer study was conducted to express CTB antigen in an edible plant via chloroplast genetic engineering.

2.Antibiotics selectable markers were replaced by safe selectable marker (BADH) expressing salt and drought resistance.

3.Chloroplast genetic engineering is sufficient in expressing new foreign protein (quantity and quality) in plants.

4.Use of gene gun technology as gene transfer method produced high number of transgenic plants.

5.Salt and drought resistance in lettuce depends on several mechanisms, one of them is production of osmoprotectant material like betaine aldehyde to overcome high salanity.

6.Use of regulatory elements (*rbcl*, *Ppsp*A, *Tpsp*A) and strong promoter (*Prrn*) in cassette vectors is essential for efficient production of CTB and BADH in lettuce plant.

7. The chloroplast integration of *CTB* was confirmed by PCR and homoplasmy was confirmed by Southern blot analysis. The expression of CTB, a 22 kDa protein in transgenic chloroplasts was confirmed by immunoblot analysis using anti- CTB antibody. Maximum accumulation of the CTB protein was up to 6.2% of TSP which was observed in the old leaves.

4.2 Recommendations

1.Development of other edible transgenic plants which carry *CTB* gene like carrot root and attempting to express this protein in chromoplasts.

2.Using CTB protein in immunological studies to test its ability to stimulate immune response in mice.

3.Study the expression of another therapeutic proteins for another disease using chloroplast genetic engineering.

4.Exploring chloroplast genetic engineering to produce transgenic plants able to remove environmental pollutions.

5.Desgining new chloroplast vectors with new promoters and regulatory elements to improve the production of new traits.

6.Transforming plants and other organisms with mitochondrial vector using gene gun technology.

7.Using molecular marker to find new genes for stress tolerance to explore them as selectable markers for transgenic plants.

8.Making fusion between CTB and other therapeutic proteins to enhance immunity for more than one disease in one transgenic plant.

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الخلاصة

نبات الخس من النباتات المهمة في كل أنحاء العالم، وهو نبات مرشح لحمل اللقاحات للإنسان كونه من النباتات المأكولة. يعد الجزء B من سم بكتريا الكوليرا *Vibrio cholera* المسببة لمرض الكوليرا من البروتينات المرشحة لتكون لقاح ناجح للانسان. صممت الدراسة الحالية لنقل جين الكوليرا B(*CTB*) الى البلاستيدات الخضراء لنبات الخس لانتاج لقاح ماكول. نميت بذور الخس في وسط Murshige and Skoog, (MS) بنصف القوة بعد ان عقمت البذور سطحيا واعطت نسبة إنبات عالية. طبقت تقانة الزراعة النسيجية في الاخلاف المباشر لاجزاء اوراق الخس بعد زراعتها على وسط MS ذو القوة الكاملة الحاوي على منظمي النمو NAA بمقدار 0.1 ملغم/لتر وBAP بمقدار 0.2 ملغم/لتر. ثبت مقدرة أجزاء الاوراق على الاخلاف المباشر بوجود منظمي النمو اعلاه. اختبرت مقدرة النبات المدروس على تحمل ملح NaCl ومركب البيتايين الديهايد BA تحت ظروف الإخلاف وتبين ان لاوراق نبات الخس المزروعة على وسط ملائم القدره على تحمل 75 ملي مولر و 10 ملي مولر من كلا المادتين وعلى التوالي. وظفت تقانة تفاعل سلسلة البوليميريز Polymerase Chain Reaction (PCR) للحصول الجينات المطلوبة لانشاء ناقل جيني جيد باستعمال بادئات جينية مختلفة وحسب الغرض من التفاعل. عزل جين لقاح الكوليرا (*CTB*) وجين تحمل الملوحة (*BADH*) والباديء الخاص بالبلاستيدات (*Prrn*) كما وعزلت بعض الجينات من نواقل كلونة سابقة مستخدمة في مختبر البايولوجي الجزيئي في كلية الطب التابعة لجامعة سينترال فلوريدا في الولايات المتحدة الأمريكية. استثمرت كل الجينات المعزولة بطرق مختلفة لبناء ناقل استنسال ملائم pLS-BADH-LS-CTB باستخدام تقنيات متعددة للربط وتقنيات اتجاه الجينات باستعمال الانزيمات القاطعة وتسلسل الجينات. استعمل هذا الناقل لنقل جيني تحمل الملوحة و لقاح الكوليرا الى البلاستيدات الخضراء لنبات الخس. كانت نسبة التحول للنبات عالية باستخدام طريقة مسدس الجينات اذ وجد ان افضل بعد لورقة النبات المدروس عن مصدر اطلاق الجينات كانت 6 سم وافضل ضغط لغاز الهيليوم المستخدم 900 باوند /انج مربع وأعطت نسبة تحول وصلت الى 10% نبات متحول من بين الاجزاءالنباتية المزروعة على وسط MS الحاوي على عامل انتخابي BAبتركيز 35 ملي مولر كما وجد ان افضل عمر للنبات لعملية التحول كان عند عمر 40 يوم من زراعته على الوسط الغذائي MS. يعد CTB من البروتينات المرشحة كلقاح ضد مرض الكوليرا وفي هذا الدراسة تم نقل جيني *CTB* و

BADH إلى البلاستيدات الخضراء لنبات الخس وتم اختبار النبات المهندس وراثيا من خلال انماءه على وسط MS حاوي على العناصر الانتخابية BA و NaCl وبدون اضافة مضادات الحيوية كعناصر انتقائية للنبات المتحول وراثيا. نتج عن عملية اندماج الجين الاصلي لبروتين الكوليرا الممنع CTB في البلاستيدات الخضراء لنبات الخس (اكثر من 100 بلاستيده في الخلية الواحدة للنبات كل بلاستيدة خضراء تمتلك 100 مجين يمكن ان يحمل الجينات) اعطاء نسبة عالية جدا من التعبير الجيني وصلت الى 6.2 % من وزن البروتين الكلي الذائب وهي نسبة عالية جدا مقارنة بالتعبير الجيني لنفس البروتين في DNA النواة بحيث وصلت النسبة الى 620 مرة اكثر في التعبير الجيني في البلاستيدة الخضراء مقارنة بالتعبير الجيني للنواة. استخدمت تقنيات متعددة مثل تفاعل استطالة السلسلة ووصمة ساوذرن لتاكيد الاندماج الفعلي لناقل الاستنسال في البلاستيدة الخضراء للنبات المدروس كما واستخدمت وصمة ساوذرن لتاكيد الاندماج بالشكل الصحيح وفي الموضع الصحيح من البلاستيدة الخضراء. فيما اكدت النتائج المستحصلة من وصمة ويسترن عن التعبير الجيني للجينات المنقولة وتاكيد مطابقتها لبروتين CTB المستخلص من البكتريا المسببة لمرض الكوليرا. أظهرت النباتات المتحولة وراثيا قدرتها على تحمل 150ملي مولر و 35 ملي مولر من كل من NaClو BA على التوالي عند زراعتها في البيت الزجاجي. اظهرت النتائج ايضا استقرار الجينات المنقولة في الاجيال المتتابعة الناتجة من النبات المتحولة وراثيا وذلك بتحليل النباتات المتعاقبة باستخدام تفاعل استطالة السلسلة ووصمة ساوذرن. إن عملية إنتاج CTB في النباتات كلقاح وكحامل لبروتينات مناعية أخرى يمكن ان يكون مفتاح لإنتاج لقاحات كاملة ماكولة في النباتات كما ان توفر نباتات خالية من الجينات الانتقائية الضارة والحفاظ على التركيب الرباعي للبروتينات في النباتات يجعل عملية إنتاج لقاحات مأكولة تطبيقية وقابلة للانتشار.

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إلى من أضاء لي طريق العلم والمعرفة ، إلى من له الفضل في وضعي بأول الطريق.... أبي

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أحمد

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

اللَّهُ الَّذِي رَفَعَ السَّمَاوَاتِ بِغَيْرِ عَمَدٍ تَرَوْنَهَا
ثُمَّ اسْتَوَى عَلَى الْعَرْشِ وَسَخَّرَ الشَّمْسَ
وَالْقَمَرَ كُلٌّ يَجْرِي لِأَجَلٍ مُسَمًّى يُدَبِّرُ الْأَمْرَ
يُفَصِّلُ الْآيَاتِ لَعَلَّكُمْ بِلِقَاءِ رَبِّكُمْ تُوقِنُونَ (2)
وَهُوَ الَّذِي مَدَّ الْأَرْضَ وَجَعَلَ فِيهَا رَوَاسِيَ
وَأَنْهَارًا وَمِنْ كُلِّ الثَّمَرَاتِ جَعَلَ فِيهَا
زَوْجَيْنِ اثْنَيْنِ يُغْشِي اللَّيْلَ النَّهَارَ إِنَّ فِي ذَلِكَ
لَآيَاتٍ لِقَوْمٍ يَتَفَكَّرُونَ (3) وَفِي الْأَرْضِ قِطَعٌ
مُتَجَاوِرَاتٌ وَجَنَّاتٌ مِنْ أَعْنَابٍ وَزَرْعٌ
وَنَخِيلٌ صِنْوَانٌ وَغَيْرُ صِنْوَانٍ يُسْقَى بِمَاءٍ
وَاحِدٍ وَنُفَضِّلُ بَعْضَهَا عَلَى بَعْضٍ فِي الْأُكُلِ
إِنَّ فِي ذَلِكَ لَآيَاتٍ لِقَوْمٍ يَعْقِلُونَ(4)

صدق الله العظيم

الرعد 2-4

جمهورية العراق
وزارة العلمي والبحث العالي التعليم
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

انتاج لقاح مأكول من نبات الخس *Lactuca sativa* المتحمل للملوحة والمتحول بجين سم الكوليرا B Cholera Toxin B

أطروحة دكتوراه

مقدمة الى مجلس كلية العلوم ،جامعة النهرين وهي جزء من متطلبات نيل درجة دكتوراه فلسفة علوم في التقانة الاحيائية

من قبل

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