Towards generation of transplastomic plants expressing antigens against human pathogens, for use as vaccines

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ABSTRACT

Chloroplast transformation has gained strong interest in regard to production of vaccines in plants. Numerous advantages are pledging for the expression of vaccine antigens through the chloroplast. Environmental friendliness, the possibility of multiple transgene expression, precision of transgene integration and lack of epigenetic effects are the main advantages provided by plastid transformation. Due to economic limitations in the availability of vaccines against infectious diseases, it is necessary to develop improved vaccines which are more efficient and cost effective and hence, easily affordable to most patients in resource-poor countries.

To reach this goal, the current study has investigated the generation of transplastomic plants expressing antigens against two pathogens: Human Papilloma virus (HPV) and *Mycobacterium leprae.* A modified HPV L1 gene with GST as a fusion protein was transformed into tobacco plastids. Expression of this recombinant protein was shown with an antigen capture ELISA by binding of conformation-specific antibodies. This assay confirmed correct assembly of the GST-L1 fusion protein which is necessary for positive immune reaction in future use. To test if the plants are completely transformed, they were examined by Southern Blot analysis. This test confirmed homoplasmy of all transformants in all three generations T_0-T_3.

The second transformation was performed with a vector covering the *mmp*I gene encoding a 35kDa protein which confers cross-protection against leprosy and *Mycobacterium avium*. The plant material was tested by PCR positively; however, the transformants did not reach homoplasmic state. One explanation for heteroplasmy could be a putative detrimental effect of constitutive expression of *mmp*I which might counter-select against transformed plant cells. To solve these problems of counter-selective transgenes it was considered to express them only after the plants have passed their sensitive regeneration phase. For this purpose a chemical induction system was tested which allows regulation of transgene expression according to a time schedule. This alternative was analyzed by expression of the GUS reporter gene under control of the promoter for a nuclear-encoded, chloroplast imported, ethanol-inducible T7 RNA polymerase in transplastomic plants. The MUG test confirmed inducible protein expression after application of 0.5% ethanol onto transformed plants. This result indicates the trans-activation system can later be used for the regulated expression of *mmpI*. Taken together, this data contribute another step forward towards the development of chloroplast-derived antigen expression to produce cost-efficient and easily available vaccines in low-income countries.

KURZFASSUNG

Die Technologie der Chloroplasten-Transformation hat auf dem Gebiet der Produktion von Impfstoffen in Pflanzen starkes Interesse gewonnen. Vielfältige Vorteile sprechen für die Expression von Vakzin-Antigenen in Chloroplasten: Deren Umweltfreundlichkeit, die Möglichkeit mehrere Transgene zu exprimieren, die Präzision der Transgen-Integration sowie das Fehlen epigenetischer Effekte sind die wichtigsten Vorteile dieser Technologie. Aufgrund ökonomischer Beschränkungen in der Verfügbarkeit von Impfstoffen gegen Infektionskrankheiten besteht die Notwendigkeit, verbesserte Vakzine zu entwickeln, welche effizienter und preiswerter, und damit für die meisten Patienten in einkommensschwachen Ländern leichter leistbar sind.

Um dieses Ziel zu erreichen, untersuchte die vorliegende Studie die Herstellung transplastomer Pflanzen zur Expression von Antigenen gegen zwei Erreger: Das Humane Papilloma Virus (HPV) und *Mycobacterium leprae*. Ein modifiziertes HPV L1 Gen mit GST als Fusionsprotein wurde in Tabak-Plastiden transformiert. Die Expression dieses rekombinanten Proteins wurde mittels Antigen Capture ELISA gezeigt, indem konformationsspezifische Antikörper am GST-L1 Fusionsprotein binden. Dieser Assay bestätigte das korrekte Assembly, welches die Voraussetzung für eine positive Immunreaktion in einer zukunftigen Nutzung ist. Um zu testen, ob die Pflanzen komplett transformiert sind, wurden sie in einer Southern Blot Analyse untersucht. Dieser Test bestätigte die Homoplasmie aller Transformanten in allen drei Generationen T_0-T_3.

Die zweite Transformation wurde mit einem Vektor durchgeführt, welcher das *mmp*I Gen beinhaltet. Dieses codiert ein 35kDa Protein, welches gegen *Mycobacterium leprae* und *M. avium* immunisiert. Das Pflanzenmaterial wurde mittels PCR positiv getestet; allerdings erreichten die Transformanten nicht ihr homoplastomes Stadium. Eine Erklärung für deren Heteroplasmie könnte ein möglicher schädlicher Effekt der konstitutiven Expression von MMPI sein, welcher einen Gegenselektionsdruck gegen transformierte Pflanzenzellen aufbauen könnte. Um derartige Probleme von gegen-selektiven Transgenen zu lösen, kommt in Betracht, solche Transgene erst dann zu exprimieren, nachdem die Pflanzen ihre sensitive Regenerationsphase überwunden haben:

Zu diesem Zweck wurde ein chemisches Induktionssystem getestet, welches die Regulation der Transgenexpression zu einem gewählten Zeitpunkt erlaubt. Diese Alternative wurde mittels Expression des GUS Reportergens in transplastomen Pflanzen analysiert. Dieses stand unter Kontrolle des Promoters für eine kern-kodierte, chloroplasten-importierte, ethanol-induzierbare T7 RNA Polymerase. Der darauffolgender MUG Test bestätigte die induzierbare Protein-Expression nach Anwendung von 0.5% Ethanol auf die transformierten Pflanzen. Dieses Ergebnis zeigte, daß das trans-Aktivierungssystem für die regulierbare Expression des mmpI genutzt werden könnte. Zusammengenommen tragen diese Ergebnisse einen weiteren Schritt dazu bei, mittels der Entwicklung von chloroplasten-exprimierten Antigenen kosten-effiziente und einfach verfügbare Impfstoffe in Ländern mit niedrigem Einkommen zu produzieren.

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CHAPTER 1
INTRODUCTION

1.1 Plants for pharmaceutical production

Since more than 6 millennia man is using plants as medicines to treat a wide range of diseases **(**Pasquale 1984). For the last two decades plants do offer the unique opportunity to be engineered as bio-factories for the production of different antibodies, enzymes, hormones and vaccines (Hiatt et al. 1989). The ability of plants to produce complex proteins in an efficient, safe and economical way is the reason why biopharming is gaining an increasing importance in plant biotechnology. The world health organization (WHO) recognizes plant made vaccines as a promising avenue to ensure the widest availability of vaccines in resource poor countries with lower production costs and less need of medical personnel to administer the vaccines in case of oral vaccines (WHO 2005). Plants require only water, sunlight, carbon dioxide and some nutrients, but in return they provide a valuable system for production of recombinant proteins **(**Cunningham and Porter 1998). Plants are the most efficient producers of biomass and proteins on the whole earth and in comparison to all existing methods of production, they have definitely the most advanced ability to produce complex protein molecules that can be used to manufacture effective therapeutics. The two most prominent processes for transgene expression in plants are the transient expression and the stable expression system. The transient system involves viral vectors or in some cases bacterial vectors to insert the foreign gene into the host plant cell (Komarova et al. 2010). This foreign gene is then expressed for a short time span, while the stable transformation system involves insertion of the gene of interest (*goi*) in the host plant genome. This in return will result in the maintenance of the *goi* in the host genetic progeny over multiple generations (Sainsbury and Lomonossoff 2008).

The technology of vaccine and therapeutic protein production in plants is yet very young. Only few years ago Barta et al. (1986) produced a human growth hormone in tobacco and sunflower to understand mammalian RNA processing signals in plants. In 1992 the first antigen for hepatitis B virus was expressed in tobacco (Mason et al. 1992). Growing vaccines in fields has remarkable advantages in comparison to conventional fermenters. It is a cheap

and efficient production system with high scalability, very low upstream costs, minimum of human or animal pathogens risk, and capability to produce required proteins with desired structures and biological functions (Yusibov et al. 2011). Food delivered subunit vaccines are engineered to contain single antigens but do not bear genes that would enable the formation of a whole pathogen. Additionally bacteria undergo numerous cycles of genetic changes during the fermentation process. Plant genes also go through recurrent multiplication but are exposed to far less stress than microbes in fermenters and are therefore extremely stable (Shuler and Kargi 2001).

1.2 Suitable plants for plant-derived vaccine production

A key issue in transgenic plant vaccine production is the selection of suitable plant species, which can be easy to transform, express high levels of a chosen vaccine candidate and suitable for extensive storage (Daniell et al. 2005b). An assortment of strategies for the best output of vaccines from plant sources includes the use of strong and organ-specific plant promoters, targeting of the protein to a specific compartment in the cell for e.g. mitochondria, endoplasmic reticulum (ER), plastids etc. Successful development of vaccine antigens against human or animal pathogens in plants also requires selection of one or more immuno-protective antigens i.e. cross protective antigen vaccines (Ohara et al. 2000, 2001).Various transformation methods are then utilized for introducing the gene to the target plant species. An optimal plant for vaccine production should have the following characteristics (Tiwari et al. 2009):

a) Amenability to transformation
b) Cell compartment of the plant which is to be targeted should be rich in proteins
c) Targeted cell compartment should not contain any toxic molecules
d) Cell compartment should allow correct folding of the antigen protein and post translational modifications.

Tobacco (*Nicotiana tabacum*) is one of the few plants that can address the technical and regulatory consideration of biopharmaceutical industry. Tobacco is an easily transformed plant and it has been the model system for plant transformation for a long time (Horsch et al. 1985). It is a good model system for evaluating the production of recombinant proteins, low

cost preserving system, easy to purify, high level of harvesting (Webster et al. 2002). A single tobacco plant can produce millions of seeds and single acre of cultivated tobacco can produce about forty metric tons of leaves per year (Cramer 1999, Arlen et al. 2007). In addition to the protection presented by maternal inheritance the tobacco leaves are harvested before flowering which offers almost complete transgene containment. Tobacco is a non food and non feed crop, which ensures that a transformed line expressing a highly potent drug will not cause any threat to human or animals in the environment (Fischer and Schillberg 2004). With these prominent features, there is a major role for tobacco as a vehicle for the production of recombinant proteins.

In 1997 for the first time Tacket et al. presented the concept of vaccines from transgenic plants and since then considerable developments have been made in the area (Walmsley and Arntzen 2000, Fischer and Emans 2000). A variety of foreign proteins including serum albumin, human alpha-interferon, human erythroprotein and murine IgG and IgA immune globulin have been effectively expressed in plants (Tamas 2010). Novel efforts have been made to produce various antigens in plants and these plant made vaccines can be administered orally as a part of the plant, intramuscularly or as intravenous injection after isolation and purification from the plant tissues (Roy et al. 2010). Until 2010 the production of more than 60 antigenic and 30 biopharmaceutical proteins were to be expressed and were reported in about 200 scientific papers. (Daniell et al. 2009). A wide range of plant made antibodies, vaccines and therapeutics are at the moment in clinical development and some are even on market.

Table 1. Plant based vaccines, antibodies and therapeutic proteins in clinical development or on market (Yusibov et al. 2011)

Product	Plant Host	Expression System	Indication	Product Development Stage
Vaccines				
E.coli l-TB	Potato	Transgenic	Diarrhea	Phase 1
	Maize	Transgenic		Phase 1
Norwalk virus CP	Potato	Transgenic	Diarrhea	Phase 1
HBsAg	Potato	Transgenic	Hepatitis B	Phase 1
	Lettuce	Transgenic		Phase 1
Rabies virus GP/NP	Spinach	Transient (viral vector)	Rabies	Phase 1

Newcastle disease virus HN	Tobacco cell suspension	Transgenic	Newcastle disease (poultry)	USDA approved (not marketed)
Personalized antiidiotype scFVs	*Nicotiana benthamiana*	Transient (viral vectors)	Non-Hodgkin's lymphoma	Phase 1
Personalized antiidiotype dcFVs	*Nicotiana benthamiana*	Transient (magniCON vectors)	Non-Hodgkin's lymphoma	Phase 1 (ongoing)
H5N1 influenza HA VLP	*Nicotiana benthamiana*	Transient (agrobacterial binary vector)	H5N1 "avian" influenza	Phase 1 (ongoing) Phase 2 (Health Canada approved and enrolling volunteers)
H5N1 influenza HAl1	*Nicotiana benthamiana*	Transient (launch vector)	H5N1 "avian" influenza	Phase1(initiation in fall 2010)
H1N1 influenza HAC1	*Nicotiana benthamiana*	Transient (launch vector)	H1N1 "swine" influenza	Phase 1(ongoing)
Antibodies				
Anti-CD20	Duckweed	Transgenic	arthritis	Pre Clinical
Anti-Streptococcus surface antigen i/ii	Tobacco	Transgenic	Dental caries	Phase 2; EU approved
Anti-αCCr5	*Nicotiana benthamiana*	Transient vector	HIV	Pre-clinical
Anti-HBsAg scFV	Tobacco	Transgenic	Hepatitis B vaccine purification	On market (in Cuba)
Therapeutic and dietary proteins				
Glucocerebrosidase	Carrot cell suspension	Transgenic	Gaucher disease	Phase 3 (marketing expected in 2011)
Insulin	Safflower	Transgenic	Diabetes	Phase 1/2 (marketing expected)
Gastric lipase	Maize	Transgenic	Cystic fibrosis, pancreatitis	Phase 2 (marketed as analytical reagent)
Lactoferrin	Maize	Transgenic	Gastrointestinal infections	Phase 1 (marketed as analytical reagent)
Intrinsic factor	*Arabidopsis thaliana*	Transgenic	Vitamin B12 deficiency	Phase 2

Subunit vaccination includes formulations comprising protein antigens with adjuvants improving the immunogenicity. Such vaccines have to be capable to stimulate and activate T cell responses (Rodriguez et al. 2003, Holmgren and Czerkinsky 2005). Consumption of plant-based vaccines already allows acquiring immunogenicity against various viral and bacterial diseases. Many studies were focused on viral pathogens. Immunogenicity against a hepatitis antigen was achieved in mice fed with transgenic potatoes developed by groups Thanavala et al. (1995), Richter et al. (2000) and Kong et al. (2001). Successful experiments with plant derived vaccines have also been carried out with Norwalk virus (Mason et al. 1996, Tacket et al. 2000), Rabies (Yusibov et al. 2002), Hepatitis C (Zhou et al. 2006), Rotavirus (Birch et al. 2004), Foot and mouth disease virus (Li et al. 2006), Vaccinia virus (Rigano et al. 2009) and Newcastle disease virus (Sim et al. 2009). Also many bacterial pathogens were targeted. Immunogenicity was achieved in humans by consumption of transgenic potatoes containing enterotoxin subunit B from E. coli (Mason et al. 1998). Potato was also used to achieve protective efficacy towards cholera and enterotoxic E. coli by Yu et al. (2001) and Lauterslager et al. (2001), that in principle is also possible in corn (Streatfield et al. 2000). Tobacco was an important plant to produce foreign protein and even today many scientists use it due to its easy genetic manipulation and considerable growth rate. Tobacco was used to attain immunogenicity against bacterial pathogens like Tetanus (Tregoning et al. 2005), Anthrax (Watson et al. 2004), *Bacillus burgdorferi* (Hennig et al. 2007), *Yersinia pestis* (Arlen et al. 2008).

More recently a variety of recombinant subunit vaccine candidates, therapeutic proteins, and monoclonal antibodies has been produced using the plant genome. Some of these products have been tested in early phase clinical trials, and show safety and efficacy as referred to in Table 2.

Table 2. Recent antigens expressed through the plant nuclear genome that have reported immunogenicity or protection, modified according to Daniell et al. 2009.

Protein product	Expression system	Functional Evaluation	Refs.
Bacterial antigens	Carrots	Immunogenic and protective against CT challenge	Rosales Mendoza, S. et al. (2008)
Enterotoxigenic E. coli Heat labile toxin B subunit (LTB)			
Cholera toxin B subunit (CTB)	Tomato	Immunogenic by oral delivery to mice	Jiang, X.L. et al. (2007)
Viral antigens	Potato	Immunogenic response in humans following oral administration	Thanavala, Y. et al. (2005)
Hepatitis B virus surface antigen (HBsAg)			
Hepatitis B virus surface antigen fused with preS1 epitope	Rice	Immunogenic by intraperitoneal delivery to mice	Qian, B. et al. (2008)
Human group A rotavirus (VP6) protein	Alfalfa	Immunogenic in mice	Dong, J.L. et al. (2005)
Rotavirus (VP7)	Potato tubers	Immunogenic in mice following oral delivery.	Li, J.T. et al. (2006)
SARS–CoV S protein (S1)	Tomato tobacco leaf	Immunogenic to mice following oral administration	Pogrebnyak, N. et al. (2005)
Smallpox recombinant vaccine virus B5 antigenic domain (pB5)	Tobacco and collard leaf	Antibody response in mice immunized parenterally and protects against lethal dose of vaccinia virus	Golovkin, M. et al. (2007)
Japanese encephalitis virus (JEV) envelope protein (E)	Rice	JEV specific neutralizing antibody detected in mice following oral administration	Wang, Y. et al. (2009)
Human vaccines (viral /transient expression)			
Bacterial antigens	Tobacco leaf tissue	Immunogenic and protective in monkeys against Y. pestis following subcutaneous injection	Mett, V. et al. (2007)
Yersinia pestis F1 and LcrV			
Yersinia pestis F1–V antigens	Tobacco leaf	Immunogenic and protection in vaccinated guinea pigs against Y. pestis aerosol challenge	Santi, L. et al. (2006)

Viral antigens	Tobacco leaf	Antibody response in mice immunized parenterally and protective against lethal dose of vaccinia virus	Golovkin, M. et al. (2007)
Smallpox recombinant vaccine virus B5 antigenic domain (pB5)			
Encoding domain III of the dengue 2 envelope protein (D2EIII)	Tobacco	Retains antigenicity and immunogenicity as well as inducing neutralizing antibodies in animals	Kim, T.G. et al. (2008)
HIV entry inhibitors red algal protein griffithsin (GRFT)	Tobacco (TMV)	Active against HIV at picomolar concentrations, directly virucidal via binding to HIV envelope glycoproteins and capable of blocking cell-to-cell HIV transmission	O'Keefe, B.R. et al. (2009)
Pathogenic avian influenza virus (H5N1 subtype)	Tobacco	Immunogenic in mice and ferret and also protects ferrets against challenge infection with virus	Shoji, Y. et al. (2009)
Avian Influenza vaccine	Tobacco	Phase II	Medicago (2010)
Diarrhea	Rice	Oral immunization in mice confirmed	Tokuhara et al. (2010)

With improved plant transformation techniques first successful steps have already been undertaken, but there are some limitations and safety concerns about the use of nuclear transformed vaccines. The technology is limited by very low expression levels of nuclear transformants and in most cases, immunogenic doses cannot be delivered in reasonably small portions. Until 2010 only few plants derived vaccines have reached clinical tests, with most of them failing due to low antibody response.

According to Mason et al. (2002) in order to address these problems we markedly need improvements in plant expression technology and inducible expression of transgenes. One questionable alternative to plant transformation for vaccine synthesis was plant-viral infection so far (Wigdorovitz et al. 1999, Belanger et al. 2000, Yusibov et al. 2002). However, in its first generation this transient system suffered from instability and frequent gene loss (Mason et al. 2002). And above all there is still the serious problem of transgene spread. Most approaches so far were carried out by nuclear transformation bearing a high risk of transgene propagation (Losey et al. 1999, Dale 1999). Out-crossing via pollen mediated gene flow needs to be substantially controlled and reduced (Jank and Gaugitsch 2001).

Currently there are only three reasonable ways to keep the transgenes away from neighbour field plants. The one way is the usage of plants with male sterility as characterised in potato by Lössl et al. (2000). Use of plants that are infertile and clonally propagated could facilitate management of quality control and production of vaccines. The second way is transient expression system for use in green house containment. And the third way to prevent transgene transmission is integration of these transgenes into the chloroplast genome. Valuable improvements can be provided to the current state of transgenic plants by application of the transplastome technology (Svab et al. 1990, Koop et al. 1996, Ruf et al. 2002).

Chloroplast transformation has emerged as a very precise alternative technique. Presence of multiple copies of plastid genome per cell and target specific integration is a huge advantage over nuclear transformation. The use of transplastomic plants addresses the risk of transgene spread by pollen as the chloroplasts are maternally inherited in most plants (Corriveau and Coleman 1988) and therefore pollens don't contain any transgenes.

1.3 The Chloroplast and its genome

A unique advantage of plants over animals is their capability to convert solar light into energy rich molecules and the plastid type which is necessary for this characteristic is called chloroplast. Plastids are double membraned intracellular organelles that contain their own DNA as well as transcription and translation machinery. Plastids can be differentiated in to several different types; some of them are proplastids, pigment carrying plastids, amyloplasts and elaioplasts.

According to the endosymbiont theory chloroplasts are derived from cynobacteria engulfment, a process which took million of years and evolved into the compartmentalization of the reactions of photosynthesis and biosynthesis of starch, amino acid, lipid and pigment in higher plants (Maliga 2004, Lopez and Pyke 2005). Similarly to the evolution of mitochondria, which are thought to originate from engulfment of proteobacteria, the endosymbiotic uptake of the chloroplast cyanobacterial antecedent was accompanied by a slowly increasing division of labor between host cell and the acquired organelle followed by an abolition of redundant and dispensable genes. In contrast to a cyanobacterial genome which encodes about 3200 genes, an average higher plant chloroplast genome encodes almost

130 genes, which indicates a drastic reduction as well as a reorganization of information storage during evolution (Bock 2006). From its bacterial origin chloroplasts have still retained many useful prokaryotic features.

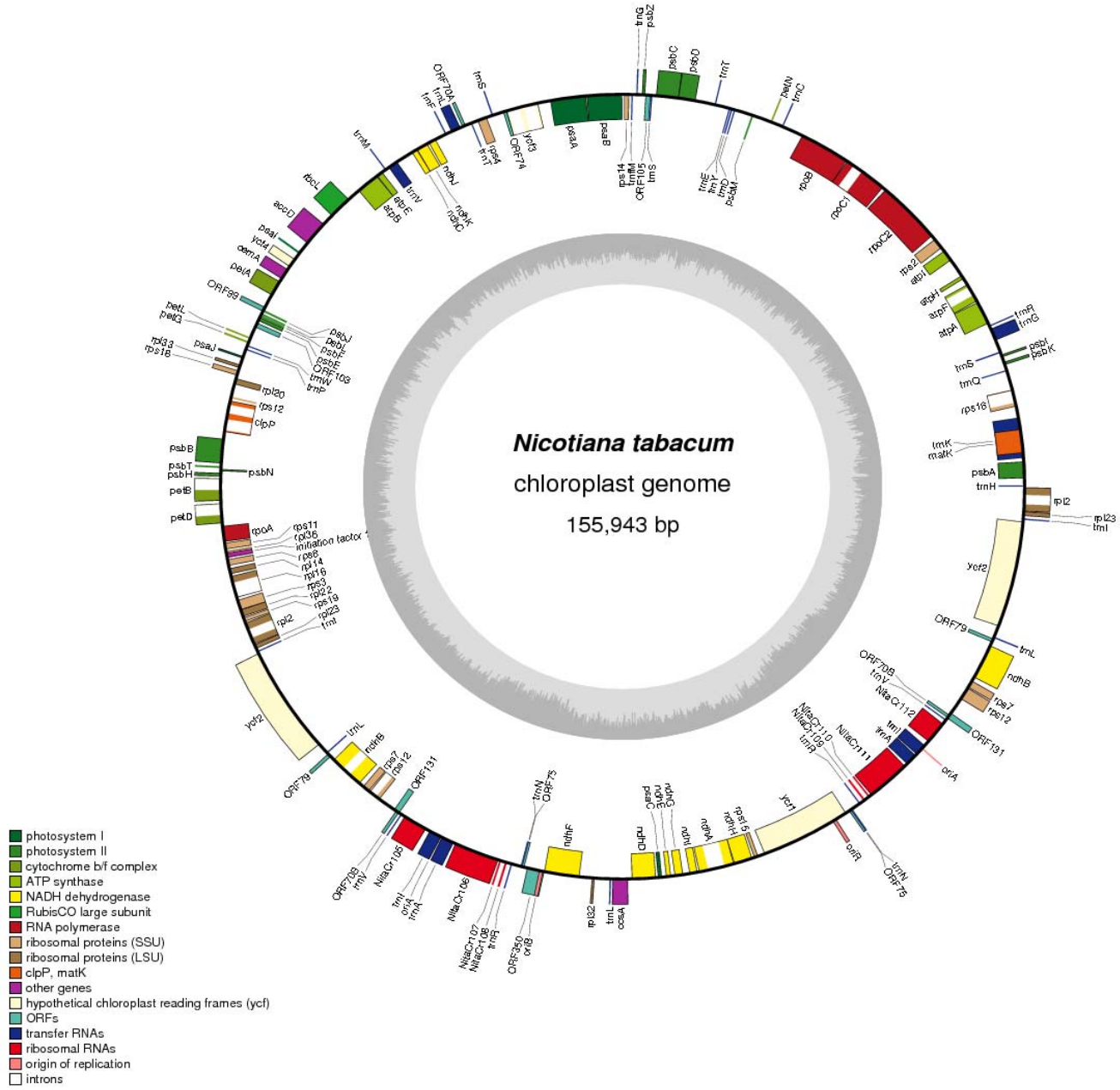


Fig. 1 Plastome map from *Nicotiana tabacum*. Genes on the outside of the circle are transcribed counter clockwise whereas those on the inside of the circle are transcribed clockwise. Genes for transfer RNAs are represented by the one-letter code of amino acids (Lohse et al. 2007).

The chloroplast genome is a circular double-stranded DNA molecule called plastid or "ptDNA", which varies in its size depending upon the different plant species from 120 to160 kb and contain approximately 130 genes. The number of chloroplasts and ptDNA copies per cell varies depending on the cell type. In most cases, photosynthetic cells have the highest number of plastids per cell, with the highest genome copy number (Bendich 1987). A typical dicot leaf cell may contain around 100 chloroplasts with up to 100 ptDNA molecules each, for a total of app.10,000 ptDNA copies per cell. The number can rise to almost 500 ptDNA copies per leaf cell in monocots (Staub 2002). By translation of polycistronic mRNAs the expression of entire biosynthetic pathways from operons is feasible and by plastid genome transformation it is possible to express several genes with a single transformation vector (Lössl et al. 2003). Chloroplast transformation allows integration of multiple antigens and this allows several vaccinations in one step.

The organization and structural characteristics of plastid genomes are conserved among eukaryotic photosynthetic organisms (Raubeson and Jansen 2005). The process of plastid genetic engineering that was developed in tobacco leaves has been the basis for development of the technology in several additional plant species (Staub 2002). Thus, the chloroplast DNA from tobacco (*Nicotiana tabacum*) has served as a reference for other plastid genomes. The complete nucleotide sequence and gene map of tobacco chloroplast genome was first published by Shinozaki et al. (1986).

Many land plants have a quadripartite structure of their genomes with two copies of a large inverted repeat (IR) separating two single copy regions. The IR region is characterized by presence of the ribosomal RNA genes and a variable number of additional genes depending on the plant species (Staub 2002). If the gene is introduced into the inverted repeat (IR) region, integration within one inverted repeat causes the phenomenon of copy-correction which replicates the introduced transgene into the other inverted repeat as well (Daniell et al. 2004).

Plastid encoded transgenes confer high levels of transgene expression and foreign protein accumulation which is because of the polyploidy nature of the plastid's genetic system. Usually a high stability of foreign peptides is obtained with an accumulation of novel protein of up to 70-80% of total soluble protein (Oey et al. 2009, Ruhlman et al. 2010).

1.4 Transplastomic plants

Several advantages are pledging for expression of vaccine antigen in the chloroplast genome for plastid transformation. In contrast to conventional plant transformation methods plastid transformation is a very precise technology of genetic engineering. Site directed insertion of designed transgenes into the plastome allows predicting transformation results more precise than for nuclear transformations (Svab et al. 1990, Maliga 2001). Since transgene integration into the plastome takes place via homologous recombination, position effects can be excluded which in contrast happen in nuclear transformation. Epigenetic effects in transplastomic plastids are absent, co-suppression or gene silencing do not occur (Koop et al. 1996, Heifetz 2000), methylation in the plastid genome is rarely found (Ngernprasirtsiri et al. 1988a, b Ahlert et al. 2009).

Most importantly chloroplast transformation is safe to the environment. Due to the apparent danger for transgenes out crossing with their wild relative species, field production of many transgenic crops engineered through nuclear transformation has grown strong opposition in numerous countries. Hence, transplastomic plants are ideal for harvesting transgene products which are expressed in leaves before the any of plant reproductive organs grow (Clarke and Daniell 2011). Also in contrast to nuclear transformants by plastid transformation, spread of transgenic pollen to the environment can be reduced drastically, as pollen rarely contains plastids. By maternal inheritance of the plastid pollen-mediated gene flow is reduced to a very small probability (Bock 2008, Hagemann 2010). Thus a much higher security is ensured compared to antigens transformed to the cell nucleus. The following list shows the most important advantages of chloroplast transformation that bypasses some of the major hurdles like expensive production and regulatory concerns of plastid based vaccines:

- Eco-friendliness due to gene containment in chloroplast. Their maternal inheritance minimizes spread of transgenic pollen and out-crossing to the related crops or wild species (Daniell et al. 2002, Koop et al. 2007, Lutz et al. 2007, Bock and Timmis 2008, Cardi et al. 2010).
- Possibility to express multiple transgenes in prokaryont like operons (Lössl et al. 2003, Cardi et al. 2010).

- Most foreign proteins are non-toxic in chloroplast (Ruf et al. 2007, Zhoe et al. 2008)
- Lack of epigenetic effects like positional effect, gene silencing (Maliga 2004, Daniell et al. 2009) or methylation (Ahlert et al. 2009).
- Foreign genes integrated into the chloroplast genome by homologous recombination eliminate variation of expression among independent transgenic lines (Maliga 2004, Verma and Daniell 2007).
- Existence of numerous plastids in each cell and various copies of plastid genome in every single cell assists a lot in higher amount of protein expression (Streatfield 2006, Bock and Warzecha 2010, Cardi et al. 2010).

The very first foreign gene expression in higher plant plastids was reported by Daniell and McFadden in 1987. Since then chloroplast transformation has been extensively employed in the model plant tobacco (Svab and Maliga 1993, Dhingra and Daniell 2006). Plastid transformation is regularly used in tobacco but has rapidly expanded to diverse crops including *Solanum lycopersicum* (Zhou et al. 2008), *Solanum tuberosum* (Sidorov et al. 1999), *Lactuca sativa* (Lelivelt et al. 2005, Kanamoto et al. 2006), carrots (Rosales-Mendoza et al. 2009) *Glycine max* (Dufourmantel et al. 2004, Moravec et al. 2007), *Brassica oleracea* (Nugent et al. 2006) and *Populus alba* (Okumura et al. 2006) but the efficiency of transformation is extremely low. Chloroplast expressions of several antigens against different viral and bacterial diseases are summarized in Table 3.

Table 3. A summary of vaccine antigens expressed in the chloroplast genome, against various human diseases (Lössl and Waheed 2011) updated and modified.

Vaccine antigen (Disease)	Expression system	Immunological investigation	References
Viral antigens HPV-16 L1 VLPs (Cervical cancer)	Tobacco	Induced systemic immune response in mice	Fernandez et al. (2008)
HPV-16 L1 VLPs (Cervical cancer)	Tobacco	Not tested	Lenzi et al. (2008)
HPV-16 L1 Capsomers (Cervical cancer)	Tobacco	Not tested	Waheed et al. (2011a)
Vaccinia virus envelope protein (A27L) (Small pox)	Tobacco	Plastid-derived A27L protein formed oligomers	Rigano et al. (2009)
Hepatitis E virus (HEV E2) (Hepatitis)	Tobacco	Antibodies reacting with pE2 found in serum samples of mice immunized	Zhou et al. (2006)

Hepatitis C virus core protein (Hepatitis)	Tobacco	In process of testing	Madesis et al. (2010)
Rotavirus (VP6) (Diarrhoea)	Tobacco	Not tested	Birch-Machin et al. (2004)
HIV (p24) (AIDS)	Tobacco	Not tested	McCabe et al. (2008)
HIV-1 Gag structural poly-protein (Pr55gag) (AIDS)	Tobacco	Not tested	Scotti et al. (2009)
Human b-site APP cleaving enzyme (BACE) (Alzheimer disease)	Tobacco	Mice exhibited a slight induction of primaryanti-BACE antibody upon oral administration.	Youm et al. (2010)
Bacterial antigens CTB (Cholera)	Tobacco	Binding to the intestinal membrane GM1-ganglioside receptor.	Daniell et al. (2001)
CTB-AMA1 and CTB-MSP1 (Cholera)	Tobacco	Long-term protection, Fifty% mouse lifespan in both orally & subcutaneously (89%) immunized mice.	Davoodi-Semiromi et al. (2010)
CTB-2L21 (cholera, Canine parvovirus)	Tobacco	GM1-ganglioside binding assay	Molina et al. (2004)
Escherichia coli LTB (Diarrhoea)	Tobacco	GM1-ganglioside binding assay	Kang et al. (2003)
Mutant of E. coli heat-labile enterotoxin (LTK63) (Diarrhoea)	Tobacco	GM1-ganglioside binding assay	Kang et al. (2004)
E. Coli heat-labile enterotoxin subunit B fused with heat-stable toxin (LTB-ST) (Diarrhoea, Cholera)	Tobacco	GM1-ganglioside binding assay Oral immunization of mice .	Rosales-Mendoza et al. (2009)
Tetanus toxin fragment C (TetC) (Tetanus)	Tobacco	Immunization of mice with the plastid-derived protein against tetanus toxin challenge.	Tregoning et al. (2003, 2005
Anthrax protective antigen (pagA) (Anthrax)	Tobacco	Macrophage lysis assay	Watson et al. (2004)
Anthrax protective antigen (pagA) (Anthrax)	Tobacco	Mice totally survived with lethal dose of toxin.	Koya et al. (2005
Anthrax protective antigen (pagA) (Anthrax)	Tobacco, Lettuce	Not tested	Ruhlman et al., 2010
Borrelia burgdorferi outer surface lipoprotein A	Tobacco	Mice produced protective antibodies against bacteria.	Glenz et al. (2006)

(OspA, OspA-T) (Lyme disease)			
Yersinia pestis F1-V antigen (CaF1-LcrV) (Plague)	Tobacco	Mice were immunized orally and subcutaneously when they exposed to 50-times lethal Y. pestis.	Arlen et al. (2008)
Multi-epitope DPT fusion protein (Diphteria, Pertussis, Tetanus)	Tobacco	Upon oral immunization in mice production of IgG and IgA antibodies was detected in serum and mucosal tissues.	Soria-Guerra et al. (2009)
Fibronectin extra domain A (EDA) (as Adjuvant)	Tobacco	Successful TNF production .	Farran et al. (2010)
CTB-AMA1 and CTB-MSP1 (Malaria)	Tobacco, Lettuce	Mice got immuned against malaria	Davoodi-Semiromi et al. (2010)
Autoantigens Cholera toxin B–proinsulin fusion protein (CTB-Pins) (Diabetes type 1)	Tobacco, Lettuce	Upon oral immunization, non obese diabetic sffected mice showed lower inflammation (insulitis).	Ruhlman et al. (2007)
Cholera toxin B–proinsulin fusion protein (CTB-Pins) (Diabetes type 1)	Tobacco Lettuce	Not tested	Ruhlman et al. 2010
L1-LTB fusion protein against HPV	Tobacco	Not tested	Waheed et al. 2011b

1.5 Inducible expression of transgene

Constitutive expression of transgenes in chloroplast has in some cases been reported to produce abnormal phenotypes i.e. growth retardation, paleness and male sterility. During an experiment to constitutively express the phb operon in tobacco chloroplast, male sterile plants with some abnormal phenotypic characters were observed (Lössl et al. 2003). In other studies pale leaf colouration was observed (Tregoning et al. 2003, Ruf et al. 2007, Waheed et al. 2011b). A solution to this problem can be provided by the introduction of controlled antigen expression system similar to an approach first reported by McBride et al. (1994). Novel inducible expression systems could serve as a useful tool to express transgenes and to control production of foreign protein at any specific stage or even after harvesting. In particular this system will also prevent any over-uptake of antigens by unintended

consumption of transgenic crop. Lössl et al. (2005) have successfully demonstrated an inducible system for chloroplast expression (FIG. 2).

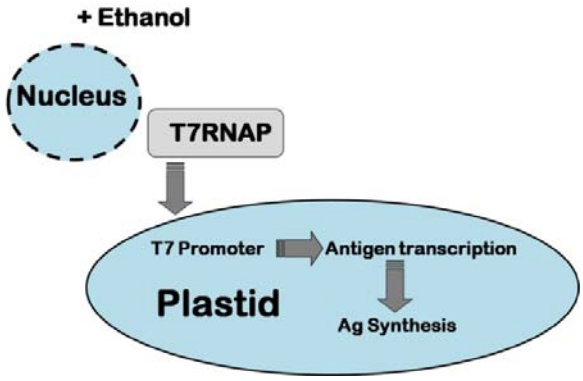


Fig 2. Regulation of Ag synthesis by an inducible antigen expression. Transcription of T7RNAP is initiated through the irrigated with 5% ethanol (Lössl et al. 2005)

The very first trans-activation system in plastids was constructed for an inducible expression of the phb operon which was regulated by a plastid targeted, nuclear located T7-RNA polymerase. Induction of transgene expression was achieved by activation of an inducible promoter. This transgene control method through ethanol induction was based on the alc-A promoter and *Aspergillus nidulans*'s transcription factor AlcR for the alcohol dehydrogenase regulon (Caddick et al. 1998, Salter et al. 1998, Roslan et al. 2001). Thus, in our application the risk of unintended antigen uptake can be largely reduced as the plants express the transgene only when irrigated with 5% ethanol. To test feasibility of this approach for vaccine production in the current study I report a quantitative analysis of this trans-activation system of transgene expression using the nuclear encoded, chloroplast imported, ethanol-inducible T7 RNA polymerase which transcribes the plastid located GUS gene under control of the T7 promoter.

1.6 Subunit vaccine production

The capability of plant-based production systems to provide cost effective and easily administrable (mucosal immunization) recombinant biopharmaceuticals free from any pathogen contamination and almost unlimited scalability are the strengths of biopharming. A

major decision in producing transplastomic plant vaccines is the selection of appropriate plant material, which can both express high levels of a chosen vaccine candidate and be suitable for extensive storage. During these experiments we have used tobacco *Nicotiana tabacum* which is one of the few platforms that can address the technical and regulatory consideration of biopharmaceutical industry. It is easily transformable, and is often used for genetic engineering due to its easy genetic manipulation and its considerable growth rate (Horsch et al. 1989, Watson et al. 2004).

In the following study I have investigated the possibility of transplastomic expression of transgene in tobacco plants to produce efficient and cost effective vaccines against HPV-virus and mycobacterium. Mycobacteria are very diverse pathogen present commonly in our environment and are an important cause of infections in humans worldwide (WHO 2008). Most often mycobacterium is associated with the disease Tuberculosis caused by *Mycobacterium tuberculosis*, however some atypical species as *Mycobacterium leprae* and *Mycobacterium avium* cannot be ignored as they cause severe and chronic illness i.e Crohn's Disease and leprosy (Prince et al. 1989).

1.6.1 Human papilloma virus

Human Papillomavirus (HPV) is a major source in causing cervical cancer with almost 95% of cervical cancer worldwide (Munoz et al. 2003). It is the second highest cancer disease in women after breast cancer and it is the most common cause of death in females. Each year 500,000 new cases are reported of cervical cancer worldwide and majority of them are from developing countries (Clifford et al. 2003). The major capsid L1 protein self-assembles in Virus like Particles (VLPs), which are highly immunogenic and suitable for vaccine production (Rose et al. 1998).

Papilloma viruses are distributed worldwide and have been reported in a wide variety of animals as well as in humans. HPV associated diseases have affected mankind since ancient times, but the viral origin of skin and genital warts (GWs) or cervical cancer (CC) was not discovered until the 20th century (Hausen, 2009). Papillomaviruses are highly host specific and are usually named after the species they infect. In Human beings, more than 100 HPV types have been described (deVilliers et al. 2004). HPV is a papillomavirus that infects

the epidermis and mucous membranes of humans. HPV infections are associated with about 10% of the worldwide cancer burden, most of which are anogenital cancers and HPV DNA is present in about 80% of cervical tumors worldwide (Bosch FX. et al. 1995).

Due to the common occurrence of HPV, health professionals recommend to vaccinate before the individuals become sexually active. Even though the recommended age for the vaccination is between 11 and 12 years of age, catch-up vaccines are available through the age of 26. Individuals that receive the vaccine prior to initial sexual activity are immunized against the four major types of HPV and the ones who receive the vaccine after becoming sexually active will only be protected from the types of HPV they have not yet acquired (Markowitz et al. 2007).

The HPV virion is approximately 55 nm in diameter which consists of an icosahedral capsid made up of 72 capsomers containing a closed circular double stranded DNA genome. (Williams et al. 1961). The circular double-stranded DNA genome is approximately 8kb in length encoding a total of eight proteins (Cheung et al. 2004). The protein coding sequences (open reading frames, ORFs) are located on one strand of the double-stranded DNA and are divided into two main groups early E and late L, based on their location in the genome (Howley and Lowy 2001). The proteins: E1, E2, E4, E5, E6, E7 are nonstructural proteins, which are mainly involved in replication, transcription, transformation and facilitation of viral escape. The remaining two, L1 and L2 are the structural proteins that compose the capsid (Fehrmann and Laimins, 2003). Interestingly it is found that L1 capsomers alone can form virus-like particles (VLPs) that are morphologically identical to the real viral capsids (Müller et al. 1997), but which lack viral DNA or RNA. Therefore the VLPs are completely noninfectious and non-oncogenic and are currently the most attractive candidate for developing a prophylactic vaccine against HPV infections.

There are two recently licensed commercial vaccines have been introduced to the market for the prevention of HPV infections, both of which are based upon HPV L1 virus-like particles. Gardasil® by Merck & Co. (NJ, USA) is a quadrivalent vaccine targeting HPV 6, 11, 16 and 18 while Cervarix® by GlaxoSmithKline (London, UK) is a bivalent vaccine against HPV 16 and HPV 18 (Garcia-Pineres et al. 2009). However, these vaccines have substantial manufacturing costs due to their expression and purification from eukaryotic cells

i.e. insect cells or yeast. Therefore for resource-poor countries these vaccines remain unaffordable where in fact 83% of all cervical cancer cases occur (Agosti and Goldie 2007). Thus, there is a pressing need for second generation vaccines that are cheaper to produce and affordable.

In the current study I report the possibility of producing a plant made vaccine by the expression of a modified L1 gene (L1_2xCysM) fused to glutathione S-transferase (GST) in tobacco chloroplast. The fused GST allows efficient one-step purification of L1 fusion protein by an enzyme linked immunosorbent assay (ELISA) system.

1.6.2 Mycobacteria

For hundreds of years leprosy has been affecting humans as an individual and also as a society. The affected have often been hated and shunned by their communities and families (Britton and Lockwood 2004). Leprosy has been characterized as a disease responsible for serious deformities and disabilities resulting psychological and social suffering. Today leprosy has been eradicated form the most parts of the world but in some parts of Asia Africa and South America, particularly in Brazil, Nepal, and Mozambique it is still present (Sinsimar et al. 2010).

Leprosy is a dermatological and neurological disease caused by the intracellular infection of M. leprae which can cause nerve damage and can lead to severe disabilities. (Monot et al. 2010). Despite intensive study, it is still unclear how leprosy is acquired and what elements cause the development of active disease or the level of severity of the disease in different individuals (Sinsimar et al. 2010). However, it is clear that the host immune response to *Mycobacterium leprae* determines the clinical manifestation of disease and various severity levels can arise (Ridley et al. 1966). Official figures from the World Health Organization (WHO) show that more than 213 000 people mainly in Asia and Africa are infected, with approximately 249 000 new cases reported in 2008. According to reports and statistics from 121 countries the number of new cases detected globally has fallen by 9126 (a 4% decrease) during 2008 compared with 2007 (Accession date, 17 August 2011, Leprosy today, WHO, Geneva, Switzerland. http://www.who.int/lep/en/).

Vaccination has always been an effective tool against viral and bacterial diseases. For many years the only licensed vaccine present against *Mycobacterium leprae* and *Mycobacterium tuberculosis* was Bacille Calmette-Guérin (BCG). BCG was developed in the early 1900s by Albert Calmette and Camille Guèrin. It is made of a live, vitiated strain of M. bovis. BCG was first administrated in 1921 to a newly born baby whose mother was a tuberculosis patient. This immunized individual remained free of tuberculosis throughout his life (Rosenthal et al. 1945). In 1928 United Nations (The Leauge of Nations) suggested widespread vaccination with BCG. Today about 80% of the world population is vaccinated with BCG. The vaccine is effective against disseminated and meningeal TB in infants and young children, however, unfortunately the protective efficacy of BCG vaccination against *Mycobacterium leprae* has not proved very effective (Fine 1995).

Due to the poor performance of multidrug therapy (MDT) and BCG vaccination, especially in the developing areas of Asia and Africa, it is necessary to explore new immunoprotective vaccines (Andersen 2005) and the identification of protective antigens is particularly important for the subunit vaccine approach (Sanchez and Holmgren 2008). Immunization with DNA encoding mycobacterial antigens has already been proven to stimulate successful protective cell mediated immune responses against *Mycobacterium tuberculosis* (Kammath et al. 1999) and M. avium infection (Faircloth et al. 1999) and is a new strategy for leprosy control.

An efficient cross-protective antigen against *Mycobacterium* consists in a 35kDa protein encoded by *mmp*I gene in *Mycobacterium leprae* (Triccas et al. 1996, Triccas et al. 1998, Martin et al. 2001), which is also efficiently protective against *Mycobacterium avium* (Martin et al. 2000). The 35kDa-antigen, the major membrane protein I, in native form exists as a multimer of 950 kD (Winter et al. 1995). In order to retain its correct folding capacity it is indicated to express it as a single peptide without additional sequences. In this study *mmp*I gene product has been cloned into the high-expression vector and transiently transformed into tobacco chloroplasts.

1.7 Aim and focus of the work

Due to increasing number of patients suffering from different contagious diseases especially in resource poor countries it is important to produce efficient and cost effective alternative vaccines. Plants provide us with a unique system of foreign protein production which has potential to address the problems mentioned above. Among biopharming, chloroplast transformation system has great advantages. Besides its eco-friendliness and high containment due to maternal inheritance, it offers the opportunity to use multiple copies of plastid genome per cell resulting in high level of protein expression with minimum or no risk of epigenetic effects.

The following list gives the objectives addressed in the current study on chloroplast transformation and vaccine production through plants.

- Cloning of transformation vectors for tobacco chloroplast genome
- Transformation of tobacco with expression cassettes for vaccine subunit antigens
- Regeneration of transformed tissues and transplastomic tobacco lines
- Proof of accurate insertion of transgene by PCR and southern analysis
- Proof of transgenic protein expression an correct assembly by ELISA
- Confirmation of the stability of transformants in to next generation, homoplasmy
- Study of the feasibility of induced transplastomic expression by ß-Glucuronidase (GUS) reporter gene

Thus the aim of this study is the evaluation of the potential of tobacco chloroplast as a production platform for vaccines against HPV and Mycobacterium. Production of transplastomic lines, their expression of protein, possible effects of foreign protein within plant and an efficient trans-activation system is covered by this analysis.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

2.1 BACTERIAL STRAINS

- E. coli Sure
- E. coli JM110
- E. coli XL1 Blue
- E. coli DH5α

2.2 PLANT MATERIAL

Sterile seeds of *Nicotiana tabacum* cv. Petit Havana (Wild type, Wt)

Constructed transgenic plants:

• *Nicotiana tabacum* line expressing GST-L1
• *Nicotiana tabacum* line expressing GUS
• *Nicotiana tabacum* line expressing LTB and *mmpI*

2.3 REAGENTS

Table 4. Kits and reaction systems

Name/ Description	Company
QIAquick® PCR Purification Kit, No. 28104	Qiagen GmbH, Hilden, Germany
Qiagen MinElute® Reaction cleanup Kit, No. 28204	Qiagen GmbH, Hilden, Germany
QIAquick® Gel Extraction Kit, No. 28704	Qiagen GmbH, Hilden, Germany
QIAquick® Nucletide removal Kit, No 28304	Qiagen GmbH, Hilden, Germany
QIAprep® Miniprep Kit, No 27106	Qiagen GmbH, Hilden, Germany
Qiagen® Plasmid Midi Kit, No 12143	Qiagen GmbH, Hilden, Germany
Qiagen® Qiafilter Plasmid Maxi Kit, No 12263	Qiagen GmbH, Hilden, Germany
Dneasy® Plant Maxi Kit	Qiagen GmbH, Hilden, Germany
Dneasy® Plant Mini Kit	Qiagen GmbH, Hilden, Germany

Dneasy® Plant DNA extraction Kit	Qiagen GmbH, Hilden, Germany
Fermentas ® Rapid DNA ligation Kit, No 1422	MBI Fermentas, Vilnius, Lithuania
Fermentas ® Rapid DNA ligation and Transformation Kit, No 1431	MBI Fermentas, Vilnius, Lithuania
DIG High Prime DNA labeling and detection Kit II, No 585614910	MBI Fermentas, Vilnius, Lithuania

Table 5. List of Enzymes

Name of Enzyme	**Manufacturer**
Cellulase R-10	YAKULT Pharm., Tokyo, Japan
Fermentas ApaI, AflII, BamHI	MBI Fermentas, Vilnius, Lithuania
BglII, BsiWI, Eco911, EcoRV	
EcoRI, HpaI, KpnI, MluI, NheI	
Fermentas Taq DNA polymerase	
NcoI, SacI, SexAI, Xba I, XhoI	
Macerase R-10	YAKULT Pharm. , Tokyo, Japan
RNase	Carl Roth, Karlsruhe, Germany

Table 6. Readymade Reagents

Reagents	**Manufacturer**
6 x Loading Dye	MBI Fermentas, Vilnius, Lithuania
10 X PCR Buffer without MgCl2	MBI Fermentas, Vilnius, Lithuania
Chloroform	Carl Roth, Karlsruhe, Germany
dATP-Solution 100mM	MBI Fermentas, Vilnius, Lithuania
dCTP-Solution 100mM	MBI Fermentas, Vilnius, Lithuania
dGTP-Solution 100mM	MBI Fermentas, Vilnius, Lithuania
dTTP-Solution 100mM	MBI Fermentas, Vilnius, Lithuania
Ethanol, absolute	Australco, Spillern, Austria
Ethidium bromide 1% in water	Carl Roth, Karlsruhe, Germany
Isopropanol (2-prepanol), absolue	Carl Roth, Karlsruhe, Germany

Lambda DNA/Eco130I/Mlul Marker, 17	MBI Fermentas, Vilnius, Lithuania
LyseBlue (Color indicator for QIAGEN)	QIAGEN Vertriebs GmbH, Austria
Magnesiumchloride 25 mM	MBI Fermentas, Vilnius, Lithuania
Microcarriers 0.6 $m (Gold Particles)	Bio-Rad, CA, USA
Nitrogen, liquid	TU Wien, Austria
RNase A for QIAGEN Plasmid Kits	QIAGEN Vertriebs GmbH, Austria

2.4 CHEMICALS, BUFFERS AND SOLUTIONS

Table 7. List of Chemicals

Chemicals	Purity grade	Manufacturers
Acetic acid	p.a.	Carl Roth, Karlsruhe, Germany
Agar for media	p.a.	Sigma, Dreisenhofen, Germany
Agarose for electrophoresis		Carl Roth, Karlsruhe, Germany
Ammonium chloride	p.a.	Merck, Darmstadt, Germany
Ammonium succinate	p.a.	Carl Roth, Karlsruhe, Germany
Ammonium Sulphate	p.a.	Merck, Darmstadt, Germany
B5 salt	p.a.	Sigma, Dreisenhofen, Germany
Bacto Agar		Carl Roth, Karlsruhe, Germany
Bacto Tryptone	p.a.	Carl Roth, Karlsruhe, Germany
BAP (6-benzylaminopurine)	p.a.	Sigma, Dreisenhofen, Germany
Biotin	p.a.	Duchefa Biochemie, Haarlem, NL
Boric acid	p.a.	Carl Roth, Karlsruhe, Germany
Calcium Chloride Dihydrate	p.a.	Merck, Darmstadt, Germany
Calcium nitrate tetrahydrate	p.a.	Carl Roth, Karlsruhe, Germany
Cobalt chloride hexahydrate	p.a.	Merck, Darmstadt, Germany
Copper Sulphate Pentahydrate	p.a.	Merck, Darmstadt, Germany
CTAB (Cetyl trimethylammonium bromide)	p.a.	Duchefa Biochemie, Haarlem, NL
Cupric Sulfate Pentahydrate	p.a.	Carl Roth, Karlsruhe, Germany
EDTA (Ethylenediaminetetraacetic acid)	p.a.	Fluka, Buchs, Switzerland
EDTA Ferric (III) Sodium salt	p.a.	Duchefa Biochemie, Haarlem, NL
Glucose	p.a.	Duchefa Biochemie, Haarlem, NL

Glycerol	p.a.	Carl Roth, Karlsruhe, Germany
Glycine	p.a.	Carl Roth, Karlsruhe, Germany
Inositol	p.a.	Sigma, Dreisenhofen, Germany
Magnesium chloride hexahydrate	p.a.	Carl Roth, Karlsruhe, Germany
Magnesium Sulfate heptahydrate	p.a.	Carl Roth, Karlsruhe, Germany
Mannitol	p.a.	Carl Roth, Karlsruhe, Germany
Monosodium Phosphate	p.a.	Carl Roth, Karlsruhe, Germany
MS (Murashig & Skoog) = MSMO	p.a.	Sigma, Dreisenhofen, Germany
MS-Salt		Sigma, Dreisenhofen, Germany
Myo-inositol	p.a.	Duchefa Biochemie, Haarlem, NL
Nicotinic acid (Niacin)	p.a.	Carl Roth, Karlsruhe, Germany
Nitrocalcite	p.a.	Sigma, Dreisenhofen, Germany
PEG (Polyethylene glycol)	p.a.	Merck, Darmstadt, Germany
Polyvinylpyrrolidone (PVP)	p.a.	Carl Roth, Karlsruhe, Germany
Potassium acetate	p.a.	Carl Roth, Karlsruhe, Germany
Potassium Chloride	p.a.	Carl Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate	p.a.	Merck, Darmstadt, Germany
Potassium hydroxide-Pellets	p.a.	Merck, Darmstadt, Germany
Potassium iodide	p.a.	Merck, Darmstadt, Germany
Potassium nitrate	p.a.	Duchefa Biochemie, Haarlem, NL
Pyridoxine-HCl	p.a.	Duchefa Biochemie, Haarlem, NL
SDS (Sodium lauryl sulfate)	p.a.	Carl Roth, Karlsruhe, Germany
Sodium hydroxide	p.a.	Carl Roth, Karlsruhe, Germany
Sodium Molybdate	p.a.	Riedel-de Haen, Seelze, Germany
Spectinomycin	p.a.	Duchefa Biochemie, Haarlem, NL
Spermidine	p.a.	Duchefa Biochemie, Haarlem, NL
Succinic acid	p.a.	Carl Roth, Karlsruhe, Germany
Thiamine Hydrochloride	p.a.	Duchefa Biochemie, Haarlem, NL
Tris-HCl	p.a.	Carl Roth, Karlsruhe, Germany
Yeast extract	p.a.	Carl Roth, Karlsruhe, Germany
Zinc sulphate heptahydrate	p.a.	Merck, Darmstadt, Germany

2.4.1 Buffers and Solutions

Following are the recipes of buffers and solutions prepared in the laboratory.

Ammonium succinate 2M

Succinic acid 23.6 g, NH_4Cl 10.6 g, KOH-Pellets, 22.4 g. Ingredients were dissolved in 100 ml distilled water, adjusted the pH to 5.8, sterilized by filtering and stored at -20 °C.

Ampicillin (25mg/ml)

250 mg ampicillin dissolved in10 ml distilled water then sterilized by filtering and stored at -20 °C.

B5-Medium

B5-Salt 3.1 g, B5-Vitamine (100X) 10 ml, $MgSO_4 \times 7H_2O$ 0.983 g, sucrose 20 g, Agar purified 8g. Mixed and dissolved in 1L distilled water, pH to 5.8 (1M KOH) and sterilized by autoclaving.

B5-Macro 10X

KNO_3 25 g, $CaCl_2 \times 2H_2O$ 1.5 g, $MgSO_4 \times 7H_2O$ 2.5 g, $NaH_2PO_4 \times H_2O$ 5 g, $(NH4)_2SO_4$ 1.34 g. Mixed and dissolved in 1L distilled water, stored in 100 ml plastic beakers at -20 °C. Dispensed into 100 ml aliquots and stored at -20°C.

B5-Micro 100X

EDTA Ferric (III) Sodium Salt 4 g, Potassium iodide (KI) 75 mg, Boric acid (H3BO3) 300 mg, $MnSO_4 \times H_2O$ 1 g, $ZnSO_4 \times 7H_2O$ 200 mg, $Na_2MoO_4 \times 2H_2O$ 25 mg, $CuSO_4 \times 5H_2O$ 2.5 mg, $CoCl_2 \times 6H_2O$ 2.5 mg. Mixed and dissolved in 1L distilled water, stored in 10 ml tubes at -20 °C. Dispensed into 10 ml aliquots and stored at -20°C.

B5-Vitamine (1 L)

Myo-inositol 10 g, Pyridoxine-HCl 100 mg, Thiamine Hydrochloride 1 g, Nicotinic acid 100 mg. Mixed and dissolved in 1L distilled water, stored in 10 ml tubes at -20 °C

BAP (1 mg/ml)

BAP (6-benzylaminopurine) 100 mg, NaOH 1M 0.5 ml. BAP was dissolved in 1M NaOH then filled up to 100 ml with distilled water, filter sterilized, dispensed into 1 ml aliquots and stored at -18°C.

Ca2+ -Agar

MES 0.97 g, $CaCl_2$ x 2 H_2O 1.47 g, Mannitol41 g, Agar purified 5 g. Mixed and dissolved in 500 ml distilled water, adjusted the pH to 5.8 with 1M KOH and autoclaved.

Calcium chloride 2.5 M

$CaCl_2$ x 6 H_2O 10.8 g. Dissolved in 20 ml distilled water. Filter sterilized and stored at -20°C.

Cellulase R10

Cellulase R10 1g, sucrose 1.37 g. Mixed and dissolved in 10 ml distilled water, filtrated through a sterilizing filter. Stored 1 week at 4°C, or 3 months at -20°C.

CTAB solution

CTAB 4 g, Tris HCl 1M 40 ml, EDTA 0.5 M 8 ml, NaCl 16.36 g, Polyvinylpyrrolidone (PVP) 2g. Mixed and dissolved in 200 ml distilled water and sterilized by autoclaving.

dNTPs 25 mM

dATP 25 µl, dCTP 25 µl, dGTP 25 µl, dTTP 25 µl. Dispensed into 5 µl aliquots and stored at -20°C.

EDTA 0.5 M (250 ml)

EDTA disodium salt dihydrate 43.53, NaOH-Pellets 5 g. Adjusted the pH to 8.0 with g NaOH pellets , the volume was filled up to 250 ml with distilled water and sterilized by autoclaving.

F-Alginate (100 ml)

MES 0.137 g, MgSO4 x 7H_2O 0.25 g, MgCl2 x 6 H_2O 0.204 g, Mannitol 7.7 g (550 mOsm) Alginic acid 2.4 g. Mixed and dissolved in 100 ml distilled water, PH 5.8 and sterilized by autoclaving.

F-PCN

MS-Macro 10X 100 ml, MS-Micro 100X 10 ml, PC-Vitamin 100X 10 ml, Ammonium succinate 2M 10 ml, BAP (conc. 1mg/ml) 1 ml, NAA (conc. 1mg/ml) 0.1 ml, MES or Polypuffer 1.952 g, sucrose 20 g, glucose 65 g. Mixed and dissolved in 1 L distilled water, pH 5.8, filter sterilized and stored at -20°C.

F-PIN

MS-Macro 10X 100 ml, MS-Micro 100X 10 ml, PC-Vitamin 100X 10 ml, Ammonium succinate 2M 10 ml, BAP (conc. 1mg/ml) 1 ml, NAA (conc. 1mg/ml) 0.1 ml, MES or Polypuffer 1.952 g, sucrose 130 g. Mixed and dissolved in 1 L distilled water, pH 5.8, filter sterilized and stored at -20°C.

Glucose 1M

18 g Glucose was mixed and dissolved in 100 ml distilled water and filter sterilized.

Glycerol 50%

Glycerol 100% 50 ml, ddH_2O 50 ml. Total volume 100 ml using distilled water and sterilized by autoclaving.

IAA 1 mg/ml (Indole-3-acetic acid)

IAA 50 mg, EtOH or 1N NaOH 5 ml. Filled up to 50 ml with distilled water, filter sterilized and dispensed into 200 µl aliquots and stored at -20°C.

LB-Medium

Bacto Tryptone 10 g, Yeast extract 5 g, NaCl 10 g, Bacto-Agar (if preparing solid LB-medium) 10 g. Mixed and dissolved in 1 L of distilled water, adjusted pH to 7.0 and sterilized by autoclaving.

Macerase R10

Macerase R101 g, sucrose 1.37 g. Mixed and dissolved in 10 ml of distilled water, filtrated through a sterilizing filter. Stored 1 week at 4°C, or 3 months at -20°C.

Magnesium chloride 2M

19 g $MgCl_2$ x $6H_2O$ mixed and dissolved in 100 ml distilled water and sterilized by autoclaving.

MMM

$MgCl_2$ x $6H_2O$1,02 g, $MgSO_4$ x $7H_2O$1,25 g, MES1,952 g, Mannitol 85 g. Mixed and dissolved in 1L distilled water, adjusted pH to 5.6 with and filter sterilized. Stored 1 week at 4°C, or 3 months at -20°C.

MS-Macro 10X

KNO3 10.12 g, CaCl2 x 2 H_2O 4.4 g, MgSO4 x 7 H_2O 3.7 g, KH2PO4 1.7 g. Filled up to 1 L with distilled water and sterilized by autoclaving. Dispensed into 100 ml aliquots and stored at -20°C.

MS medium

MS-Salt 4.4 g, MS-Vitamin 100X 10 ml, sucrose 10 g, $MgSO_4$ 1 g, Agar purified 8 g. Mixed and dissolved in 1 L distilled water, adjusted pH to 5.8 and sterilized by autoclaving. When the solution was cooled, 10 ml NH4-Succinat was added to the solution, shortly before using.

MS medium

MS-salt 2.2 g, MS-Vitamin 100X 5 ml, sucrose 15 g. Mixed and dissolved in 1 L distilled water, adjusted pH to 5.8 and sterilized by autoclaving. When the solution was cooled, 10 ml NH4-Succinat was added to the solution, shortly before using.

MS-Micro 100X

Na-EDTA-Fe (III) 4 g, KJ 75 mg, H3BO3 300 mg, $MnSO_4$ x H_2O 1 g, $ZnSO_4$ x $7H_2O$ 200 mg, Na_2MoO_4 x $2H_2O$25 mg, $CuSO_4$ x $5H_2O$ 2.5 mg, $CoCl_2$ x $6H_2O$ 2.5 mg. Filled up to 100 ml distilled water and sterilized by autoclaving.

NAA (1mg/ml)

Naphthaleneacetic acid 100 mg, Absolute EtOH 10 ml. NAA was dissolved in EtOH then filled up to 100 ml with distilled water. Filter sterilized and then dispensed into 200 µl aliquots and stored at -20°C.

NT-Vitamin 100X

Inosit 10 g, Thiamin-HCl 100 mg. Filled up to 1L distilled water, filter sterilized, dispensed into 10 ml aliquots and stored at - 20°C.

PC-Vitamin 100X

Inosit 20 g, Pyridoxin-HCl 200 mg, Thiamin-HCl 100 mg, Biotin 2 mg, Nicotinic acid (Niacin) 200 mg. Filled up to 1L distilled water, filter sterilized, dispensed into 10 ml aliquots and stored at - 20°C.

PEG 40% (26 ml)

$Ca(NO_3)_2$ X $4H_2O$ 0.413 g, Mannitol 1.275 g, PEG1500 10 g. Dissolved in distilled water and filled up to 17.5 ml, pH was adjusted to 9.75 with and filter sterilized aliquoted and stored at -20°C.

Potassium acetate 3M

29.44 g K_OAc was dissolved in 100 ml distilled water and pH (5.2)

Potassium Chloride 250 mM

1.86 g KCl was dissolved in 100 ml distilled water.

Potassium hydroxide 1M

28.05 g KOH was dissolved in 500 ml distilled water.

RMOP medium

MS-Macro 10X 100 ml, MS-Micro 100X 10 ml, NT-Vitamin 10 ml, BAP (1mg/ml) 1 ml, NAA (1mg/ml) 100 µl, sucrose 30 g, $MgSO_4$ 1 g, Agar purified 8 g, Spec (100 mg/ml) 10 ml. Filled up to 1L with distilled water, adjusted to pH 5.8 and sterilized by autoclaving. When the solution was cooled to 50 °C, 10 ml NH_4-Succinat was added, shortly before using.

SDS 10%

10 g SDS was added in 100 ml distilled water and heated at 68 °C. pH was adjusted to 7.2 and sterilized by autoclaving.

SOB-medium

Bacto tryptone 20 g, Yeast Extract 5 g, NaCl 0.5 g, KCl 250 mM 10 ml. Filled up to 1 L with distilled water and mixed well, adjusted to pH 7.0 (5M NaOH) and sterilized by autoclaving. When the solution was cooled to 45-50°C, 5 ml sterile $MgCl_2$ 2M was added, shortly before using.

SOC-medium

Bacto tryptone 20 g, Yeast Extract 5 g, NaCl 0.5 g, KCl 250 mM 10 ml, Glucose 1M 20 ml. Filled up to 1 L with distilled water and mixed well, adjusted to pH 7.0 (5 N NaOH) and sterilized by autoclaving. When the solution was cooled to 45-50°C, 5 ml sterile MgCl2 2 M was added, shortly before using.

Sodium chloride 1M (NaCl)

5.84 g NaCl, distilled water was added to 100 ml final volume, sterilized by autoclaving and stored at room temperature.

Sodium hydroxide 1 M (NaOH)

20 g NaOH, distilled water was added to 500 ml final volume. Stored at room temperature.

Sodium hydroxide 5 N (NaOH)

20 g NaOH, distilled water was added to 100 ml final volume. Stored up to 1 month at room temperature.

Spectinomycin 100 mg/ml

10 g Spec. was dissolved in 100 ml distilled water, filter sterilized. Dispensed into 10ml aliquots and stored at -20°C.

Spermidine 0.1 M

0.255 g Spermidine Was dissolved in 10 ml distilled water, stored at -20°C.

TAE 50X

Tris 242 g, Acetic acid 57.1 ml, EDTA 0.5 M 100 ml. Filled up to 1 L with distilled water and dissolved.

TAE 1X

TAE 50X was diluted 1:50 (20 ml TAE 50X / 980 ml distilled water)

TE buffer

Tris-HCl 1 M 1 ml, EDTA 0.5 M 200 µl. Filled up to 100 ml with distilled water and pH adjusted to 5.6.

TENS buffer

Tris-HCl 1 M 0.5 ml, EDTA 0.5 M 0.1 ml, NaOH 5 M 1 ml, SDS 10% 2.5 ml. Filled up to 50 ml distilled water and pH adjusted to 8.0

Transformation-medium

MgCl2 x 6 H_2O 3.045 g, MES 1g, Mannit 85 g . 1 L distilled water was added and pH adjusted to 5.6 filter sterilized and Stored at -20 °C.

Tris-HCl 1M

12.11 g was dissolved in 100 ml distilled water; pH adjusted to 8.0 and filter sterilized.

2.5 Laboratory equipments and appliances

Table 8. Laboratory Equipments and Materials

Product Name	Manufacturer
Glass flasks 250, 500,1000 ml	Bellco, USA
Centrifuge tubes 1.5 ml	Bilatec AG, Germany
Centrifuge tube rack	Carl Roth, Karlsruhe, Germany
Mesh copper grid	Plano, Germany
Ultra micro pipette tips 0.5 - 20 µl	Eppendorf, Hamburg, Germany
Pipette tips 10,100,200,1000µl	Eppendorf, Hamburg, Germany

Bottle Top Filter Cellulose Acetate, 22µm	Corning incorporated, USA
Centrifuge tubes 2 ml	G. Kisker GbR, Germany
Water distillation apparatus	Quartz glass GmbH, Germany
Centrifuge tubes, Nalge Nunc Oak Ridge	Nalgene, Germany
Glass Funnel	Sigma Aldrich, Germany
Glass screw cap bottles 100, 250, 500 ml 1000 ml	Greiner bio-one, Germany
Centrifuge tubes 15, 50 ml	Greiner bio-one, Germany
Dialysis filter 0.025 $m	Millipore, USA
Filter papers, Whatman 3MM-paper	Schleicher & Schuell, Germany
Hemocytometer	Carl Roth, Karlsruhe, Germany
Electroporation cuvette, 0.2 cm	Bio-Rad, CA, USA
Latex gloves	Semperit, Austria
Nesco-Parafilm	Pechiney plastic packaging, USA
Nitrocellulose-Membrane 0.45$m 6x9 cm	Schleicher & Schuell, Germany
Macrocarriers	Bio-Rad, CA, USA
Magneta box	Laborhandel 2000, Austria
Magnetis stirrer M22/1	Framo, Germany
PCR-tubes 0.5 ml	Bilatec AG, Germany
Pipette 10 ,50, 100, 200, 1000 µl	Scorex, Switzerland
Pipette tips 10 ml	Carl Roth, Karlsruhe, Germany
pH indicator strip	Carl Roth, Karlsruhe, Germany
Polypropylene-Net	Scrynel PP2000, K. H. Büttner GmbH,
Rotor F2850	DuPont, Germany
Rotor RW 20	Janke & kunkel, Germany
QIAfilter Midi & Maxi Cartridge	QIAGEN Vertriebs GmbH, Austria
QIAGEN-tips 100, 500	QIAGEN Vertriebs GmbH, Austria
Test tube (Culture tube)	Carl Roth, Karlsruhe, Germany
Ultra-Clear Centrifuge tubes 14 x 89 mm	Beckman instruments, USA
Vinyl Gloves	Semperit, Austria

Table 9. Laboratory Appliances

Appliances	Manufacturer
Autoclave	Labortechnique Gmbh, Germany
Bombardment chamber	PDS1000He, Bio-Rad, CA, USA
Centrifuge 54145	Eppendorf, Germany
Centrifuge Rotina 35R	Hettich Centrifuges, Germany
Centrifuge bottle 250 ml	Hettich Centrifuges, Germany
Electric Microscope EM 10A	Zeiss, Germany
Electrophoresis Chamber EasyCast #B2	UniEquip, Germany
Electrophoresis Power Supply	Gibco BRL, Germany
Electroporation Apparatus E. coli Pulser®	Bio-Rad, CA, USA
Heating block	Grant boekel, Germany
Ice Machine Compact Ice Apexa	Bartscher GmbH, Germany
Incubator Chamber	Memmert, Germany
Labophot microscope	Nikon, Japan
Laminar flow cabinet	Ceag Schirp, Germany
Microwave OM007G	Orion, Germany
Minishaker MS1	IKA Works Inc., USA
Overhead-Shaker	Heidolph, Germany
Particle-gun PDS-1000/He	BioRad, München
PCR PX2 Thermal Cycler	Thermo Electron Corporation, USA
PH-Meter G103	Seibold, Austria
Precision Balance, Satorius 2001 MP2	Sartorius GmbH, Germany
Refrigerator and Freezer	Labotect, germany
Refrigerator Whirlpool	Philips, Germany
Shaker KS 15	Edmund Bühler, Germany
Spectrophotometer DU-6	Beckman, USA
Ultrasonic device sonifier 250	Branson, USA
Water bath Haake SWB 20	Haake, Germany

2.6 MARKERS AND PRIMERS

Marker

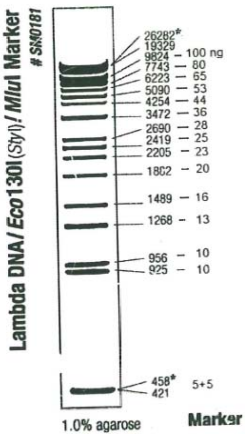


Fig 3. Lambda DNA/ Eco 1301 Marker (SM0181) was used supplied by MBI Fermentas, Vilnius, Lithuania

Primers

Following are the primers used in the current study. All primers were first designed in the lab according to the special needs of the constructs. The designed sequence was then sent to Eurofins MWG Operon© who provided us with the designed primer as given below in Table 10.

Table 10. List of Primers

Primer	Sequence	Manufacturer
p196 Forward	GCCCACCCCAAGATGAGTGC	Eurofins MWG Operon©
p251 Forward	CCAGTATCAGCCCGTCATAC	Eurofins MWG Operon©
p252 Forward	AGACAGCGACGGGTTCTCTG	Eurofins MWG Operon©
p253 Reverse	GAAATTCTATGGCTCGGATC	Eurofins MWG Operon©
p266 Reverse	CTAGCCAACGCGACAAAAAC	Eurofins MWG Operon©
p267 Reverse	GCACGATTTCGACGTTGCTA	Eurofins MWG Operon©

METHODS

2.7 Plant growth conditions

2.7.1 Seed sterilization

Seeds of *Nicotiana tabacum* were washed with 5% Dimanin C along with few drops of washing liquid and were gently stirred for 10 min. The solution was pipetted out and the seeds were then soaked in 70% ethanol for 1 min, washed three times in distilled water and air dried overnight at room temperature. The dried seeds were transferred to a new sterile petri dish sealed tightly with a parafilm and stored in refrigerator at 4 °C.

2.7.2 In vitro Plant growth in sterile culture

For seeds culture, ten healthy sterilized seeds were cultivated on B5 medium containing 2% sucrose and 0.8 % bactoagar, which were then placed in growth chamber at 25 °C (Universal lamps with white fluorescence, light intensity; 0.5–1 W/m^2 Osram L85 W/25). After two weeks of culture, seeds started to germinate. The healthy seedlings were transferred to fresh MS medium in jar glasses. Seedlings developed healthy and mature leaves after four weeks of culture which were later used for protoplast isolation and particle gun transformation.

2.8 Bacteria growth conditions

Escherichia coli cells were cultured on LB medium at 37 °C for 16 hours. Transformed *E. coli* cells were selected by its antibiotic resistance, which was obtained through the transformation with the antibiotic resistant marker gene. Only cells containing these plasmids were able to grow on selection media.

2.9 Preparation of DNA

2.9.1 Plasmid-DNA preparation (Tens Miniprep)

For isolation of plasmid DNA *E. coli* culture was prepared in incubator at 37 ºC, 220 rpm overnight. The next day, bacteria were pelleted from culture by centrifugation at 15000 rpm for 3 min and supernatant was removed. Pellets were resuspended in 350 µl TENS PH 8.0 and inverted 6-8 times to mix. 150 µl 3M potassium acetate (PH 5.2) was added until a white precipitate formed then was centrifugated at room temperature/15000 rpm for 5 min. The supernatant was transferred by pouring into a fresh tube with 500 µl isopropanol, vortexed to mix and spun at 15000 rpm 8-10 minutes to pellet cell debris and chromosomal DNA, then was washed with 900 µl 100% EtOH (ethanol). Pellet was spinned again at 15000 rpm 10 minutes, washed with 500 µl 70% EtOH. Supernatant was removed. DNA was air dried for 5-10 min, resuspended in 50 µl TE containing RNAse. The solution was incubated at 70ºC for 10 min to inactivate contamination of RNAse and stored at 4ºC.

2.9.2 Plasmid-DNA preparation (QIAfilter Plasmid Midi and Maxi Kits)

For transformation, plasmid DNA was isolated by using QIAfilter Plasmid Midi Kit which yielded 100 µg of plasmid DNA and up to 500 µg using the QIAfilter Plasmid Maxi Kit. In these methods by using alkaline/SDS lysis procedure high concentration of DNA can be isolated. DNA is denatured by these alkaline reagents, while addition of potassium acetate to the denatured lysate plasmid DNA, which remains in solution, can revert to its native supercoiled structure. Using QIAfilter Cartridges plasmid DNA is bound to anion-exchange resin and eluted with an alkaline buffer after a number of washing steps. The eluted DNA is precipitated with isopropanol and dissolved in sterile distilled water.

2.9.3 Isolation of genomic DNA from plant tissue (CTAB method)

Liquid nitrogen was used to quick freeze the plant tissue and then quickly ground to fine powder, keeping tissue frozen the entire time. 700 µl of pre-warmed CTAB solution (65°C) was added to each sample in a 1.5ml eppendorf tube. Solution was incubated 65°C for 1-1.5 h in re-circulating water bath, gently mixed by inverting from time to time. After

incubation, 700 µl chloroform was added to each sample and gently mixed by inverting tubes for 30 min. The mixture was centrifugated for 15 min at 10.000 rpm at 4 °C and the supernatant was transferred into a new eppendorf tubes. Isopropanol was used to precipitate the DNA and the solution was spun down for 10 min at 10.000 rpm at 0 °C. Pellet was washed twice with ice cold 70% ethanol by for 10 min at 10.000 rpm at 0°C and then air dried for 15 minutes. DNA was resuspended in dissolved in sterile distilled water and stored for later use.

2.10 Cloning and *E. coli* transformation

2.10.1 DNA digestion with restriction enzymes

Digestion of appropriate amounts of plasmid DNA with corresponding restriction endonucleases (reaction volume 30 µl) was performed in appropriate buffer systems provided by the manufacturers (MBI Fermentas, Vilnius, Lithuania) at the recommended temperature.

2.10.2 Ligation of vector and DNA fragments

To ligate DNA fragments, Fermentas ® Rapid DNA ligation Kit, No 1422 was used provided by MBI Fermentas, Vilnius, Lithuania. Following reaction mixture was prepared in a reaction tube and incubated at 16 °C over night.

Linear vector DNA ~	50 ng
Insert DNA	3:1 molar ratio of insert to vector DNA
10x ligation buffer	1.5 µl
Ligase enzyme	1.0 µl
H_2O	add to 15.0 µl

2.10.3 Electrocompetent *E. coli* cells

E. coli strains Sure, JM110, XL1 Blue and DH5α were streaked on LB Medium plate carrying no antibiotic, to isolate colonies and were incubated overnight at 37°C for 16-20 hours. The next day, 12 test-tubes were filled each with 4 ml of LB medium without any

antibiotic. Each tube was inoculated with 5 isolated colonies and incubated at 37°C on a shaker at 220 rpm for 3.5 hours. All the culture was mixed in a beaker to equalize cell density and transferred to eight conical flasks containing 125 ml of fresh liquid LB medium, flasks were placed on a vigorous shaking at 37°C, 220 rpm until OD600 ~ 0.6 (OD Checked every 30 minutes, when reached 0,6 then chilled on ice). The culture was chilled on ice for 10 min before transferred to centrifuge tubes. The culture was transferred to 4 centrifuge tubes and was centrifuged at 4°C, 4000 g for 15 min. The supernatant was carefully discarded and the pellet was gently resuspended in 2 ml cold distilled water and later diluted to 100 ml in cold sterile-distilled water. The suspension was centrifuged for 15 min at 4000 g / 4°C. Washing of the pellet was done in two steps and the cell pellet was resuspended in 2 ml cold sterile-distilled water and then diluted 1:1 into cold 50% glycerin. The cells were then dispensed in 100 µl aliquot each into chilled and sterile eppendorf tubes and stored at -80°C.

2.11 Tobacco protoplast isolation

2.11.1 Plant Material

Sterilized seeds of *Nicotiana tabacum* L. cv. Petit Havana were germinated on a B5 medium. Seedlings were shifted to fresh MS medium in growth chamber after two weeks.

2.11.2 Incubation

Young tobacco leaves (4-5 weeks old), were incubated for 1 h in the dark with 9.5 ml of F-PIN. After one hour, 250 µl cellulase and 250 µl macerase enzymes were added. Solution was incubated at room temperature on slow shaking for 20 min and digestion was performed at room temperature for overnight (16-20 hours) in the dark.

2.11.3 Protoplasts purification

For the protoplast purification, the incubation mixture was filtered through a 100 µm stainless steel sieve to remove the debris. Rest of the solution was collected in a 12 ml centrifuge tube, 2 ml of MMM solution was carefully added as the top layer and the mixture was centrifuged at 70 g (rcf) for 10 min at 24 °C. After the centrifuge three clear layers were

formed in the centrifuge tube, damaged protoplasts and the debris in the bottom, large unsuited protoplast on the top layer and the optimal protoplast in the middle layer. Protoplasts were carefully collected from the interphase and transferred to a fresh centrifuge tube.

2.11.4 Protoplasts culture

Protoplasts were pellet by centrifugation at 50 g (rcf) for 10 min at room temperature. Pelleted protoplasts were suspended in MMM and MES media containing $MgCl_2$ and mannitol for osmotic stabilization. An equal volume of 2.4% alginate was used to adjust the protoplasts density to 4 x 10^5 PP/ml. Alginate embedding was performed on thin layers of plastic mesh sheet by spreading 625 µl of protoplast/alginate mixture on an area of a 10 x 10 meshes sheet, placed on Ca-Agar plate. The grids mechanically stabilize the gel layer thus facilitate the transfer of embedded cells/colonies to different dishes during further steps of culture. It also facilitates defining the location of individual cells for tracking their development during further culture steps. After solidification on Ca- Agar in the dark, grids were carefully removed and placed into 7 ml F-PCN. Next day, the cultures were transferred to 7 ml fresh F-PCN and shifted to the growth chamber. Cell wall started to develop in couple of days.

2.11.5 Regeneration and Cultivation

First micro colonies were formed after one week on the grid. After the first microcolony formation grids were shifted to solid RMOP without antibiotic. Green calli started to grow after two weeks which were transferred to fresh RMOP media after every 15 days. Small shoots started to grow on the calli after three to four weeks of culture. Shoots were removed from the grids and rooted on MS-medium containing appropriate hormones.

2.12 Transformation techniques

2.12.1 PEG mediated protoplast transformation

For transformation, 100µl of protoplast suspension was transferred to a sterile petri dish and the protoplasts were allowed to settle for few minutes. 18 µl of 50µg plasmid DNA

was added drop by drop to the petri dish. The solution was gently mixed and then 12µl FPCN and 125µl of 40%PEG solution was added slowly mixed and incubated for 8 minutes. For a second time 120µl FPCN was added, mixed and incubated for 8 minutes, followed by another 2.6 ml FPCN, mixed and incubated for 2 minutes. Protoplast density was adjusted to 8.5 x 10^4 pp/ml using F-alginate. 625µl of protoplast-alginate mixture was applied to plastic mesh grid present on the surface of Ca agar medium and let it to solidify in dark for two hours. After solidification the grids were transferred to 7 ml liquid FPCN for equilibration in the dark for 1hour. Finally the grids were transferred to 7ml of fresh FPCN solution in a new petri dish, wrapped with parafilm and stored.

Transformed protoplasts were incubated at room temperature in dark for three days before transferring to normal light conditions. After one week of transformation the embedded protoplasts transferred to RMOP medium containing 500 mg/L spectinomycin for selection. Grids containing protoplasts were transferred to fresh medium every three weeks. First resistant colonies started to grow after 7-8 weeks of transformation. Transgene integration into the plastid genome was confirmed by PCR analysis.

2.12.2 Biolistic transformation (Particle delivery system)

For the biolistic transformation method PDS-1000/He Particle Delivery System by Bio-Rad Laboratories was used. The system uses the pressurized helium gas to bombard DNA coated gold particles on leaf tissues present in a vacuum chamber. Young tobacco leaves grown on MS media in sterile conditions were used as the target tissues for the gene gun bombardment. Young healthy leaves were placed on RMOP medium with abaxial side up for one night before the bombardment.

a) Sterilization of microcarriers

30mg of gold particles were accurately weighed and transferred to 1.5 ml eppendorf tube. 1 ml of 70% ethanol was added to the tube, vortexed for 15 minutes and centrifuged for 10 seconds at maximum speed. Supernatant was removed and the gold palette was washed again with 70% ethanol, washing was repeated for two more times. After third washing the

supernatant was discarded and the gold particles were resuspended in 500µl of 50% glycerol with a final conc. of 60 mg/ml.

b) DNA coating of microcarriers

50 µl of sterile microcarriers resuspended in glycerol were removed to a new 1.5 ml eppendorf tube and vortexed for 5 minutes. After 5 minutes of vortexing 5 µl DNA (1µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl 0.1M spermidine were added while vortexing. The mixture was incubated on ice for 10 minutes and then centrifuged for 1 min at 8 000 rpm. The supernatant was removed and the pellet was washed with 140µl 70% ethanol by centrifuging 1 min at 10,000 rpm. In the next washing step pellet was washed with 140 µl of 100% ethanol for 1 min at 10, 000 rpm. Now the supernatant was removed and the DNA-coated microcarriers were carefully resuspended in 48 µl of 100% ethanol and put on ice ready to use for bombardment.

c) Bombardment

All the equipments along with the chamber walls of particle gun were sterilized with 70% ethanol. 6µl of freshly prepared DNA coated gold particles were loaded on macro carries in macro carrier holder. Sterile rupture disk of 1100 psi was placed in retaining cap and secured to the gas acceleration tube. Plant tissues were delivered with DNA coated gold microcarriers in the vacuum chamber by a pressure of 1100 psi.

The bombarded leaf tissues were placed on RMOP medium in dark for two days. After two days the leaves were cut into small pieces (~ 5 mm), were transferred to RMOP medium containing 500 mg/l spectinomycin and kept at 26°C in growth chamber under standard light conditions. After three weeks of transformation the resistant shoots started to regenerate which were transferred to new and fresh medium. In order to obtain homoplasmic plants transplastomic shoots were subjected to 2-4 additional rounds of regeneration on RMOP medium containing 500 mg/l spectinomycin.

2.13 Molecular analysis

2.13.1 Polymerase chain reaction (PCR) analysis

The PCR amplification of DNA fragments were performed in a 50 µl reaction mixture using thin walled PCR tubes in a PCR thermocycler (PCR PX2 Thermal Cycler, Thermo Electron Corporation, USA)

Reaction mixture:

DNA Template (1-10 ng)	3µl
Primers (10 pmol/µl)	1 µl (each)
dNTPs (25 mM)	1 µl
$MgCl_2$ (25 mM)	4 µl
PCR buffer (10 x)	5 µl
Taq polymerase (5U/µl)	1 µl
H_2O	added to 50µl

2.13.2 Southern Blot analysis

Southern blotting is the transfer of DNA fragments from agarose gel to a nylon membrane through capillary action. After immobilization, the DNA is subjected to hybridize with a specific labeled probe to visualize the sequence of interest. DIG high prime DNA labeling and detection starter kit by Roche, Germany was used in the experiment. 2µg of transgenic and wild type DNAs were restricted by *Bgl*II restriction enzyme and were run overnight on 0.8% Agarose gel at low voltage. DNA was transferred onto N+ nylon membrane (GE Healthcare, Munich, Germany) using 0.6M NaCl and 0.4 M NaOH solution through whatman paper and paper towels by capillary action overnight. Membrane was washed with SSC solution and baked at 120°C in an oven for 30-45 minutes to cross link DNA to the membrane. Pre hybridization of the membrane was done with DIG Easy Hyb (supplied with labeling kit) for 3 hours on a water bath at 45C. For the hybridization of membrane with appropriate probes (purified PCR products) previously labeled probe with DIG labeling mix using the DIG high prime DNA labeling kit (Roche, Germany) according to the manufacturer's protocol. Hybridization was performed at 45C for 17 hours. Afterwards

membranes were washed twice with SSC and wash buffer (0.1 M Maleic acid, 0.1 M NaCl, 3% Tween) in order to increase hybridization stringency and thereby eliminating the non-specific background.

Membranes were incubated for 30 minutes with blocking solution (100 ml for 100 cm^2 membrane) and incubated appropriate antibody solution for 30 minutes. After the incubation the antibody solution is discarded and the membranes are washed two times for 15 minutes each in washing buffer. 30 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) was used to equilibrate membranes for 2-5 minutes at room temperature with gentle agitation. 1 ml CSPD ready to use (supplied with the kit) was applied to the membrane and the membrane was then exposed to X-ray film in an exposing folder. Developer and fixer solution were used to develop the X-ray film.

2.13.3 Western Blot analysis

Young tobacco leaves (100mg) were obtained from the transplastomic plants grown under sterile conditions and were ground using liquid nitrogen. Powdered leaves were homogenized in SDS-buffer containing 32% glycerol, 13% beta-mercaptoethanol,185 mM Tris-HCl (with 6.8 pH), 6.5% SDS, 0.02% bromophenol blue and was incubated for 5 minutes at 95ºC. SDS-PAGE was used to separate the proteins, which were then transferred on to the nitrocellulose membrane, Hybond C (GE Healthcare) and blocked with 5% of skimmed milk for half an hour. Membrane was washed three times with PBS containing tween and was incubated for 1 hour with peroxidase conjugated goat anti mouse IgG (sigma) as secondary antibody diluted 1:3000 in skimmed milk. Proteins were spotted by chemiluminescence and bands were visualized on X-Ray film.

2.13.4 MUG assay

Young tobacco leaves from the transplastomic plants grown under sterile conditions were homogenized in 100 µl of extraction buffer (50 mM $NaPO_4$, pH 7.0 , 10 mM dithiothreitol (DTT), 1 mM Na_2EDTA , 0.1% Sodium Lauryl Sarcosine, 0.1% Triton X 100). The mixture was centrifugated at 15000 rpm, 4C for 5 min. 22 mg of 4 Methyl umbelliferyl B D glucuronide (MUG) was dissolved in 50 ml GUS extraction buffer in a 50 ml disposable

polypropylene tube which can be stored at 4 C for up to two weeks. Assay buffer (1mM MUG in extraction buffer) was incubated at 37°C to pre warm the buffer. 50ul of extract was added to 0.5 ml assay buffer and mixed thoroughly on a vortex. At different desired time intervals solution was transferred to new tubes and stop buffer (0.2 M $Na_2CO_{3)}$ was added.

2.13.5 Enzyme-linked immunosorbent assay (ELISA)

Young tobacco leaves (100mg) were obtained from the transplastomic plants grown under sterile conditions. Leaves were grounded in fine powder using liquid nitrogen and homogenized in the extraction buffer which contained 5 mM $MgCl_2$, 5mM $CaCl_2$, 1M Sodium chloride, 0.01% Triton X-100, 20 mM Hepes (with 7.4 pH) and 1 mM PMSF. The homogenized mixture was centrifuged at 18 000 g for 5 min and the supernatant was collected in a separate clean tube. The 96-well microtitre plate (Costar Corning, Corning, NY, USA) was coated with 50 µL of HPV-16 L1 conformation specific mouse monoclonal antibody Ritti01 (Thones et al. 2008) with a dilution of 1 : 300 in PBS and left for overnight at 4C. Plates were rinsed with PBS-T and blocked with PBSTM for 1 h at 37°C. Homogenized plant extract was added to micro titre plate and incubated for one hour at 37°C. 50 µL of polyclonal rabbit antiserum (1 : 3000 in PBSTM) raised against HPV-16 L1 was added in each well and plates were again incubated for 1 h at 37°C. Plates were washed three times and incubated for 1 h at 37°C after the addition of 50 µL of goat anti rabbit peroxidase conjugate supplied by Sigma (1: 3000 in PBSTM) in each well. Plates were washed thoroughly before adding 100 µL of staining solution in each well. After 15 minutes the measurements at 405 nm were carried out.

2.14 Vector construction

2.14.1 pPNGST-$L1_M$-T

For the construction of the vector first a precursor vector pPNG1014_MCS120 was constructed with slight modifications (also used by Waheed et al. 2011a). This precursor vector included the plastid encoded polymerase (PEP) promoter from *rrn* 16 gene (P*rrn*) as reported by Svab and Maliga (1993), with the ribosomal binding site (RBS) from the leader sequence of gene 10 (G10L) of the λ-phage T7 (Studier et al. 1990), nuclear encoded

polymerase (NEP) promoter, Prrn^{-62}NEP, nuclear encoded polymerase promoter, G10L RBS, ribosomal binding site from gene 10 leader sequence, multiple cloning site (MCS) and 5´-untranslated region (5′ UTR) consisting of synthetic ribosomal binding site. Restriction sites Nhe1 and AflII was used to clone modified L1 gene (L1_2xCysM) and restriction sites NcoI and Nhe1 to clone GST gene in the precursor vector to get the plasmid pPNGST-L1$_M$. To achieve final transformation vector plasmid pT7PHB-N (Lössl et al. 2005) was used which already contained *aad*A gene which conferred resistance against spectinomycin and streptomycin, 3´UTR from large subunit of ribulose-bisphosphate carboxylase gene (T*rbc*L) and the flanking regions INSL and INSR, homologous to the respective loci *trn*N and *trn*R in the inverted repeats (IR) of the plastid genome. This plasmid was cut with restriction enzymes *Sac*II and *Bam*HI and the whole construct of pPNGST-L1$_M$ was inserted in the vector using restriction enzymes *Sac*II and *Bgl*II. All cloning techniques were performed using the standard protocols as explained by Sambrook et al. (1989).

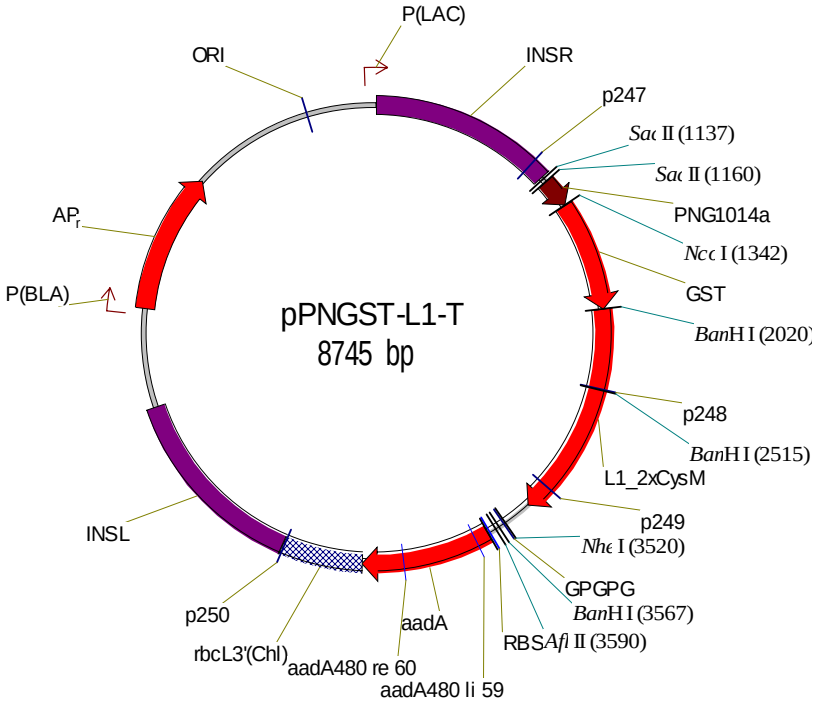


Figure 4. Complete gene map of transformation vector pPNGST-L1M-T

2.14.2 p2PNGL-mmpI-T

For the construction of this vector a pre constructed intermediate vector pPNGL-mmp (Dr. Lössl lab, unpublished) served as a precursor vector. The vector already contained *Escherichia coli* heat-labile enterotoxin subunit B (LTB) and major membrane protein (mmpI) under the effect of psbA promoter. Restriction sites EcoRV and Xba1 were used to to remove the LTB and mmpI from this vector. To achieve final transformation vector plasmid pPNG1014-glpk (Dr. Lössl lab, unpublished) was used. This vector already contained the plastid-encoded polymerase (PEP) promoter from *rrn* 16 gene (P*rrn*) (Svab and Maliga 1993) with the ribosomal binding site (RBS) from the leader sequence of gene 10 (G10L) of T7 lambda phage as reported by Studier et al. (1990), nuclear encoded polymerase (NEP) promoter, Prrn^{-62}NEP, nuclear encoded polymerase promoter, G10L RBS, ribosomal binding site from gene 10 leader sequence and 5´-untranslated region (5′ UTR) consisting of synthetic ribosomal binding site, *aad*A gene providing resistance against spectinomycin and streptomycin, terminator from large subunit of ribulose-bisphosphate carboxylase gene (T*rbc*L) and the bordering regions INSL and INSR, homologous to the respective loci *trn*N and *trn*R . Restriction enzymes EcoRV and Xba1 were used to replace glpk with the construct LTB mmpI. All cloning techniques were performed using the standard protocols as explained by Sambrook et al. (1989).

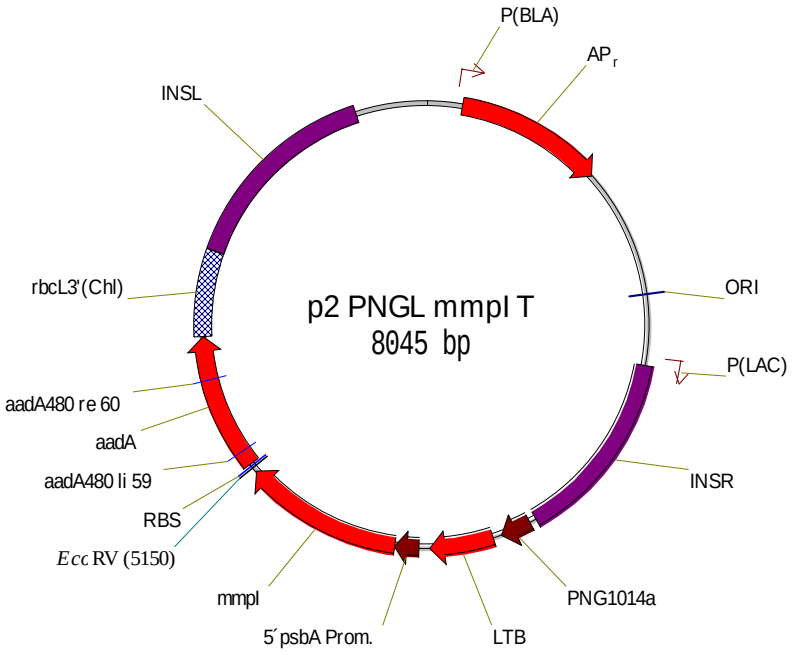


Figure 5. Complete gene map of transformation vector p2PNGL-mmpI-T

CHAPTER III
RESULTS

The present work contains the generation of transgenic tobacco plants and the assessment of antigen expression against human papillomavirus (HPV) and Mycobacterium spp. by chloroplast transformation. To increase security of plant made pharmaceuticals and to improve the selection process of transformants an inducible expression system is recommendable (Lössl and Waheed 2011). Therefore this study is accompanied by a quantitative analysis of the expression of an inducible system for transplastomic plants.

3. 1 Chloroplast derived vaccine against human papillomavirus (HPV)

To allow one-step purification of L1 fusion protein from transformed plants, the major capsid protein L1 from human papillomavirus (HPV) was planned to be fused C-terminally to glutathione S-transferase (GST) and transformed into tobacco plastids.

3.1.1 Plastid Transformation vector design

The vector pPNG1014_MCS120 served as a precursor to construct a transplastomic vector pPNGST-$L1_M$-T (Fig 6), containing L1 gene fused to glutathione S-transferase (GST). Using the restriction sites NcoI, AflII and NheI the GST and L1 was cloned into precursor vector. The fused L1-GST gene was expressed by a cassette containing both, the promoters for the nuclear and plastid encoded RNA polymerases, the P*rrn*PEP and P*rrn*$^{-62}$NEP respectively. For selection of transformants the aminoglycoside 3`-adenyltransferase (*aad*A) gene was co-expressed with the same promoter. For homologous recombination within the tobacco chloroplast genome the trnR served as right insertion site and trnN as left insertion site.

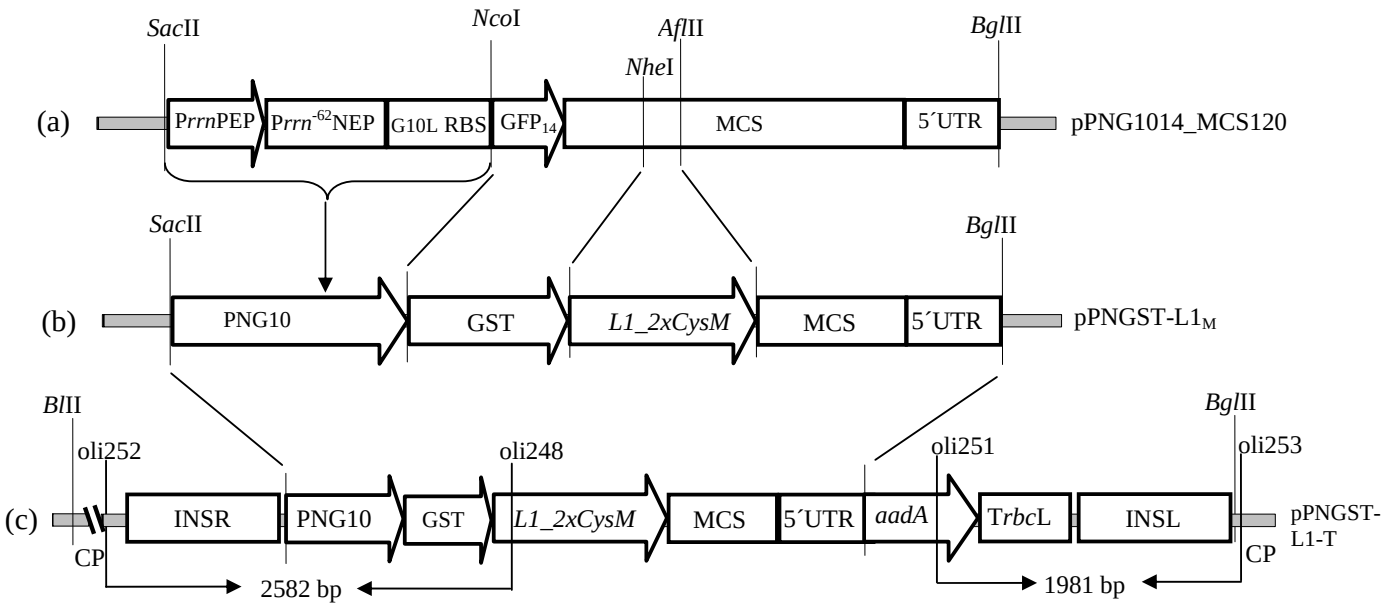


Figure 6. Schematic diagram of transformation vectors development.

(a) Precursor vector pPNG1014_MCS120 was used to clone transgenes. **(b)** Plasmid pPNGST-$L1_M$ obtained after the insertion of transgenes GST and L1_2xCysM in the precursor vector. **(c)** Final transformation vector pPNGST-$L1_M$-T for the transformation of plants with fused GST-L1_2xCysM gene, showing transgenes along with plastome flanks inserted within the tobacco plastid genome. PrrnPEP, plastid encoded polymerase promoter from rrn 16 gene; $Prrn^{-62}$NEP, nuclear encoded polymerase promoter; G10L RBS, ribosomal binding site from gene 10 leader sequence; GFP14, first fourteen amino acids of the green fluorescent protein; MCS, multiple cloning site; 5´ UTR, 5´ untranslated region; *aad*A, aminoglycoside 3´-adenyltransferase; PNG10, cassette containing PrrnPEP, $Prrn^{-62}$NEP and G10L RBS; CP, chloroplast DNA; GST, glutathione S-transferase gene; L1_2xCysM, modified L1 gene; TrbcL, terminator from large subunit of ribulose-bisphosphate carboxylase gene; INSR, right insertion site (trnR); INSL, left insertion site (trnN).

3.1.2 Transformation of tobacco plants.

Three weeks old tobacco (Petit Havana) leaflets were biolistically transformed using BIORAD's PDS-1000/He Particle Delivery System. Leaves were cut into 5mm pieces and transferred to RMOP medium containing 500 mg/l spectinomycin for cultivation at 26°C in a growth chamber under standard lighting conditions. Wild type plants served as control. After three weeks of incubation inoculated leaves from control plants and all untransformed explants on selective medium became bleached. While the successfully transformed leaflets produced green micro calli, they looked healthy and showed very rapid growth even on selective medium. The leaf surface started to get covered with calli on its edges and after two more weeks small shoots were induced from calli. These transformed shoots were subjected to selective media and to the same conditions for further one and a half month to achieve homoplasmy. After 5weeks of vigorous selection the shoots were transferred to the B5 rooting medium. Soon after root development the plants were transferred to the green house in pots.

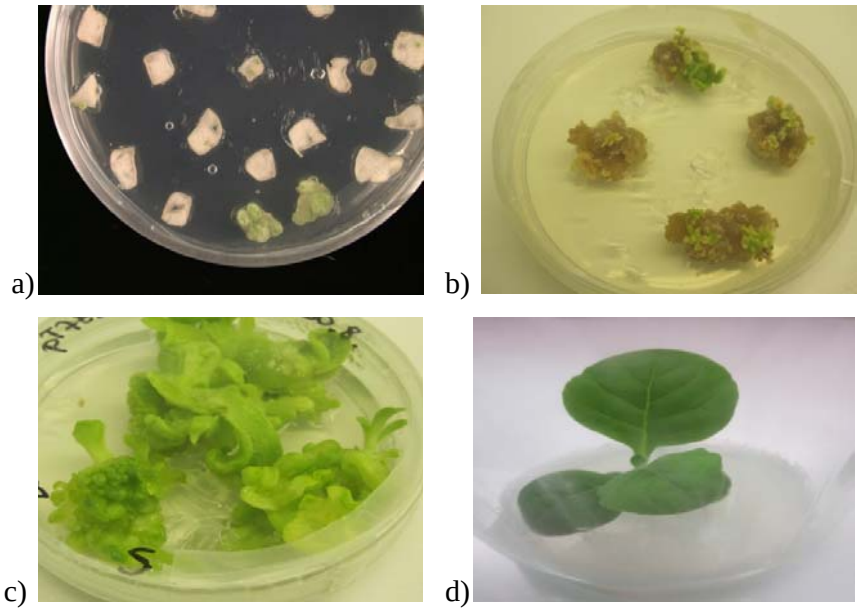


Figure 7. Tobacco leaf cut into small pieces after transformation; **a)** Callus starting to develop in transformed parts of the leaf; **b and c)** Transformant shoots which emerged from calli on selection medium; **d)** Fully grown shoot transferred to B5 rooting media.

3.1.3 Morphology of Transformed Plants

Plants transformed with GST-L1 gene appeared healthy and normal. Morphologically they look liked wild type. Earlier in a different experiment we have observed male sterility in transplastomic plants (Waheed et al. 2011a) but surprisingly all the transplastomic lines carrying GST-L1 gene produced healthy flowers and were fully fertile. The plants were checked for two generations through their F1 and F2 and all of them were morphologically sound and fertile. Growth of seeds was completely uniform on antibiotic media which confirmed their homoplasmy.

a)  **b)** 

Figure 8. Transplastomic plants carrying transgene GST-L1 were morphologically healthy and produced viable flowers.

3.1.4 PCR confirmation of the transgene cassette

Integration of GST-L1_2xCysM gene into transplastomic plant genome was confirmed by PCR analysis. The primers oli248 (in L1, reverse) and oli252 (forward at 5` end in INSR, trnR) were designed to amplify a DNA fragment of 2582 bp in all seven lines as shown in FIG 9a. A second PCR with the primers oli251 (in *aad*A, forward) and oli253 (reverse, at 3` end, INSL or trnN) amplified the 1981 bp DNA fragment, confirming the site specific integration of *aadA* gene within the plastid genome (FIG. 9b). The results were identical to the PCR analysis of generation F1 and F2 transformants.

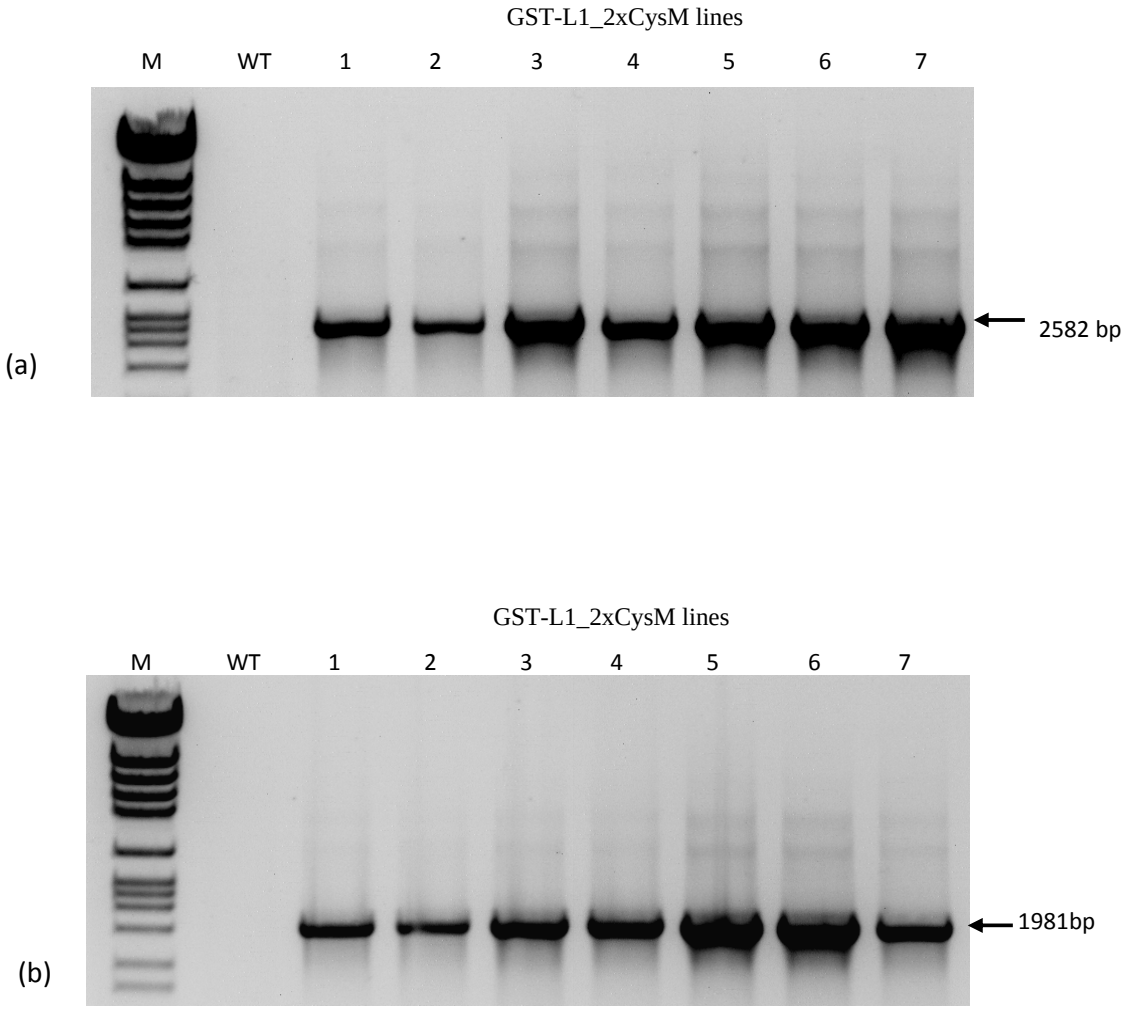


Figure 9. PCR analysis of the transgenes inserted correctly within the plastid genome, **a;** Amplification of GST-L1_2xCysM gene (2582 bp) with the primers oli248 in the L1_2xCysM gene and oli252 located within the plastome, **b;** Amplification of the *aad*A gene (1981 bp) with primers oli251 in the *aad*A gene and oli253 present within the plastid genome. Seven independently generated transplastomic lines (Lanes 1,2,3,4,5,6,7) were analyzed. M: marker, WT: wild type.

3.1.5 Confirmation of the transgene cassette by Southern analysis

Southern blot analysis performed to confirm homoplasmy of all selected PCR positive plants. Plant DNA was digested with the enzyme BglII and a site specific probe served to confirm the transgene integration. Transformed plants containing GST-L1_2xCysM were confirmed by a 9.7 kb DNA fragment (FIG 10) while in the case of wild type plant a 5.9kb fragment was produced (FIG 10). Homoplasmy was confirmed by the fact that the wild type specific 5.9kb band was absent in all seven transplastomic lines. Homoplasmy was also tested and confirmed in transformed plants from F1 and F2 generations.

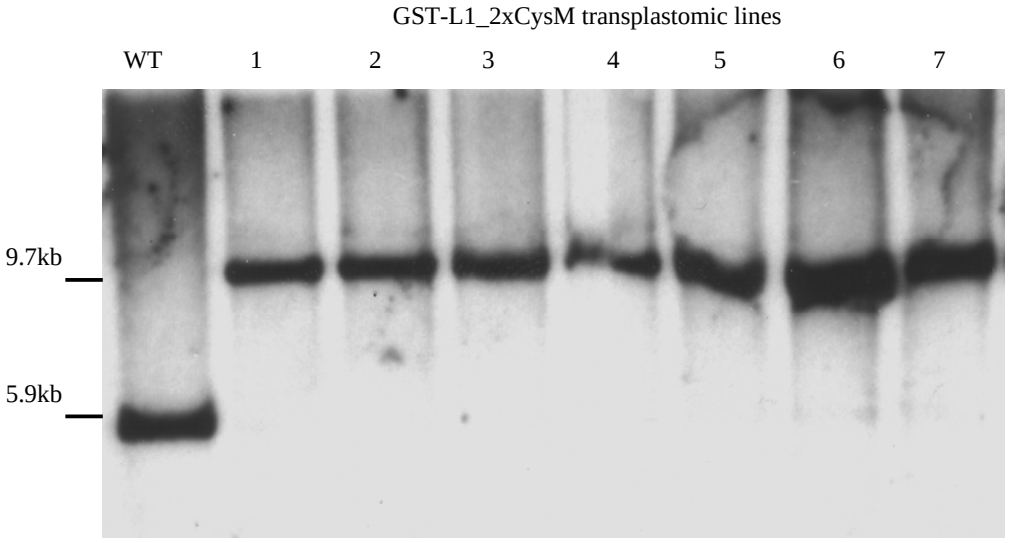


Figure 10. Southern blot analysis of GST-L1_2xCysM transplastomic plants. Seven independently generated transplastomic lines were analyzed for the transgene integration. WT, wild type. Total plant DNA was digested with BglII. The DNA sequence P (773 bp) located within left insertion site (INSL) of plastid genome, was amplified by PCR and served for probing in the Southern analysis.

3.1.6 Antigen capture ELISA and protein estimation

Enzyme-linked immunosorbent assay **(**ELISA) for all seven positive lines was performed to confirm the GST-L1 protein accumulation in the leaves of transplastomic plants. A ninety six well plate was coated with the conformational specific monoclonal antibody *Ritti01*. The plate was incubated with soluble protein from all seven transplastomic lines. The antibody bound to the L1 protein which confirmed the correct conformation of the recombinant protein. As positive control baculo-virus derived VLPs were used. All seven GST-L1_2xCysM transplastomic lines showed significant signals relative to the VLPs which served as positive control (FIG 11). Correct assembly of the protein into capsomers was confirmed with the binding of the conformation specific antibody to the L1 protein.

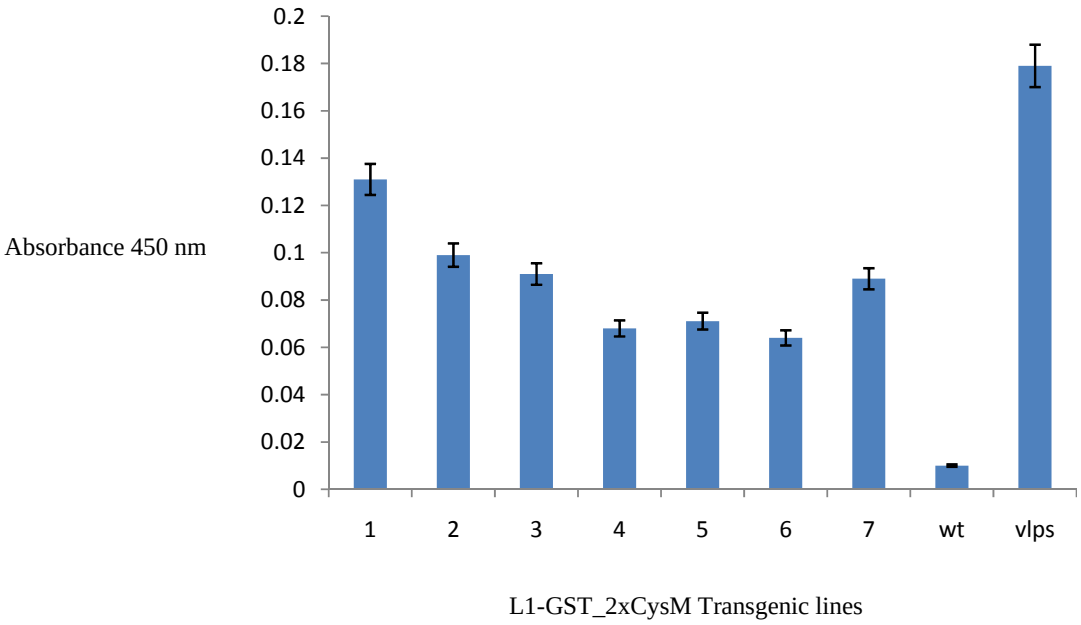


Figure 11. Antigen capture Enzyme-linked immunosorbent assay **(**ELISA) of the seven transplastomic lines showing the L1 protein accumulation in the leaf extracts. VLPs as positive controls for the assay (vlps). Detection of conformational epitopes was done by Monoclonal antibody Ritti01. Wild type (wt) as the negative control

3.1.7 Western blot analysis of transformants

Western blot analysis of all seven transformants was done with HPV-16 L1- specific monoclonal antibody MD2H11 from the German Cancer Research Center in Heidelberg (Schädlich et al. 2009a). Surprisingly the analysis did not detect protein with the corresponding monoclonal antibody. The experiment was repeated two more times but protein was below the detection limit in both the cases.

3. 2. Transplastomic protein expression against mycobacterium

In a second experiment the plastid transformation of an efficient cross-protective antigen against *Mycobacteria* was studied. A 35kDa protein encoded by the *mmp*I gene has been reported to be immunogenic against *Mycobacterium leprae* and also efficiently protective against *Mycobacterium avium* (Martin et al. 2000). This gene product was amplified and cloned into the high-expression conferring vector pPNG1014 for constitutive expression. Since constitutive expression of foreign proteins within the host genomes of crops is an issue of great concern, this analysis also covers the regulative expression of beta-glucuronidase (GUS) by its enzymatic reaction in an inducible expression system.

3.2.1 Transformation vector design

The plastid transformation vector p2PNGL-mmpI-T was constructed from the precursor vector pPNG1014_MCS120. The *mmpI* gene encoded for a 35kDa protein having immunogenic properties against *Mycobacterium avium* as well as *Mycobacterium leprae*. Both the *mmpI* and aminoglycoside 3`-adenyltransferase (*aad*A) were transcribed by the 5` *psb*A promoter while the Escherichia coli heat-labile enterotoxin subunit B (LTB) was controlled by 16S PEP Promotor, Prrrn-62 NEP and G10L-UTR. Insertion sites *trn*R for right insertion site and *trn*N for left insertion site served for homologous recombination within the plastid genome. Complete construction of vector p2_PNGL_mmpI_T is described in the section material and methods.

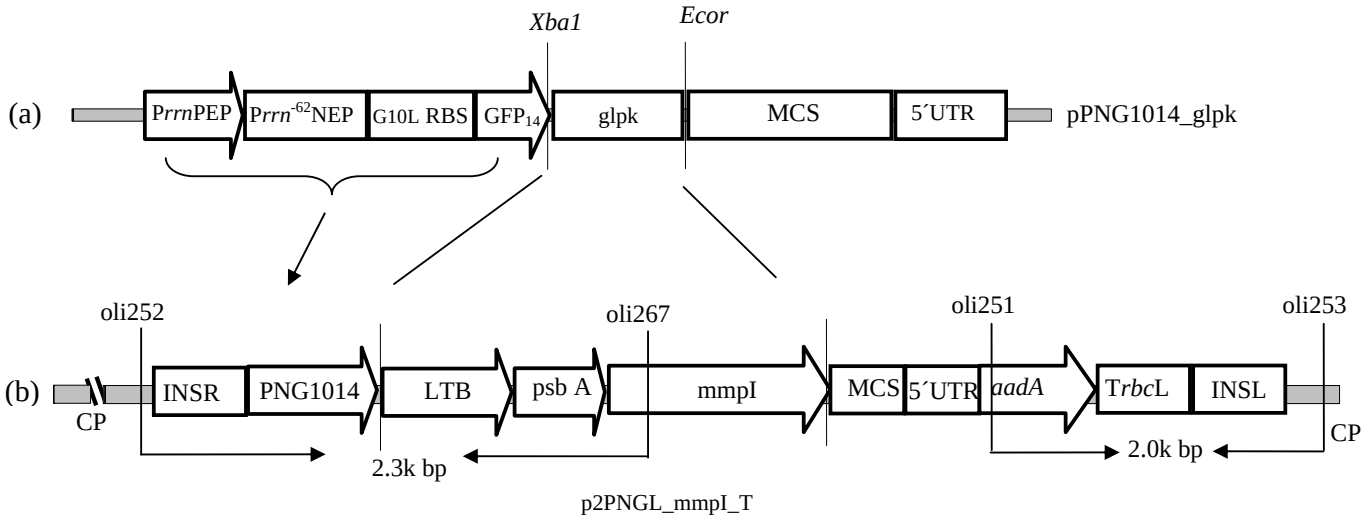


Fig 12. Schematic diagram of transformation vectors development. **(a)** Precursor vector pPNG1014_glpk was used to clone transgenes. **(b)** Plasmid p2PNGL-mmpI obtained after the insertion of transgenes LTB and MMPI in the precursor vector. Final transformation vector p2PNGL-mmpI-T for the transformation of plants with fused LTB and MMPI gene, showing transgenes along with plastome flanks inserted within the tobacco plastid genome. PrrnPEP, plastid encoded polymerase promoter from rrn 16 gene; Prrn^{-62}NEP, nuclear encoded polymerase promoter; G10L RBS, ribosomal binding site from gene 10 leader sequence; GFP14, first fourteen amino acids of the green fluorescent protein; MCS, multiple cloning site; 5´ UTR, 5´ untranslated region; *aad*A, aminoglycoside 3´-adenyltransferase; PNG1014, cassette containing PrrnPEP, Prrn^{-62}NEP, G10L RBS and GFP14 ; CP, chloroplast DNA; LTB, Escherichia coli heat labile enterotoxin subunit B; MMPI, major membrane protein I; TrbcL, terminator from large subunit of ribulose-bisphosphate carboxylase gene; INSR, right insertion site (trnR); INSL, left insertion site (trnN).

3.2.2 Polyethylene glycol (PEG) mediated transformation

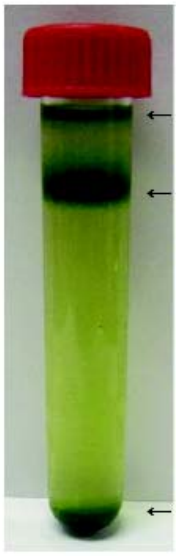
In 1996 Koop et al. first described the protoplast transformation procedure. The method was adapted to novel types of dishes, tubes and centrifuges. The PEG transformation method provides an effective and cost efficient transformation procedure and can easily be adopted in resource-poor countries. Particle gun is a very expensive instrument which may not be affordable for every research institute. It also requires a constant supply of gold particles for the gene bombardment and the gold prices have risen 5 times within the last 10

years. So we optimized the protoplast transformation method by PEG and applied this transformation technique for the following experiment.

3.2.3 Protoplast isolation

Five weeks old uncontaminated tobacco shoots grown on modified B5 media were taken to isolate protoplasts. We found in our study that the leaves on B5 medium containing high amount of calcium in comparison to the other culture media showed increase in protoplast survival and produced higher number of intact protoplasts. Protoplasts were isolated by incubation of 3-4 weeks old tobacco leaves in cellulase and macerase containing solution. The solution was filtered and centrifugated according to the adapted conditions of a swing out bucket centrifuge (Hettich labs). Due to the difference in the density the solution was clearly separated in three distant layers i.e. debris and damaged protoplasts in the bottom, large protoplast floating on the top and the intact and viable protoplasts in the middle layer of the solution (FIG 13a).

a)



b)

Fig 13. a; After density gradient centrifugation cell debris and damaged protoplasts are sedimented in the bottom layer, large and over mature protoplasts floating on the top layer while optimal protoplasts were collected from the middle layer, **b;** Healthy and fully intact protoplast isolated from tobacco leaves (Picture taken from the monitor attached to the microscope).

3.2.4 Protoplast Transformation

Healthy and viable protoplasts were subjected to Polyethylene glycol (PEG) transformation technique. Transformed protoplasts along with their alginate mixture were shifted to small pieces of SEFAR mesh of 2x2mm grids. This protoplast containing grids were first put on Ca-agar media and then to liquid F-PCN (Fig 14a). One week after transformation embedded protoplasts started to regenerate stable callus which were transferred to selective medium containing antibiotic spectinomycin (solid RMOP containing 500 mg/L). We choose to transfer the grids to fresh medium after every two week until no further regenerates appeared. The first resistant colonies were recovered approx 6–9 weeks after transformation. Shoots started to grow from the green calli regularly after two weeks of culture. These shoots were removed from the grids and rooted on B5 medium using appropriate hormones. To confirm the integration of the transgene cassette into plastid genomes, clones were analyzed by PCR analysis.

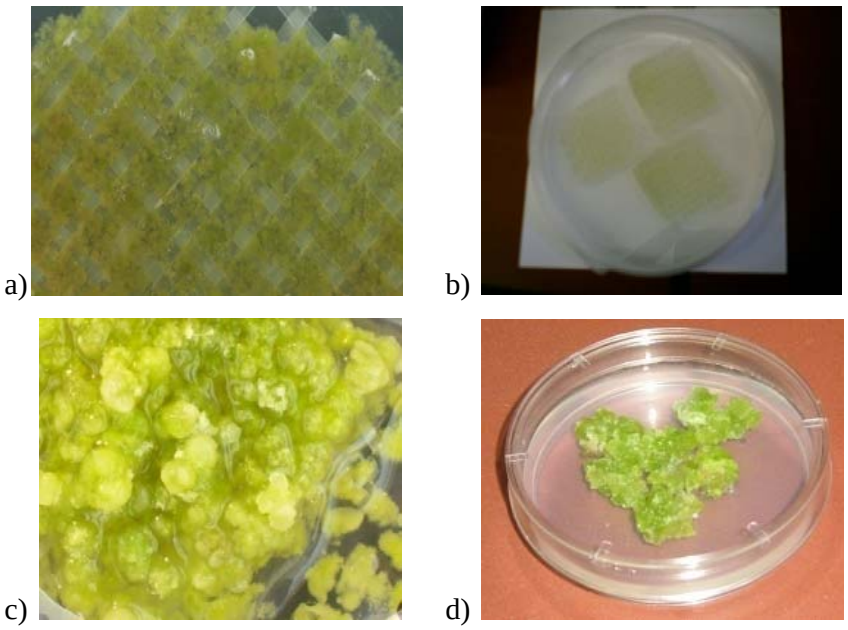


Figure 14. **a:** Transformed protoplasts present on the mesh grids in F-PCN solution, **b:** Embedding, Protoplast suspension mixed with alginate for embedding and solidified on mesh grids **c and d:** Green colonies individually picked and cultured on solid RMOP for shoot regeneration

3.2.5 Morphology of transformed plants

Plants transformed with *mmpI* gene were healthy and normal. All plants had regular growth pattern, they were able to reach maturity and produce viable seeds. All four transplastomic lines carrying *mmpI* gene were checked for the next generation, F2. All seeds grew uniformly on medium containing antibiotic and produced completely healthy and fertile plants.

a)  **b)** 

Figure 15. Morphologically healthy transplastomic plants carrying transgene LTB and *mmpI*

3.2.6 PCR confirmation of the transgene cassette

Integration of LTB and *mmpI* genes into transplastomic plant genome was confirmed by PCR analysis. The primers oli267 (in mmpl, reverse) and oli252 (in CP, Forward at 5` end in INSR, trnR) served for amplification of a DNA fragment of 2317 bp in all four lines as shown in FIG 16a. A second PCR with the primers oli251 (in *aad*A, forward) and oli253 (in CP, reverse, at 3` end, INSL or trnN) amplified the 2k bp DNA fragment, confirming the exact integration of *aadA* gene within the plastid genome (FIG. 16b). The results were identical with the PCR analysis of the generation F1 and F2 transformants.

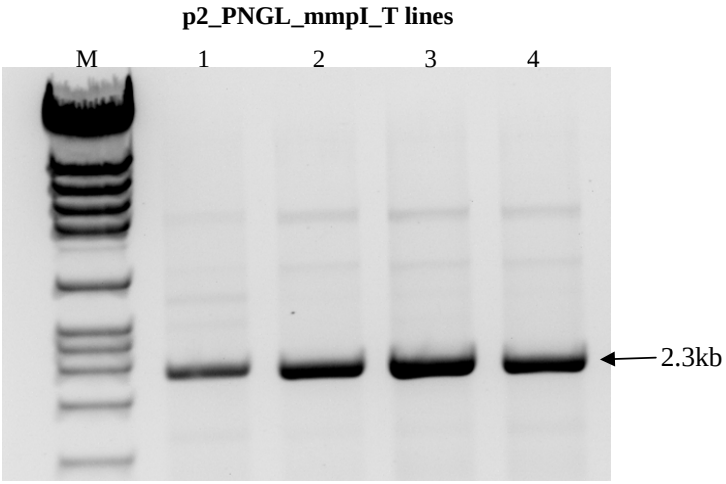


Figure 16a. PCR analysis of the transgene cassette inserted correctly within the plastid genome. Amplification of p2_PNGL_mmpI (2317 bp) with the primers oli267 in the *mmpI* gene and oli252 located within the plastome. Four independently transformed lines (Lane 1, 2, 3, 4) were evaluated. M: marker (SM0181)

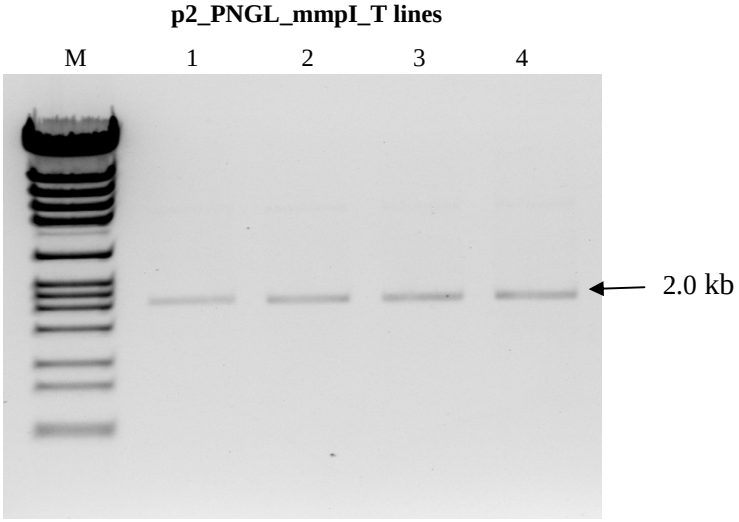


Figure 16b. Amplification of the *aad*A gene (2k bp) using primers oli251 in the *aad*A gene and oli253 present within the plastid genome. Four independently transformed lines (Lane 1,2,3,4**)** were evaluated. M: marker (SM0181)

Even though the PCR was positive, the *mmpI* transgene cassette was under the detection limit of Southern analysis. Therefore it must be considered that the plant tissue was only heterogeneously transformed. Heteroplasmy can be due to problematic transgene products which are detrimental to the plastid metabolism and hence prevent selection of transformant plant tissue. Therefore the experiment on *mmpI* expression was paused and

focus was laid on analysis of a novel method to circumvent this problem: The inducible chloroplast expression system.

3. 3 Inducible expression system

Constitutive expression of genes in chloroplasts can be of disadvantage in case the protein reacts toxically and it interferes with the plant metabolism. Interactions between foreign protein and metabolism in different growth stages potentially reduce plant productivity or even inhibit selection of primary transformants (Lössl et al. 2005). To omit this problem of counter-selection we studied a trans-activation system which allows to germinate the transformants without expression of the *goi* and which later induces the transcription of foreign gene in chloroplast. For this purpose an inducible expression cassette was tested which is detectable by GUS staining of the leaves and was based on the system described above.

3.3.1 Transformation vector

The plastid transformation vector pKCZ-GUS was constructed in cooperation with Icon Genetics (Bayer AG) from the precursor vector pKCZ according to the previously established system of Lössl et al. (2005). In this new version the beta-glucuronidase (GUS) gene was controlled by a nuclear encoded, chloroplast imported ethanol-inducible T7 RNA polymerase. GUS sequence was placed downstream of the T7 promoter sequence in the plastome, thus the GUS can only be expressed when it was sprayed with 0.3 - 5% ethanol. The selection marker gene for Aminoglycoside 3`adenyl- transferase (*aad*A) was placed under the Prrn16S promoter. For integration of the cassette the right insertion site carrying *trn*R and the left insertion site *trn*N served as flanking sites for homologous recombination into the plastid genome.

3.3.2 GUS assay and MUG test

Qualitative analysis:

Tobacco leaves were transformed with an inducible expression system as described above. The transformed leaves after induction with 5% ethanol showed developed blue color under normal white light confirming the presence of beta glucuronidase activity (FIG 17).

Figure 17. Plants leaves were stained blue under normal white light confirming the GUS expression upon ethanol induction.

Quantitative analysis:

A MUG assay was performed to estimate the quantity of protein in an inducible expression system. The healthy transformed plants were sprayed with 0.5% ethanol and after 24 hours the GUS activity was measured using MUG test (FIG 18). The assay confirmed that the induction by ethanol resulted in the activation of GUS gene however, a certain amount of background activity was also detected in not-induced transformants.

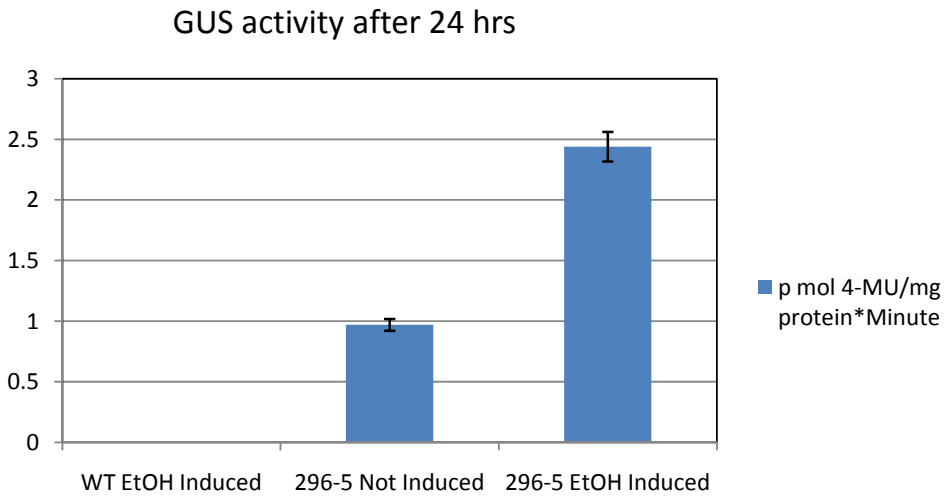


Figure 18. GUS activity of transformed plants after 24 hours of the ethanol induction, 4-MU: 4-methylumbelliferyl WT: wild type

CHAPTER IV
DISCUSSION

The present study is a report on the feasibility and analysis of vaccine-antigen production against cervical cancer and leprosy in tobacco chloroplasts. The study is also accompanied by a quantitative analysis of the expression of an inducible system for transplastomic plants.

Vaccines are playing a major role for health improvement of humans and animals in industrialized countries. They are the most potent tools in the fight against infectious diseases and have begun showing effectivity in prevention and treatment against various pathogens as reviewed by several authors (Hilleman 2002, Daniell et al. 2009, Yusibov et al. 2011). However, despite the successful performance of vaccination programs to eliminate infectious diseases still about 15 million people die each year due to these preventable causes, nearly all in low income countries (WHO 2008). Missing prevention also disables millions of people, it diminishes their quality of life and creates financial hardships. According to the World Health Organization there is a major need of developing new cost-effective vaccines against several diseases especially for resource poor countries (WHO 2009). Therefore more efforts are required to address the vaccination demand in these countries (Lössl and Waheed 2011). Currently available systems for commercial production of vaccines and therapeutic proteins mainly include use of bacteria, yeast, insect and mammalian cell cultures. These systems have their specific benefits, but their overall application is limited by insufficient scalability, expensive production, high distribution costs and safety issues (Yusibov 2008). It is difficult to retain the cold chain throughout from producer to consumer in resource poor countries and also have very high processing costs.

As a potential solution to these problems, plants as production platform have won considerable attention in the last decade (Rybicki 2010). Plants offer the unique opportunity to be engineered as biofactories for the production of foreign proteins and secondary metabolites (Hassan et al. 2011) and they are considered as an alternative and attractive source for the vaccine production (Wagner et al. 2004). Unlike nuclear transformation of plants, chloroplast genetic engineering is considered more valuable due to numerous

advantages which are mainly associated to the cost effective production and the regulatory issues of plant made vaccines (Lössl and Waheed 2011). Plastid transformation offers transgene confinement (Maliga 2004), high level of protein expression (Oey et al. 2009), opportunity to co express multiple genes (Lössl et al. 2003) and inducibility of expression (Lössl et al. 2005).

To examine the possibility of vaccine production in tobacco chloroplasts this study analyzes three related topics:

1. GST-L1 expression in tobacco chloroplast against human papillomavirus (HPV)
2. Transplastomic expression of *mmpI* gene conferring cross-protection against *Mycobacterium leprae* and *Mycobacterium avium*.
3. Inducible expression system for regulation of antigen synthesis

In the first part of the current study I have reported the possibility of producing a plant made vaccine against cervical cancer with expression of a modified L1 gene fused to glutathione S-transferase (GST) in tobacco chloroplasts. Cervical cancer is the second most common cancer type in women worldwide after breast cancer (Parkin and Bray 2006). In meta-analyses of HPV type distribution Smith et al. (2007) revealed that HPV16 and HPV 18 are the predominant causes of invasive cervical cancer worldwide contributing to 70 % of all cases. L1 is a major capsid protein of HPV which is capable of self-assembling into more complex molecular structures for instance virus like particles (VLPs) and capsomers (Sapp et al. 1998). Every VLP consists of 72 capsomers arranged on an icosahedral lattice and all 72 capsomers have distinct five-fold symmetry which confirms that they are pentamers of the major capsid protein, L1(Schädlich et al. 2009b).

VLP based HPV vaccines are very immunogenic and have shown to prevent HPV infection (Villa et al. 2006). Prophylactic human papillomavirus (HPV) L1 virus like particle (VLP) vaccines have been shown, in many clinical trials, to stimulate high titer anti-HPV serum antibody levels, well-tolerated and highly successful against this genital disease caused by the different types of HPV (Harper et al. 2006). However at present these vaccines are very expensive, they need delivery by intramuscular injection and require a continuous cold chain. As a result these vaccines in their current form will be largely unavailable to people in

resource poor countries where more than 80% of cervical cancer cases occur (Parkin and Bray 2006). Therefore there is a growing demand to develop cost effective second generation vaccines that are easy to administer and provide long term protection.

Recently capsomers have emerged as a potential cost saving alternative to VLP based vaccines against HPV (Stanley et al. 2008). Pentameric capsomers are considered to be thermo-stable and have shown elevated titers of neutralizing antibodies and L1 specific cytotoxic-T-lymphocytes (CTLs) upon hypodermic, intranasal or oral immunization (Schädlich et al. 2009a). In this study I opted for a modified L1 gene (L1_2xCysM): the replacement of two cysteines by serines in this modified form leads to the assembly of L1 protein in to capsomers (Schädlich et al. 2009b.) Also L1 protein with glutathione S-transferase (GST) fused to their N-terminus (GST-L1) has been reported to form proper pentamers (Chen et al. 2000).

The plastid expressed GST-L1 fusion protein was expected to assemble into proper pentameric capsomer form. To confirm this fusion protein assembly an antigen capture ELISA was carried out with the conformation specific monoclonal antibody “Ritti01”. Conformation specific antibodies only bind to protein with accurately presented epitopes. Baculovirus derived L1 protein served as a positive control for binding confirmation. The GST-L1 capture ELISA detected HPV antibodies with very high specificity and all of the seven transplastomic lines depicted significant signals relative to the positive control in ELISA (FIG 11, lanes 1-7). This result is rather promising, as it confirmed the formation of chloroplast derived immunogenic GST-L1 capsomers in recognizable conformational form: Capsomers of HPV which have been reported to react with conformation-specific antibodies have also induced neutralizing antibodies in rabbits and dogs which indicates that capsomers present conformational immunogenic epitopes of virions (Yuan et al. 2001, Thönes and Müller 2007, Schädlich et al. 2009a).

During the process of plastid transformation it is very much possible that only a single or few plastid genome copies were successfully transformed among thousands of ptDNA copies. Therefore as a first stage the transplastomic cell line will be carrying a mixed population of transformed and non-transformed genomes. Such cells, tissues or plants are called as “heteroplasmic” (Bock 2001). This implies clearly that genetic stability of

transformed plants require homoplasmy. During the experiment the tissue samples obtained from the regenerated transformants were re-exposed to 500 mg/l spectinomycin for repeated cycles to achieve homoplasmy. Homoplasmy of transplastomic plants was confirmed by Southern blotting (FIG 10).

The transformed plants expressing GST-L1 protein showed normal morphological characters like Wild types with no pleiotropic phenotypes. There have been some reports that expression of foreign protein in chloroplasts has effected plant growth and morphology (Lössl et al. 2003, Hasunuma et al. 2008, Tissot et al. 2008). In many of the reported cases, the causes are associated to some specific genes, though in a few studies the reason remains uninvestigated. Waheed et al. (2011a) has recently reported detrimental effects including male sterility, yellow coloration of leaves and stunted growth of transplastomic plants with the L1 fused to the LTB protein. Fortunately there are no pleiotropic phenotypes in our experiment with GST-L1 plants.

Western blot analysis of all seven transformants was done with HPV-16 L1- specific monoclonal antibody. Unexpectedly the analysis couldn't detect protein against the corresponding monoclonal antibody. One of the reasons could be the shorter half life of this chloroplast derived protein. A plastidial protein with a half life less than one hour has previously been reported by Whitney and Andrews (2001). Rapid turnover and degradation products after the first minutes of plastidial protein synthesis could also be an explanation of quick degradation of protein as been reported by Kim et al. (1994). ELISA assays are much more sensitive than immuno blot which is the most possible reason that the ELISA confirmed protein remained undetected in Western analysis.

In conclusion it is suggested that the data obtained from the current study will help other researchers for a possible development of chloroplast based, low cost thermostable vaccine. Data have shown that the capsomers are promising candidates against cervical cancer in producing cost effective novel vaccines accessible to resource-poor countries. I foresee chloroplast derived capsomers based vaccines paving the way to affordable vaccines against cervical cancer and also for a variety of contagious diseases.

The second part of the current study has reported the possibility to generate transplastomic plants carrying antigens which confer cross-protection against two different mycobacterial diseases. An efficient cross-protective antigen against leprosy consists in a 35kDa protein encoded by *mmpI* gene in *Mycobacterium leprae* (Triccas et al. 1996, Triccas et al. 1998, Martin et al. 2001), which is also effectively cross-protective against *Mycobacterium avium* (Martin et al. 2000). The *mmpI* gene was cloned into the high-expression conferring vector pPNG1014.

Throughout the history leprosy has been affecting humans as individuals and also as a society. Human beings are the only known host of this pathogen (Monot et al. 2010). It is a chronic dermatological and neurological disease which is caused from an infection by pathogen M. leprae and causes nerve damage which may lead to severe disabilities (Britton and Lockwood 2004). In recent years the World Health Organization (WHO) has reported a decrease in global numbers of new leprosy cases per annum but underdeveloped countries are still affected from high infection rates. In the year 2010 approximately 228,474 new leprosy cases worldwide were reported with pockets of high endemicity in Angola, Brazil, Central Africa, Congo, India, Madagascar, Mozambique, Nepal, and Tanzania (WHO Report 2011).

Vaccination against several viral and bacterial pathogens has proven highly effective and has shown great prospects for disease control. Unfortunately, immunization with BCG against tuberculosis and leprosy has not been as effective (Fine 1995). Poor performance of multidrug therapy (MDT) and BCG vaccination has made it necessary to explore new immune-protective vaccines against mycobacterial infections (Andersen 2001). Immunization with DNA encoding mycobacterial antigens has already been shown to stimulate successful protective cell mediated immune responses against *Mycobacterium tuberculosis* (Kammath 1999) and *M. avium* infection (Velaz-Faircloth 1999) and can provide healthy prospects of developing cost effective vaccines against leprosy.

The 35-kDa protein has been reported as an immunodominant Ag in humans against M. leprae (Mohagheghpour et al. 1990, Triccas et al. 1996) and M. avium (Gelber et al. 1990, Triccas et al. 1998). The protein first identified in M. leprae has 95% amino acid homology with M. avium but not M. tuberculosis. Vaccines incorporating the 35 kDa antigen have the potential for promoting protective immunity against leprosy, as this antigen is widely

recognized by the immune system of leprosy patients (Triccas et al. 1998). Cell-mediated immune response against M. leprae has been reported using a DNA vaccine expressing the 35 kDa antigen (DNA-35) in Swiss albino mice (Martin et al. 2001).

Therefore we have used the *mmpI* gene in our vector pNGL-1014 to produce this 35-kDa protein conferring cross-protective resistance against both pathogens. Protein expression rates can be enhanced as demonstrated by Ye et al. (2001) by use of synthetic promoters containing components of different polymerase promoter sequences. For this purpose nuclear and plastid encoded polymerase promoter sequences (nep, pep) in addition to a leader sequence of gene 10 from lambda phage (g10L) were fused in tandem. This *mmpI* containing vector was applied for transformation of tobacco chloroplasts by the Polyethylene glycol (PEG) mediated transformation method.

Transformed plants grew abundantly on media containing spectinomycin (proof of *aad*A presence) and were positive when PCR analyzed. However, surprisingly the *mmpI* transgene cassette was under the detection limit of Southern blot analysis for transgene confirmation. Two reasons could explain this result: Either these plants were false positive, or the transgenes MMPI and LTB exert a counter-selective effect for transformed chloroplasts:

False positive regenerates have been previously reported in Arabidopsis, potato and tomato (Svab und Maliga 1993, Sikdar et al. 1998, Sidorov 1999, Ruf et al. 2001). To solve this problem of false positives, a double selection pressure of transformed plants on spectinomycin and streptomycin has been exerted (Koop et al. 1996, Kavanagh et al. 1999). While repeating the experiment we carried this new approach and selected transformants on both, spectinomycin and streptomycin. Unfortunately again the transformants produced from double selection of spectinomycin and streptomycin were not true positives. Another reason could be a mutation near the rRNA binding region of tobacco plastid which confers resistance of spectomycine (Fromm et al. 1987). Point mutation in transformed tobacco plastid 16SrDNA and 23SrDNA genes resulting in streptomycin resistance have been reported by Svab and Maliga (1991).

The expression vector for *mmpI*, p2_PNGL_mmpI_T also contains the LTB gene for E. coli heat-labile enterotoxin subunit B. Therefore, a putative counter-selection could be caused by either the LTB or the *mmpI* transgene. LTB however, has already been shown by Kang et al. (2004) and Waheed et al. (2011b) to be compatible with the chloroplast environment. Therefore the *mmpI* protein is more likely to be a challenging compound for the chloroplast environment: As a "major membrane protein" *mmpI* is able to insert into the lipid double layers and hence it is possible that the chloroplast membrane could be affected by this particular transgene product of mmpI.

Somaclonal variations, interference of introduced transgene with cytoplasmic metabolic system or lower levels of ATP production could also be factors affecting transplastomic plants (Lössl and Waheed 2011). Another possible reason could be the constitutive expression of the newly introduced foreign gene. Oey et al. (2009) has reported that the over expression of foreign protein was causing phenotypic alterations in transformed plants. Activation of the transgene expression according to a time schedule could be a solution to this problem. The recombinant protein could be expressed after highly sensitive regeneration phase. Inducible expression systems have this capability to regulate the expression of foreign protein in transformed plants. Hence, in the last part of the current study quantitative analysis of the expression of an inducible system for transplastomic plants is reported.

For this purpose we generated chloroplast transformed lines carrying the GUS gene under control of an ethanol inducible promoter. Considerable steps forward have been taken in the development of inducible expression systems in recent few years (Lössl and Waheed 2011). In the study we have reported the quantitative analysis of trans-activation system of transgene activation using a nuclear-encoded, chloroplast imported ethanol-inducible T7 RNA polymerase. Direct application of 0.5% of ethanol to the plant induced the GUS gene expression.

The MUG test showed 2.4 p mol /mg protein expression in transformed plants after the application of ethanol indicating the functionality of this trans-activation system. However, some background activity was also found in transformed plants which were not sprayed with ethanol (FIG. 18). Leaky expression of the nuclear encoded T7RNAP even in

the absence of ethanol could be due to either permeable plastid transcription from the T7 promoter by any other RNA polymerase present in the plastids or due to a read-through transcription from any upstream plastid promoter (Lössl et al. 2005). This report also suggests that surplus protein synthesis can be due to a non-specific plastid background transcription activity which is present in all transformants.

Lössl et al. (2005) have demonstrated functionality of this trans-activation system which allows overcoming growth reduction and male sterility, by constitutively expressing the phb operon in tobacco chloroplasts. External control of the transgene expression in tobacco chloroplast has also been reported by Mühlbauer et al. (2005). More recently, Verhounig et al. (2010) reported a synthetic riboswitch that assists controlled expression of transgenes by translational regulation of expression in plastids. The inducible expression system analyzed can avoid the observed negative effects of transgene expression in chloroplast. For nuclear encoded vaccines, similar systems have already been used by Alvarez et al. (2008). Use of trans-activation systems in chloroplast transformation can become a useful mean to manage transgene expression and regulate foreign protein production at any particular developmental phase or even after harvesting.

Taken together the present study has accomplished a couple of milestones regarding chloroplast transformation and plant made vaccines: Two novel chloroplast transformation vectors, pPNGST-L1$_M$-T and p2PNGL-mmpI-T were constructed to express the GST-L1 fusion protein and *mmpI*. Transformation of tobacco was carried out by protoplast and biolistic transformation techniques. Accuracy of transgene insertions was confirmed by PCR and Southern analysis. Expression of the recombinant protein in GST-L1 lines was confirmed by antigen capture ELISA which proved the proper assembly of the chimeric capsomeres necessary for immunogenicity. Complete homoplasmy of the transplastomic lines was achieved in all transformants of GST-L1 in generations T_0-T_3. To investigate the feasibility of an inducible transplastomic expression system, the trans-activation of a plastid transformed GUS reporter gene was proven.

In conclusion the data presented in the current study will be useful for the progress of plant-based vaccine production on low cost. Even though a couple of hurdles need to be taken yet, the inducible trans-activation system could serve as an alternative to constitutive expression of transgenes like *mmpI* in the plastid genome, thus solving the problem of inhibitory transgene expression. Plastid expressed capsomers are a valuable step forward to pave the way in the development of highly immunogenic and easily administrable vaccines for resource-poor countries.

CHAPTER V
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