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# Towards Generation of Transplastomic Tobacco, Expressing a 35 kda Protein as an Antigen: A Step towards Affordable Plant made Vaccine against Mycobacterium

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## Abstract

Plants offer the unique opportunity to be engineered as bio-factories for the production of different antigens, enzymes and vaccines. Recently chloroplast transformation has gained strong interest in the field of Plant Made Vaccines (PMVs). An efficient cross-protective antigen against Mycobacterium is a 35 kDa protein encoded by *mmpl* gene *in Mycobacterium leprae*. Although vaccines against Mycobacterium infections are commercially available, they are neither affordable nor available for patients in resource poor countries. Thus, alternative cost-effective vaccine production approaches need to be developed. In the current study we have reported the possibility to generate transplastomic tobacco carrying 35-kDa protein conferring cross-protective resistance against two different pathogens *Mycobacterium leprae* and *Mycobacterium avium. The mmpl* gene along with an adjuvant Lymphotoxin-beta (*LTB*) was successfully transformed into tobacco chloroplast by the Polyethylene glycol (PEG) mediated transformation method. The PEG transformation method provides an effective and cost efficient transformation procedure and can easily be adopted in resource-poor countries in comparison to biolistic transformation. Integration of *LTB* and *mmpl* genes into transplastomic plant genome was confirmed by PCR analysis. In total four transplastomic lines were generated which were healthy and normal. All plants had regular growth pattern, they were able to reach maturity and produce viable seeds. Taken together, the data presented in the study is a valuable step forward to pave the way in the development of cost effective and easily administrable PMVs for resource-poor countries.

**Keywords:** Chloroplast transformation; *mmpl; Mycobacterium leprae; LTB;* Plant made vaccines

## Introduction

For many centuries leprosy has been affecting humans as an individual and also as a society. The patients have often been hated and shunned by their communities and families [1]. 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 [2].

First licensed vaccine against *Mycobacterium leprae* and *Mycobacterium tuberculosis* was BCG (Bacille Calmette-Guérin), which was successfully administrated to a young boy in 1921 [3]. 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 [4]. 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 immune protective vaccines and the identification of protective antigens is particularly important for the subunit vaccine approach [5]. Immunization with DNA encoding mycobacterial antigens has already been proven to stimulate successful protective cell mediated immune responses against *Mycobacterium tuberculosis* and *M. avium* infection [6] and is a new strategy for leprosy control.

Since more than 6 millennia human are using plants as medicines to treat a wide range of diseases [7]. 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 [8]. 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 PMVs 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 [9]. Plants require only water, sunlight, carbon dioxide and some nutrients, but in return they provide a valuable system for production of recombinant proteins.

In 1998 for the first time Tacket et al. [10] presented the concept of vaccines from transgenic plants and since then considerable developments have been made in the area [11]. 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 [12]. Novel efforts have been made to produce various antigens in plants and these PMVs can be administered orally as a part of the plant, intramuscularly or as intravenous injection after isolation and purification from the plant tissues [13,14]. The ability of plants to produce complex foreign proteins and secondary metabolites in an efficient, safe and economical way is the reason why biopharming is gaining an increasing importance in plant biotechnology [15]. 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 [16].

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A serious concern about Plant made vaccines is the transgene spread of the transformed plant. Most approaches so far were carried out by nuclear transformation bearing a high risk of transgene propagation [17]. Out-crossing via pollen mediated gene flow needs to be substantially control and reduce [18]. Chloroplast transformation has emerged as a very precise alternative technique. 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 [19]. 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 [20] methylation in the plastid genome is rarely found [21]. Most importantly the use of transplastomic plants addresses the risk of transgene spread by pollen as the chloroplasts are maternally inherited in most plants [17] and therefore pollens don't contain any transgenes.

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 [22]. 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 various groups [23,24]. Successful experiments with plant derived vaccines have also been carried out with Norwalk virus [25], Rabies [26], Human Papilloma Virus [27], Newcastle disease virus [28] and many more. Immunogenicity has been reported in humans by consumption of transgenic potatoes containing enterotoxin subunit B from E.coli [29]. Potato was also used to achieve protective efficacy towards cholera and enterotoxic E. coli that in principle is also possible in corn [30]. 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 [31], Anthrax [32] and *Yersinia pestis* [33].

An efficient cross-protective antigen against Mycobacterium consists in a 35kDa protein encoded by *mmpI* gene in *Mycobacterium leprae* [34-36] which is also efficiently protective against *Mycobacterium avium* [37]. The 35 kDa-antigen, the major membrane protein I, in native form exists as a multimer of 950 kD [38]. In order to retain its correct folding capacity it is indicated to express it as a single peptide without additional sequences. In the following study we have investigated the possibility of transplastomic expression of *mmpI gene* cloned into the high-expression vector and stably transformed into tobacco chloroplasts.

## Material and Methods

### Buffers and solutions

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

**B5-vitamine (1 L):** Myo-inositol 10 g, Pyridoxine-HCl 100 mg, Thiamine Hydrochloride 1 g, Nicotinic acid and dissolved in 1L distilled water, stored in 10 ml tubes at -20°C

**Cellulase R10:** Cellulase R10 1 g, 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.

**Ca2+ -agar:** MES 0.97 g, $CaCl_2 \times 2\ H_2O$ 1.47 g, Mannitol 41 g, Agar purified 5 g. Mixed and dissolved in 500 ml distilled water, adjusted the pH to 5.8 with 1M KOH and autoclaved.

**F-PIN:** MS-Macro 10X 100 ml, MS-Micro 100X 10 ml, PC-Vitamin 100X 10 ml, Ammonium succinate 2M 10 ml, BAP (conc. 1 mg/ml) 1 ml, NAA (conc. 1 mg/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.

**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.

**Macerase R10:** Macerase R101 g, sucrose 1.37g. 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.

**MMM:** $MgCl_2 \times 6H_2O$1,02 g, $MgSO_4\ 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:** $KNO_3$ 10.12 g, $CaCl_2 \times 2\ H_2O$ 4.4 g, $MgSO_4 \times 7\ H_2O$ 3.7 g, $KH_2PO_4$ 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, $MgSO_4$ 1g, Agar purified 8g. Mixed and dissolved in 1 L distilled water, adjusted pH to 5.8 and sterilized by autoclaving.

When the solution was cooled, 10 ml $NH_4$-Succinat was added to the solution, shortly before using.

**MS-Micro 100X:** Na-EDTA-Fe (III) 4 g, KJ 75 mg, $H_3BO_3$ 300 mg, $MnSO_4 \times H_2O$ 1 g, $ZnSO_4 \times 7H+O$ 200 mg, $Na_2MoO_4\ 2H_2O$ 25 mg, $CuSO_4 \times 5H_2O$ 2.5 mg, $CoCl_2 \times 6H_2O$ 2.5 mg. Filled up to 100 ml distilled water and sterilized by autoclaving.

**PC-Vitamin 100X:** Inosit 20g, Pyridoxin-HCl 200mg, Thiamin-HCl 100mg, Biotin 2mg, 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):** $Ca(NO_3)_2 \times 4H_2O$ 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 aliquated and stored at -20°C.

**RMOP medium:** MS-Macro 10X 100 ml, MS-Micro 100X 10ml, NT-Vitamin 10ml, BAP (1mg/ml) 1ml, 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.

## Vector Construction

For the construction of this vector a pre constructed intermediate vector pPNGL-*mmpI* (Dr. Andreas Lössl's 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 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 [39] from *rrn* 16 gene (P*rrn*) with the Ribosomal Binding Site (RBS) from the leader sequence of gene 10 (G10L) of T7 lambda phage [40], 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 reported previously [41]. Diagrammatic representation of the vector construction is given in Figure 1.

## Transformation and regeneration of transplastomic plants

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

**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.

**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, 2ml 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.

**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 4×105 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 10×10 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.

**PEG mediated protoplast transformation:** For transformation, 100 µl of protoplast suspension was transferred to a sterile petri dish and the protoplasts were allowed to settle for few minutes. 18 µl of 50 µg plasmid DNA was added drop by drop to the petri dish. The solution was gently mixed and then 12 µl F-PCN and 125 µl of 40% PEG solution was added slowly mixed and incubated for 8 minutes. For a second time 120 µl F-PCN was added, mixed and incubated for 8 minutes, followed by another 2.6 ml F-PCN, mixed and incubated for 2minutes. Protoplast density was adjusted to 8.5×104 pp/ml using F-alginate. 625 µl 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

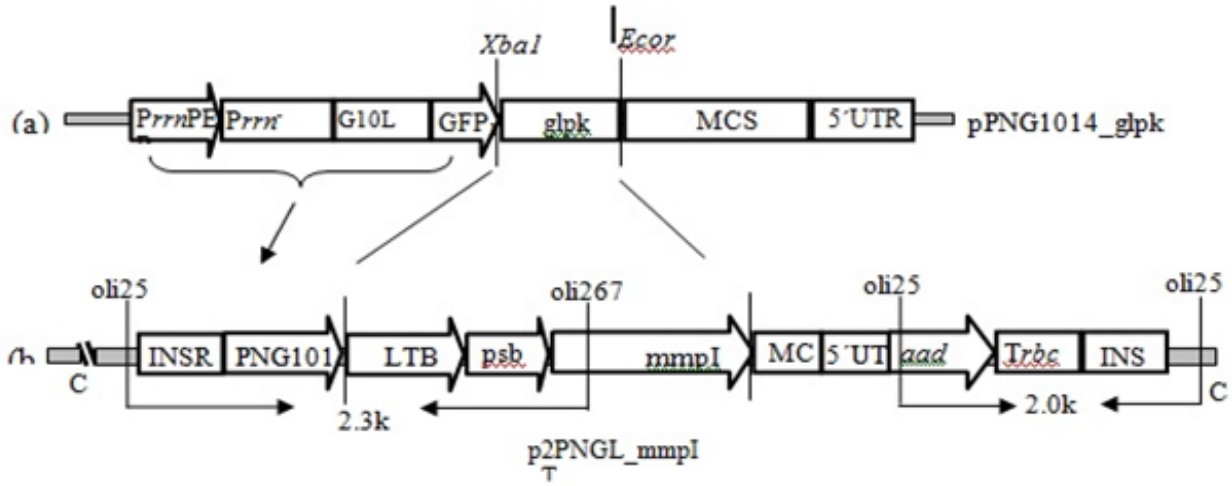


**Figure 1:** Schematic diagram of transformation vectors development: (a) Precursor vector pPNG1014_glpk was used to clone transgenes. (b) Plasmid p2PNGL-*mmpI* obtained after the insertion of transgenes *LTB* and *mmpI* in the precursor vector. Final transformation vector p2PNGL-*mmpI*-T for the transformation 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).

for two hours. After solidification the grids were transferred to 7 ml liquid F-PCN for equilibration in the dark for 1hour. Finally the grids were transferred to 7 ml of fresh F-PCN solution in a new petri dish, wrapped with parafilm and stored.

**Regeneration and cultivation:** 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 calli started to grow after 7-8 weeks of transformation. Transgene integration into the plastid genome was confirmed by PCR analysis.

**Polymerase Chain Reaction (PCR) analysis:** Insertion of transgenes within the chloroplast genome was confirmed by PCR anylysis. DNA was extracted from 80 mg of leaves by Cetyltrimethylammonium Bromide (CTAB) method. For the confirmation of *LTB-mmpI* integration, forward (oligo252) and reverse (oligo267, binding site located within *mmpI*) primers were used. The binding site of oli252 was located within the plastome (Figure 1). The sequences of oli252 and oli267 were: 5′-AGACAGCGACGGGTTCTCTG-3′ and 5′-GCACGATTTCGACGTTGCTA-3′, respectively. For amplification of antibiotic resistant *aad*A gene, oligo251 with the sequence 5′-CCAGTATCAGCCCGTCATAC-3′ and oligo253 with sequence 5′-GAAATTCTATGGCTCGGATC-3′, were used as forward and reverse primers.

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:** Reaction mixture consists of following composition.

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

## Results

### Plastid transformation vector

The plastid transformation vector p2PNGL-*mmpI*-T was constructed from the precursor vector pPNG1014_MCS120. The *mmpI* gene encoded for a 35 kDa protein having immunogenic properties against *Mycobacterium avium* as well as *Mycobacterium leprae*. The *mmpI* was transcribed by the 5`*psb*A promoter. Aminoglycoside 3`-adenyltransferase (*aad*A) a marker gene, which confers resistance against spectinomycin and streptomycin was also transcribed by *psb*A promoter. While the Escherichia coli heat-labile enterotoxin subunit B (*LTB*), used as a subunit protein to enhance the immunogenic response of the antigen, was controlled by 16S PEP Promoter, 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.

### Chloroplast transformation, regeneration and seed production of tobacco plants

**Polyethylene glycol (PEG) mediated transformation:** In 1996 Koop et al. [20] 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.

**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 (Figure 2a).

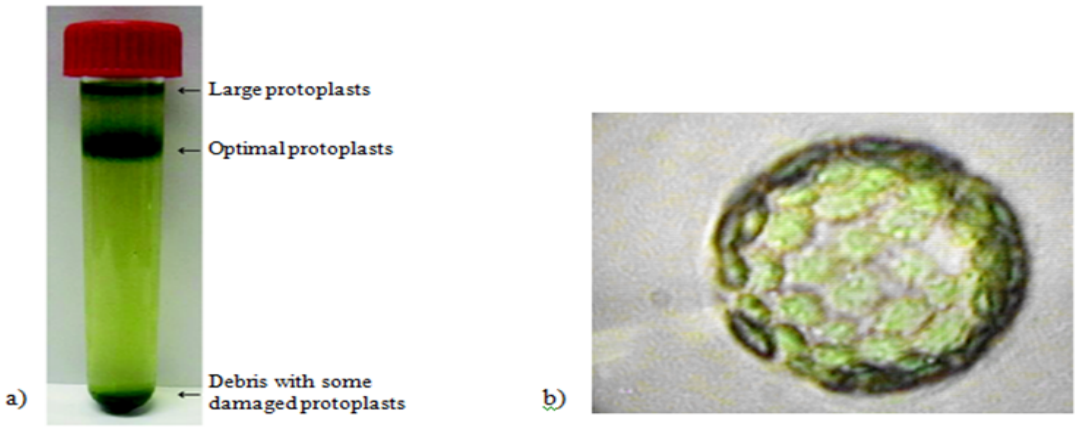


**Figure 2: Protoplast Isolation: (a)** After density gradient centrifugation cell debris and damaged protoplasts are sedimented in the bottom layer, large and over mature protoplasts floating on the top layer while optimal protoplasts were collected from the middle layer, **(b)** Healthy and fully intact protoplast isolated from tobacco leaves (Picture taken from the monitor attached to the microscope).

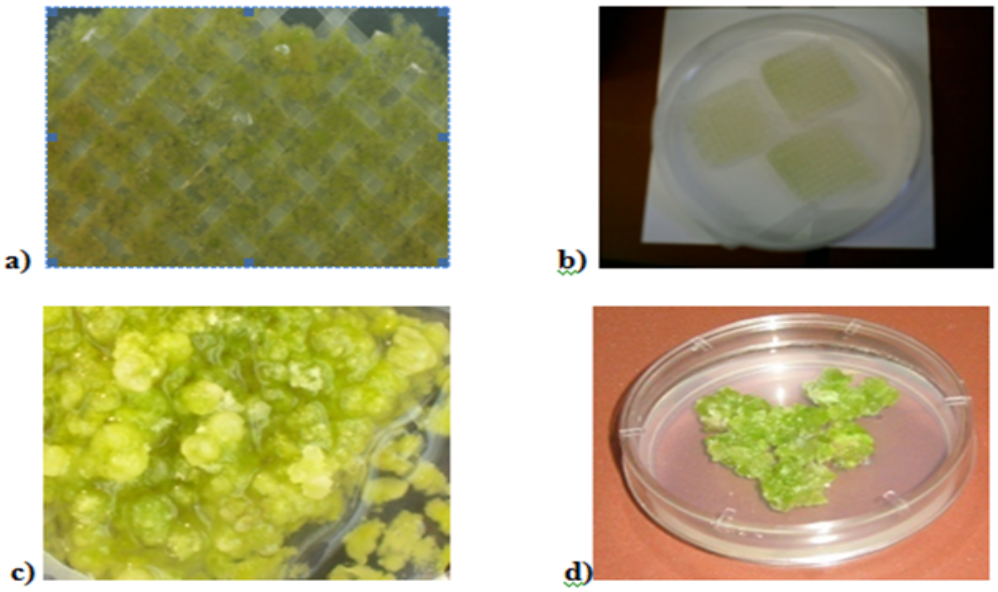


**Figure 3: Transformed protoplast's regeneration: (a)** Transformed protoplasts present on the mesh grids in F-PCN solution, **(b)** Embedding, Protoplast suspension mixed with alginate for embedding and solidified on mesh grids **(c & d)** Green colonies individually picked and cultured on solid RMOP for shoot regeneration.

**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 2×2 mm grids. This protoplast containing grids were first put on Ca-agar media and then to liquid F-PCN (Figure 3a). One week after transformation embedded protoplasts started to regenerate stable callus which were transferred to selective medium containing antibiotic spectinomycin (solid RMOP containing 500mg/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 (Figures 3c and 3d). 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.

**Confirmation of the transgene integration:** 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 Figure 4a. 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 *aad*A gene within the plastid genome (Figure 4b). The results were identical with the PCR analysis of the generation F1 and F2 transformants.

**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 (Figure 5).

## Discussion

In the current study we have 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 35 kDa protein encoded by *mmpI* gene in *Mycobacterium leprae* [34-36] which is also effectively cross-protective against *Mycobacterium avium* [37]. 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. 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 [1]. 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 [42].

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 [4]. Poor performance of Multidrug Therapy (MDT) and BCG vaccination has made it necessary to explore new immune-protective vaccines against mycobacterial infections [6]. Immunization with DNA encoding mycobacterial antigens has already been shown to stimulate successful protective cell mediated immune responses against *Mycobacterium tuberculosis* [43] and *M. avium* infection

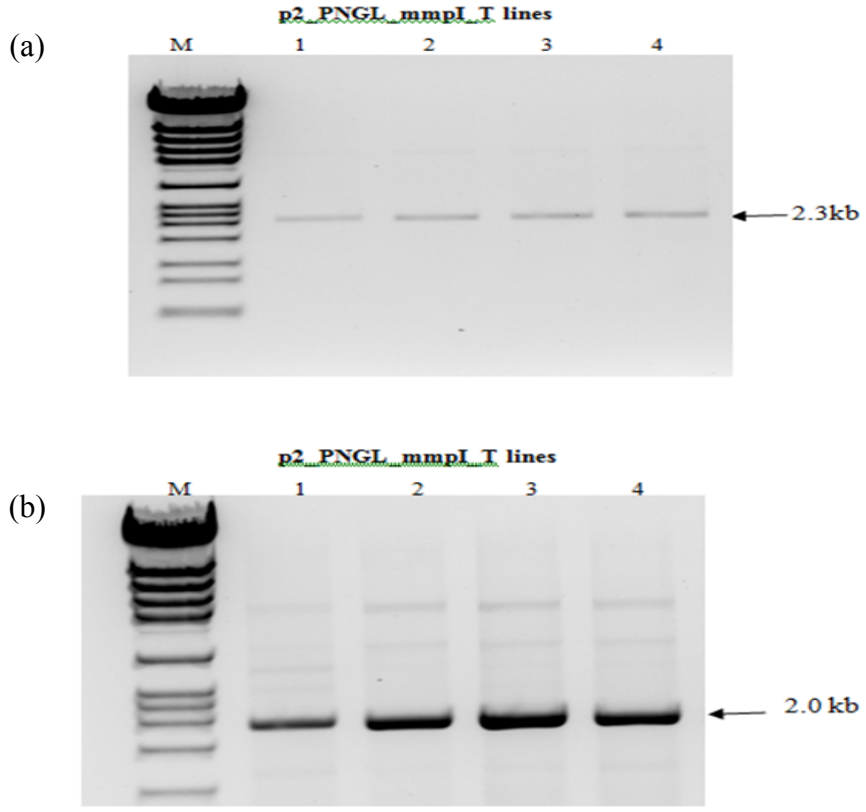


**Figure 4: (a) PCR confirmation of transgene presence:** PCR analysis of the transgene cassette inserted correctly within the plastid genome. Amplification of p2_PNGL_*mmpI* (2317 bp) with the primers oli267 in the *mmpI* gene and oli252 located within the plastome. Four independently transformed lines (Lane 1, 2, 3, 4) were evaluated. **(b) PCR confirmation of transgene presence:** Amplification of the *aad***A** gene (2k bp) using primers oli251 in the *aadA* gene and oli253 present within the plastid genome. Four independently transformed lines (Lane 1,2,3,4) were evaluated.

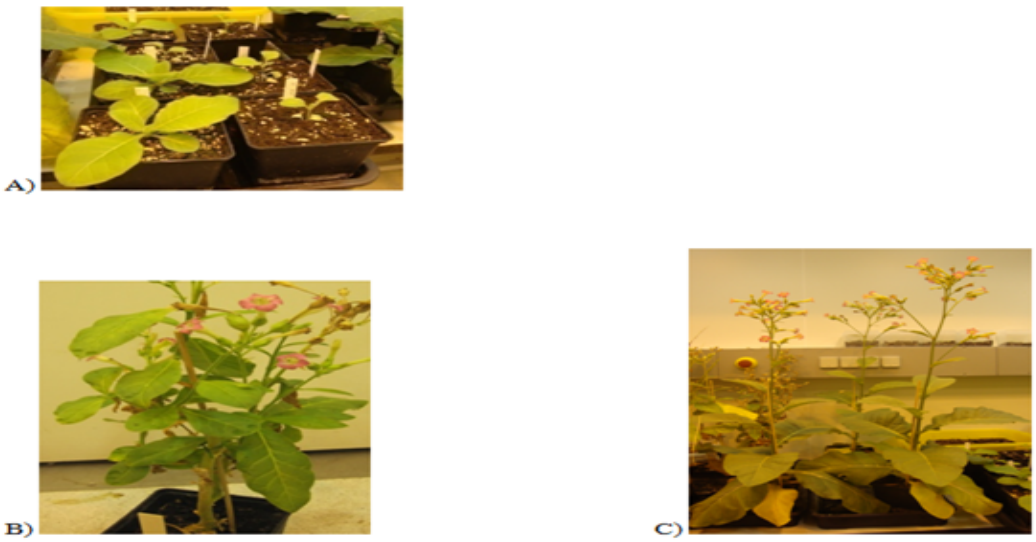


**Figure 5: Morphological features of transplastomic plants: (a)** Morphologically healthy transplastomic plants carrying transgene *LTB* and *mmpI* **(b)** Transplastomic tobacco producing viable flowers **(c)** Wild Type tobacco.

[6] and can provide healthy prospects of developing cost effective vaccines against leprosy.

The 35-kDa protein has been reported as an immune dominant Ag in humans against *M. leprae* [34,44] and *M. avium* [35]. The protein first identified in *M. leprae* has 95% amino acid homology with *M. avium* but not M. tuberculosis. Vaccines incorporating the 35kDa antigen have the potential for promoting protective immunity against leprosy, as this antigen is widely recognized by the immune system of leprosy patients [35]. 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 [36].

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 [45] 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 [39,46,47]. To solve this problem of false positives, a double selection pressure of transformed plants on spectinomycin and streptomycin has been exerted [20,48]. 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 [49]. Point mutation in transformed tobacco plastid 16SrDNA and 23SrDNA genes results in streptomycin resistance [50].

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, that can be compatible with the chloroplast environment [27,51]. 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*. Another possible reason could be the constitutive expression of the newly introduced foreign gene. 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 [52].

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, plant made vaccines are a valuable step forward to pave the way in the development of highly immunogenic and easily administrable vaccines for resource-poor countries.

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