Expression of human papillomavirus-16 L1 capsomeres in chloroplasts of *Nicotiana tabacum*: a novel approach towards cost-effective second-generation vaccines against cervical cancer

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Which is it , of the favours of your Lord that ye deny?

(Al-Qur'an: 55:13)

Dedicated To

My Beloved Family

Preliminary remarks

This thesis has been prepared as cumulative, in the framework of the Department of Crop Sciences, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria. This cumulative thesis consists of four publications including one expert commentary, one review article and two scientific articles. A general collaborative introduction, methodology and discussion is also included that correlates the individual scientific publications.

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Contents

1. Abstract

Several types of human papillomaviruses (HPVs) are causatively associated with cervical cancer, the second most common cancer in women worldwide. Among these, HPV-16 is responsible for approximately 50% of cases. Mainly due to high costs, currently available virus-like particles (VLP)-based vaccines would be largely unavailable for developing countries, where the majority of cases occur. Hence, there is need for affordable vaccines against HPV for these countries. Capsomeres are a cost-effective alternative for second-generation HPV vaccines, recently shown to be highly immunogenic in mice. To further enhance the immune response against a vaccine antigen, an adjuvant is often co-administered with the antigen. However, separate production and administration of adjuvants is laborious and expensive. Production of an adjuvant-antigen couple as a fusion protein can reduce the costs related to adjuvants and additionally augment its efficacy.

In the present study, we have opted for two approaches: chloroplast-based expression of a modified HPV-16 L1 (L1_2xCysM) gene alone and in fusion with *E. coli* heat-labile enterotoxin subunit B (LTB), as an adjuvant. L1_2xCysM gene confines the assembly of L1 to capsomeres. Transgene integration within the plastome and homoplasmy was confirmed by PCR and Southern blotting. L1 and LTB-L1 proteins accumulated up to 1.5% and 2% of total soluble protein, respectively, as verified by Western blotting. Cesium chloride gradient centrifugation and sucrose sedimentation analysis verified that the L1 protein assembled correctly into capsomeres. Display of antigenic epitopes of L1 was confirmed by antigen capture ELISA. To prove the concept that both partners in double pentameric protein (LTB-L1) assembled to their functional conformations, we additionally carried out a GM1-ganglioside ELISA for LTB protein, which confirmed the display of immunogenic epitopes of LTB.

Taken together, these data provide a platform for the development of low-cost second-generation vaccines against HPV, affordable for developing countries. The fusion of L1 antigen with LTB as adjuvant would additionally facilitate the reduction of costs related to adjuvants. Above all, direct coupling of adjuvant to the L1 antigen will pave a way to the development of an HPV vaccine with enhanced immunogenicity.

Kurzfassung

Vielfältige Typen des Humanen Papillomavirus (HPV) verursachen Gebärmutterhalskrebs, den zweithäufigsten Krebs weltweit bei Frauen. In ca. 50% der Fälle liegt HPV-16 vor. Derzeit erhältliche virus-like particles (VLP)-basierte Impfstoffe gegen HPV sind teuer und für Entwicklungsländer kaum verfügbar. Daher besteht ein hoher Bedarf an preiswerten Vakzinen. Capsomere sind eine kostengünstige Alternative zu VLPs und zeigten im Mausmodell hohe Immunogenität. Um die Immun-Antwort gegen ein Antigen zu steigern, wird es oft zusammen mit einem Adjuvans verabreicht. Allerdings ist die separate Produktion und Administrierung von Adjuvantien aufwändig und kostenintensiv. Die Produktion eines Adjuvant-Antigen-Paars als Fusionsprotein könnte dieses Problem lösen und sogar die Effizienz des Vakzins steigern.

Die vorliegende Studie verfolgte zwei Ansätze: Chloroplasten-basierte Expression eines modifizierten HPV-16 L1 (L1_2xCysM) Genes alleine und in Fusion mit der *E. coli* hitze-labilen Enterotoxin Untereinheit B (LTB) als Adjuvans. L1_2xCysM führt zur Retention der L1 Proteinstrukturen als Capsomere. Die korrekte Insertion des Transgens im Plastom bestätigten PCR und Southern Blot. Western Analysen zeigten, dass die rekombinanten Proteine L1 und LTB-L1 die erwarteten Größen von 56.5 bzw. 68 kDa aufwiesen und dass sie sich bis zu 1.5% bzw. 2% des gesamtlöslichen Proteins anreicherten. Cäsiumchlorid Dichte-gradienten-Zentrifugation und Saccharose-Gradienten-Sedimentations-analysen verifizierten das Assembly der L1 Proteine zu Capsomer-Strukturen. Ein Antigen Capture ELISA bestätigte die korrekte Konformation der Antigen-Epitope. Als Machbarkeitsnachweis, dass beide Partner als Doppel-Pentamer (LTB-L1) korrekt in ihre richtigen Konformationen assemblieren, führten wir auch einen GM1-Ganglisoid-ELISA für das LTB Protein durch, welcher die sterisch korrekte Lage der immunogenen Epitope bestätigte.

Insgesamt bilden diese Ergebnisse eine Plattform zur Entwicklung preiswerter HPV-Impfstoffe der zweiten Generation, die auch für Entwicklungsländer leistbar sind. Die Fusion von L1 Antigen mit LTB ist dazu geeignet, Kosten für zusätzliche Adjuvantien zu senken. Darüber hinaus ebnet die direkte Kopplung eines Adjuvans an L1 den Weg, um ein HPV Vakzin mit erhöhter Immunogenität zu entwickeln.

2. Introduction

2.1 Molecular farming

In the history, plants have been used as a source of medicinally important products. Even today, many of the currently available drugs are directly or indirectly derived from plants (Ma and Wang 2011). In the modern era, the application of biotechnology and genetic engineering has enabled the production of commercially and pharmaceutically important proteins by plants. Production of such compounds by genetically engineered plants is called as molecular farming or biopharming.

Traditionally, *Escherichia coli,* yeast and animal cell cultures have been used for the production of pharmaceuticals. However, these conventional fermenter-based systems are difficult to scale up and associated with high costs of production, complex processes of purification and risk of contamination with endotoxins or human pathogens (Fischer *et al*., 2004; Daniell *et al*., 2009). In contrast, plants have gained importance as bio-factories because of many advantages, which include: the low production cost, high yield of expressed proteins, easy to scale up and safety of plant-derived pharmaceuticals (Bock 2007). Another crucial advantage of plants is that they are capable of carrying out the post-translational modification of the expressed proteins, which is necessary for their proper function (Lössl and Waheed 2011). In some cases, expression of antigenic proteins in *Escherichia coli* is not feasible due to the lack of a variety of post-translational modifications and folding requirements. Some of these essential mammalian-type post-translational modifications of proteins take place in yeast and insect cell lines. However, there are immunologically significant differences in the patterns of post-translational modifications which limit their use as expression platforms for vaccine antigens (Streatfield and Howard, 2003a,b; Houdebine 2009).

Since the last two decades, many biologically important compounds have been produced in plants which include vaccines, antibodies, plasma proteins, enzymes, cytokines and growth factors (Basaran and Rodríguez-Cerezo 2008). Many plant-derived pharmaceuticals are undergoing clinical trials and few are already in market or expected to be approved soon (Yusibov *et al*., 2011).

2.2 Production of cost-effective vaccines through plants

In the year 2000, at the UN millennium summit, world leaders set eight Millennium Development Goals, aimed at improving social and economic conditions in developing countries. These goals cover various issues associated with education, health and sustainable development (Penney *et al*., 2011). In the context of health related goals, plant-derived vaccines have the potential to address many issues. Plants are potentially a cost-effective source of vaccine production because plants can be grown at site where production is needed, thus eliminating the costs related to transportation and maintenance of cold chain (Lössl and Waheed 2011). There are several other advantages of plants which can be employed for vaccine production as reviewed by Bock and Warzecha (2010).

Various vaccine antigens have been successfully expressed in plants by the integration of antigenic genes either into nuclear or chloroplast genome (Rybicki 2010). However, to date, no plant-derived vaccine against human diseases has succeeded to reach the market. Nevertheless, there are few therapeutics that are passing through clinical trials (Daniell *et al*., 2009; Rybicki 2009). Recently, a plant-derived vaccine against Newcastle disease of poultry has been approved by the USDA. Initially, plant transformation was restricted to few species only. However, in the recent past, this technology has expanded to important edible plant species such as potato, tomato, lettuce, cabbage, alfalfa, spinach and carrot (Tiwari *et al*., 2009). Transformation of these plant species has opened a way to consolidate the idea of ‘edible vaccines’ produced in plants (Lössl and Waheed 2011).

2.3 Tobacco for vaccine production

Tobacco is a very well established expression host for which efficient transformation procedures are available (Fischer and Emans 2000). Tobacco is a good candidate for vaccine production due to its high biomass yields and rapid scalability. Since tobacco is a non-food, non-feed crop, it poses a reduced risk of contaminating feed and human food chains (Stöger *et al*., 2000, Fischer *et al*., 2004). Moreover, vaccine antigens can be expressed in tobacco and grown in contained facilities to assure biosafety. Compared to nuclear transformation, expression of vaccine antigens in tobacco chloroplasts is more advantageous to achieve transgene containment due to the fact that plastids are maternally inherited in tobacco (Daniell 2007; Ruf et *al*., 2007).

However, due to high contents of nicotine and other toxic alkaloids, transgenic or transplastomic tobacco can not be used as edible material. Hence, downstream processing is necessary for the extraction and purification of proteins from transformed tobacco plants.

2.4 Plant transformation

For accomplishing the expression of a desired protein, plants can be transformed in two ways: either stably *via* nuclear or chloroplast transformation or transiently. These transformation methods are described below.

2.4.1 Nuclear transformation

In this method, target genes are integrated in a plant's nuclear genome. Currently, there are two main techniques used for nuclear transformation: The *Agrobacterium*-mediated transformation and particle bombardment, also known as biolistic transformation. In this method of DNA delivery, an expression cassette with foreign DNA containing gene of interest (GOI) and an antibiotic resistance gene is coated on small particles of gold or tungsten and shot into the plant tissues by biolistic gun (Sanford 1990). The transformed tissues are regenerated into complete plants by selection on a regeneration medium containing the selective antibiotic. Compared to transient expression, nuclear transformation has certain advantages. One main benefit is that the nuclear transformed plants can be scaled up for the production of recombinant proteins. In addition, the stable integration of transgenes is inherited to the next generations and thus assures the long term continuous production (Tremblay *et al*., 2010). However, the yield of recombinant protein obtained by nuclear transformation is low (an average of 0.01–0.4% of total soluble protein; Daniell *et al*. 2001). Moreover, transgenes can accidentally escape into wild populations through cross pollination and thus pose a risk to the environment. Because of this reason, it is recommended to choose plastid transformation, especially for those plant species that are transformable and show maternal inheritance of plastids.

2.4.2 Chloroplast transformation

Transgenes can be stably integrated in the chloroplast genome of certain plant species by the biolistic transformation method. Plant tissues (leaf or callus) are transformed by biolistic bombardment and the GOI is integrated into plastome *via* homologous recombination (Maliga and Bock 2011). An antibiotic resistance gene, *aadA* (aminoglycoside 3′-adenyltransferase) is included in the foreign DNA carrying the expression cassette, for selection of transformed tissues. The *aadA* gene confers resistance to the antibiotics spectinomycin and streptomycin. Selection and regeneration of transformed tissues is carried out on revised medium for organogenesis (RMOP) containing the respective antibiotic. To achieve homoplasmy, i.e. plastids with only the transformed genomes, regenerated shoots are subjected to an additional round of selection on antibiotic containing RMOP medium (Singh *et al*., 2009). The plastid genome exhibits high polyploidy, for instance, a tobacco leaf contains as many as 100 chloroplasts in each cell and up to 100 copies of plastid genome in each chloroplast. This makes total copy number of any inserted gene to 10,000 in each cell (Davoodi-Semiromi *et al*., 2009). Due to this characteristic feature of plastids, high yield of recombinant protein can be achieved. Recently, Oey *et al*. (2009) and Ruhlman *et al*. (2010) obtained an exceptionally high yield of protein *via* plastid transformation (approximately 70% of total soluble protein and 72% of total leaf protein, respectively). An additional advantage of plastid transformation is biosafety. Transgenes can not escape into environment because in most plant species, plastids are not spread *via* pollen (Daniell 2006; Bock and Warzecha 2010). These two characteristic features of plastid transformation make this system very attractive for vaccine production. Moreover, due to the prokaryotic nature of gene expression system of plastids, multigenes can be expressed by linking them together in operons (Lössl *et al*., 2003; Bock and Warzecha 2010). This characteristic of plastids is very useful for the production of those subunit vaccines which require more than one epitope for their function. Multigene expression can also be utilized to co-express an adjuvant-antigen couple to circumvent separate production and administration of adjuvants. Plastid biotechnology is not only helpful in pharmaceutical production but it has also been applied to engineer metabolic pathways in plants, for the production of biofuels and to develop crops with high levels of resistance to insects, fungal,

bacterial and viral diseases and different herbicides (Bock 2007; Clarke and Daniell 2011).

2.4.3 Transient expression

In transient expression, transgenes are inserted and expressed in host cells for a short period of time. Transient expression is achieved either through agroinfiltration or infection with viral vectors. In agroinfiltration, agrobacteria with T-DNA harbouring the GOI are introduced into plant leaves through vacuum infiltration or syringe injection. This method has been successfully used for rapid production of clinical grade biopharmaceuticals (Pogue *et al*., 2010). In the viral infection method, plant viruses are used to deliver foreign genes in plants. Different viral vectors are used for this purpose such as tobacco mosaic virus (TMV) or cowpea mosaic virus (CPMV) (Rybicki 2009). A very promising technique for transient expression (Magnifection) system has been developed by Icon Genetics (Bayer Crop Science). This system uses agroinfiltration to systematically deliver a TMV-based transient expression vector, which significantly amplifies the mRNA levels compared to agroinfiltration (Gleba *et al*., 2005; Rybicki 2009). The main advantage of transient expression is rapid protein production in matter of days or weeks (Komarova *et al*., 2010). Another advantage of transient expression lies in its use to verify expression constructs before proceeding to transgenic plants (Twyman *et al*., 2003) However, there are certain drawbacks of transient expression: in case of agroinfiltration, there is a low capacity to scale up while additional concerns of gene containment arise for viral vectors (Twyman *et al*., 2003).

2.5 Cervical cancer

Cervical cancer is the second most common cancer in women, across the globe (Parkin and Bray 2006). Every year, approximately 493,000 new cases and 274,000 deaths occur in the world (Parkin and Bray 2006). By 2030, the number of deaths due to cervical cancer is expected to increase approximately to 410,000 (Kamangar *et al*., 2006; Mathers *et al*., 2008). In developing countries, incidence and mortality rate due to cervical cancer is very high. In resource poor countries, approximately 85% of overall cases of cervical cancer occur and it is the leading cause of death in these countries (Ferlay *et al*., 2010).

2.5.1 Human papillomavirus (HPV)

Different types of human papillomaviruses (HPV) are causatively associated with cervical cancer. These can be divided into high and low-risk types. High-risk types of HPV include HPV-16, 18, 31, 33, 45, 52 and 58 (Muńoz *et al*., 2004). HPV-16 and 18 have been identified to be responsible for approximately 50% and 20% of cervical cancer cases, respectively (Bosch *et al*., 1995; Clifford *et al*., 2003; Clifford *et al*., 2006).

HPVs are non-enveloped viruses containing double stranded circular DNA and a spherical capsid of icosahedral symmetry. The viral DNA encodes for two sets of genes; early and late genes. The early proteins are E1, E2, E4, E5, E6 and E7 and the late proteins are the capsid proteins L1 and L2 (zur Hausen 2002). L1 is the major capsid protein of HPV, which self assembles *in vitro* into empty virus-like particles (VLPs), which are similar to native virions in size and shape. Each VLP of HPV is composed of 72 capsomeres and each capsomere is a pentamer of L1 protein arranged in an icosahedral lattice (Baker *et al*., 1991). The VLPs have a diameter of 55 nm and cryo-electron microscopic analysis shows their assembly by 60 hexavalent and 12 pentavalent capsomeres (Baker *et al*., 1991).

2.5.2 Modified HPV-16 L1 gene

VLPs are stabilized by disulfide bonds between L1 protein molecules of adjacent capsomeres. Reduction of these disulfide bonds leads to the disassembly of VLPs to capsomeres (Sapp *et al*., 1995). Two cysteines have been reported to be important for efficient VLP assembly. These cysteine residues are located at position 175 and 428 and replacement of either of these two highly conserved cysteines by serines prevents VLP assembly and retains the conformation of L1 protein to capsomeres (Sapp *et al*., 1998). There are several other ways of modifying native L1 gene to retain its assembly to capsomeres (Schädlich *et al*., 2009b). In our studies, we have used modified L1 gene (L1_2xCysM), in which two cysteines were replaced by serines at position 175 and 428 (Schädlich *et al*., 2009b).

2.5.3 Second-generation vaccines against HPV

Second-generation vaccines are those vaccine candidates that are less costly, more stable, have more or equal immunogenicity and easy to produce, compared to vaccines already in use (Giorgi *et al*., 2010, Lössl and Waheed 2011). In case of HPV, currently available vaccines are either produced by yeast or insect cell expression systems (Madrid-Marina *et al*., 2009). These are VLP-based vaccines targeting major capsid protein L1 of different HPV types. However, there are certain disadvantages related to currently available prophylactic vaccines against HPV. These vaccines target only a limited number of HPV types (Waheed *et al*., 2012). Moreover, these vaccines are expensive due to complex processes of production. In case of currently available vaccines, other aspects such as maintenance of cooling chain, separate administration of adjuvant, need of sterile needles and trained medical staff for injectable delivery add more to the costs. All these factors make these vaccines largely unaffordable for developing countries, where most of cervical cancer cases occur (Stanley *et al*., 2008). Hence, there is an urgent need of low-cost vaccines for the women in developing countries. Currently used vaccines against HPV are prophylactic, i.e. used prior to the infection to generate neutralizing antibodies to block future infection. In this context, second-generation vaccines may also include those vaccine candidates that can have therapeutic effect against HPV infection.

The pentameric capsomeres are promising candidates for second-generation prophylactic vaccines against HPV. There are many reports about the immunogenicity of capsomeres (Rose *et al*., 1998; Fligge *et al*., 2001; Yuan *et al*., 2001; Öhlschläger *et al*., 2003; Dell *et al*., 2006). Recently, capsomeres have been found to be highly immunogenic in mice, comparable to the levels of VLPs (Schädlich *et al*., 2009b). In contrast to VLPs, a major advantage of capsomeres is that they are considered more thermo-stable and are easy to produce (Stanley *et al*., 2008; Tuma 2009; Ma *et al*., 2010). Keeping in mind the difficulty to maintain the cold chain in developing countries, this advantage of capsomeres is very attractive for the development of cost-effective vaccines against HPV. To further reduce the costs related to expensive conventional methods of production, capsomeres can be expressed in plants.

2.6 Adjuvants

An adjuvant is an agent, which upon co-administration with a given antigen, helps to increase or prolong the immune response against that antigen. Compared to conventional whole-cell or virus-based vaccines, new subunit vaccines have more defined composition. This makes these vaccines more secure; however, these are often linked to lower immunogenicity. Therefore, adjuvants are required to assist the immune response of subunit vaccines (Guy 2007). There are a number of compounds that can be used as adjuvants such as alum, saponins, liposomes, cholera toxin subunit B (CTB), *Escherichia coli* heat-labile enterotoxin subunit B (LTB) and interleukin (Millar *et al*., 2001; Guy 2007; Sánchez and Holmgren 2008).

2.6.1 *Escherichia coli* heat-labile enterotoxin subunit B (LTB)

Enterotoxigenic *Escherichia coli* (ETEC) is the cause of infectious diarrhoea, which is due to the release of either heat-labile toxin (LT) or heat-stable toxin (ST) or both. LT consists of a receptor binding (B) oligomer, formed by five identical B subunits (LTB). LTB is the nontoxic subunit and is highly immunogenic upon oral delivery (Field 1979). Moreover, LTB is a powerful mucosal and parenteral adjuvant and induces strong immune responses against co-administered or coupled antigens (Dickinson 1996). Adjuvant and immunogenic properties of LTB have been well documented in literature (Sánchez and Holmgren 2008).

2.6.2 Coupling of adjuvants with antigens

There are many advantages of coupling an antigen with an adjuvant. In case of CTB and LTB, the most important advantage is that their coupling can facilitate the transport of the antigen from gut lumen to the gut associated lymphoid tissues. Here, the adjuvants bind to receptors such as GM1-ganglioside and target the antigens to the relevant sites and/or activate antigen-presenting cells (APCs) to elicit protective immunity (Granell *et al*., 2010; Salyaev *et al*., 2010). In addition, co-expression of an antigen-adjuvant couple can eliminate the costs related to the separate production and administration of adjuvants. In addition to its adjuvanticity, LTB can also act as an immunogen and protect against the infectious diarrhoea.

LTB alone has been expressed in plastids (Kang *et al*., 2003) and its functional conformation has been proved *in vitro* by GM1-ganglioside binding assay. However, there are very few reports about the expression of LTB coupled with a vaccine antigen, particularly against a human disease (Lössl and Waheed 2011). Only two studies report the expression of LTB as fusion protein in plastids (Sim *et al*. 2009; Rosales-Mendoza *et al*. 2009). Sim *et al*. (2009) expressed a synthetic LTB fused with hemagglutinin-neuraminidase neutralizing epitope (HNE) of Newcastle disease virus. While Rosales-Mendoza *et al*. (2009) expressed LTB fused with *E. coli* heat stable toxin (ST) and showed the induction of serum and mucosal LTB-ST specific antibodies upon oral immunization of mice with freeze-dried leaves of transplastomic tobacco.

2.7 Chloroplast-based expression of vaccine antigens against HPV

As described earlier, chloroplasts can be used as an effective platform for the production of vaccines. Very high expression of proteins in chloroplasts makes this system ideal for vaccine production. To meet the requirement of several doses of vaccines necessary to vaccinate large populations in resource poor countries, high expression of vaccine antigens is a pre-requisite. Regarding the development of a chloroplast-derived prophylactic vaccine against HPV, there are few reports about the expression of HPV-16 major capsid protein L1 in chloroplasts (Lössl and Waheed 2011). Fernández-San Millán *et al*. (2008) expressed L1 protein in tobacco plastids and obtained fairly high levels (24%) of total soluble protein (TSP). These authors tested the immunogenicity of protein in mice and showed that plastid-derived L1 antigen was highly immunogenic. Lenzi *et al*. (2008) expressed the native viral L1 and synthetic codon-optimized L1 genes in tobacco chloroplasts. However, in contrast to Fernández-San Millán *et al*. (2008), the yield of plastid-expressed L1 was low (1.5% of TSP). In both above mentioned studies, L1 protein was expressed in the form of VLPs. Recently, our group expressed a modified HPV-16 L1 gene (L1_2xCysM) in tobacco chloroplasts (Waheed *et al*., 2011a), which led to the formation of only capsomeres. In another study (Waheed *et al*., 2011b), we expressed L1 protein leading to the formation of capsomeres, in fusion with LTB as adjuvant. In the above mentioned two reports, the recombinant proteins L1 and LTB-L1 accumulated up to 1.5% and 2% of TSP, respectively. For the development of therapeutic vaccines

against HPV, HPV-16 oncoprotein E7 has been expressed in chloroplasts by Morgenfeld *et al*. (2009). They expressed E7 antigen alone and as a fusion protein with potato virus X coat protein (CP). However, the expression of E7 and E7CP proteins was very low with 0.1% and 0.5% of TSP, respectively.

2.8 Objectives

There were three main objectives of the present study.

2.8.1 Establishment of a cost-effective expression system for HPV-16 L1 capsomeres

Since more than 80% of cervical cancer cases occur in developing countries and currently available VLP-based vaccines against HPV are expensive, there is an urgent need for the development of low-cost second-generation vaccines against HPV. These vaccines should be cheap to produce and more stable than the existing vaccines. Due to this reason, we chose the expression of a modified HPV-16 L1 gene (L1_2xCysM) in plants. Expression of this gene leads to the retention of L1 assembly to capsomeres. Capsomeres are considered more thermo-stable than VLPs and this characteristic can potentially reduce the costs related to the maintenance of cooling chain. Furthermore, we have selected tobacco chloroplasts as an expression platform. This is advantageous for developing cost-effective vaccines, as tobacco has high biomass production capacity and very high yield of recombinant proteins can be obtained from tobacco chloroplasts. Another reason to choose tobacco plastids for transformation is that plastids are only maternally inherited in tobacco, which helps to assure the biosafety.

2.8.2 Expression of L1_2xCysM gene coupled with LTB as adjuvant

After the expression of capsomeres in chloroplasts, we were interested to express L1_2xCysM gene and LTB as fusion protein in tobacco chloroplasts. We directly coupled L1 with LTB, as LTB can act as adjuvant and increase the immunogenicity of HPV-16 L1 capsomeres. Aim of this coupling was to establish a basis for the production of a possible vaccine against HPV, with enhanced immunogenicity due to direct fusion of an adjuvant with the HPV-16 L1 antigen. Direct fusion of an adjuvant with the antigen is advantageous because this approach can circumvent separate production and administration of adjuvants. In addition to its adjuvant properties, LTB

can also act as immunogen, providing protection against ETEC infection. Since both LTB and L1 lead to the formation of their respective pentameric forms, we were also interested to investigate and confirm that the functional conformation of both proteins is retained after coupling.

2.8.3 Expression of LTB in tobacco chloroplasts to investigate pleiotropic effects

In the second study, when LTB-L1 fusion protein was expressed in tobacco chloroplasts, phenotypic alterations were observed in transplastomic plants. All transplastomic plants were chlorotic, male sterile and showed stunted growth. In the first study, when tobacco chloroplasts were transformed with modified L1 gene, no such effects were observed, except male sterility. Therefore, we wanted to examine whether these pleiotropic effects were due to the expression of LTB-L1 fusion protein or LTB protein. For this reason, a third study was conducted in which tobacco plastids were transformed with LTB gene only.

3. Methodology

Detailed experimental procedures are given in the respective publications (articles 3 and 4). A general outline of methodology is described here.

3.1 Vectors used for tobacco chloroplast transformation

Three vectors were constructed for the transformation of tobacco chloroplasts.

3.1.1 PNGL1$_M$T

PNGL1$_M$T was the final transformation vector used in the first study (article 3, Waheed *et al*., 2011a). This vector consisted of L1_2xCysM gene having two cysteines replaced by serines at position 175 and 428. First 14 amino acids of green fluorescent protein (GFP$_{14}$) were attached to the N-terminus of modified L1 gene to enhance the expression of L1_2xCysM gene in the chloroplasts, as reported by Ye *et al*. (2001). Expression of transgenes was driven by combined promoters for the plastid and nuclear encoded polymerases, *Prrn*PEP and *Prrn*$^{-62}$NEP, respectively (Svab and Maliga 1993; Hajdukiewicz *et al*., 1997). Between the promoter and GFP$_{14}$, the vector contained ribosomal binding site (RBS) from the leader sequence of gene 10 (G10L) of the lambda phage T7 (Studier *et al*., 1990). The *aadA* gene was used for the selection of transplastomic plants. The 5′-UTR, consisting of a synthetic RBS, was linked to the N-terminus of the *aadA* gene. The terminator sequence from the large subunit of the ribulose-bisphosphate carboxylase gene (T*rbc*L) from *Chlamydomonas reinhardtii* was used as terminator. The *Prrn*PEP-*Prrn*$^{-62}$NEP promoter served as a bicistronic promoter, driving the expression of both the L1_2xCysM and *aadA* genes. The *trn*N (INSL) and *trn*R (INSR) loci were used as insertion sites for the homologous recombination within the tobacco plastid genome.

3.1.2 PNGLTB-L1$_M$T

For transformation of tobacco plastids with coupled LTB-L1 genes in the second study (article 4, Waheed *et al*., 2011b), the transformation vector PNGLTB-L1$_M$T was constructed. This vector consisted of all the same components as PNG-L1$_M$T, only the LTB gene was additionally inserted between GFP$_{14}$ and L1_2xCysM. To minimize the potential steric hindrance between LTB and L1 proteins, the spacer sequence GPGPG

was used between LTB and L1_2xCysM genes. All genes (LTB-L1_2xCysM and *aadA*) were under control of *Prrn*PEP-*Prrn*$^{-62}$NEP promoter.

3.1.3 PNGLTB-T

In the second study, certain pleiotropic effects were observed in transplastomic plants. To investigate these effects, another transformation vector was constructed. This vector PNGLTB-T consisted of a transformation cassette with only the LTB gene for transformation of tobacco plastids. The *aadA* gene was used as selection marker and the rest of the components were identical to the vector PNGL1$_M$T, used in the first study.

3.2 Transformation and regeneration of transformed plants

Leaves of *Nicotiana tabacum* cv. Petit Havana were used as explants for transformation. Seeds were grown *in vitro* in the growth chamber (25°C, light intensity: 0.5–1 W/m^2 Osram L85 W/25 universal-white fluorescent lamps, 16 h light/8 h dark cycle) on agar-solidified MS medium (Murashige and Skoog 1962) and leaves were harvested when they were at 5-7 leaf stage of plant growth. Tobacco leaves, with abaxial side facing up, were placed on RMOP medium (for 1 litre: MS salts, 100 mg myo-inositol, 1 mg thiamine HCl, 1 mg BAP, 0.1 mg NAA, 30 g sucrose, pH 5.8 and 6g agar). Leaves were bombarded with DNA coated gold particles (0.6 µm) by a biolistic gun (PDS1000He; Bio-Rad, Hercules, CA), as described by Verma *et al*. (2008) and incubated in dark for two days on RMOP medium in climatic chamber at 25°C. After two days, leaves were cut into small sections (5 mm × 5 mm) and transferred to RMOP medium containing 500 mg/l spectinomycin for selection of transformed tissues (Svab *et al*., 1990) under the same growth conditions. Green microcalli appeared on the bleached leaves, which were further subcultured on the same spectinomycin containing selection medium for shoot formation. After 4-6 cycles, regenerated shoots were shifted to B5 medium (Gamborg *et al*., 1968) supplemented with spectinomycin, for root formation. After rooting, transplastomic plants were shifted to green house in pots, for further growth and seed production.

3.3 Confirmation of transgenes integration by polymerase chain reaction

Polymerase chain reaction (PCR) is a standard method to verify the insertion of transgenes in the plant genome. During the regeneration of transformed plants, transplastomic leaves were tested for transgene integration by PCR. DNA was extracted from 100-150 mg of leaves from regenerating shoots by the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson 1980). Primer pairs were designed to confirm the insertion of the transformation cassettes from both left and right insertion sites, INSL and INSR, respectively. One primer on either side of the integrated cassette was inserted within the plastid genome. In this way the specific amplified fragment could only be obtained from the plastomes of transformed plants. Description and sequence of primers and the sizes of the amplified fragments are given in the respective publications (articles 3 and 4).

3.4 Southern blot analysis

Under optimal conditions, there are more than 100 copies of plastid genome in each chloroplast of tobacco. After transformation, wild type copies of plastid genome are also present. Homoplasmy refers to the state when no wild type copy of plastid genome is left and the chloroplast exclusively contains the transformed DNA (Maliga 2004). To achieve homoplasmy, the regenerating tissues are subjected to a further round of selection on RMOP medium containing spectinomycin. Southern blotting is carried out to confirm the homoplasmy of transformed plants. The technique was performed by standard procedure described by Sambrook and Russell (2001). DNA was extracted from 100-150 mg of leaves from *in vitro* grown plants by CTAB method. A probe with the size of 773bp, located within INSL was amplified by PCR. The amplified fragment was purified using QIAquick kit (Qiagen, Hilden, Germany). Probe labeling, hybridization and detection were performed using the DIG High Prime DNA Labeling and Detection Starter kit II for chemiluminescent detection with CSPD, as instructed by the manufacturer (Roche, Mannheim, Germany). Details of primers, probe and sizes of fragments obtained by Southern blotting are given in the respective publication (article 4).

3.5 Extraction, purification and determination of assembly forms of proteins

3.5.1 Protein extraction

Protein was extracted from the leaves of transplastomic plants by the procedure described by Verma *et al*. (2008). Leaf discs from transplastomic and wild type plants were obtained from plants grown under sterile conditions. To extract soluble proteins, 100 mg leaves were ground in liquid nitrogen and homogenized in protein extraction buffer (PEB, for composition see article 3 and 4). The homogenized samples were centrifuged at 24,000 g for 10 minutes at 4°C and supernatant was collected as soluble fraction of leaf proteins. The protein concentrations were determined by Bradford protein assay (Sigma, MO, USA).

3.5.2 Western blotting

To verify the expression of recombinant proteins, the isolated soluble proteins were subjected to Western blotting. Soluble proteins were heated at 95°C for 5 minutes and separated by SDS-PAGE. Concentration of polyacrylamide was according to the expected sizes of proteins (article 3 and 4). Western blotting was carried out by the procedure described by Verma *et al*. (2008). HPV-16 L1-specific monoclonal antibody MD2H11 (DKFZ, Heidelberg, Germany) was used as primary antibody. As secondary antibody, peroxidase-conjugated goat antimouse IgG (Sigma) was used. Detection of proteins was carried out by chemiluminescence and bands were visualized on X-ray film. For quantification of TSP, a dilution series of baculovirus-derived purified VLPs (DKFZ, Heidelberg, Germany) as reference. Details of Western blotting for individual L1 and LTB-L1 proteins are given in the articles 3 and 4, respectively.

3.5.3 Cesium chloride density gradient centrifugation

L1 protein obtained from transplastomic plants was purified by cesium chloride (CsCl) density gradient centrifugation. By this method L1 protein can be analyzed for higher order structures such as capsomeres and VLPs. In this method, as described by Thönes *et al*. (2008), plant material was ground in liquid nitrogen and homogenized with extraction buffer. After sonication, the lysate was cleared by centrifugation. The cleared lysate was transferred onto a two step gradient consisting of sucrose at top and

CsCl at bottom and centrifuged at high speed. The particles move through the sucrose phase and are caught in the interphase between the sucrose and CsCl layer. The interphase was mixed with the CsCl layer and centrifuged at high speed. After centrifugation, tube was punctured at the bottom and 14 fractions (1 ml each) were collected. Assembly forms of L1 protein (capsomeres or VLPs) were expected in the middle fractions. All these fractions were tested by Western blotting and antigen capture ELISA. The specific details of the CsCl density gradient centrifugation protocol are given in the article 3.

3.5.4 Sucrose gradient sedimentation analysis of L1 protein

Sucrose gradient sedimentation was used to verify that L1 protein assembled into capsomeres rather than into VLPs. Tobacco leaves were ground in liquid nitrogen and homogenized with extraction buffer (for composition see article 3). Proteins were extracted by sonication and the lysate was cleared by centrifugation. The cleared lysate was loaded onto a linear sucrose gradient (5-50%) in extraction buffer and centrifuged at high speed. In this method, VLPs, capsomeres and other assembly forms of L1 protein are separated according to their sedimentation coefficients. The tube was punctured at the bottom and 20 fractions (600 µl each) were collected and their refractive indices were determined. Due to low sedimentation coefficient (~10-11S), capsomeres were expected in the last fractions. All the collected fractions were analysed by Western blotting and antigen capture ELISA. The specific details of the procedure are described in the article 3.

3.6 Confirmation of proper conformation of recombinant proteins

3.6.1 Antigen capture Enzyme-Linked Immunosorbent Assay (ELISA)

Antigen capture ELISA was used to confirm that the plastid-derived L1 protein was correctly folded and retained antigenic epitopes. In this technique a HPV-16 L1 conformation specific mouse monoclonal antibody Ritti01 (Thönes *et al*., 2008) was used. The wells of a 96-well microtitre plate were coated with Ritti01 at 4°C overnight. After washing and blocking, plant extracts were added to the wells and the plate was incubated for 1 h at 37°C. After washing, polyclonal rabbit antiserum raised against HPV-16 L1 was added to the wells and the plate was incubated for 1 h at

37°C. The plate was washed and goat anti-rabbit peroxidise conjugate was added to the wells and incubated for 1 h 37°C. After incubation, staining solution was added to the wells and absorption was measured at 405 nm. The detailed protocol of the capture ELISA, composition of solutions and antibody dilutions are illustrated in the article 3 and 4. All the antibodies for antigen capture ELISA were provided by DKFZ, Heidelberg, Germany.

3.6.2 GM1-ganglioside binding assay

A GM1-ganglioside binding assay was used to verify the correct folding of LTB protein in plastid-derived LTB-L1 protein. In this method, a 96-well microtitre was coated with monosialoganglioside-GM1 (Sigma G7641) and incubated at 4°C overnight. After washing, the wells were blocked for 1 h at 37°C. Protein extracts from transplastomic leaves were added to the wells and incubated at 37°C for 1 h. After incubation, the wells were washed and rabbit anti-LTB antibody (Immunology Consultants Lab, Newberg, OR, USA) was added to the wells and incubated for 1 h at 37°C. After washing, peroxidise conjugated anti-rabbit antibody was added and the plate was incubated for 1 h at 37°C. Staining solution was added to the wells after washing, and the measurement was taken at 405 nm. The compositions of solutions and the dilutions of antibodies are described in detail in the article 4.

4. Publications

4.1 Expert Commentary

Plant-derived vaccines: An approach for affordable vaccines against cervical cancer

Plant-derived vaccines

An approach for affordable vaccines against cervical cancer

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Abstract

Several types of human papillomavirus (HPV) are causatively associated with cervical cancer, which is the second most common cancer in women worldwide. HPV-16 and 18 are among the high risk types and responsible for HPV infection in more than 70% of the cases. The majority of cervical cancer cases occur in developing countries. Currently available HPV vaccines are expensive and probably unaffordable for most women in low and middle income countries. Therefore, there is a need to develop cost-effective vaccines for these countries. Due to many advantages, plants offer an attractive platform for the development of affordable vaccines. These include low cost of production, scalability, low health risks and the potential ability to be used as unprocessed or partially processed material. Among several techniques, chloroplast transformation is of eminent interest for the production of vaccines because of high yield of foreign protein and lack of transgene transmission through pollen. In this commentary, we focus on the most relevant aspects of plant-derived vaccines that are decisive for the future development of cost-effective HPV vaccines.

Key words: plant-derived vaccines, chloroplast transformation, biosafety, cost-effective vaccines, human papillomavirus, L1, adjuvant, LTB.

Background

Cervical cancer is the second most common cancer in women and the seventh overall in the world. Every year, approximately half a million new cases occur across the globe.[1] In 2008, an estimated 529,000 new cases and 275,000 deaths were reported, about 88% of which occurred in developing countries: 159,800 in Asia, 53,000 in Africa and 31,400 in Latin America and the Caribbean.[2] The number of deaths from cervical cancer is expected to increase to approximately 410,000 by 2030.[2,3] Overall, in developing countries, cervical cancer accounts for more than 85% of the total global burden and is the leading cause of cancer-related deaths among women.[4] Those regions that are at high risk include South-Central Asia, South America and whole Africa with exception of North. In Eastern Africa, South-Central Asia and Melanesia, cervical cancer remains the most common cancer in women.[4] During the last three decades, the number of cervical cancer cases has been increasing for all regions except in high income countries, while for east and South Asia, Eastern Europe, and Southern Latin America, the number of new cases has been constant.[5]

Almost all cases of cervical cancer are caused by one of 15 types of oncogenic human papillomavirus (HPV). Among these, HPV-16 and 18 are responsible for approximately 70% of invasive cervical cancer cases while HPV-16 alone contributes to 54% of the total cases.[6] Other high-risk HPV types include 31, 33, 35, 45, 52 and 58.[7] HPVs are non-enveloped icosahedral viruses containing double stranded circular DNA. The DNA genome encodes for two classes of genes: the early and late. The early gene products are E1, E2, E4, E5, E6 and E7 and the late proteins are L1 and L2. The later proteins are the structural components of the viral capsid (for review see ref. 8).

Vaccines can either be prophylactic, which generate neutralizing antibodies to block HPV infection or the therapeutic vaccines, which eliminate infection by inducing a virus-specific T cell-mediated response. To date, no therapeutic vaccine is available for the treatment of already existing HPV infections. Current strategies for the development of safe and effective prophylactic vaccines are based on the induction of neutralizing antibodies against the major (L1) and minor (L2) capsid proteins of HPV.[9] To improve the existing strategies for broad protection, a conserved and cross-protective antigen such as L2 can be included in vaccine formulations. However, to accelerate the control of cervical cancer and treat currently infected patients, it is

important to develop HPV vaccines that are therapeutic. For this purpose, research is focused on early proteins encoded by the HPV genome. A recent report shows promising results for the development of such therapeutic vaccine (Pentarix) directed at the E7 proteins from five of the most prevalent high-risk genotypes of HPV (HPV16, 18, 31, 45 and 52).[10] In the above mentioned report, upon administration, mice elicited strong, multi-genotype specific CD8T cell immunity. Moreover, large and established E7-expressing TC-1 tumors showed rapid and complete regression after therapeutic vaccination of mice with Pentarix.[10]

Are currently available HPV vaccines affordable for women in developing countries?

Currently, two prophylactic vaccines, Gardasil and Cervarix manufactured by Merck and GlaxoSmithKline (GSK), respectively, are commercially available for the prevention of cervical cancer. Both vaccines contain the HPV L1 capsid protein in the form of virus-like particles (VLPs). Gardasil targets HPV types 6, 11, 16 and 18, while Cervarix targets types 16 and 18.[11,12,13] In the USA, the drug company's recent prices for Gardasil and Cervarix are almost similar, i.e., approximately 130$ per dose and 390$ for three shots for private health providers.[14] This price does not include the cost of receiving the injection. World Bank data estimates that the number of people living on less than $2 a day is over 2.5 billion worldwide (World Bank 2008). Due to the low resources in most developing countries, government health care programs can not provide HPV vaccination. As a result, people need to acquire the vaccine through private health care providers, which is not affordable for a large percentage of people in low-income countries. In addition, the price of HPV vaccine from both companies is high for private use. Although, both companies have introduced price tiering systems for their respective HPV vaccines, so that they can offer vaccine to lower income countries at lower prices, the costs associated with the vaccination are still not affordable for most women in developing countries. Therefore, it is necessary to develop low-cost, stable and effective preventive vaccines that are suitable for developing countries.

Plants as a platform for the production of cost-effective vaccines

The majority of the currently available vaccines are expensive because of their complex manufacturing processes through various cell culture systems, requirement of fermenters and purification by complex technologies. Moreover, additional expenses are associated with adjuvant, transportation, cold storage and sterile delivery.[15] In contrast, plants offer a cost-effective platform for the production of low-cost vaccines. Production of vaccines from plants has many advantages: low cost, scalability, low health risks and the potential ability to be administered as unprocessed or partially processed material (for review see refs. 16-18). Plant-derived vaccines can be produced either stably by nuclear and chloroplast transformation or transiently by tobacco mosaic virus (TMV) based expression. Each technique has its own advantages and disadvantages. However, regarding vaccine production in plants, chloroplast transformation suits well, because of high yield of recombinant protein.[19] Moreover, chloroplast transformation is safe for the environment as transgenes can not spread via pollen in most plant species[20] Various vaccine antigens against different human diseases have been successfully expressed in chloroplast.[19] However, none of the chloroplast-derived vaccine candidate has yet entered into clinical trials. So far, few plant-derived vaccines against human diseases, expressed either by nuclear transformation or TMV based expression, have shown promising results in phase I and II clinical trials upon oral delivery (for review see refs. 21-22).

HPV-16 L1 antigen has been expressed in plants by nuclear and chloroplast transformation, either as VLPs or capsomeres,[23-29] and the plastid-derived VLPs were shown to be highly immunogenic in mice.[27] Recently, our group expressed HPV-16 L1 protein along with *Escherichia coli* heat labile enterotoxin subunit B (LTB), as a fusion protein.[30] LTB can act as an adjuvant and by this facilitate the transport of the antigen from the gut lumen to the gut associated lymphoid tissues. In addition to L1, many studies have been performed regarding the expression of E6 and E7 in plants (for review see refs. 31). Currently, research is underway for the development of a therapeutic vaccine against HPV from plants, by a renowned research group in South Africa.[32]

There are several advantages of plant/plastid transformation that can be exploited for cost-effective production of HPV vaccines. These are:

- Very high yields of recombinant proteins can be achieved by chloroplast transformation. In two recent reports,[33,34] approximately 70% and 72% of total soluble protein (TSP) has been obtained by plastid transformation, respectively. Since stimulation of the mucosal immune system generally requires much higher doses of the antigen than injection, plastid transformation can be optimized to express L1 or other antigenic proteins in large amounts to meet the prerequisite for the initiation of proper immune responses. In addition, with the increase in the yield of the recombinant protein, the downstream processing costs can be decreased.[35]
- Scalability: growth of plants can be scaled up according to the required amount of protein.
- Biosafety issues can be covered by the application of chloroplast transformation and/or growing the plants in contained facilities. Furthermore, an inducible system can be used to control the transgene expression when required.[19,36]
- Transgenic plants can be grown at the site where the vaccine is needed. This advantage can save the costs related to transportation and cold storage.
- Plant-derived vaccines have the potential to be used as oral vaccine, thus evading the costs related to sterile needles and trained medical staff.
- Stability: plants-derived vaccines are likely to be more stable. A recent report[37] shows that a chloroplast-derived vaccine candidate was stable at room temperature for 20 months. Moreover, mice immunized with the vaccine stored at room temperature showed similar IgA/IgG levels as those of mice immunized with the vaccine stored at 4°C. [36] This characteristic is very important for the development of a vaccine for developing countries where cold chains are difficult to maintain in remote areas.
- Due to the prokaryotic nature of the gene expression system of the plastid, multiple genes can be linked in operons and co-expressed simultaneously. This advantage of multigene expression in plastids can be adopted for the production of vaccines with improved immunogenicity by coupling the antigens with specific adjuvants. By this, costs related to separate production and administration of adjuvants can be saved. Moreover, this feature of plastid

transformation can be used to co-express L1 antigens from different HPV types and/or combine L2 for broad protection against different types.

In conclusion, plants have a great potential to produce cost-effective vaccines against HPV. There are many advantages of plant-derived vaccines that can be used to produce low-cost vaccines for low and middle income countries. Exceptionally high yields obtained through plastid transformation make this system very promising for vaccine production. Moreover, the issue of biosafety can be covered by using chloroplast as an expression system. However, for plant-derived vaccines, the major hurdles are the costs related to clinical trials. Limited resources in research and lack of interest/investment from companies are also further reasons for the slow advancement of plant-based vaccines. Nevertheless, a couple of plant-derived pharmaceuticals has entered the clinical trials[38,39] and these successful developments markedly increase the chances that further plant-derived vaccines will soon be available in the market to serve patients in low and middle income countries.

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4.2 Review article

Chloroplast-derived vaccines against human diseases: achievements, challenges and scopes

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Review article

Chloroplast-derived vaccines against human diseases: achievements, challenges and scopes

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Keywords: chloroplast, adjuvant, pleiotropic effects, inducible expression, cost-effective vaccines, human infectious diseases.

Summary

Infectious diseases represent a continuously growing menace that has severe impact on health of the people worldwide, particularly in the developing countries. Therefore, novel prevention and treatment strategies are urgently needed to reduce the rate of these diseases in humans. For this reason, different options can be considered for the production of affordable vaccines. Plants have been proved as an alternative expression system for various compounds of biological importance. Particularly, plastid genetic engineering can be potentially used as a tool for cost-effective vaccine production. Antigenic proteins from different viruses and bacteria have been expressed in plastids. Initial immunological studies of chloroplast-derived vaccines have yielded promising results in animal models. However, because of certain limitations, these vaccines face many challenges on production and application level. Adaptations to the novel approaches are needed, which comprise codon usage and choice of proven expression cassettes for the optimal yield of expressed proteins, use of inducible systems, marker gene removal, selection of specific antigens with high immunogenicity and development of tissue culture systems for edible crops to prove the concept of low-cost edible vaccines. As various aspects of plant-based vaccines have been discussed in recent reviews, here we will focus on certain aspects of chloroplast transformation related to vaccine production against human diseases.

Introduction

According to the World Health Organization (WHO), there is a need to develop cost-effective vaccines against several infectious diseases (WHO, 1999). In developing countries, the morbidity and mortality rates due to infectious diseases are very high: over 9.5 million people die every year in these countries (WHO, 2008). Therefore, much more efforts are needed to fulfil the needs of vaccines for these countries. Currently used vaccines against several infectious diseases face the problem of safety and/or their production and distribution costs are high. Production of these vaccines from bacteria, yeast or insect cells require fermenters, which are expensive and lack scalability (Daniell *et al.*, 2009). In addition, production is often accompanied by contamination due to endotoxins or pyrogens, which requires an extensive purification of these vaccines. Moreover, the downstream processing costs of these vaccines are very high: maintenance of the cold chain from producer to consumer, the need of trained medical staff for injectable delivery and sterile needles are the parameters that are difficult to maintain in the developing world (Tregoning *et al.*, 2004). Keeping in view the burden of infectious diseases in developing countries, it is necessary to explore alternate production methods that should facilitate cost reduction at different levels.

Plants present an alternative and attractive source for this purpose, as they can be grown at the site where production is needed and yield can be scaled up according to the demand. In the past 20 years, there has been extensive research on plant-based vaccines. Innovative efforts include nuclear as well as chloroplast transformation technologies. In contrast to nuclear transformation, chloroplast genetic engineering is considered as more valuable owing to several advantages. The following are the most important of these advantages related to the cost-effective production and regulatory issues of plant-based vaccines:

1. Transgene confinement offered by plastid transformation. The release of plastome integrated genes to the environment is restricted due to the maternal inheritance of plastids, in most plant species (Daniell, 2007; Ruf *et al.*, 2007).
2. Existence of many chloroplasts in each cell and multiple copies of plastid genome per cell, making the high level of protein expression possible (Maliga, 2002; Koop *et al.*, 2007; Bock and Warzecha, 2010).
3. Possibility to co-express multiple genes in operons, which is suitable for those vaccines that require multiple epitopes for their proper function.

Transgene containment is one of the key features of plastid transformation that provides increased biosafety and covers the regulatory issue related to the dispersal of any unwanted gene into the environment. In most plant species, plastids show uniparental maternal inheritance (Hagemann, 2004), which minimizes the risk of pollen transfer from transgenic to conventional varieties or related species. Maternal inheritance and prevention of gene flow via pollen has been shown in tobacco and tomato plants (Daniell *et al.*, 1998; Ruf *et al.*, 2001). Some recent studies show that there is a small degree of paternal transmission of

plastids in tobacco (Ruf *et al.*, 2007; Svab and Maliga, 2007). Ruf *et al.* (2007) assessed the strictness of maternal inheritance and the extent to which the plastid transformation technology confers an increase in transgene confinement. They set up a stringent selection system for paternal transmission by using male-sterile maternal parents and transplastomic pollen donors, conferring plastid-specific antibiotic resistance and green fluorescence for visual screening. This selection system identified six among 2.1 million seedlings screened (frequency of 2.86×10^{-6}) that showed paternal transmission of transgenes. Svab and Maliga (2007) investigated whether alien cytoplasm contributes to rare paternal plastid transmission. They reported low frequencies (10^{-4}–10^{-5}) of paternal plastids in both normal and alien cytoplasm. However, this observation was facilitated by tissue culture selection, and therefore transgenes are less likely to get into the germ-line under field conditions. Although transgenic plants can be grown under controlled conditions to assure the gene containment, this might add to the costs related to vaccine production via plants. In this respect, chloroplast transformation is a competitive system that provides an opportunity of growing transplastomic plants in the fields.

Plastid transformation has been explored for the expression of various compounds of biological and pharmaceutical importance (Daniell *et al.*, 2009). History, advantages, developments and milestones of the chloroplast transformation and plant-derived vaccines have been covered in the recent reviews (Davoodi-Semiromi *et al.*, 2009; Rigano *et al.*, 2009a; Tiwari *et al.*, 2009; Cardi *et al.*, 2010; Rybicky, 2010). In the present review, we will specifically focus on the chloroplast-derived vaccine candidates against human diseases that can potentially aid in the development of relatively cheaper and safer vaccines for the resource-poor countries. We will focus on the main advantages and achievements of plastid transformation, which are helpful for the production of cost-effective vaccines. Moreover, some important challenges and scopes for potential plastid-derived vaccine candidates will also be discussed.

Achievements

As mentioned earlier, there are certain characteristic advantages and achievements of the plastid transformation technology, which not only enable cheap production of vaccines but also cover some of the regulatory concerns related to vaccine production in plants. Here, we discuss two major achievements of plastid transformation that are important for the production of vaccines by plants.

High-level expression of proteins in plastids

Attainment of high expression level of foreign proteins in plastids is a major breakthrough, which makes this system ideal for large-scale vaccine production (Chebolu and Daniell, 2009). Tregoning *et al.*, 2003 for the first time reported fairly high expression of a vaccine candidate *TetC* in tobacco plastids, accumulating up to 25% of total soluble protein (TSP). Many other reports exist about high expression of vaccine antigens in chloroplasts; for instance, Molina *et al.* (2004), 31.1% TSP; Fernandez-San Millan *et al.* (2008), 26% TSP; Zhou *et al.* (2008), 40% TSP; Dreesen *et al.* (2010), 23% TSP. For other proteins, plastids have been reported to accumulate foreign proteins to even higher levels [De Cosa *et al.*, 2001, 46.1% TSP; Oey *et al.*, 2009; approximately 70% TSP; (Ruhlman *et al.*, 2010), 72% of total leaf protein (TLP)]. Although physiological alterations were observed in the reports of a couple of studies (discussed later under pleiotropic effects), in rest of the studies, plants grew normally. Generally, 1% of TSP is considered as threshold to allow commercial or economical production (Fischer *et al.*, 2004; Meyers *et al.*, 2008). It is obvious from the Table 1 that in most of these reports, the expression is higher than the expected threshold of 1% TSP. Hence, in the context of protein expression, chloroplast-derived vaccines have very promising and competitive potential for commercialization.

Marker excision from transplastomic plants

Marker genes are necessary for the selection of transformants after the transformation. In case of plastid transformation, the most commonly used marker gene is *aadA*, which confers resistance to the antibiotics spectinomycin and streptomycin. However, integration of antibiotic resistance genes in transformed plants raises environmental and health concerns towards the commercialization of transgenic plants. Strategies adopted to overcome this hurdle are either by excision of marker genes or by using plant-derived markers for selection. Different methodologies have been used to remove marker genes from the transplastomic plants (Lutz and Maliga, 2007). One of these approaches is based on the deletion of marker genes by homologous recombination via direct repeats flanking the marker gene (Iamtham and Day, 2000). However, homology-based marker excision relies on secondary recombination and segregation of plastid DNA, which makes the attainment of marker-free transplastomic plants difficult. The second approach is an antibiotic/phenotypic selection system that utilizes plastid mutants i.e. knockout of a photosynthetic gene that produces a chlorophyll-deficient phenotype with pale-green leaves (Klaus *et al.*, 2004). The third approach relies on marker excision by the *Cre–loxP* site-specific recombination system (Corneille *et al.*, 2001). A more recent report about the removal of plastid marker genes is based on the excision by the phiC31 phage site-specific integrase (Int) that mediates recombination between bacterial (attB) and phage (attP) attachment sites (Kittiwongwattana *et al.*, 2007). Alternatively, the issue of selectable marker genes can be overcome by using plant genes that confer a metabolic or developmental advantage to the transformed cells by manipulation of the plant's biosynthetic pathways. Recently, Barone *et al.* (2009) showed successful generation of transplastomic plants by using a selection system based on the feedback-insensitive anthranilate synthase α-subunit gene of tobacco (ASA2) as a new selective marker. In their study, they used the indole analogue 4-methylindole or the tryptophan analogue 7-methyl-DL-tryptophan as selection agents. Although there are certain shortcomings in some of the studies listed above (Lutz and Maliga, 2007), chloroplast transformation technology has yet been shown to overcome the issue of biosafety related to the antibiotic resistance genes.

Vaccine antigens expressed in chloroplasts

A large variety of compounds and proteins have been successfully expressed by plastid transformation, including biopharmaceuticals, enzymes, vaccine antigens, plasma proteins and antibodies. Expression of recombinant antigen proteins in *Escherichia coli* is sometimes not feasible owing to the lack of a variety of post-translational modifications and folding

Table 1 Summary of vaccine antigens against different human diseases expressed in the plastid genome

Vaccine antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
Viral antigens				
HPV-16 *L1* VLPs (Cervical cancer)	Tobacco	26% TSP	Induced systemic immune response in mice after intraperitoneal injection, and neutralizing antibodies were detected.	Fernandez-San Millan *et al.* (2008)
HPV-16 *L1* VLPs (Cervical cancer)	Tobacco	1.5% TSP	Not tested	Lenzi *et al.* (2008)
HPV-16 *L1* Capsomeres (Cervical cancer)	Tobacco	1.5% TSP	Not tested	Waheed *et al.* (2011)
Potato virus X coat protein (CP) fused with HPV-16 E7 oncoprotein (*E7-CP*) (Cervical cancer)	Tobacco	0.5% TSP	Not tested	Morgenfeld *et al.* (2009)
Vaccinia virus envelope protein (*A27L*) (Small pox)	Tobacco	18% TSP	Plastid-derived A27L protein formed oligomers, suggesting correct folding and quaternary structure, and was recognized by serum from a patient recently infected with a zoonotic orthopoxvirus.	Rigano *et al.* (2009b)
Hepatitis E virus (*HEV E2*) (Hepatitis)	Tobacco	1.09 ng/μg TSP	Antibodies cross-reacting with pE2 were detected in serum samples of mice immunized.	Zhou *et al.* (2006)
Hepatitis C virus core protein (Hepatitis)	Tobacco	approximately 0.1% TLP	Expressed protein detected anti-core antibodies in infected human sera	Madesis *et al.* (2010)
Rotavirus (*VP6*) (Diarrhoea)	Tobacco	3% TSP	Not tested	Birch-Machin *et al.* (2004)
Epstein–Barr virus (VCA) (infectious mononucleosis, T-cell lymphoma, gastric carcinoma)	Tobacco	0.002%–0.004%, TSP	Not tested	Lee *et al.* (2006)
HIV (*p24*) (AIDS)	Tobacco	4.5% TSP	Not tested	McCabe *et al.* (2008)
HIV (*p24-Nef*) (AIDS)	Tobacco, Tomato leaves	40% TSP	Not tested	Zhou *et al.* (2008)
	Green fruits	2.5% TSP		
HIV-1 Gag structural poly-protein ($Pr55^{gag}$) (AIDS)	Tobacco	8% TSP	Not tested	Scotti *et al.* (2009)
Human β-site APP cleaving enzyme (BACE) (Alzheimer disease)	Tobacco	2.0% TSP	Mice exhibited a slight induction of primary anti-BACE antibody upon oral administration with extracts from transplastomic tobacco.	Youm *et al.* (2010)
Bacterial antigens				
CTB (Cholera)	Tobacco	4.10%	Binding to the intestinal membrane GM1-ganglioside receptor.	Daniell *et al.* (2001)
CTB-AMA1 and *CTB-MSP1* (Cholera)	Tobacco	13.17% and 10.11% TSP	Long-term protection (50% mouse lifespan) in both orally (100%) and subcutaneously (89%) immunized mice.	Davoodi-Semiromi *et al.* (2010)
	Lettuce	7.3% and 6.1% TSP		
CTB-2L21 (cholera, Canine parvovirus)	Tobacco	31.1% TSP	GM1-ganglioside binding assay	Molina *et al.* (2004)

Table 1 Continued

Vaccine antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
CTB fused with fibronectin-binding domain (D2) of *Staphylococcus aureus* (*CTB-D2*) (Skin infections, Bacteraemia)	Chlamydomonas	23% TSP	GM1-ganglioside binding assay, Upon immunization, induced specific mucosal and systemic immune responses in mice. Significantly reduced the pathogen load in the spleen and the intestine of treated mice and protected 80% of them against lethal doses of *S. aureus*.	Dreesen *et al.* (2010)
Escherichia coli LTB (Diarrhoea)	Tobacco	2.5% TSP	GM1-ganglioside binding assay	Kang *et al.* (2003)
Mutant of *E. coli* heat-labile enterotoxin (LTK63) (Diarrhoea)	Tobacco	3.7% TSP	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	2.3% TSP	GM1-ganglioside binding assay Oral immunization of mice with freeze-dried transplastomic tobacco leaves led to the induction of both serum and mucosal LTB-ST specific antibodies against cholera toxin challenge.	Rosales-Mendoza *et al.* (2009)
LTB fused with Hemagglutinin–neuraminidaseneutralizing epitope (*LTB-HNE*) (Diarrhoea, Newcastle disease)	Tobacco	0.5% TSP	GM1-ganglioside binding assay.	Sim *et al.* (2009)
Tetanus toxin fragment C (*TetC*) (Tetanus)	Tobacco	25 and 10% TSP	Immunization of mice with the plastid-derived protein induced protective levels of antitoxin antibodies, and mice were protected against tetanus toxin challenge.	Tregoning *et al.* (2003, 2005)
Anthrax protective antigen (*pagA*) (Anthrax)	Tobacco	18.1% TSP	Macrophage lysis assay	Watson *et al.* (2004)
Anthrax protective antigen (*pagA*) (Anthrax)	Tobacco	14.2% TSP	Mice survived (100%) challenge with lethal doses of toxin.	Koya *et al.* (2005)
Anthrax protective antigen (*pagA*) (Anthrax)	Tobacco Lettuce	approximately 29% TSP approximately 22% TSP	Not tested	Ruhlman *et al.*, 2010
Borrelia burgdorferi outer surface lipoprotein A (*OspA*, *OspA-T*) (Lyme disease)	Tobacco	10% TSP	Mice produced protective antibodies against bacteria.	Glenz *et al.* (2006)
Yersinia pestis F1-V antigen (*CaF1-LcrV*) (Plague)	Tobacco	14.8% TSP	Mice were immunized orally (88%) and subcutaneously (33%) when exposed to 50-fold lethal dose of *Y.pestis*.	Arlen *et al.* (2008)
Multi-epitope DPT fusion protein (Diphteria, Pertussis, Tetanus)	Tobacco	0.8% TSP	Upon oral immunization in mice with freeze-dried transplastomic leaves, production of IgG and IgA antibodies specific to each toxin was detected in serum and mucosal tissues.	Soria-Guerra *et al.* (2009)

Table 1 Continued

Vaccine antigen (Disease)	Expression system	Maximum expression level	Immunological investigation	References
Fibronectin extra domain A (*EDA*) (as Adjuvant)	Tobacco	2% TCP	Purified protein was able to induce the production of TNF-α either by bone marrow-derived dendritic cells or by THP-1 monocytes. Induced up-regulation of CD54 and CD86 maturation markers on dendritic cells.	Farran *et al.* (2010)
Protozoan antigens				
Gal/GalNAc lectin of *Entamoeba histolytica* (*LecA*) (Amoebiasis)	Tobacco	6.3% TSP	Subcutaneous immunization of mice with crude extracts of transgenic leaves resulted in high IgG titres.	Chebolu and Daniell (2007)
CTB-AMA1 and *CTB-MSP1* (Malaria)	Tobacco Lettuce	13.17% and 10.11% TSP 7.3% and 6.1% TSP	Significant levels of antigen-specific antibody titres of immunized mice completely inhibited proliferation of the malarial parasite.	Davoodi-Semiromi *et al.* (2010)
Autoantigens				
Cholera toxin B–proinsulin fusion protein (CTB-Pins) (Diabetes type 1)	Tobacco Lettuce	16% TSP 2.5% TSP	Upon oral immunization, nonobese diabetic (NOD) mice showed a decrease in inflammation (insulitis). Insulin producing β-cells in the pancreatic islets were significantly preserved. Increased expression of immunosuppressive cytokines (IL-4 and IL-10) was observed.	Ruhlman *et al.* (2007)
Cholera toxin B–proinsulin fusion protein (CTB-Pins) (Diabetes type 1)	Tobacco Lettuce	72% TLP, approximately 24% TLP	Not tested	Ruhlman *et al.*, 2010
Glutamic acid decarboxylase 65 (hGAD65) (Diabetes type 1)	Chlamydomonas	0.3% TSP	Immunoreactivity to diabetic sera and induction of proliferation of spleen cells from NOD mice.	Wang *et al.* (2008)

HPV, Human papillomavirus; HIV, Human immunodeficiency virus; AIDS, Acquired immunodeficiency syndrome; *CTB,* Cholera toxin subunit B; *LTB,* heat-labile enterotoxin subunit B; TLP, total leaf protein; TSP, total soluble protein; TCP, total cellular protein.

requirements. Some of these required mammalian-type post-translational modifications of proteins take place in yeast and insect cell lines. However, immunologically significant differences in the patterns of post-translational modifications limit their use for the expression of vaccine antigens (Streatfield and Howard, 2003a,b; Houdebine, 2009). In contrast, plastids allow post-translational modifications and correct folding of the proteins by formation of disulphide bonds, a characteristic that is required for functional tertiary and quaternary structures and hence for antigenicity of many proteins (Chebolu and Daniell, 2009; Cardi *et al.*, 2010). This feature of chloroplasts makes them an attractive platform for the expression of vaccines. Various vaccine antigens have been successfully expressed in plastids. Among these, major viral and bacterial antigens against different human diseases are discussed below.

Viral antigens

To date, there are different promising reports on chloroplast-derived vaccine antigens against several viral infectious diseases, such as hepatitis C and E, cervical cancer and smallpox. Owing to their high mutation rates, viral pathogens are expected to become a global challenge in the near future. Human immunodeficiency virus (HIV), which causes human acquired immunodeficiency syndrome (AIDS), is one of the viruses that show high genetic variability. HIV is the leading cause of death in Africa, and the fourth worldwide. Like other infectious diseases, a safe, effective and largely accessible vaccine is ultimately required to complement and enhance the effectiveness of existing prevention strategies to control the HIV/AIDS pandemic, particularly in developing countries. Different antigens against

HIV have been expressed in chloroplasts but none of them has been tested for immunogenicity (see Table 1). In addition, to develop an effective vaccine against HIV, the medical research still needs to overcome many barriers: for example, the high genetic variability of HIV, the lack of knowledge of immune correlates of protection and the absence of relevant and predictive animal models.

Hepatitis is an inflammation of the liver, most commonly caused by a viral infection. There are five main hepatitis viruses, referred to as A, B, C, D and E. Zhou *et al.* (2006) expressed the *E2* fragment of hepatitis E virus (HEV) open reading frame 2 in tobacco plastids, and they showed that this subunit vaccine was immunogenic in mice when injected subcutaneously. Recently, hepatitis C virus core protein has been expressed in plastids by Madesis *et al.* (2010). There is no report about the expression of antigenic proteins against other types of hepatitis viruses.

Cervical cancer is the second most common cancer in women worldwide, which is caused by different types of human papillomavirus (HPV). Among these types, HPV-16 and 18 are responsible for approximately 70% of invasive cervical cancer cases (Smith *et al.*, 2007). Fernandez-San Millan *et al.* (2008) and Lenzi *et al.* (2008) expressed HPV-16 L1 antigen (the major capsid protein of HPV) in tobacco chloroplasts as VLPs, which were shown to be highly immunogenic in mice injected intraperitoneally (Fernandez-San Millan *et al.*, 2008). Recently, an interesting approach for the development of a cost-effective, second-generation vaccine against HPV was reported by our group (Waheed *et al.*, 2011). We expressed a modified HPV-16 *L1* gene, which retains the assembly of L1 protein to capsomeres, in tobacco chloroplasts. Owing to various advantages, capsomeres are promising candidates to develop second-generation vaccines against HPV. For example, capsomeres are considered relatively thermostable and are able to induce the immunogenicity to a level as that of VLPs (Schädlich *et al.*, 2009). In our study, we showed that the capsomeres were correctly assembled and exhibited the conformational epitopes necessary for immunogenicity. An additional vaccine candidate against cervical cancer, the E7 oncoprotein, was recently expressed in tobacco chloroplasts as a fusion protein (Morgenfeld *et al.*, 2009).

Smallpox is another viral infectious disease that caused a high mortality rate in the past. Since the eradication of that disease, routine vaccination of general population has ceased. However, the threat of potential use of smallpox and related viruses in bioterrorism has urged different countries to renew the production of traditional vaccines and develop new-generation vaccines against these viruses (Wiser *et al.*, 2007). The existing vaccine against smallpox contains live attenuated vaccinia virus. Although this vaccine has been very effective, it has a high incidence of adverse side effects: for instance, a high reactogenicity with cardiac adverse events and a wide range of contraindications. These risk factors make this vaccine non recommendable and thus hinder the vaccination of certain groups of people such as pregnant women, patients with immune disorders and patients with eczema and atopic dermatitis (Wiser *et al.*, 2007). This problem can be solved by the development of alternative vaccines against smallpox. In this respect, an approach to develop a subunit vaccine from plants is reported by Rigano *et al.* (2009b). This group expressed the vaccinia virus envelope protein (*A27L*) in tobacco chloroplasts up to 18% of TSP and demonstrated that it was correctly folded and recognized by serum obtained from a human infected with zoonotic orthopoxvirus (OPV). For the eventual breakthrough of plastid-produced vaccines, it will be necessary not only to ensure such high yields but also to focus on novel antigens, so-called second-generation vaccine candidates. These are referred to those vaccines that are produced by genetic engineering or recombinant DNA technology. In those cases where subunit vaccines are already in use, second-generation vaccines correspond to the vaccine candidates that have comparatively equal or more immunogenicity, are relatively cheaper, are thermostable and can be used as broad-spectrum vaccines (Giorgi *et al.*, 2010).

Bacterial antigens

Concerning bacterial pathogens, a couple of reports emerged about the expression of cholera toxin subunit B (*CTB*), the first vaccine antigen expressed in chloroplasts. Daniell *et al.* (2001) showed that the chloroplast-derived CTB was able to bind to the intestinal membrane GM1-ganglioside receptor. A CTB fusion protein has recently been reported by Dreesen *et al.* (2010). In this study, *CTB* was fused with fibronectin-binding domain (*D2*) of *Staphylococcus aureus*, a bacterium responsible for skin infections and bacteraemia, which may lead to life-threatening secondary infections such as endocarditis (Moreillon and Que, 2004). In the latter study, the fusion protein was expressed in chloroplasts of *Chlamydomonas reinhardtii* and CTB was functionally evaluated by GM1-ganglioside assay. Mice immunized by this fusion protein showed specific mucosal and systemic immune responses, and 80% of mice survived against lethal dose of *S. aureus*. Another study from Davoodi-Semiromi *et al.* (2010) has shown that even dual immunity was induced in mice by a fusion protein containing *CTB* and the antigens *AMA1* and *MSP1* of malaria. Here, mice were reported to be completely protected against cholera toxin (CT) challenge upon oral immunization. On the other hand, against malaria challenge, significant levels of antigen-specific antibody titres of immunized mice completely inhibited proliferation of the malarial parasite and cross-reacted with the native parasite proteins in immunoblots and immunofluorescence studies. Although malaria belongs to the 'big three' diseases (together with TB and HIV), this is the only report about the expression of malarial vaccine antigens in plastids.

The heat-labile enterotoxin subunit B (LTB) from *E. coli*, a close homologue of CTB, was first expressed in chloroplasts by Kang *et al.* (2003). A different approach towards the development of a subunit vaccine was adopted by Rosales-Mendoza *et al.* (2009) who expressed a protein containing the LTB fused with the heat-stable toxin (ST) in chloroplasts. Oral immunization of mice with dried tobacco leaves led to the induction of both serum and mucosal LTB-ST specific antibodies and protected mice against CT challenge.

In addition to all the above-mentioned diseases, rare and nearly eradicated diseases are also relevant to show proof of concept, as is e.g. plague. A report about the expression of the plague antigen *F1-V* in chloroplasts was presented by Arlen *et al.* (2008). In this study, mice were immunized with chloroplast-derived F1-V fusion protein either subcutaneously or orally. The results indicated higher survival rate of mice in case of oral immunization (88%) than in case of subcutaneous delivery (33%), against a high dose of aerosolized *Yersinia pestis*.

One of the most common vaccines, provided throughout the globe, contains three combined components against diphtheria,

pertussis and tetanus (DPT). Global immunization coverage against these diseases has greatly increased since 1974. In 2003, global DPT coverage was 78%. However, 27 million children worldwide were not reached by DPT, including 9.9 million in South Asia and 9.6 million in sub-Saharan Africa (WHO, 2010). However, DPT vaccine is expensive because of the manufacturing process, which involves both scale-up production and purification of recombinant proteins from three different bacteria. Owing to certain side effects of DPT vaccine, most of the developed world has shifted to the DTaP, which contains an acellular component of pertussis. This vaccine is safer with significantly fewer side effects (Lin *et al.*, 2008). However, cost related to the production and purification is still high. A single dose of DTaP vaccine in USA costs approximately $20 [Centers for Disease Control and Prevention (CDCP), USA]. These expenses could be sufficiently reduced by the production of DPT vaccine in plastids. A multi-epitope DPT fusion protein has been expressed in tobacco chloroplasts (Soria-Guerra *et al.*, 2009). Upon oral immunization with freeze-dried transplastomic tobacco leaves, mice produced IgG and IgA antibodies specific to each toxin in serum and mucosal tissues. Chloroplast expression of several other antigens against different viral and bacterial diseases and autoantigens is also reported. All these studies are summarized in Table 1.

Challenges

Transplastomic plants produced via chloroplast genetic engineering are considered safe for the environment, because of the transgene containment mediated through maternal inheritance in most species. Moreover, plants can be produced at large scale, a feature that can enable the abundant production of vaccines to meet the criterion of their supply to the developing countries. However, there are many confrontations that lie in issues that still need to be addressed and solved: these apply mainly for protein degradation, evaluation of efficacy of plastid-derived vaccines, regulatory issues and development of plastid transformation system for edible plant species. Many of these challenges are discussed in detail in the previous and recent reviews (Faye *et al.*, 2005; Rybicki, 2009). Therefore, here we will focus on some important concerns as given below.

Pleiotropic effects

Compared to nuclear transformation, chloroplast genetic engineering is considered a 'plant safe' strategy. In most reports, expression of a foreign protein in chloroplasts does not seem to effect plant growth and morphology. This characteristic feature of plastid transformation is important for safe and large-scale production of vaccine antigens. However, few reports indicated that pleiotropic effects occur upon insertion of transgenes in plastids: for example, Lössl *et al.* (2003), Tregoning *et al.* (2003), Magee *et al.* (2004), Aziz *et al.* (2005), Koya *et al.* (2005), Hasunuma *et al.* (2008), Tissot *et al.* (2008), Waheed *et al.* (2010). The observed detrimental effects included male sterility, yellow leaves and stunted growth of transplastomic plants. In an attempt to investigate male sterility, Ruiz and Daniell (2005) showed that this effect was related to the specific expression of *phaA* gene in chloroplasts. In many of the above-listed reports, the causes are linked to the specific genes, while in some studies the causes remain uninvestigated. Nevertheless, in most of the reports, low protein expression was observed, and hence in these cases, pleiotropic effects apparently are not the consequence of overexpression of foreign proteins. Recently, Ruhlman *et al.* (2010) achieved overexpression of a fusion protein (CTB-Pins) up to 72% of TLP in plastids, without any negative effects on the growth of plants. However, overexpression of a foreign protein, causing phenotypic alterations in transformed plants, is reported by Oey *et al.* (2009), where high expression of lysin protein affected plant development. The observed detrimental effects of foreign proteins on the growth of transplastomic plants can be attributed to various other factors such as the interference of novel open reading frames within the cytoplasmic metabolism or to lower levels of ATP production (Chase, 2007; Pelletier and Budar, 2007). Somaclonal variation can also be a reason for male sterility in transplastomic plants. However, this factor can be precluded if the similar effects are observed in all transplastomic lines, which appears to be the case in the above-mentioned studies reporting the pleiotropic effects. To overcome the negative effects related to the expression of specific genes, detailed studies are needed: for example, investigations of metabolic pathways affected within chloroplasts. Furthermore, use of inducible expression systems in chloroplast transformation technology can circumvent the observed detrimental effects in plants. Significant progress has been made in the development of such systems over the past few years. Below, we describe the basic methodologies and advancements made in this area.

Inducible expression of transgenes in plastids

As described earlier, constitutive expression of transgenes in plastids occasionally resulted in aberrant phenotypes. Inducible expression systems can be used as a tool to control transgene expression and allow production of foreign protein at any specific developmental stage or after harvesting. In particular, this methodology can protect the consumers from inadvertent uptake of pharmaceutically active proteins. Different inducible systems have been reported, which are summarized below.

For the first time, our group (Lössl *et al.*, 2005) demonstrated successful expression of an inducible system in plastids. In an attempt to express the *phb* operon constitutively in tobacco plastids, we observed growth reduction and male sterility (Lössl *et al.*, 2003). To avoid this problem, we constructed a transactivation system in which the expression of the *phb* operon in plastids was regulated by a nuclear-located, plastid-targeted T7RNA polymerase (T7RNAP) under control of an inducible promoter. The ethanol-inducible gene-control mechanism was based on the *alcA* promoter and transcription factor ALCR for the alcohol dehydrogenase regulon of *Aspergillus nidulans* (Caddick *et al.*, 1998). Spraying of ethanol on transformed plants induced the expression of T7RNAP in the nucleus, which in turn switched on the transcription of *phb* operon in plastids. Without ethanol induction, transformed plants grew normally and could produce fertile flowers. Transgenes were activated by spraying with 5% ethanol, and transplastomic plants produced polyhydroxybutyric acid (PHB) after induction. Nevertheless, in this study, some degree of leakiness was observed and the transformation of plastid as well as nuclear genome was required. In another approach (Tungsuchat *et al.*, 2006), the activation of green fluorescent protein (GFP) was combined with the removal of the *aadA* marker gene by CRE recombinase-mediated excision. However, in this report, again a transgene expressed from the nucleus is required to trigger plastid

expression and thus requires *Agrobacterium*-mediated gene transfer. In contrast to above-mentioned studies, an inducible system that is entirely based on the plastid components was reported by Mühlbauer and Koop, (2005). This system was based on the constitutive repression of a plastid transgene by the *lac* repressor, and the expression was induced by isopropyl-ß-D-galactopyranoside (IPTG). This system is attractive as it allows post-harvest induction of the transgene. However, in this case, low background expression was observed in the noninduced state. Later, for the selective transgene expression, Buhot *et al.* (2006) created a hybrid transcription system in which the transgene was placed under the control of an eubacterial promoter that does not exist in the plastid genome and hence not recognized by the plastid endogenous transcriptional machinery. In this system, selective transcription of the transgene was achieved by the supply of a chimeric transcription factor that interacts with plastid-encoded plastid RNA polymerase (PEP) and directs it specifically to the foreign eubacterial-type transgene promoter. Recently, an interesting approach about the controlled expression of transgenes in plastids is reported by Verhounig *et al.* (2010). They identified a synthetic riboswitch that functions as an efficient translational regulator of gene expression in plastids in response to its exogenously applied ligand theophylline. In this study, after the induction by theophylline, the expression of GFP in tobacco plastids was very low i.e. 0.01%–0.02% of TSP. However, this method provides a novel tool that can facilitate tightly regulated inducible expression of transgenes in chloroplasts.

For the production of vaccines and other biopharmaceuticals in plastids, high levels of protein expression are important to consolidate the idea of cost-effective production from plants. Hence, it is interesting to compare the level of protein expression in different inducible approaches. Magee *et al.* (2004) attempted the induction of an antibody fragment in plastids by a salicylic acid-inducible transgene located in nucleus, encoding a plastid-targeted T7RNAP. In this report, the transcript was found at a high level even without induction, yet an increase in accumulation of transcript after induction could be shown. Accumulation of antibody was not detected by immunoblotting or enzyme linked immunosorbent assay. This effect may be attributed to the reason that T7RNAP can transcribe endogenous plastid genes at elevated levels, resulting in low-level accumulation of foreign protein. Mühlbauer and Koop (2005) presented the quantification of GFP obtained from plants transformed with Prrnlac2 construct. However, they did not report the exact amount of accumulated protein. It appears in their report (figure 4a, Mühlbauer and Koop, 2005) that the GFP accumulation could be estimated approximately up to 1% of TSP; yet; some level of expression in noninduced form is also notable. No data about the quantification of expressed protein was provided by Buhot *et al.* (2006). Tungsuchat *et al.* (2006) reported the accumulation of GFP up to 0.3% of total cellular protein. Our group (Lössl *et al.*, 2005) evaluated the inducible expression of the *phb* operon in terms of synthesized polyester. In our above-mentioned report, we showed the transcript level induced by T7RNAP but absolute protein expression level was not determined. Here, we provide the protein expression obtained in our ethanol-inducible system of PHB synthesis. In Figure 1, the accumulation of protein by inducible and constitutive expression is shown. The data are evaluated for the expression of PHB synthase after 7 days of induction from three transplastomic lines (Lanes 1, 2 and 3). Maximum expression

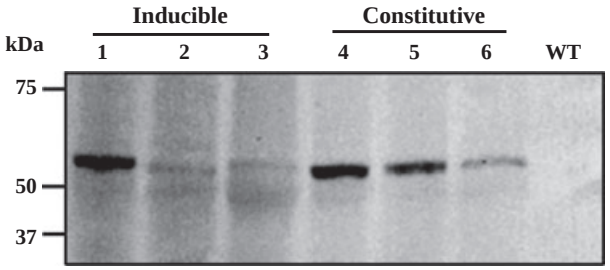

Figure 1 Protein expression of PHB synthase by an ethanol-inducible system. Three independently generated transplastomic lines were tested for inducible expression (lanes 1, 2 and 3) and three for constitutive expression (lanes 4, 5 and 6). Five micrograms of total soluble protein was loaded in each lane. WT, wild type.

was obtained for line 1. Although actual amount of protein cannot be quantified from this Western blot, it clearly shows that the expression of PHB after 1 week reached the level equal to that obtained from constitutive expression of three different transformed lines (Lanes 4, 5 and 6).

There are certain limitations in the inducible methods developed so far in plastid genetic engineering. First, two inducible methods (Lössl *et al.*, 2005; Tungsuchat *et al.*, 2006) need transformation of two compartments, i.e. nucleus and plastid, posing a risk of nuclear transgene dispersal through pollen, which counteracts with the biosafety advantage of chloroplast transformation. Secondly, all the above-mentioned studies have been exclusively carried out in plastids of tobacco, a plant that is not suitable for edible vaccine production. To proffer plastid transformation technology with minimal detrimental effects, establishment of inducible expression systems for plastid transformation of edible crops will be needed in the future. Third, till now, these systems yielded only low amounts of proteins. In contrast to accumulation of protein to high levels in plastids by constitutive expression (Oey *et al.*, 2009; Ruhlman *et al.*, 2010), achievement of high protein levels by inducible systems still remains a challenge, which needs further research efforts.

Choice of plant species

The plastid transformation system has been well established and optimized over the last decade. However, expression of most vaccine antigens in plastids is still confined to tobacco. Although use of tobacco for production of biopharmaceuticals and other beneficial products can be an alternative employment for this plant, yet it cannot be used for oral administration because of its toxic alkaloid contents. Therefore, it is necessary to choose those plant species that can be consumed as raw material and meet the basic requirement of producing edible vaccines derived from plants. Among the species of choice, most considerable are those that can be used as either leaves or fruits, such as lettuce, spinach, cabbage, tomato, carrot and grapes. Some advances in this perspective are reported for lettuce and tomato (Ruhlman *et al.*, 2007; Zhou *et al.*, 2008; Davoodi-Semiromi *et al.*, 2010). However, in case of tomato, higher expression was detected for green fruits (Ruf *et al.*, 2001; Zhou *et al.*, 2008). This is because of the conversion of chloroplasts to chromoplasts during ripening, resulting in the down-regulation of transgene expression. To address this problem, tomato cultivars bearing green mature fruits can be used

for plastid transformation. Recently, a rapid and efficient tissue culture regeneration system for different commercially available cultivars of lettuce has been developed (Ruhlman *et al.*, 2010). This advancement is very attractive for vaccine production in plastids, as lettuce is an edible crop and can be consumed in raw form. Among other species, cabbage and eggplant have recently been documented to be promising alternative crops (Liu *et al.*, 2008; Singh *et al.*, 2010). This adaptation of plastid transformation technology is a step forward to achieve the goal of 'plants as edible vaccines'. However, lack of efficient regeneration system for most plant species and in some cases chloroplast genome sequence data are the major limitations for extending the plastid transformation technology to other crops.

Clinical trials

The ultimate goal of the plant-derived vaccines to attain the status of approved and affordable vaccines against human diseases is still to be achieved. Few plant-produced vaccines have entered the clinical trials (for review see Rybicki, 2009). These vaccines were derived from plants either through nuclear transformation or recombinant tobacco mosaic virus (TMV). To date, none of the chloroplast-derived human vaccines has reached the clinical trials. To accomplish this goal, all regulatory issues related to clinical trials (phase I–IV) need to be pursued to assess efficacy, safety and reliability. As these regulatory requirements are more demanding for the vaccines against human diseases, it is more challenging for these vaccines to advance. Regarding biosafety issues, the removal of antibiotic resistance marker genes is a very promising step (Iamtham and Day, 2000; Klaus *et al.*, 2004; Kittiwongwattana *et al.*, 2007), which covers the concerns related to contamination of food chain. Additionally, owing to maternal inheritance, plastids serve to accomplish transgene containment. Some of the plant-produced vaccines against human diseases have shown promising results in phase I and II clinical trials upon oral delivery (for review, see Daniell *et al.*, 2009; Tiwari *et al.*, 2009). However, these vaccine antigens were produced by either nuclear or TMV-based transformation. It is surprising that although the chloroplast transformation system has competent potential and chloroplast-derived vaccines have shown promising immunological results in test animals, till date none of the plastid-produced vaccine has entered the clinical trials. One of the main reasons is the lack of significant investment and support from pharmaceutical industries in this specific area of research. In addition, for currently assessed vaccines moving through clinical trials, emphasis is given to the efficacy and safety of plant-produced vaccines compared with other production systems, thus ruling out the potential advantage of oral administration of plants. For this reason, in future research, the degree of product purity needs to be illustrated in parallel to the expression and functional evaluation of vaccine antigens in chloroplasts.

Glycosylation

Plants offer an effective platform for the production of biopharmaceuticals over other expression systems such as *E. coli* and yeast. However, in plastids, the lack of glycosylation is a limitation that confines the choice of antigens to nonglycosylated proteins (Ma *et al.*, 2003). As reviewed by Apweiler *et al.* (1999) and Walsh and Jefferis (2006), more than 50% of the proteins in eukaryotes and one-third of approved biopharmaceuticals are glycoproteins. Although the activity of many proteins is not affected by glycosylation, it can be critical in case of other proteins (Rice *et al.*, 2005). Thus, the expression of many vaccine antigens, which require glycosylation for their function, is challenging: for instance, certain antigenic proteins from measles, rabies, hepatitis B and C viruses. In case of measles, glycosylation is necessary for the antigenicity of haemagglutinin protein of measles virus (Hu *et al.*, 1994). Many studies on the expression of rabies virus glycoprotein (G) in different expression systems and the evaluation of immunogenicity of this protein in animal models show that glycosylation may be essential for the function of rabies virus G protein (Wunner *et al.*, 1983; Yelverton *et al.*, 1983; Kieny *et al.*, 1984; Wiktor *et al.*, 1984; Prehaud *et al.*, 1989). Glycosylation of hepatitis B surface (HBS) antigen is also necessary (Xing *et al.*, 2008). In the later study, authors investigated the effect of deglycosylation on the immunogenicity of HBS antigen and showed that deglycosylation of S protein resulted in a significant decrease in S-specific cell-mediated immune responses in mice. In case of hepatitis C, it has been shown that the absence of glycans on hepatitis C envelope glycoproteins E1 and E2 leads to their misfolding, demonstrating the essential role played by glycosylation for the folding of these proteins (Goffard and Dubuisson, 2003; Goffard *et al.*, 2005). However, this problem can be solved by selecting specific proteins that do not require glycosylation for their function. This aspect has recently been shown for hepatitis C by Madesis *et al.* (2010). This group expressed hepatitis C virus core polypeptide in tobacco plastids and showed that the expressed protein could detect the anti-core antibodies in infected human sera. However, such choice should be based on the antigens that are already proved as immunogenic to a considerable level by immunological research groups.

Scopes

Plant-based vaccines in general and chloroplast-derived in specific have opened many prospects that could be helpful to adopt and develop strategies and methodologies for the production of vaccines with improved immunogenicity. Chloroplast transformation has been well established in the last decade. High expression of proteins has been shown, and the safety aspects have been addressed. Further on, for vaccine production in plastids, it is necessary to give more emphasis on the selection of already-optimized parameters that are reported to give high yields. Some important focal opportunities for vaccine production in plastids are figured out below.

Choice of vaccine antigens and expression system

For developing countries, there is an urgent need for low-cost vaccines against several human infectious diseases. In this respect, the choice of vaccine antigens is very vital. Moreover, the preference of a plastid expression system is also important for biosafety and large-scale production to further reduce the cost-related burden. For vaccines with low stability at room temperature, selection of alternative antigens can be more advantageous. For instance, in case of cervical cancer, capsomeres offer an effective alternative to VLPs. Capsomeres are considered more thermostable and have been proved to be highly immunogenic in animal models (Yuan *et al.*, 2001; Schädlich *et al.*, 2009). Such approaches can reduce the costs

related to maintain continuous cold chains in developing countries. Concerning stability of plastid-derived vaccines, a promising study with *Chlamydomonas reinhardtii* was recently provided by Dreesen *et al.* (2010). In this report, vaccine obtained from chloroplasts was stable at room temperature for 20 months. In addition, mice immunized with the vaccine stored at room temperature showed similar IgA/IgG levels as those of mice immunized with the vaccine stored at 4 °C. The overview in Table 1 shows that the number of vaccine candidates expressed in plastids is very limited. Many important infectious disease antigens are not yet reported in chloroplasts: this applies to tuberculosis, leishmaniasis, several types of hepatitis, dengue fever, measles and rabies. Some of these pathogens still require investigations to figure out which of their antigens are feasible to express in their functional conformations in plastids.

Evaluation of immunogenic properties of plastid-produced antigens

To develop plant-derived vaccines, functional evaluation of the recombinant proteins is indispensable. For vaccine development, it is essential to consider the two pillars of chloroplast-produced vaccines: correct expression and immunogenicity. In many reported antigens expressed in plastids (Table 1), functional investigations are either not conducted or confined to *in vitro* assays. To obtain a more realistic prospect, *in vivo* studies should be preferred to test the immunogenicity of vaccine antigens in suitable animal models. Chloroplast-produced proteins very often are expressed as N-terminal fusion proteins with stabilizing peptides such as GFP or five extra amino acids (MASIS-box) to enhance protein stability within the plastids (Herz *et al.*, 2005; Lenzi *et al.*, 2008; Waheed *et al.*, 2011). Subsequent functional analysis of proteins have to ensure that the respective fusion partners do not affect assembly and correct folding of the protein and presentation of epitopes for recognition by the immune system. In such cases or in those where two antigenic proteins are jointly expressed, evaluation of immunogenic epitopes is of eminent importance.

Expression of antigens coupled with adjuvants

In vaccine formulations, antigens are often co-administered with adjuvants to boost the immune response against a given antigen. Several adjuvants have been reported to enhance immunity in tested animals against many infectious diseases (for review see Nashar *et al.*, 1993; Holmgren *et al.*, 2003). Some of the important adjuvant candidates are CTB, LTB and interleukin that can be coupled with the antigen and expressed as fusion protein in chloroplasts. Co-expression of an adjuvant with an antigen usually is more valuable because it can facilitate the transport of the antigen from the gut lumen to the gut-associated lymphoid tissues. Here, the adjuvants bind to receptors such as GM1-ganglioside and target the antigens to the relevant sites and/or activate antigen-presenting cells (APCs) to elicit protective immunity (Granell *et al.*, 2010; Salyaev *et al.*, 2010). Moreover, in an adjuvant-antigen couple, the antigen itself can increase or counteract adjuvant efficacy (Guy, 2007; Sánchez and Holmgren, 2008). A recent report emphasizes the expression of another adjuvant candidate, which can be used for co-expression with antigens in chloroplasts: Farran *et al.* (2010) expressed fibronectin extra domain A (*EDA*) in chloroplasts and showed that the plastid-derived protein was able to induce up-regulation of CD54 and CD86 maturation markers on dendritic cells. However, the choice of adjuvants for co-expression with antigens in chloroplasts depends upon the proved efficacy of the specific adjuvant with a selected antigen. One of the major hurdles related to plant-based vaccines is the weak antigenicity of many proteins (Rybicki, 2009). The expression of adjuvant–antigen as fusion protein will help to remove this obstacle from the way of chloroplast-derived vaccines to their future application.

Conclusion

To date, 27 vaccine antigens have been expressed in chloroplasts against 17 different human diseases. In contrast to vaccines against viral or other pathogens, most efforts have been made to combat bacterial diseases. Out of these 27 reported antigens, nearly half are tested in animal models. Although various advantages of chloroplast transformation technology can help to cover the regulatory concerns related to plant-based vaccines, many issues are still to be resolved. For this reason, evaluation of efficacy and purity of plastid-derived vaccines should be the preferred goal. Vaccine production in plastids needs a close intellectual bridge with the medical research groups for the selection of preferred antigens. Immunological investigations of second-generation vaccine candidates have shown promising results in animal models. These antigens with established immunogenicity can be expressed in plastids for the development of low-cost vaccines. Moreover, the advantage of multigene expression in plastids can be adopted for the production of vaccines with improved immunogenicity by coupling antigens with specific adjuvants. Furthermore, promising recent achievements in plastid transformation of edible crops such as lettuce and cabbage provide a platform for the development of vaccines in edible plant species.

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4.3 Scientific article

Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines

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ORIGINAL PAPER

Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines

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Abstract Certain types of human papillomaviruses (HPV) are causatively associated with cervical carcinoma, the second most common cancer in women worldwide. Due to limitations in the availability of currently used virus-like particle (VLP)-based vaccines against HPV to women of developing countries, where most cases of cervical cancer occur, the development of a cost-effective second-generation vaccine is a necessity. Capsomeres have recently been demonstrated to be highly immunogenic and to have a number of advantages as a potential cost-effective alternative to VLP-based HPV vaccines. We have expressed a mutated HPV-16 L1 (L1_2xCysM) gene that retained the ability to assemble L1 protein to capsomeres in tobacco chloroplasts. The recombinant protein yielded up to 1.5% of total soluble protein. The assembly of capsomeres was examined and verified by cesium chloride density gradient centrifugation and sucrose sedimentation analysis. An antigen capture enzyme-linked immunosorbent assay confirmed the formation of capsomeres by using a conformation-specific monoclonal antibody which recognized the conformational epitopes. Transplastomic tobacco plants exhibited normal growth and morphology, but all such lines showed male sterility in the T_0, T_1 and T_2 generations. Taken together, these results indicate the possibility of producing a low-cost capsomere-based vaccine by plastids.

Keywords HPV-16 · L1_2xCysM gene · Capsomeres · Plastids · ELISA · Male sterility

Introduction

Cervical cancer is the second most common cancer in women worldwide, with approximately 493,000 new cases and 274,000 deaths occurring annually around the globe (Parkin and Bray 2006). Human papillomavirus (HPV) types 16 and 18 have been identified to be responsible for approximately 70% of invasive cervical cancers, with HPV-16 being by far the most prevalent type and found in about 54% of all cervical cancer cases (Smith et al. 2007).

L1 is the major capsid protein of HPV and self assembles into higher order molecular structures, such as capsomeres and virus-like particles (VLPs). Each VLP consists of 72 capsomeres, and each

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capsomere is a pentamer of the L1 protein arranged in an icosahedral surface lattice (Baker et al. 1991). The formation of disulphide bonds between L1 protein molecules in adjacent capsomeres is essential for VLP stability, and the reduction of these bonds leads to the disassembly of VLPs into capsomeres (Sapp et al. 1995). Replacement of either of the two highly conserved cysteines in L1 protein by serine prevents VLP assembly and leads to the retention of L1 capsomeres (Sapp et al. 1998).

VLPs are highly immunogenic as they can induce high titres of neutralizing antibodies (Rose et al. 1998) that have been shown to successfully prevent HPV infections (Koutsky et al. 2002; Villa et al. 2006). The first two VLP-based vaccines using L1 protein as the selected antigen have recently been introduced onto the market (Harper et al. 2004; Villa et al. 2006; Müller and Gissmann 2007). One of these vaccines, obtained from a yeast expression system, is a tetravalent that covers HPV types 16, 18, 6 and 11, while the second is a bivalent vaccine covering HPV types 16 and 18 and is obtained from insect cells. However, relatively high production and distribution costs are associated with these two vaccines. Furthermore, both require a continuous cold chain and sterile needles for intramuscular administration. Consequently these vaccines are cost-intensive and, as such, they will likely be unavailable (=unaffordable) for people in developing countries where more than 80% of all cervical cancer cases occur (Parkin and Bray 2006; Stanley et al. 2008). Therefore, there is an urgent need for second-generation cost-effective vaccines (Stanley et al. 2008).

Capsomeres represent a potentially cost-saving alternative to VLPs, as they are considered to be thermo-stable, which is advantageous for use in developing countries where cold chains are difficult to maintain (Stanley et al. 2008). Capsomeres have been shown to induce high titres of neutralizing antibodies and L1-specific cytotoxic T-lymphocytes (CTLs) upon oral, intranasal and subcutaneous immunization, and they have also been reported to protect dogs against viral challenge (Rose et al. 1998; Fligge et al. 2001; Yuan et al. 2001; Öhlschläger et al. 2003; Dell et al. 2006; Thönes and Müller 2007; Schädlich et al. 2009a).

The use of plants as an alternate system for the production of biopharmaceuticals, therapeutic proteins and recombinant vaccines is associated with a number of advantages, such as low cost, ease to scale up for mass production and low health risks; there is also the additional potential benefit that the products thus produced can be administered unprocessed (e.g plant leaves) or partially processed material (Daniell et al. 2001; Fischer et al. 2004; Ma et al. 2005). HPV-16 L1 protein has been expressed in plants following integration of the transgene into the nuclear genome (Biemelt et al. 2003; Varsani et al. 2003; Liu et al. 2005; Maclean et al. 2007). However, in all of these studies, the antigen expression level was <1% of total soluble protein (TSP) with the exception of that of Maclean et al. (2007), who obtained 11% TSP by expressing a human codon-optimized gene linked to a chloroplast-targeting signal. The low level of antigen expression (an average of 0.01–0.4% of TSP; Daniell et al. 2001) is one of the main drawbacks of transgene integration into the nuclear genome.

This constraint can be solved by engineering the plastid genome, which in terms of developing applications involving the production of plant-made pharmaceuticals has many advantages, including high levels of transgene expression due to high copy number, absence of epigenetic effects, transgene containment via maternal inheritance and multi-gene expression in a single transformation event (for review, see Maliga 2002; Bock 2007; Koop et al. 2007; Chebolu and Daniell 2009). Various vaccine antigens against several human and animal diseases have been successfully expressed in plastids, with an average expression level in the range of 4–31% of TSP (Chebolu and Daniell 2009). The highest transgene expression in chloroplasts obtained to date is 70% of TSP for an antibacterial lysin (Oey et al. 2009). In two recent studies, HPV-16 L1 VLPs were expressed in tobacco chloroplasts, obtaining 24% (Fernandez-San Millan et al. 2008) and 1.5% (Lenzi et al. 2008) of TSP, respectively. These VLPs obtained from the chloroplast were subsequently shown to be immunogenic in mice (Fernandez-San Millan et al. 2008).

The aim of the study reported here was to demonstrate the successful production of capsomeres in plastids through the expression of a mutated L1 gene (L1_2xCysM) having two cysteines replaced by serines. We found that the expression of the L1_2xCysM gene confined the assembly of L1 protein to pentameric capsomeres. The assembly and correct formation of capsomeres was confirmed by sucrose gradient analysis and further verified by an

enzyme-linked immunosorbent assay (ELISA), using a conformation-specific antibody. The considerable expression of the modified L1 protein and its assembly into capsomeres makes this plastid expression system suitable for developing a cost-effective second-generation vaccine against HPV.

Materials and methods

Vector construction

A precursor vector pPNG1014_MCS120 was constructed as introduced by Ye et al. (2001), with slight modifications. This plasmid contained the plastid-encoded polymerase (PEP) promoter from the *rrn* 16 gene (P*rrn*; Svab and Maliga 1993) and the ribosomal binding site (RBS) from the leader sequence of gene 10 (G10L) of the lambda phage T7 (Studier et al. 1990). Nuclear encoded polymerase (NEP) promoter (P*rrn*$^{-62}$NEP; Hajdukiewicz et al. 1997) was fused with P*rrn*PEP immediately downstream, followed by G10L fusion. Thereafter, the sequence encoding for first 14 amino acids of the green fluorescent protein (GFP) (GFP_{14}) was fused with the 5′ end of the gene of interest. Cleavage space was left for the multiple cloning site (MCS), and the 5′-untranslated region (5′ UTR) was linked to the gene of interest to allow downstream insertion of the *aad*A gene. This 5′ UTR consisted of synthetic ribosomal binding site.

The mutated L1 gene (L1_2xCysM), in which two cysteines, 175 and 428, were replaced by serine residues (Schädlich et al. 2009b), was amplified by PCR using forward primer (oli) 5′-AAAAgctag-cATGTCTACTTGCCTCCTGTC-3′ and reverse primer 5′-TTTTTTgcatgcTTACAGCTTACGTTTTTTGC-3′ (restriction sites in lower case). The L1_2xCysM gene was cloned in pPNG1014_MCS120 by *Cla*I and *Mlu*I to obtain plasmid $pPNGL1_M$. Plasmid pT7PHB-N (Lössl et al. 2005) was used to obtain the final transformation vector $pPNGL1_MT$. Plasmid pT7PHB-N contained the *aad*A gene (conferring resistance to spectinomycin and streptomycin), the terminator from the large subunit of ribulose-bisphosphate carboxylase gene (T*rbc*L) from *Chlamydomonas reinhardtii* and the flanking regions INSL and INSR, homologous to the loci *trn*N and *trn*R, respectively, in the inverted repeats (IR) of the tobacco plastome. The flanking sequences included nucleotides 109,230–110,348 and 110,349–111,520 of IR-A as well as nucleotides 131,106–132,277 and 132,278–133,396 of IR-B. Plasmid pT7PHB-N was cut with *Sac*II and *Bam*HI, and the whole cassette from $PNGL1_M$ was cloned in pT7PHB-N by *Sac*II and *Bgl*II. The figure in the Electronic Supplementary Material (ESM) shows the vector construction pattern. Nucleotide positions for transgene insertions are given according to the plastome sequence data for EMBL accession no. Z00044, as initially reported by Shinozaki et al. (1986) and later updated by Wakasugi et al. (1998) and Yukawa et al. (2005). All cloning procedures were carried out using the standard methods described by Sambrook et al. (1989).

Transformation and regeneration of transplastomic plants

Nicotiana tabacum (Nt) cv. Petit Havana plantlets (Surrow Seeds, Sakskøbing, Denmark) were grown from seeds in vitro at 25°C (light intensity: 0.5–1 W/m^2 Osram L85 W/25 universal-white fluorescent lamps) on agar-solidified MS medium (Murashige and Skoog 1962) containing MS salts and sucrose (30 g/l). Leaves were transformed using a particle gun (PDS1000He; Bio-Rad, Hercules, CA) by coating plasmid DNA on gold particles (0.6 µm; Svab et al. 1990). Following bombardment, leaves were cut into 5-mm pieces and placed on RMOP medium containing 500 mg/l spectinomycin (Svab et al. 1990). After four to six cycles of shoot regeneration on the same medium with spectinomycin, shoots were transferred to B5 medium (Gamborg et al. 1968) containing spectinomycin, for rooting. Transformed plants were then shifted to the greenhouse for further studies and seed production. These plants were pollinated with pollen from wild-type plants and seeds were collected. Seeds from the T_0 generation were grown on agar-solidified MS medium (with MS salts and 30 g/l sucrose) containing 500 mg/l spectinomycin to generate T_1 progeny. The T_2 generation was obtained in the same way from the seeds of T_1 progeny.

Confirmation of transgene integration

The PCR assay was performed to confirm transgene integration within the plastome in the T_0 and T_1 generations. DNA was extracted from 100–150 mg of plant leaves by the cetyltrimethylammonium bromide

(CTAB) procedure (Murray and Thompson 1980). To confirm correct insertion of the L1_2xCysM gene within the plastid genome, PCR was carried out with the forward primer oli252 (positioned within the plastome outside the vector flanks; sequence 5′-AGACAGCGACGGGTTCTCTG-3′) and reverse primer oli248 (within L1_2xCysM gene; sequence 5′-GTACTTGGGGATCCTTTGCC-3′). For confirmation of the correct insertion of the *aad*A gene within the plastid genome, forward primer oli251 (5′-CCAGTATCAGCCCGTCATAC-3′) located within the *aad*A gene and reverse primer oli253 (5′-GAT CCGAGCCATAGAATTTC-3′) located in the plastid genome outside the vector flanks were used. All primer positions are shown in the ESM (figure).

Western blot analysis

Soluble proteins were extracted from leaf discs collected from the plants grown under sterile conditions. Leaf samples (100 mg) were ground in liquid nitrogen, homogenized in sodium dodecyl sulphate (SDS) buffer (187.5 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 15% ß-mercaptoethanol, 0.03% bromophenol blue), and the extract heated at 95°C for 5 min. The proteins were separated by SDS–polyacrylamide gel electrophoresis (12.5% polyacrylamide gel), transferred onto nitrocellulose membrane (Hybond C; GE Healthcare, Little Chalfont, UK) and blocked with 5% skimmed milk for 30 min. The membrane was incubated for 1 h with HPV-16 L1-specific monoclonal antibody MD2H11 (DKFZ, Heidelberg, Germany) diluted 1:4,000 in 5% skimmed milk, washed three times (10 min each time) with PBS containing 0.3% of Tween-20 (PBS-T) and incubated a second time for 1 h with peroxidase-conjugated goat antimouse immunoglobulin G (IgG; Sigma, St. Louis, MO) as secondary antibody diluted 1:3,000 in skimmed milk. After washing the membrane with PBS-T, proteins were detected by chemiluminescence, and the bands were visualized on X-Ray film (Kodak). Fractions from a sucrose gradient and CsCl centrifugation were also analyzed as described above.

Antigen capture ELISA

Tobacco leaves were obtained from the transgenic plants grown in sterile conditions. Leaves (100 mg) were ground in liquid nitrogen and homogenized in extraction buffer containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 M NaCl, 0.01% Triton X-100, 20 mM Hepes, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were centrifuged at 13,000 rpm for 5 min and the supernatant collected. Each well of 96-well microtitre plates was coated overnight at 4°C with 50 µl of HPV-16 L1 conformation specific mouse monoclonal antibody Ritti01 (Thönes et al. 2008) diluted 1:300 in PBS. The plates were washed with PBS-T and blocked for 1 h at 37°C with a blocking solution containing PBS, 3% skimmed milk and 0.3% Tween-20 (PBS-TM). Plant extracts were added to the wells and incubated for 1 h at 37°C. After three washing steps, 50 µl of polyclonal rabbit antiserum (1:3,000 in PBS-TM) raised against HPV-16 L1 was added to each well, and the plates were incubated for 1 h at 37°C. The plates were then washed three times, incubated for 1 h at 37°C after the addition of 50 µl of goat anti-rabbit peroxidase conjugate (Sigma; 1:3000 in PBS-TM) to each well and were washed thoroughly before 100 µl of staining solution {100 mM sodium acetate, 44 mM NaH_2PO_4, pH 4.2, 1 mg ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)]/ml, 0.012% H_2O_2} was added to each well. After 10–20 min, the measurement was carried out at 405 nm. Fractions from a sucrose gradient and CsCl centrifugation were analyzed in the same manner as described above.

Sucrose gradient sedimentation analysis of protein

Tobacco leaves (500 mg) were ground in liquid nitrogen and homogenized with extraction buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 M NaCl, 0.01% Triton X-100, 20 mM Hepes, pH 7.4, 1 mM PMSF). The proteins were extracted by sonication for 30 s. The lysate was cleared by centrifugation at 13,000 rpm for 15 min at 4°C, loaded onto a linear 5–50% (w/v) sucrose gradient in extraction buffer and centrifuged at 36,000 rpm for 3 h at 4°C using a Beckman SW41 Ti rotor (Beckman Coulter, Brea, CA). A total of 20 fractions (600 µl each) were collected from the bottom of the tube, and their refractive indices were determined. These fractions were then analyzed by Western blotting and antigen capture ELISA.

Protein purification by cesium chloride gradient centrifugation

Leaf material (5 g) from transgenic plants was ground in liquid nitrogen and homogenized with extraction buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 M NaCl, 0.01% Triton X-100, 20 mM Hepes, pH 7.4, and 1 mM PMSF). After sonication, the lysate was cleared by centrifugation at 17,000 rpm for 30 min at 4°C. The cleared lysate was transferred onto a two-step gradient consisting of 7 ml of sucrose (30% w/v) on top of 7 ml of CsCl (58% w/v), and then centrifuged at 27,000 rpm for 3 h at 10°C. The interphase between sucrose and the CsCl was mixed with a CsCl layer and centrifuged at 50,000 rpm for 16 h at 20°C using a Sorvall TFT 65.13 rotor (Thermo Fisher Scientific, Waltham, MA). A total of 14 fractions (1 ml each) were collected from the bottom of the tube and analyzed by ELISA. A small volume of fractions was dialyzed against distilled water using a filter with a pore size of 0.025 μm (Millipore, Bedford, MA) and used for Western blot analysis.

Results

Chloroplast transformation vectors

The constructed plastid transformation vector $PNGL1_MT$ contained the mutated L1 gene (L1_2x CysM). This gene was expressed by a cassette containing the promoters for the plastid and nuclear-encoded polymerases (P*rrn*PEP and Prrn^{-62}NEP, respectively) in combination with the terminator sequence from the large subunit of the ribulose-bisphosphate carboxylase gene (T*rbc*L) from *C. reinhardtii*. The DNA sequence for the first 14 amino acids of the GFP protein (GFP_{14}) were fused with the transgene to enhance expression, as demonstrated by Ye et al. (2001). The *aad*A gene was used to select transplastomic plants. The P*rrn*PEP-Prrn^{-62}NEP promoter served as a bicistronic promoter, driving the expression of both the L1_2xCysM and *aad*A genes. The *trn*N and *trn*R loci were used for homologous recombination within the tobacco plastid genome. The figure in the ESM shows the complete construct within the tobacco chloroplast genome, including the primers used to confirm the insertion of the transgenes within the plastid genome.

Plastid transformation and morphology of transplastomic plants

Tobacco leaves were transformed by the biolistic method and then placed on RMOP medium containing 500 mg/l spectinomycin for selection and regeneration. Control plants and all untransformed explants bleached out, while green microcalli developed from transformed explants that continued to grow on selection medium. Homoplastomy was achieved by subjecting resistant shoots to three regeneration cycles under the same conditions. For rooting, transgenic shoots were placed on B5 selection medium and then transferred to the greenhouse into pots.

All transplastomic lines transformed with the L1_2xCysM gene were male sterile and shed flowers (Fig. 1). Flowers were produced but showed senescence before maturity and did not produce seeds. However, plants had normal growth and morphology, like those of the wild type. Male sterility and flower senescence persisted in the successive T_1 and T_2 generations obtained by pollinating transgenic plants with wild-type plants.

Confirmation of transgene integration in transgenic plants

Tobacco plants with the L1_2xCysM gene were analyzed for transgene integration in the plastid

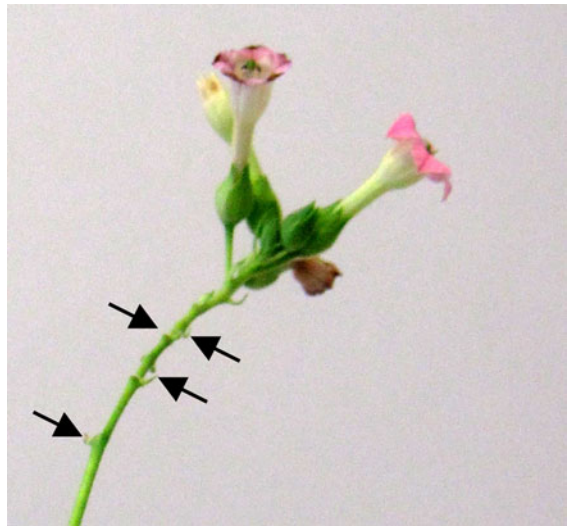

Fig. 1 A branch of transplastomic tobacco plant showing senescence of flowers and male sterility. *Arrows on the lower section of branch* Marks of shed flowers, *upper part* of figure flowers without seeds

genome by PCR. The primers oli248 (within L1_2xCysM) and oli252 (within cp at 5′ end) amplified a 1,939-bp DNA fragment, as expected for L1_2xCysM. This PCR-generated fragment confirmed the correct insertion of L1_2xCysM at the right insertion site (INSR) within the plastome. To prove the correct insertion of the *aad*A gene at the INSL within the plastome, a PCR was carried out with primers oli251 (within *aad*A) and oli253 (within chloroplast at 3′ end), amplifying the expected 1,981-bp fragment. Figure 2 depicts the amplified products from both flanks. Similar results were obtained by the PCR analysis of T_1 plants (data not shown). Seeds of the T_1 and T_2 generations uniformly germinated on spectinomycin (500 mg/l) containing MS medium, as expected for homoplastomic plants.

Expression and quantification of the L1 protein

Expression of recombinant L1 protein was detected by Western blot using MD2H11 antibody specific for HPV-16 L1 protein (Schädlich et al. 2009a). Three independently generated transplastomic plant lines were analyzed (Fig. 3a, lanes 1–3). Each line showed an expected band of 56.5 kDa corresponding to the calculated size of GFP_{14}-L1 protein (Fig. 3a). Baculovirus-derived purified VLPs (DKFZ) were used as the positive control. No protein was detected in samples extracted from wild-type tobacco control plants. All three transplastomic lines generated signals in the antigen capture ELISA using the conformation-specific monoclonal antibody Ritti01 (Fig. 3b), thereby demonstrating the presence of conformational epitopes in chloroplast-derived L1 protein. VLPs and samples from wild type served as positive and negative controls, respectively. In addition, PBS was used as a negative control for each sample in ELISA.

The amount of L1 protein was quantified by Western blotting using the MD2H11 antibody (Fig. 3c) and a dilution series (1, 2 and 3 μg) of total leaf protein extracted from transplastomic line 1. A dilution series of baculovirus-derived purified L1 VLPs (2, 4 and 8 ng) was used as reference. Other bands, in addition to that of GFP_{14}-L1, were also observed, probably due to protein degradation. The amount of total recombinant protein was estimated to be up to 1.5% of TSP, as shown in Fig. 3c.

Chloroplast-derived L1 protein self assembles to capsomeres

Western blot analysis using monoclonal antibody MD2H11 and antigen capture ELISA with conformation-specific monoclonal antibody Ritti01 revealed the successful expression of L1 protein in all three transgenic lines. These data suggested that recombinant protein was present in higher order molecular structures. To verify these structures, cleared plant extracts were sedimented through a sucrose cushion followed by a CsCl density gradient centrifugation. L1 protein was analyzed in collected fractions (14 in total, fraction 1 from the bottom of the tube) by ELISA (Fig. 4a) using conformation-specific monoclonal antibody Ritti01. Most of the L1 protein was concentrated in fractions 6–10 as expected. Western blot analysis of these fractions also detected the GFP_{14}-L1 protein (56.5 kDa) using HPV-16 L1-specific antibody MD2H11, as shown in Fig. 4b. These results suggested that chloroplast-derived L1 protein displayed conformational epitopes and assembled into higher order structures, such as capsomeres or VLPs.

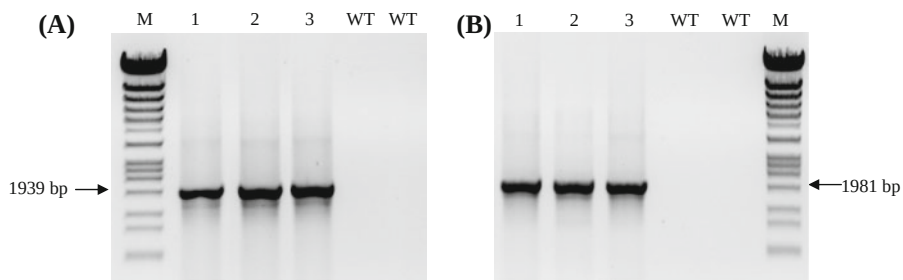

Fig. 2 PCR analysis of the transgenes inserted within the plastome. **a** Amplification of L1_2xCysM gene (1,939 bp) with the primers oli248 and oli252. **b** Amplification of the *aad*A gene (1,981 bp) with the primers oli251 and oli253. Three independently generated transplastomic lines (*Lanes 1, 2, 3*) were analyzed. *M* Marker, *WT* wild type

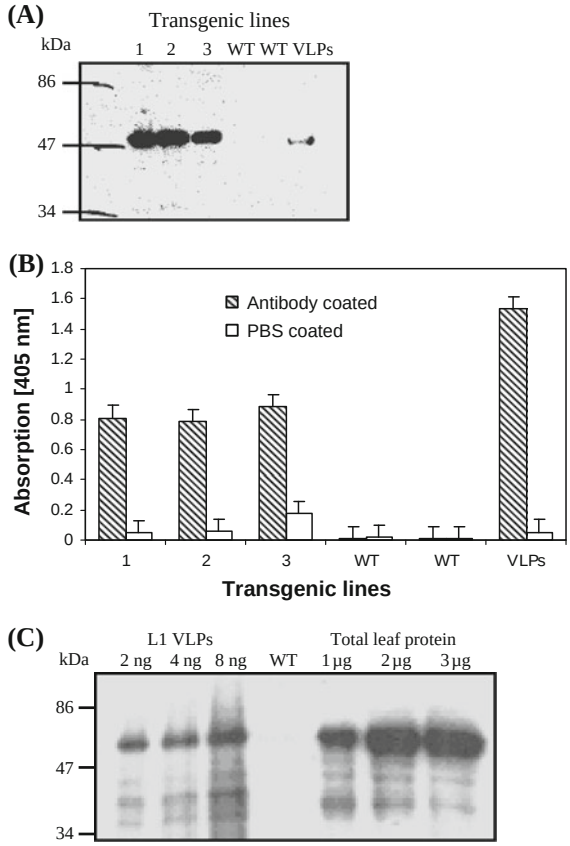

Fig. 3 L1 protein expression and quantification. **a** Western blot indicating the expression of plant-derived L1 protein (56.5 kDa) from three independent transplastomic lines in comparison with baculovirus-derived purified virus-like particle (*VLPs*). MD2H11 monoclonal antibody specific for HPV-16 L1 protein was used. Wild-type (*WT*) protein served as the control. *Lanes 1, 2, 3* Protein samples from three independent transplastomic lines (transgenic lines 1, 2, 3, respectively). **b** Antigen capture enzyme-linked immunosorbent assay (ELISA) of the three transplastomic lines showing the accumulation of L1 protein in leaf extracts. VLPs were used as the positive control. Monoclonal antibody Ritti01 was used for the detection of conformational epitopes. Protein extracted from WT leaves and phosphate buffered saline (*PBS*) (for each sample) were the negative controls. **c** Protein quantification by western blot analysis based on a dilution series of baculovirus-derived L1 VLPs as reference (2, 4 and 8 ng). Total soluble leaf protein from transplastomic line 1 (1, 2 and 3 µg) was loaded in lanes. Monoclonal antibody MD2H11 was used

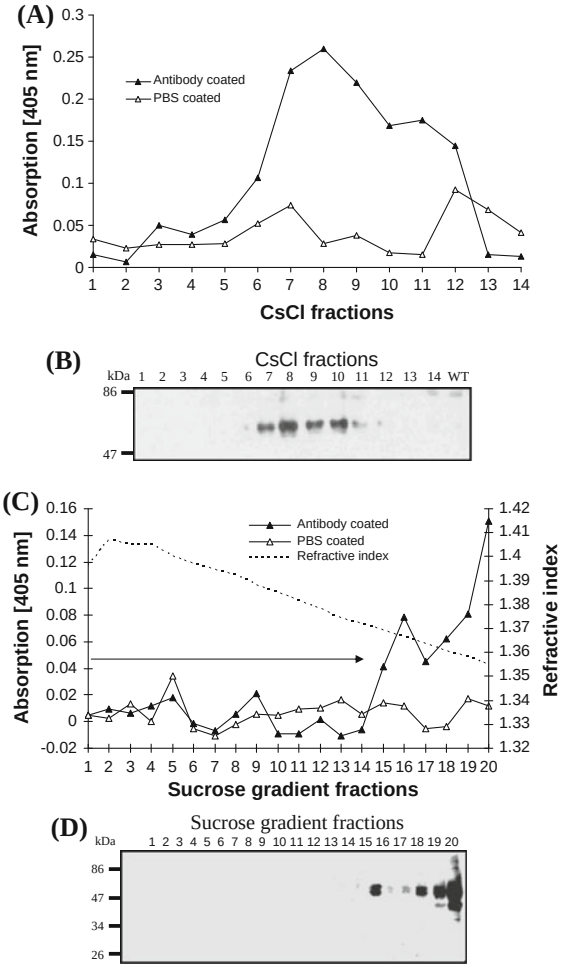

Fig. 4 Purification of chloroplast-derived L1 protein and the analysis of different assembly forms. **a** ELISA analysis of purified protein fractions obtained from CsCl density gradient centrifugation. Conformation-specific monoclonal antibody Ritti01 was the primary antibody for the detection of conformational epitopes. PBS was the control used for each collected protein fraction (*lanes 1–14*; fraction 1 corresponds to the tube bottom). **b** Western blot of CsCl fractions using MD2H11 monoclonal L1-specific antibody. The tobacco wild type was used as the control. **c** ELISA of 20 fractions obtained from sucrose gradient sedimentation (fraction 1 corresponds to tube bottom) using conformation-specific monoclonal antibody Ritti01. PBS served as the negative control for each fraction. *Broken line* Refractive index of the different fractions, *arrow* direction of sedimentation. **d** Western blot of the sucrose gradient sedimentation fractions using HPV-16 L1-specific monoclonal antibody MD2H11 for the detection of linear epitopes

To determine different assembly forms and to verify the assembly of L1 protein to capsomeres, we subjected crude extracts of tobacco leaves to sucrose sedimentation analysis. Among the 20 fractions collected (fraction 1 is from the bottom of the tube), capsomeres were expected in last fractions due to their low sedimentation coefficient. An ELISA of the sucrose sedimentation samples, using conformation-specific monoclonal antibody Ritti01, showed that

most of the L1 protein was indeed concentrated in the final fractions (18–20; Fig. 4c), indicating conformational epitopes. The presence of a larger portion of L1 protein with a low sedimentation coefficient in the last fractions verified that most of the L1 protein assembled into capsomeres only. However, the detection of L1 protein in fractions 15–16 suggested the formation of other higher order structures. Western blot analysis by MD2H11 antibody further confirmed the presence of recombinant protein—mainly in fractions 18–20 (Fig. 4d). These data indicated that approximately 60% of the chloroplast-derived L1 protein assembled into capsomeres, as expected, while 40% of the L1 protein assembled into higher forms. In addition, some small amount of remaining unassembled L1 protein could still be present in the top fraction.

Discussion

We report here the possibility of producing a potentially cost-effective second-generation vaccine in tobacco chloroplasts through the expression of a mutated L1 gene (L1_2xCysM) that retains the assembly of L1 protein to capsomeres. The results presented here demonstrate that a reasonable amount of TSP (up to 1.5%) was obtained from the transplastomic tobacco plants. We also show that the correct formation of capsomeres was maintained, confirming their proper assembly by CsCl gradient centrifugation, sucrose gradient sedimentation analysis and ELISA with a conformation-specific monoclonal antibody. The expression of the L1_2xCysM gene appeared to induce male sterility in the plants, while rest of growth and morphological characteristics were observed to be normal.

There have been many arguments on the likelihood of being able to use VLP-based vaccines in developing countries due to their high cost, requirement of a cold chain and the necessity of sterile needles for intramuscular administration (Stanley et al. 2008). Keeping in mind that more than 80% of the cervical cancer cases occur in developing countries (Parkin and Bray 2006), there is a great need for a second-generation vaccine that is inexpensive to produce and thermostable (to circumvent cold chain) (Stanley et al. 2008), for these very properties would increase the availability of such a vaccine to developing countries. One possible alternative can be the capsomeres, which are considered to be relatively stable at room temperature and can be produced from *Escherichia coli* in large amounts (Li et al. 1997; McCarthy et al. 1998; Chen et al. 2001). As such, capsomeres represent an advantageous alternative to VLP-based vaccines for developing countries. However, there is still the question of cost-effectiveness as production of capsomeres from *E. coli* requires fermenters and the purification of protein (Daniell et al. 2009). This problem can be solved by choosing plants as the expression system: on the one hand, plants can be used for large-scale production, and on the other hand, plants can be ingested directly as a source of oral vaccines (Daniell et al. 2001).

In the study reported here, we opted chloroplast-based expression of a mutated L1 gene (L1_2x CysM), which due to the replacement of two highly conserved cysteine residues retains the assembly of L1 protein to capsomeres. The L1_2xCysM gene showed significant expression, with TSP estimated to be up to 1.5% in tobacco leaves. We used N-terminal fusion of the downstream box (DB), which consists of the first 14 amino acids of GFP (GFP_{14}); this fragment has been reported to accumulate to high levels in plastids (Sidorov et al. 1999). The significant expression of L1 protein can either be due the protection of recombinant protein from degradation due to N-terminal fusion of DB or to an increased rate of translation, as indicated by Ye et al. (2001). Similar results were obtained when the L1 gene was linked to the DB consisting of a nucleotide sequence encoding the first 14 amino acids of Rubisco large subunit (*rbc*L) (Lenzi et al. 2008). Our results illustrate that the fusion of GFP_{14} to the L1_2xCysM gene did not affect the native conformation of L1 protein, as shown by the binding of L1 protein with conformation-specific antibody in ELISA.

The L1 gene has been expressed in tobacco plants following nuclear transformation, but only a very low protein accumulation was achieved (Biemelt et al. 2003; Varsani et al. 2003; Liu et al. 2005). However, Maclean et al. (2007) achieved a high yield (up to 11% of TSP) by targeting human codon-optimized L1 protein to the chloroplast. This increased yield may be due to a different protein hydrolyzing machinery in the plastids as well as to different protein stability conditions as a result of the presence of protective chaperones. In two recent studies, HPV-16 L1

protein-forming VLPs were expressed in the tobacco chloroplast, achieving 24% (Fernandez-San Millan et al. 2008) and 1.5% (Lenzi et al. 2008) of TSP, respectively. In contrast to our approach, Lenzi et al. (2008) used native viral L1 and a synthetic codon-optimized L1 gene. Lenzi et al. (2008) compared various constructs and found that L1 was only detectable when the N-terminus of the L1 protein was translationally fused with the first 14 amino acids of the N-terminal domain of the ATPase beta subunit (*atp*B) or the Rubisco large subunit (*rbc*L). Fernandez-San Millan et al. (2008) suggested that the fairly high accumulation of L1 protein achieved in their system may have been due to the use of the light-regulated *psb*A 5′-UTR.

All three transplastomic lines obtained in our study were found to be sterile. Although the plants produced flowers, these were either shed before maturity or they did not produce seeds. Seeds were obtained when the transplastomic lines were pollinated with wild-type tobacco plants, thereby demonstrating that sterility was restricted to the male organs only. However, in all transplastomic lines, the morphological characteristics of the vegetative organs were identical to those of wild-type plants. Subsequent studies on the T_1 and T_2 generations revealed the persistence of sterility in the transplastomic lines. Various factors can account for this persistence of sterility, such as the interference of novel open reading frames (ORFs) with metabolism in the cytoplasm, possibly due to lower levels of ATP production (Chase 2007; Pelletier and Budar 2007), or somaclonal variation induced during tissue culture (Larkin and Scowcroft 1981; Jain 2001). Male sterility in transplastomic tobacco plants was detected by Lössl et al. (2003), where the expression of poylydroxybutyrate (PHB) in tobacco chloroplasts resulted in male-sterile phenotypes. This effect was subsequently studied in detail by Ruiz and Daniell (2005) who found that the male-sterile phenotype had shortened stamens and produced nonviable pollen. These researchers attributed this effect to an alteration in chloroplast fatty acid metabolism due to the expression of *pha*A gene. A detailed study on the development and morphology of the male organs and backcrosses of the sterile phenotypes for several rounds would reveal the reasons for male sterility reported here. However, male sterility is advantageous as it facilitates hybrid seed production (Chase 2007). Additionally, male sterility can be used as a tool for further transgene containment by reducing the outcrossing risk of very small fraction of paternally transmitted plastids (Ruiz and Daniell 2005, Ruf et al. 2007).

We also investigated the proper assembly of L1 protein to higher order structures by CsCl density centrifugation. In this method, the particles move through the sucrose phase and are caught in the interphase between the sucrose and CsCl layer. As expected, L1 protein was detected in fractions 6–10 by western blot analysis. These fractions also immunoreacted with the conformation-specific monoclonal antibody Ritti01 in the ELISA, thereby indicating the presence of L1 protein in the form of higher order structures, such as capsomeres or VLPs. To verify that the expression of the L1_2xCysM gene actually did lead to the formation of capsomeres, we analyzed crude plant extracts by sucrose sedimentation analysis. This method allows quantitative separation of different assembly forms on the basis of their sedimentation coefficients. The ELISA and western blot analysis revealed that the L1 protein was largely concentrated in the final fractions (18–20), a result that confirmed that most of the L1 protein assembled into capsomeres with a low sedimentation coefficient. A second peak in ELISA was also observed in fractions 15–16, suggesting the formation of assembly forms other than capsomeres—ones with relatively higher sedimentation coefficients. Both CsCL density centrifugation and sucrose sedimentation analysis are standard techniques that have been previously used for the purification and quantitative separation of different assembly forms of plant-derived L1 protein by Biemelt et al. (2003), Maclean et al. (2007) and Fernandez-San Millan et al. (2008). All of these researchers reported two peaks pertaining to VLPs and capsomeres in the region of higher and lower sedimentation coefficients, respectively. However, with the modified L1 gene used in our study, VLP formation was unlikely because of the abolishment of cysteine residues. Consequently, the second peak observed in our sucrose gradient analysis may be due to small VLPs as it appeared in a sedimentation region between VLPs and capsomeres. In their sedimentation analysis of the modified L1 protein in which serines had replaced two cysteines, Schädlich et al. (2009b) observed the formation of capsomeres as well as higher assembly forms with higher sedimentation coefficients than capsomeres. In the

same study, electron microscopy revealed that these higher assembly forms showing the sedimentation pattern related to small VLPs took the form of heterogeneous aggregates. The formation of heterogeneous rod-shaped structures for modified L1 protein having one cysteine replaced by serine at position 175 has also been reported (Ishii et al. 2003).

Many studies have focused on the immunogenicity of capsomeres that show the induction of neutralizing antibodies and T-cell responses (Rose et al. 1998; Fligge et al. 2001; Öhlschläger et al. 2003; Dell et al. 2006; Schädlich et al. 2009a). A promising approach towards capsomere-based vaccine was reported by Yuan et al. (2001) who demonstrated that dogs could be completely protected against papillomavirus infection by capsomeres. These researchers suggested that neutralizing epitopes do not necessarily need to be displayed in a complex, assembled structures (e.g., VLPs) for effective recognition by the host immune system. Rather, as suggested by Chen et al. (2000), such epitopes may be properly configured within the context of the pentameric capsomeres. Initially, in a direct comparison of different assembly forms (Thönes et al. 2008), VLPs were found to be more immunogenic than capsomeres. However, a recent study by Schädlich et al. (2009b) exemplified that capsomeres obtained by the expression of a mutated L1 gene induced antibody titres equivalent to those generated by VLPs. Although the mutated L1 gene (L1_2xCysM) used in our study is different from the mutated L1 gene reported to have higher immunogenicity by Schädlich et al. (2009b), it opens the door for the development of low-cost second-generation vaccine. Overall, these data imply that capsomeres are a promising candidate as a second-generation vaccine against HPV that can be affordable to developing countries, where cervical cancer is the leading cause of death among women (Parkin and Bray 2006).

Given the feasibility of chloroplast-derived VLP-based vaccines (Fernandez-San Millan et al. 2008; Lenzi et al. 2008) as well as the prospect of developing a cost-effective capsomere-based vaccine, we suggest that plastid expression of capsomeres is a valuable step towards the development of a second-generation vaccine against HPV.

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Title: Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: A step towards cost effective second generation vaccines

Journal: Transgenic Research

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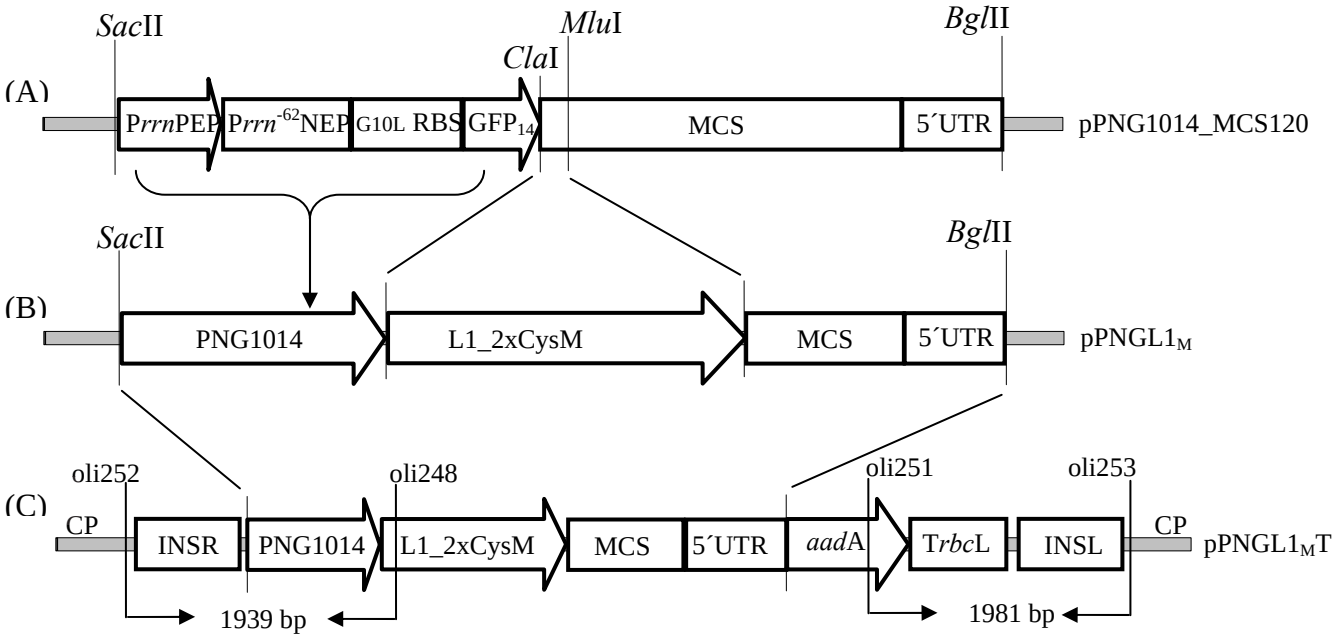
Online Resource Material 1:



Online Resource Figure. Schematic representation of the precursor and transformation vectors (constructs not drawn to scale). (A) Components of precursor vector pPNG1014_MCS120 used to clone transgenes. (B) Plasmid pPNGL1$_M$ obtained after the insertion of L1_2xCysM gene in pPNG1014_MCS120. (C) Final transformation vector pPNGL1$_M$T, containing transgenes along with plastome flanks inserted within the tobacco plastid genome. Primers oli251 and oli253 were used to amplify *aad*A gene at 3´ end (1981 bp) to prove insertion in the plastid region *trn*R on the left insertion site (INSL). On the right insertion site (INSR) primers oli248 and oli252 were used to confirm integration of L1_2xCysM (1939 bp) within the targeted plastid *trn*R region. P*rrn*PEP: 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, GFP$_{14}$: first 14 amino acids of the green fluorescent protein, MCS: multiple cloning site, 5′ UTR: 5′ untranslated region consisting of synthetic ribosomal binding site, *aad*A: aminoglycoside 3′-adenyltransferase, PNG1014: cassette containing P*rrn*PEP, Prrn^{-62}NEP, G10L RBS and GFP$_{14}$, CP: chloroplast DNA, L1_2xCysM: mutated L1 gene, T*rbc*L: terminator from large subunit of ribulose-bisphosphate carboxylase gene, INSR: right insertion site (*trn*R), INSL: left insertion site (*trn*N)

4.4 Scientific article

Plastid expression of a double-pentameric vaccine candidate containing human papillomavirus-16 L1 antigen fused with LTB as adjuvant: transplastomic plants show pleiotropic phenotypes

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Plastid expression of a double-pentameric vaccine candidate containing human papillomavirus-16 L1 antigen fused with LTB as adjuvant: transplastomic plants show pleiotropic phenotypes

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Keywords: HPV-16 *L1*, capsomeres, adjuvant, *LTB*, plastid transformation, low-cost vaccine.

Summary

Human papillomavirus (HPV) causes cervical cancer in women worldwide, which is currently prevented by vaccines based on virus-like particles (VLPs). However, these vaccines have certain limitations in their availability to developing countries, largely due to elevated costs. Concerning the highest burden of disease in resource-poor countries, development of an improved mucosal and cost-effective vaccine is a necessity. As an alternative to VLPs, capsomeres have been shown to be highly immunogenic and can be used as vaccine candidate. Furthermore, coupling of an adjuvant like *Escherichia coli* heat-labile enterotoxin subunit B (*LTB*) to an antigen can increase its immunogenicity and reduce the costs related to separate co-administration of adjuvants. Our study demonstrates the expression of two pentameric proteins: the modified HPV-16 *L1* (*L1_2xCysM*) and *LTB* as a fusion protein in tobacco chloroplasts. Homoplasmy of the transplastomic plants was confirmed by Southern blotting. Western blot analysis showed that the LTB-L1 fusion protein was properly expressed in the plastids and the recombinant protein was estimated to accumulate up to 2% of total soluble protein. Proper folding and display of conformational epitopes for both LTB and L1 in the fusion protein was confirmed by GM1-ganglioside binding assay and antigen capture ELISA, respectively. However, all transplastomic lines showed chlorosis, male sterility and growth retardation, which persisted in the ensuing four generations studied. Nevertheless, plants reached maturity and produced seeds by pollination with wild-type plants. Taken together, these results pave the way for the possible development of a low-cost adjuvant-coupled vaccine with potentially improved immunogenicity against cervical cancer.

Introduction

Various types of human papillomavirus (HPV) are causatively associated with cervical cancer which is the second most common cancer in women. Each year approximately half a million new cases and 274 000 deaths occur among women across the globe due to cervical cancer, the majority of which occurs in developing countries (Parkin and Bray, 2006). HPV types 16 and 18 have been found to be responsible for approximately 70% of invasive cervical cancers. Overall, HPV-16 is by far the most prevalent type, found in about 54% of cervical cancer cases (Smith *et al.*, 2007).

L1 is the major capsid protein of HPV which self assembles into capsomeres and virus-like particles (VLPs). Each VLP consists of 72 pentameric capsomeres of the *L1* protein, arranged in an icosahedral surface lattice (Baker *et al.*, 1991). Formation of disulphide bonds between *L1* protein molecules in adjacent capsomeres is essential for stability of VLPs, and reduction of these bonds leads to their disassembly into capsomeres (Sapp *et al.*, 1995). Replacement of either of the two highly conserved cysteines in L1 protein by serine prevents VLPs assembly and leads to the retention of L1 capsomeres (Sapp *et al.*, 1998).

Currently used VLP-based vaccines against cervical cancer are causing relatively high expenses for production and distribution; furthermore they require a continuous cold chain and sterile needles for intramuscular administration. Thus, due to their high costs these vaccines will probably be largely unaffordable for women in developing countries, where more than 80% of all cervical cancer cases occur (Parkin and Bray, 2006; Stanley *et al.*, 2008). Alternatively, capsomeres can be used as a potential cost-saving substitute to VLPs, as pentameric capsomeres are considered thermo-stable which is advantageous for use in developing countries where cold chains are difficult to maintain (Stanley *et al.*, 2008). Capsomeres have been shown to induce high titres of neutralizing antibodies and *L1*-specific cytotoxic T-lymphocytes (CTLs) upon oral, intranasal and subcutaneous immunization and have also protected against viral challenge in the canine oral papillomavirus (COPV) model (Rose *et al.*, 1998; Fligge *et al.*, 2001; Yuan *et al.*, 2001; Öhlschläger *et al.*, 2003; Dell *et al.*, 2006; Thönes and Müller, 2007; Schädlich *et al.*, 2009a).

To improve immunogenicity of a vaccine, provision of an adjuvant can augment the strength or quality of a given response (Stevenson *et al.*, 2004). *Escherichia coli* heat-labile

enterotoxin subunit B (*LTB*), a nontoxic molecule with potent biological properties, is a powerful mucosal and parenteral adjuvant that induces a strong immune response against co administered or coupled antigens (Dickinson, 1996). There are many reports about the immunogenic and adjuvant properties of LTB as reviewed by Sánchez and Holmgren (2008). *LTB* has been shown to act as adjuvant to herpes simplex virus type 1 glycoprotein and to induce a high degree of protective immunity against ocular infection of mice with live virus (Richards *et al.*, 2001). Similarly, it can act as an adjuvant to vaccines targeting influenza virus haemagglutinin, prion protein and HPV-16 L1 (Verweij *et al.*, 1998; Wang *et al.*, 2006; Yamanaka *et al.*, 2006). Another advantage of LTB lies in its higher potent adjuvanticity than its closely related homologue CTB, the B subunit of cholera toxin (Millar *et al.*, 2001).

Plants can be used as a production system for biopharmaceuticals, therapeutic proteins and recombinant vaccines due to several advantages, such as low-cost, ease to scale up for mass production, low health risks and possibility to be administered as unprocessed or partially processed material (Daniell *et al.*, 2001b; Fischer *et al.*, 2004; Ma *et al.*, 2005). Compared to nuclear transformation, plastid genetic engineering offers many advantages which make the system ideal for applications involving plant-made pharmaceuticals. These include high levels of transgene expression due to high copy number, absence of epigenetic effects, transgene containment via maternal inheritance and multi-gene expression in a single transformation event (for review see Maliga, 2002; Bock, 2007; Koop *et al.*, 2007; Chebolu and Daniell, 2009). Various vaccine antigens against several human and animal diseases have been successfully expressed in plastids with an average expression level of total soluble protein (TSP) in the range of 4%–31% (Chebolu and Daniell, 2009). Recently, very high expression of transgenes in plastids have been reported by Oey *et al.* (2009) and Ruhlman *et al.* (2010): obtaining ~70% of TSP and 72% of total leaf protein (TLP) respectively.

Chloroplasts have been used as an expression platform for L1 and LTB proteins. Recently, HPV-16 L1 VLPs and capsomeres have been expressed in tobacco plastids (Fernandez-San Millan *et al.*, 2008; Lenzi *et al.*, 2008; Waheed *et al.*, 2011). VLPs obtained from chloroplasts were shown to be immunogenic in mice (Fernandez-San Millan *et al.*, 2008). *LTB* has been expressed in tobacco chloroplasts and shown to bind to GM1-ganglioside receptors (Kang *et al.*, 2003). However, regarding the expression of LTB as fusion protein with an antigen in chloroplasts, very few reports exist. In the context of co-administration of adjuvants for increased immunogenicity and to reduce cost, it is recommended to produce an adjuvant–antigen fusion protein (Guy, 2007; Sánchez and Holmgren, 2008).

Our current study demonstrates the possibility of producing a single plant-based vaccine consisting of an antigen coupled with an adjuvant for boosting the immunogenicity against the coupled antigen. We have chosen two pentameric proteins which were expected to be expressed as a double-pentameric structure retaining the functionalities of both partners. The chloroplast derived fusion protein accumulates approximately up to 2% of TSP. Accumulation and correct size of the protein was analysed by Western blot analysis. GM1-ganglioside binding assay and antigen capture ELISA showed that both LTB and L1 properly assembled into their pentameric conformations. This study provides the ground for the possible production of low-cost capsomeres based vaccine against HPV, with enhanced immunogenicity due to the fusion of LTB as an adjuvant.

Results

Chloroplast transformation vectors

For plastid transformation, the vector PNGLTB-L1$_M$T was constructed, by combining *LTB* with the modified HPV-16 *L1* gene (*L1_2xCysM*). The fused *LTB-L1_2xCysM* gene was expressed by a cassette containing the promoters for the plastid and nuclear encoded polymerases (P*rrn*PEP and P*rrn*$^{-62}$NEP respectively), in combination with the terminator sequence of large subunit of ribulose-bisphosphate carboxylase gene (T*rbc*L) from *Chlamydomonas reinhardtii*. To enhance the expression, the coding sequence for the first 14 amino acids of GFP protein (*GFP$_{14}$*) was fused with the transgenes (Ye *et al.*, 2001). Both genes were separated with the amino acid sequence GPGPG as spacer to reduce potential steric hindrance. The aminoglycoside 3′-adenyltransferase (*aadA*) gene was used for the selection of transplastomic plants. The fused P*rrn*PEP-P*rrn*$^{-62}$NEP promoter served as a bicistronic promoter, driving the expression of both *LTB-L1_2xCysM* and *aadA* genes. Based on the results obtained from *LTB-L1_2xCysM* transplastomic plants, another vector PNGLTB-T was constructed, which contained all the above mentioned components with only *LTB* reading frame. The *trn*N and *trn*R loci were used for homologous recombination within the tobacco plastid genome. Figure 1 shows the complete description of the construct within the tobacco chloroplast genome including primers used to confirm the insertion of the transgenes within the plastid genome and probe (P) used for Southern blot analysis.

Plastid transformation, regeneration and seed production of transformed plants

Leaves from tobacco plants were transformed by the biolistic method and placed on RMOP medium containing 500 mg/L spectinomycin, for selection and regeneration. Wild type tobacco plants were used as control. First, phenotypical differences were observed during the initial selection phase: in case of *LTB-L1_2xCysM* transformed plants, all the developing microcalli were pale, while for *LTB* transformed plants, the microcalli were green. After the second round of selection the shoots were analysed by PCR. To achieve homoplasmy, resistant shoots were subjected to three regeneration cycles under the same conditions. After rooting on B5 medium transgenic shoots were transferred to the green house for seed production and morphological studies. To investigate fertility, flowers were covered with transparent plastic bags to assure self-pollination. *LTB-L1_2xCysM* plants were only able to produce seeds by cross-fertilization with wild type tobacco pollen. Whereas for *LTB* lines seeds were obtained by self-pollination of transplastomic plants.

Morphology of transplastomic plants

LTB-L1_2xCysM transplastomic lines showed remarkable pleiotropic phenotypes. All transplastomic lines were male sterile: flowers either shed before maturity or did not produce seeds (Figure 2a). Seeds were only obtained when transplastomic plants were pollinated with pollen from wild type plants. A detailed study of flowers showed that all the flowers had short stamina (Figure 2b). In all transplastomic lines, leaves were chlorotic and pale in colour as compared to wild type tobacco control plants (Figure 2c). All transplastomic lines showed stunted growth during the *in vitro* as well as under green house conditions (Figure 2d,e). These pleiotropic effects persisted in

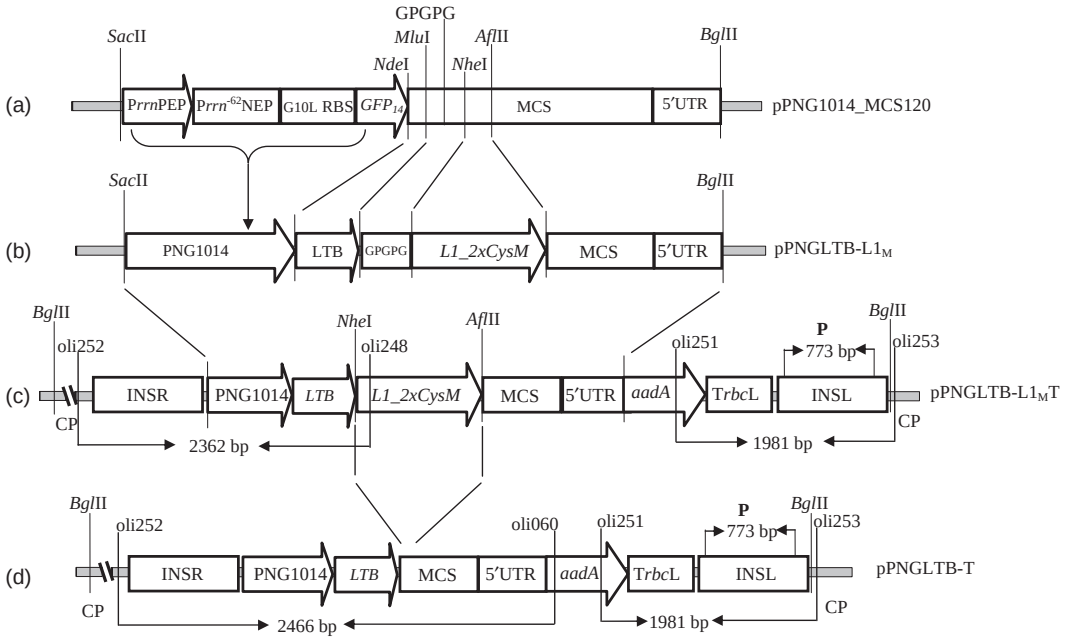

Figure 1 Schematic representation of precursor and transformation vectors (constructs not drawn to scale). (a) Components of precursor vector pPNG1014_MCS120 used to clone transgenes. (b) Plasmid pPNGLTB-L1$_M$ obtained after the insertion of *LTB* and *L1_2xCysM* genes in pPNG1014_MCS120. (c) Final transformation vector pPNGLTB-L1$_M$T for the transformation of plants with fused *LTB-L1_2xCysM* gene, showing transgenes along with plastome flanks inserted within the tobacco plastid genome. (d) Final transformation vector pPNGLTB-T for *LTB* transformation. Primers oli251 and oli253 were used to amplify *aadA* gene at 3′ end (1981 bp) to prove insertion in the plastid region *trn*R on the INSL. On the INSR primers oli248 and oli252 were used to confirm integration of *LTB- L1_2xCysM* (2362 bp) within the targeted plastid *trn*R region. Primers oli252 and oli060 were used for the confirmation of *LTB* integration (2466 bp). P*rrn*PEP, 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; *GFP$_{14}$*, first 14 amino acids of the green fluorescent protein; GPGPG, spacer; MCS, multiple cloning site; 5′ UTR, 5′ untranslated region consisting of synthetic ribosomal binding site; *aadA*, aminoglycoside 3′-adenyltransferase; PNG1014, cassette containing P*rrn*PEP, Prrn^{-62}NEP, *G10L* RBS and *GFP$_{14}$*; CP, chloroplast DNA; *LTB*, *Escherichia coli* heat-labile enterotoxin subunit B; *L1_2xCysM*, modified L1 gene; T*rbc*L, terminator from large subunit of ribulose-bisphosphate carboxylase gene; P, probe for the confirmation of homoplasmy; INSR, right insertion site (*trn*R); INSL, left insertion site (*trn*N).

the successive generations during several backcrosses with wild type tobacco plants. However, plants were able to reach maturity and produced seeds by pollination with pollen from wild type tobacco plants. To study these effects, plants were transformed with *LTB* gene only. In this case, all T_0 and T_1 plants showed normal growth, morphology and fertility like wild type tobacco plants. Figure 2 illustrates the comparison of both transformed lines with wild type tobacco plants. The morphological observations are summarized in Table 1, which compares the current data with the results obtained by Waheed *et al.* (2011) describing *L1_2xCysM* phenotypes.

Confirmation of transgene integration and homoplasmy

Spectinomycin resistant shoots were analysed for the integration of transgenes by PCR. For *LTB-L1_2xCysM* transformed plants, the primers oli248 (located within *L1_2xCysM*) and oli252 (located within plastid genome at 5′ end) and for *LTB* transformed plants, the primer pair oli252 (located within CP at 5′ end) and oli060 (located within *aadA*) were used. Similarly correct insertion of *aadA* was confirmed in *LTB-L1_2xCysM* and *LTB* transformed plants, by using the primers located within *aadA* at 5′ end (oli251) and plastid genome at 3′ end (oli253). The resulting PCR products were obtained from the plastomes of transformed plants only. Ten transplastomic shoots were screened by PCR, each for *LTB-L1_2xCysM* and *LTB* transformed plants. All lines showed positive results in T_0 and T_1 generation (data not shown). All primer positions with their expected sizes of PCR fragments are marked in Figure 1c,d. Homoplasmy of PCR positive plants was confirmed by Southern blot analysis. Total plant DNA was digested with *Bgl*II and probe (P, Figure 1) was used for the identification of site specific integration of transgenes. For *LTB-L1_2xCysM* transformed plants (data shown for three lines), a 9.4 kb fragment was detected (Figure 3), while in case of plants transformed with *LTB* (data shown for two lines), a 7.9 kb fragment was identified (Figure 3). Wild type showed 5.9 kb fragment (Figure 3). The absence of 5.9 kb band validates that all the transplastomic lines were homoplasmic and devoid of any residual wild type DNA. Transplastomic plants from T_1 progeny were tested and confirmed for homoplasmy in the same way (data not shown).

Expression and quantification of fusion protein

Transplastomic expression of fusion protein was determined and quantified by Western blotting using MD2H11 antibody specific for HPV-16 L1 protein (Schädlich *et al.*, 2009a). Data are shown for three independently generated transplastomic *LTB-L1_2xCysM* lines (lanes 1, 2, 3 in Figure 4). Transplastomic line 1 showed maximum expression of the recombinant protein. Total soluble protein from leaves of all three lines showed a

Figure 2 Morphological analysis of *LTB-L1_2xCysM* and *LTB* transplastomic plants. (a) Shows the male sterility in *LTB-L1_2xCysM* lines. Marks of shed flowers are clearly visible. (b) Comparison of floral morphology of *LTB-L1_2xCysM* and *LTB* flowers with wild type (WT) flower: showing short stamina. (c) *LTB-L1_2xCysM* chlorotic phenotype in contrast to *LTB* transformed and WT plants. (d) Growth retardation of *LTB-L1_2xCysM* lines in comparison with *LTB* and WT plants, during *in vitro* conditions. (e) Comparison of growth retardation of *LTB-L1_2xCysM* lines and WT, during green house propagation.

Table 1 Comparison of morphological characteristics of transplastomic and wild type tobacco plants

Characteristic	*LTB-L1_2xCysM* plants (current data)	*LTB* plants (current data)	*L1_2xCysM* plants (Waheed *et al.*, 2011)	Wild type plants
Leaves	Chlorotic	Green	Green	Green
Flowers	Short stamina, male sterile	Normal, fertile	Short stamina, male sterile	Normal, fertile
Growth	Stunted	Normal	Normal	Normal

band of 68 kDa expected for GFP_{14}-LTB-L1 protein (Figure 4). In addition to 68 kDa band, a 55 kDa band corresponding to L1 protein was also detected. Moreover, some bands with lower molecular weight were observed, probably due to the proteolytic degradation of fusion protein. No protein was detected in samples extracted from wild type control plants.

Protein was quantified using a dilution series (10, 20 and 30 ng) of baculovirus-derived purified VLPs (DKFZ, Heidelberg, Germany) as reference. For each transplastomic line, a dilution series of protein samples (1, 2 and 3 μg) was loaded onto the gel. The amount of fusion protein accumulated up to approximately 2% of TSP as evident from Figure 4. Since only the intact protein band (68 kDa) was considered for the quantification, the obtained expression level corresponds solely to the LTB-L1 fusion protein.

LTB and L1 in the fusion protein assemble into functional pentameric conformations

Both L1 and LTB were expected to assemble into pentameric forms. To verify the assembly of L1 protein, antigen capture ELISA was carried out by using conformation specific monoclonal antibody Ritti01. This antibody recognizes the conformational epitopes and only binds to L1 protein that properly presents these epitopes. To confirm the binding of antibody,

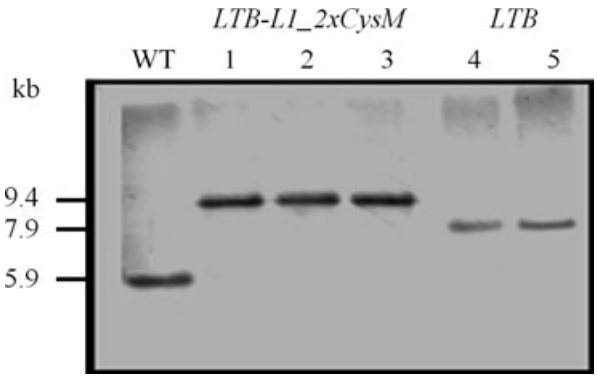

Figure 3 Southern blot analysis of *LTB-L1_2xCysM* and *LTB* transformed plants. Three independently generated transplastomic lines were analysed for *LTB-L1_2xCysM* integrated plants (lanes 1, 2, 3) and two lines for *LTB* integrated plants (lanes 3, 4). Total plant DNA was digested with *Bgl*II. Probe (P, 773 bp) located within left insertion site (INSL) of plastid genome, was amplified by PCR and used for Southern analysis. WT, wild type.

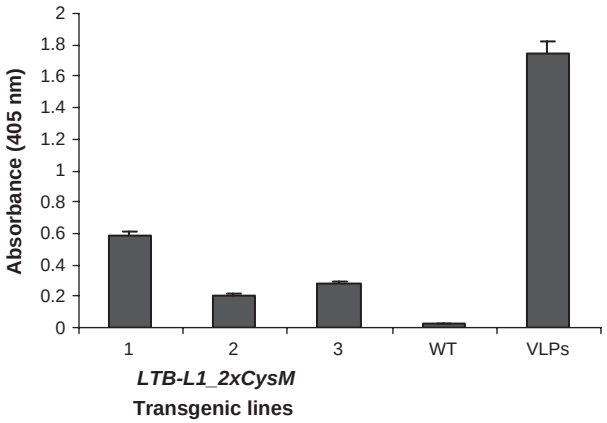

Figure 5 Antigen capture ELISA of three *LTB-L1_2xCysM* transplastomic lines showing the assembly of the L1 protein. Ninety-six well plate was coated with conformation specific monoclonal antibody Ritti01 followed by incubation with soluble protein from three transplastomic lines (lines 1, 2 and 3). Baculovirus-derived purified virus-like particles were used as positive control. WT, wild type.

baculovirus-derived L1 protein (VLPs) was used as positive control. All three *LTB-L1_2xCysM* transplastomic lines generated significant signals relative to the positive control in ELISA (Figure 5). Binding of L1 protein to the conformation specific antibody shows that the L1 protein correctly assembled into pentameric capsomeres and displayed the epitopes necessary for immunogenicity.

To prove the concept that both pentameric fusion partners, L1 and LTB, assembled accurately in their functional tertiary structure, we also investigated the folding of the LTB protein. For this purpose, GM1-ganglioside receptor binding assay was performed. The results indicate that the LTB-L1 fusion protein from all three transplastomic lines showed strong affinity for GM1-ganglioside (Figure 6). The protein extract from line 1 showed more affinity compared to lines 2 and 3. This is in accordance with the expression level of recombinant protein of the three lines observed in immunoblot analysis and antigen capture ELISA. No significant signal was detected for protein extracted from wild type tobacco. In contrast, purified LTB protein used as positive control showed high signals in GM1-ganglioside binding ELISA. The binding of LTB-L1 fusion protein to GM1-ganglioside receptor confirms that the LTB protein folded properly and exhibited the pentameric conformation, required for its functionality.

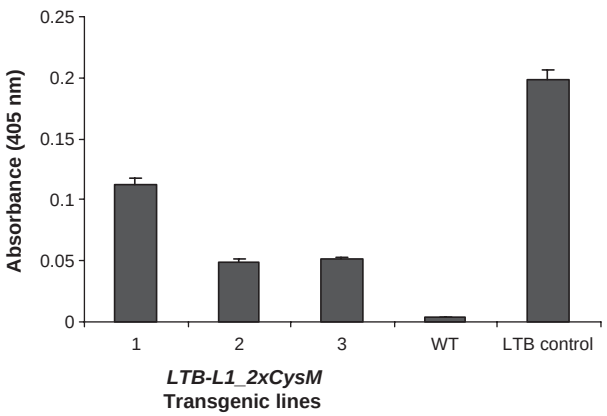

Figure 6 LTB-GM1-ganglioside binding ELISA showing the assembly of LTB protein. Wells of microtitre plate were first coated with GM1-ganglioside and then incubated with total soluble protein from transplastomic lines 1, 2 and 3. Protein from wild type (WT) tobacco plants and purified LTB served as negative and positive controls, respectively.

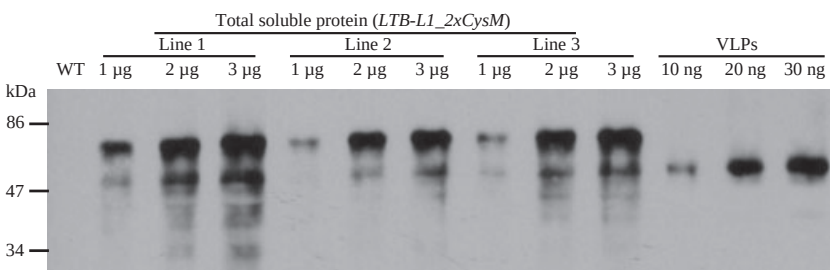

Figure 4 Expression and quantification of LTB-L1 fusion protein by Western blotting. MD2H11 monoclonal antibody specific for Human papillomavirus-16 L1 protein was used. Data are shown for three independently generated lines (line 1, 2 and 3). For each line, a dilution series of total soluble protein extracted from leaves (1, 2 and 3 µg) was loaded. A dilution series of purified baculovirus-derived L1 VLPs (10, 20 and 30 ng) was used as reference for protein quantification. WT, wild type.

Discussion

In the present study, we report the possibility of producing a recombinant fusion protein with potentially improved immunogenicity due to direct coupling of an adjuvant with an antigen. This recombinant protein consists of modified HPV-16 L1 protein C-terminally fused with LTB. Due to replacement of two cysteines by serines in the modified form, the assembly of L1 protein is retained to capsomeres. Homoplasmy of transplastomic plants was confirmed by Southern blotting. Immunoblot analysis confirmed the correct size of fusion protein and showed the accumulation of LTB-L1 fusion protein approximately up to 2% of TSP. We present the proper pentameric conformation of both LTB and L1 in the recombinant protein, as demonstrated by GM1-ganglioside binding assay and antigen capture ELISA, respectively. The transplastomic plants expressing the fusion protein showed pleiotropic phenotypes.

Due to certain limitations, currently used VLP-based vaccines against cervical cancer raise the question of their availability to developing countries where more than 80% of cases occur (Parkin and Bray, 2006; Stanley *et al.*, 2008). In contrast to VLPs, capsomeres can be used as a possible alternative because they are considered more stable at room temperature and can be produced from *E. coli* in large amounts (Li *et al.*, 1997; McCarthy *et al.*, 1998; Chen *et al.*, 2001). However, regarding the requirement of fermenters and protein purification from *E. coli*, there is still a problem of cost effectiveness (Daniell *et al.*, 2009). On the other hand, to increase immunogenicity of a given antigen, production and co-administration of adjuvants is also cost-intensive and laborious (Davoodi-Semiromi *et al.*, 2009). Moreover, adjuvanticity of LTB is much improved when coupled to antigens (Sánchez and Holmgren, 2008). These problems can be solved by selecting plants as production system due to their many advantages such as: large scale production, expression of fusion proteins and the possibility to be used directly as oral vaccines (Daniell *et al.*, 2001b; Bock and Warzecha, 2010).

In the present study, we have opted to jointly express the modified HPV-16 *L1* (*L1_2xCysM*) and *LTB* genes. In the present study N-terminal fusion of the downstream box was used, consisting of the first 14 amino acids of GFP (GFP_{14}) which confers high levels of protein accumulation in plastids (Ye *et al.*, 2001). We obtained significantly high expression of the fusion protein accumulating up to 2% of TSP in the leaves of transplastomic plants. This amount is comparable with the protein accumulation levels obtained by individual expression of LTB and modified L1 proteins (Kang *et al.*, 2003; Waheed *et al.*, 2011). However, compared to Ye *et al.* (2001), the recombinant protein accumulated to a lower level in the present study. One possible reason for low expression can be due to formation of protein aggregates, which might precipitate and remove protein from the analysed soluble fraction. Such effects have been observed in several reports, where formation of protein aggregates was considered a reason for underestimation as well as for hindering the quantification of plastid expressed protein by ELISA (Daniell *et al.*, 2001a; Ruhlman *et al.*, 2007; Arlen *et al.*, 2008). Recently, Sim *et al.* (2009) expressed a fusion protein, containing a synthetic LTB and hemagglutinin-neuraminidase-neutralizing epitope (HNE) of Newcastle disease virus. In this case aggregation of oligomers was observed and the LTB-HNE fusion protein accumulated to a low level, up to 0.5% of TSP in tobacco chloroplasts. Another factor which might account for the observed low protein yield is potential susceptibility of the foreign protein to proteolytic degradation. In our report, in addition to GFP_{14}-LTB-L1 protein (68 kDa), a 55 kDa band corresponding to L1 protein was also detected, as obvious from the Western blot analysis. In a previous approach Lenzi *et al.* (2008) expressed L1 protein alone and as fusion protein with GST in plastids. They showed that L1 was only detectable when the N-terminus of L1 protein was translationally fused with the first 14 amino acids of the N-terminal domain of the ATPase beta subunit (*atp*B) or the Rubisco large subunit (*rbc*L). In the above mentioned report, Western analysis showed that the LTB-GST fusion protein accumulated up to 0.1% of TSP and the authors observed the specific band of L1 protein in the range of 55 kDa in addition to GST-L1. In contrast to Lenzi *et al.* (2008), although showing degradation, the intact LTB-L1 fusion protein shows significantly higher expression in the current study.

In our previous study, when plants were transformed with the *L1_2xCysM* gene, male sterility was observed (Waheed *et al.*, 2011). However, other morphological characteristics of transplastomic plants were normal. In the present study, we observed persistent pleiotropic effects in the transplastomic plants expressing the LTB-L1 fusion protein. All transplastomic lines exhibited chlorotic phenotypes, were male sterile and showed stunted growth. To compare these effects in more detail, tobacco plants were transformed with the *LTB* gene alone. In this case, all transplastomic lines developed to healthy plants like wild type. These results lead to the hypothesis that the observed pleiotropic effects can be specifically due to the expression of LTB-L1 fusion protein. In the majority of reports on plastid transformation, expression of foreign protein in plastids generated normal phenotypes. In case of expression of L1 protein (Fernandez-San Millan *et al.*, 2008; Lenzi *et al.*, 2008) and LTB protein (Kang *et al.*, 2003) in tobacco plastids, no pleiotropic effects were reported. Current existing studies on the expression of LTB-fusion proteins in plastids also support these results (Rosales-Mendoza *et al.*, 2009; Sim *et al.*, 2009). However, there is a substantial number of reports in which phenotypic alterations were noted in transplastomic tobacco plants (Tregoning *et al.*, 2003; Magee *et al.*, 2004; Ruiz and Daniell, 2005; Chakrabarti *et al.*, 2006; Hennig *et al.*, 2007; Hasunuma *et al.*, 2008; Tissot *et al.*, 2008). Several reasons could account for the appearance of aberrant phenotypes in the transformed plants. For instance, it can either be due to products of specific transgenes which can interfere with certain metabolic processes (Ruiz and Daniell, 2005), or due to the integration site of transgenes. Another possible reason can be the over-expression of foreign proteins in plastids, as reported by Oey *et al.* (2009). In the latter study, recombinant lysin protein accumulated to very high levels (~70% of TSP), which affected the development of transplastomic plants. However, Ruhlman *et al.* (2010) recently reported the hyper-expression of a fusion protein (72% of TLP), without any detrimental effects on transformed plants. This shows that pleiotropic effects may not necessarily be due to the over-expression of foreign proteins. Concerning the male sterility, our study shows that flowers were characterized by short stamens. One possible reason for male sterility could be the non-viability of pollen. Male sterility in transplastomic tobacco plants has been detected by Lössl *et al.* (2003), where expression of polydroxybutyrate (*PHB*) in tobacco chloroplasts resulted in male sterile phenotypes. This effect was studied in detail by Ruiz and Daniell (2005). In the latter study, male sterile

phenotype exhibited shortening of stamens and production of nonviable pollen, an effect which was linked to the alteration of chloroplast fatty acid metabolism due to the *pha*A gene expression. In our present study, reasons for phenotypic alterations caused by the foreign protein need further investigation. As a possible way to minimize these detrimental effects, transgenes can be inducibly expressed in mature plants (Lössl and Waheed, 2011).

We selected a modified HPV-16 *L1* gene for expression in plastids. This modified gene retains the assembly of L1 protein to pentameric capsomeres. There have been many studies regarding the immunogenicity of capsomeres, showing the induction of neutralizing antibodies and T-cell responses (Rose *et al.*, 1998; Fligge *et al.*, 2001; Öhlschläger *et al.*, 2003; Dell *et al.*, 2006; Schädlich *et al.*, 2009a). A promising approach towards a capsomere-based vaccine was shown by Yuan *et al.* (2001), in which dogs were completely protected against papillomavirus infection by capsomeres. In the above mentioned report it was stated that neutralizing epitopes do not necessarily need to be displayed in complex, assembled structures (e.g., VLPs) for effective recognition by the host immune system. Rather, as suggested by Chen *et al.* (2000), such epitopes may be properly configured within the context of the pentameric capsomeres. Recently, Schädlich *et al.* (2009b) exemplified that capsomeres obtained by the expression of a modified L1 gene induced antibody titres equivalent to those generated by VLPs. These data make capsomeres a promising candidate for the development of second-generation vaccines against HPV. Immunogenicity of capsomeres can further be increased by coupling them with adjuvants such as LTB (Guy, 2007; Sánchez and Holmgren, 2008). LTB protein has been expressed in chloroplasts and shown to bind with GM1-ganglioside receptors (Kang *et al.*, 2003). In another report by Rosales-Mendoza *et al.* (2009), oral immunization of mice with dried transplastomic tobacco leaves expressing LTB-ST fusion protein (LTB coupled with *E. coli* heat stable toxin) led to the induction of both, serum and mucosal LTB-ST specific antibodies. Moreover, the adjuvanticity of LTB is also well documented in the literature (Millar *et al.*, 2001; Sánchez and Holmgren, 2008).

Both L1 and LTB proteins are expected to assemble individually into pentameric forms. In the LTB-L1 fusion protein, proper folding and the display of conformational epitopes is necessary for their adjuvanticity and immunogenicity, respectively. In the present report, *LTB* and *L1_2xCysM* genes were separated by the amino acid sequence GPGPG. Use of this spacer sequence for the recovery of functional peptide is recommended for the restoration of immunogenicity against different protein epitopes (Sette *et al.*, 2001; Livingston *et al.*, 2002). Our study demonstrates that the chloroplast-derived LTB-L1 fusion protein properly assembled into their respective functional conformations. Binding of L1 protein to conformation specific antibody in antigen capture ELISA clearly shows that the L1 protein assembled into pentameric capsomeres and contained the conformational epitopes. This confirms that the fusion of LTB did not affect the native conformation of L1 protein. In our previous study (Waheed *et al.*, 2011), using *L1_2xCysM* gene, the assembly of L1 protein to capsomeres was investigated and verified by Cesium chloride density centrifugation and sucrose sedimentation analysis. On the other hand, binding of LTB to GM1 in GM1-ganglioside binding ELISA shows that LTB protein correctly folded into pentamers. Hence, the fusion of L1 had no effect on the folding of LTB. Moreover, N-terminal fusion of first 14 amino acids of GFP protein to LTB did not obstruct its native folding. Proper assembly and display of functional epitopes for LTB and LTB-fusion proteins is evident from the reports of Kang *et al.* (2003), Rosales-Mendoza *et al.* (2009) and Sim *et al.* (2009).

In conclusion, the data presented in the current study are useful for the possible development of a plastid-based low-cost vaccine. Use of L1 capsomeres coupled with LTB would aid to further reduce the cost related to co-administration of adjuvant. Moreover, immunogenicity against the L1 antigen can additionally be enhanced by its direct fusion with the adjuvant. Taking all the advantages into account, these data will pave a way to develop a vaccine against cervical cancer, affordable for developing countries.

Experimental procedures

Vector construction

A precursor vector pPNG1014_MCS120 was constructed as introduced by Ye *et al.* (2001) with slight modifications. This vector was also used by Waheed *et al.* (2011) for plastid transformation. The above mentioned plasmid 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 the lambda phage T7 (Studier *et al.*, 1990). Nuclear encoded polymerase (NEP) promoter (P*rrn*$^{-62}$NEP, Hajdukiewicz *et al.*, 1997) was fused with P*rrn*PEP immediately downstream, followed by the *G10L*. After *G10L*, the DNA sequence encoding the first 14 amino acids of the GFP protein (GFP_{14}) was N-terminally to the gene of interest. Leaving cleavage space for multiple cloning site (MCS), 5′-untranslated region (5′ UTR) consisting of synthetic RBS to allow downstream insertion of *aadA* gene, was linked to the gene of interest.

Escherichia coli heat-labile enterotoxin subunit B (*LTB*) was amplified from enterotoxigenic *E. coli* (ETEC, Genbank accession number M17874) by PCR using forward primer (oli) 5′-TTTTTCGAAcatatgAATAAAGTAAAATTTTATGTTTTATTTAC-3′ and reverse primer 5′- TTTTTacgcgtGTTTTCCATACTGATTGCCG-3′ (restriction sites in lower case). *LTB* was inserted in pPNG1014_MCS120 by *Nde*I and *Mlu*I. The modified HPV-16 *L1* gene (*L1_2xCysM*), in which two cysteines 175 and 428 were replaced by serine residues (Schädlich *et al.*, 2009b), was amplified by PCR using forward 5′- AAAAgctagcCCTAGGGGACCAGGTCCTGGCATGCTCGTCTA-3′ and reverse 5′- TTTTTcttaagAACGTTGACGTCATTCCGGATT-3′ primers (restriction sites in lower case). *L1_2xCysM* was cloned in pPNG1014_MCS120 by *Nhe*I and *Afl*II to get plasmid pPNGLTB-$L1_M$. GPGPG spacer sequence was included between *LTB* and *L1_2xCysM* genes. To obtain the final transformation vector pPNGLTB-$L1_M$T, plasmid pT7PHB-N (Lössl *et al.*, 2005) was used. This plasmid contained the *aadA* gene conferring resistance to spectinomycin and streptomycin, the terminator from a large subunit of ribulose-bisphosphate carboxylase gene (T*rbc*L) from *Chlamydomonas reinhardtii* and the flanking regions left insertion site (INSL) and right insertion site (INSR), homologous to the respective loci *trn*N and *trn*R in the inverted repeats (IR) of the tobacco plastome. The flanking sequences included nucleotides 109 230–110 348 and 110 349–111 520 of IR-A, as well as nucleotides 131 106–132 277 and 132 278–133 396 of IR-B. Plasmid pT7PHB-N was cut with *Sac*II and *Bam*HI and the whole cassette from pPNGLTB-$L1_M$ was cloned in pT7PHB-N by *Sac*II

and *Bgl*II to obtain final transformation vector pPNGLTB-L1MT. Plasmid pPNGLTB-L1$_M$T was cut with *Nhe*I and *Afl*II and ligated to obtain *LTB* transformation vector pPNGLTB-T. Figure 1 shows the vector construction pattern. Nucleotide positions for transgene insertions are given according to the plastome sequence data for EMBL accession no. Z00044, initially reported by Shinozaki *et al.* (1986) and later updated by Wakasugi *et al.* (1998) and Yukawa *et al.* (2005). All cloning procedures were carried out using the standard methods described by Sambrook *et al.* (1989).

Transformation and regeneration of transformed plants

Seeds of *Nicotiana tabacum* (Nt) cv. Petit Havana (Surrow Seeds, Sakskøbing, Denmark) were grown *in vitro* at 25 °C (0.5–1 W/m^2 Osram L85 W/25 universal-white fluorescent lamps) on agar-solidified MS medium (Murashige and Skoog, 1962) containing MS salts and sucrose (30 g/L). Plasmid DNA was coated on gold particles (0.6 μm) (Svab *et al.*, 1990) and leaves were transformed by bombarding DNA-coated gold particles using particle gun (PDS1000He; Bio-Rad, Hercules, CA, USA). Leaves were cut into 5 mm pieces and placed on RMOP medium containing 500 mg/L spectinomycin (Svab *et al.*, 1990). After 4–6 cycles of shoot regeneration, spectinomycin resistant shoots were transferred to B5 medium (Gamborg *et al.*, 1968) supplied with spectinomycin, for rooting. Transformed plants were shifted to the greenhouse for further studies and seed production. All *LTB-L1_2xCysM* transformed plants were pollinated with wild type and seeds were collected. Seeds from *LTB* transformed plants were obtained by self-pollination. These seeds were grown on agar-solidified MS medium (containing MS salts and 30 g/L sucrose) supplied with 500 mg/L spectinomycin to generate T_1, T_2, T_3 and T_4 progenies for *LTB-L1_2xCysM* transformed plants and T_1 progeny for *LTB* transformed plants.

Southern blot analysis

DNA was extracted from 100–150 mg of leaves from *in vitro* plant material by CTAB procedure (Murray and Thompson, 1980). Three micrograms of DNA was digested with *Bgl*II, separated on 0.8% agarose gel (w/v) and transferred on to a nylon membrane (Carl Roth, Karlsruhe, Germany). Probe (P, Figure 1), located within INSL, was amplified by PCR which generated a 773 bp fragment, using forward primer oli272 (5′-TACCCGGGAATTGTGACCTC-3′) and reverse primer oli273 (5′-AGAGTCCGACCACAACGACC-3′). The amplified fragment was purified using QIAquick kit (Qiagen, Hilden, Germany). Probe labelling, hybridization and detection were performed using the DIG High Prime DNA Labelling and Detection Starter kit II for chemiluminescent detection with CSPD, as instructed by the manufacturer (Roche, Mannheim, Germany).

Western blot analysis

For protein extraction, leaf discs from transplastomic and wild type plants were collected from plants grown under sterile conditions. To extract soluble proteins, 100 mg leaves were ground in liquid nitrogen and homogenized in an extraction buffer containing 100 mM sodium chloride (NaCl), 10 mM ethylene diamine tetra-acetic acid (EDTA), 200 mM Tris–HCl (pH 8), 0.05% (w/v) Tween-20, 0.1% (w/v) sodium dodecyl sulphate (SDS), 14 mM β-mercaptoethanol, 200 mM sucrose and 2 mM polymethyl-sulfonyl-fluoride (PMSF). The homogenized samples were centrifuged at 24 000 ***g*** for 10 min at 4 °C and the supernatant was collected as soluble fraction. The extracts were heated at 95 °C for 5 min. After determining the concentrations by Bradford assay (Sigma, MO, USA), proteins were separated by SDS-PAGE (10% polyacrylamide gel), transferred onto nitrocellulose membrane (Hybond C; GE Healthcare, Little Chalfont, UK) and blocked with PBS containing 0.3% Tween-20 and 3% (w/v) skimmed milk (PBS-TM) for 1 h. The membranes were incubated for 1 h with HPV-16 *L1*-specific monoclonal antibody MD2H11 (DKFZ) diluted 1 : 4000 in PBS-TM. After washing thrice for 10 min each with PBS containing 0.3% of Tween-20 (PBS-T), membrane was incubated for 1 h with peroxidase-conjugated goat antimouse IgG (Sigma) as secondary antibody (diluted 1 : 3000 in PBS-TM). After washing with PBS-T, proteins were detected by chemiluminescence and bands were visualized on X-ray film (Kodak, Carestream, Rochester, NY, USA).

Antigen capture enzyme-linked immunosorbent assay (ELISA)

Tobacco leaves were obtained from the transplastomic plants grown under sterile conditions. Leaves (100 mg) were ground in liquid nitrogen and homogenized in extraction buffer containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 M NaCl, 0.01% Triton X-100, 20 mM Hepes (pH 7.4), and 1 mM PMSF. The extracts were centrifuged at 18 000 ***g*** for 5 min and supernatant was collected. Each well of 96-well microtitre plates (Costar Corning, Corning, NY, USA) was coated overnight at 4 °C with 50 μL of HPV-16 *L1* conformation specific mouse monoclonal antibody Ritti01 (Thönes *et al.*, 2008) with a dilution of 1 : 300 in PBS. Plates were washed with PBS-T and blocked with PBS-TM for 1 h at 37 °C. Plant extracts were added to the wells and incubated for 1 h at 37 °C. After three washing steps, 50 μL of polyclonal rabbit antiserum (1 : 3000 in PBS-TM) raised against HPV-16 *L1* was added in each well and plates were incubated for 1 h at 37 °C. Plates were washed thrice and incubated for 1 h at 37 °C after the addition of 50 μL of goat anti-rabbit peroxidase conjugate (Sigma, 1 : 3000 in PBS-TM) in each well. Plates were washed thoroughly and the measurement at 405 nm was carried out 10–20 min after adding 100 μL of staining solution {100 mM sodium acetate, 44 mM NaH_2PO_4, pH 4.2, 1 mg ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)]/mL, 0.012% H_2O_2} in each well.

GM1-ganglioside receptor binding ELISA

A 96-well microtitre plate was coated with monosialoganglioside-GM1 (Sigma G7641) by incubating the plate with 100 μL GM1/well (3.0 μg/mL) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4 °C overnight. After washing with PBS-T, the wells were blocked with PBS-TM for 1 h at 37 °C. The plate was incubated with various concentrations of soluble protein (100 μL per well) from transformed plants, wild type and LTB protein (as positive control, Sigma E8656) at 37 °C for 1 h. After three washing steps with PBS-T, 100 μL of rabbit anti-LTB antibody (Immunology Consultants Lab, Newberg, OR, USA) diluted 1 : 3000 in PBS-TM, was added in each well and the plate was incubated at 37 °C for 1 h. The remaining procedure of secondary antibody treatment and detection was same as described above for antigen capture ELISA.

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5. Summary and discussion of research articles

In the present studies, we have successfully expressed the modified HPV-16 L1 gene (L1_2xCysM) alone and in fusion with LTB as adjuvant, in tobacco plastids. Expression of L1_2xCysM gene retained the assembly of L1 to capsomeres only. Quantification of plastid-produced L1 and LTB-L1 by Western blotting showed considerably high yield, which was 1.5% and 2% of TSP, respectively. Homoplasmy of the transformed plants was confirmed by Southern blot analysis. Correct assembly of L1 to capsomeres was verified by CsCl gradient centrifugation, sucrose gradient sedimentation analysis and antigen capture ELISA, using a conformation specific monoclonal antibody. Correct folding of the LTB protein was confirmed by GM1-ganglioside receptor binding ELISA. Expression of LTB-L1 in plastids caused certain pleiotropic effects in transplastomic plants. These effects were further investigated by the expression of LTB gene in tobacco chloroplasts.

Due to many limitations, mainly high costs, currently available VLP-based vaccines against HPV would be largely unaffordable for women in developing countries. Since more than 80% of cases occur in resource poor countries (Ferlay *et al*., 2010), production of low-cost affordable vaccines is an urgent necessity. To establish a system for the development of cost-effective vaccines against HPV, we opted for two approaches. In the first approach, we expressed a modified HPV-16 gene (L1_2xCysM) in tobacco plastids (article 3). Expression of L1_2xCysM led to the retention of L1 assembly to capsomeres only. Our study is the first report about the expression of HPV-16 L1 capsomeres in chloroplasts. Capsomeres are considered to be relatively more thermo-stable and a good candidate for the development of affordable second-generation vaccines against HPV (Lössl and Waheed 2011). Moreover, capsomeres have been proved to be immunogenic and shown to induce neutralizing antibodies and T-cell responses in different animal models (Rose *et al*., 1998; Fligge *et al*., 2001; Öhlschläger *et al*., 2003; Dell *et al*., 2006; Schädlich *et al*., 2009a). A promising study about the immunogenicity of capsomeres is reported by Yuan *et al*. (2001), in which dogs were completely protected by pentameric capsomeres against papillomavirus infection. Initially, in a direct comparison of immunogenicity, VLPs were reported to be more immunogenic than capsomeres (Thönes *et al*., 2008). However, later Schädlich *et al*. (2009b) showed that capsomeres

produced by the expression of a modified HPV-16 L1 gene, induced antibody titres equivalent to those generated by VLPs.

We selected tobacco chloroplasts as expression platform for vaccine antigens. Plastid-based expression of vaccine antigens is safe for the environment as plastids are not spread through pollen. Another reason to choose plastids for expression of vaccines lies in their ability to carry out post-translational modification and formation of disulfide bonds, which is essential for functional tertiary and quaternary structures and hence for the antigenicity of many proteins (Chebolu and Daniell 2009; Cardi *et al*., 2010). Moreover, compared to nuclear transformation, plastid transformation yields fairly high levels of protein, which is very significant for low-cost vaccine production. Various vaccine antigens have been successfully expressed in plastids (Chebolu and Daniell 2009; Lössl and Waheed 2011). However, there is very limited number of reports regarding the expression of vaccine antigens in fusion with adjuvants.

In the second approach, we expressed a fusion protein, LTB-L1, in tobacco plastids (article 4). LTB was directly coupled with the L1 antigen due to the adjuvant properties of LTB. Although the adjuvant activity of LTB is less than that of holotoxins, adjuvanticity of LTB is much improved when coupled to antigens (Sánchez and Holmgren 2008). This is due to two reasons: first the increased uptake of the coupled antigen across the mucosal barrier and second, more efficient GM1-receptor-mediated uptake and presentation of the coupled antigen by antigen-presenting cells (APCs, Sánchez and Holmgren 2008). Adjuvanticity of LTB is evident from many reports. Upon administration with herpes simplex virus type 1 glycoprotein, LTB has been shown to act as an adjuvant and induced a high degree of protective immunity against ocular infection of mice with live viruses (Richards *et al*., 2001). Adjuvant activity of LTB has also been proved in many other cases such as vaccines targeting the influenza virus haemagglutinin, prion protein and HPV-16 L1 (Verweij *et al*., 1998; Wang *et al*., 2006; Yamanaka *et al*., 2006). In our study, LTB was directly coupled with L1 because separate production and administration of adjuvant is laborious and expensive (Davoodi-Semiromi *et al*., 2009). Direct coupling will not only facilitate the reduction of costs, but also enhance the immunogenicity of the coupled antigen (Guy 2007; Sánchez and Holmgren 2008). Since many plant-produced vaccines show weak antigenicity, adjuvant coupling is also vital to boost the immune response against a plant-derived antigen (Lössl and Waheed 2011). An

additional advantage of fusing LTB with L1 lies in the immunogenic properties of LTB. LTB can act as an immunogen and protect against infectious diarrhoea, caused by ETEC. Hence direct coupling would lead the LTB-L1 protein to confer dual immunity against ETEC and HPV infection. Recently, a vaccine candidate has been expressed in lettuce and tobacco plastids, offering dual immunity against cholera and malaria (Davoodi-Semiromi *et al*., 2010). In the above mentioned study, CTB (a close homologue of LTB) was fused with two malarial vaccine antigens: apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1). The authors showed that mice administered subcutaneously or orally with purified antigens or transplastomic tobacco leaves, were completely immunized against cholera toxin challenge and significant levels of antigen-specific antibody titres were observed against malaria.

Expression of recombinant L1 and LTB-L1 proteins was verified and quantified by Western blotting. L1 protein accumulated up to 1.5% of TSP. Previously, L1 protein has been expressed in chloroplasts to very high levels (24% of TSP) and this plastid-derived protein was shown to be immunogenic in mice (Fernández-San Millán *et al*., 2008). In contrast to Fernández-San Millán *et al*. (2008), yield of L1 protein obtained from plastids in our study is comparable to that of obtained by Lenzi *et al*. (2008). These authors expressed a native viral L1 and a synthetic codon-optimized L1 gene and obtained 1.5% of TSP. However, in both above mentioned studies, L1 protein assembled to VLPs, while in our study L1 protein was expressed as pentameric capsomeres. In case of LTB-L1, recombinant protein accumulated up to 2% of TSP. This amount is comparable to individual expression of LTB and L1 proteins (Kang *et al*., 2003; Waheed *et al*., 2011a). In contrast to Ye *et al*. (2001), who reported high expression of foreign protein due to the fusion of GFP_{14}, low yield was obtained in our studies. One possible reason of low protein accumulation could be the potential susceptibility of foreign proteins to proteolytic degradation. This effect was also observed by Lenzi *et al*. (2008) in case of GST-L1, which showed degradation and accumulated up to only 0.1% of TSP. In case of LTB expression, fused with a hemagglutinin-neuraminidase-neutralizing epitope (HNE) of Newcastle disease virus in plastids, the fusion protein accumulated to 0.5% of TSP (Sim *et al*., 2009). Compared to both above mentioned reports, the expression of LTB-L1 fusion protein is fairly high in our study.

In our study, the assembly of L1 protein to capsomeres was confirmed by CsCl density gradient centrifugation and sucrose gradient sedimentation analysis. In previous studies, these two techniques have been used for the purification and identification of different assembly forms of plant-derived L1 protein by Biemelt *et al*. (2003), Maclean *et al*. (2007) and Fernandez-San Millan *et al*. (2008). In all above mentioned reports, in antigen capture ELISA of sedimented samples, two peaks were obtained. These two peaks were observed in the region of high and low sedimentation coefficients, which referred to VLPs and capsomeres, respectively. In our study, due to the replacement of two cysteines, VLP formation was unlikely. Yet, in our results, two peaks were obtained: one for capsomeres and another peak in the sedimentation region between the VLPs and capsomeres. This suggested the formation of some higher assembly forms of L1 protein other than VLPs. These results obtained in our study are supported by Schädlich *et al*. (2009b): these authors expressed double cysteine mutant L1 gene (as used in our study), and they also observed other higher assembly forms in addition to capsomeres, resembling small VLPs in electron microscopy.

For immunogenicity of subunit vaccines and functionality of adjuvants, it is necessary that proteins are properly folded and retain the conformational epitopes. In case of L1 and LTB-L1 proteins, retention of immunogenic epitopes was verified by antigen capture ELISA, using conformation specific antibody Ritti01. This antibody has been used by Lenzi *et al*. (2008) to confirm the correct conformation of plastid-derived L1 protein. In case of LTB-L1 fusion protein, we additionally confirmed the folding of LTB portion. Interestingly, the native conformations of both LTB and L1 proteins are their respective pentameric forms. To prove the concept that both partners in the LTB-L1 fusion protein assembled into their proper conformations, we additionally confirmed the pentameric conformation of LTB protein by GM1-ganglioside binding ELISA. These results show that the fusion of LTB protein with L1 did not have any effect on the folding of L1 protein and *vice versa*. This indicates that the quaternary structures of both proteins were maintained due the spacer sequence GPGPG between LTB and L1, as this linker is recommended for the recovery of functional peptides and the restoration of immunogenicity against different protein epitopes (Sette *et al*., 2001; Livingston *et al*., 2002).

In the first study (expression of L1 in plastids), male sterility was observed in transplastomic plants. However, plants showed normal growth and morphology like wild type tobacco plants. In the second study, expression of LTB-L1 in tobacco chloroplasts caused detrimental effects on tobacco plants: all transplastomic lines were chlorotic, male sterile and showed stunted growth. To investigate the reason for these effects, we transformed the plants with LTB gene only. Interestingly, the LTB transplastomic plants showed healthy and normal phenotypes. In previous reports, expression of L1 (Fernandez-San Millan *et al*., 2008; Lenzi *et al*., 2008) and LTB protein (Kang *et al*., 2003) in plastids, did not cause any detrimental effects on tobacco plants. Moreover, expression of LTB fusion proteins in plastids also did not affect growth and morphology of transplastomic plants (Rosales-Mendoza *et al*., 2009; Sim *et al*., 2009). Thus our findings lead to the hypothesis that the pleiotropic effects observed in LTB-L1 transformed plants can be specifically due to the expression of LTB-L1 fusion protein in plastids. Although, in majority of reports plastid-based expression of foreign proteins generated normal phenotypes. Yet there are many studies which show the appearance of aberrant phenotypes due to plastid transformation (for review see Lössl and Waheed 2011). Several reasons could account for pleiotropic effects caused by the expression of foreign proteins in plants. These effects can be due to the interference of specific transgene products with certain metabolic processes of plants. Over-expression of proteins can also cause phenotypic alterations in plants, as evident from the report of Oey *et al*. (2009). However, recently Ruhlman *et al*. (2010) showed that the over-expression of foreign proteins (~ 72% of TSP) did not have any negative effects on plants. This shows that hyper-expression may not be the necessary cause of aberrant phenotypes of plants. Alternatively, to evade such effects on plants, the expression of proteins can be regulated by inducible expression (Lössl *et al*., 2005; Lössl and Waheed 2011). Nevertheless, in our studies, transplastomic lines reached maturity and produced seeds by pollination with pollen from wild type plants. Further investigations are needed to study these pleiotropic effects in detail.

6. Conclusions

The data presented in our studies show that the expression of modified HPV-16 L1 in chloroplasts is feasible. Chloroplasts are able to carry out the correct folding of L1 protein to capsomeres and conformational epitopes of L1 are retained that are necessary for the immunogenicity of L1 antigen. The retention of antigenic epitopes is shown *in vitro* by the binding of L1 protein to an HPV-16 L1 specific antibody in antigen capture ELISA. This study provides a platform for the development of cost-effective second-generation vaccines against HPV. Data obtained in the second study show that two pentameric proteins, LTB and L1 can be successfully expressed in plastids. Neither of the two pentameric proteins affects the folding of other. In addition to L1, LTB protein also folds to its native pentameric conformation. Both, L1 and LTB-L1 recombinant proteins are expressed to significant levels in tobacco plastids. We adapted a novel approach of directly coupling the LTB adjuvant to the L1 antigen. This approach is promising as it can reduce the costs related to separate production and administration of adjuvants. Owing to immunogenic properties of LTB, LTB-L1 fusion protein can provide dual immunity against ETEC and HPV infections, respectively. Chloroplast-based expression of LTB-L1 has the potential for the possible development of a low-cost vaccine, which due to the direct coupling of adjuvant will have enhanced immunogenicity against HPV. Nevertheless, the expression of LTB-L1 in tobacco plastids was linked to growth retardation and chlorosis of transplastomic plants, which needs further research and investigation. Taken together, the results obtained in the present studies will pave a way to the development of affordable plant-based vaccines against HPV.

7. Future Perspectives

Based on the obtained results, further research is planned as follows:

7.1 Immunogenicity of L1 and LTB-L1 *in vivo*

After the confirmation of antigenic epitopes of L1 and LTB-L1 proteins *in vitro*, it is important to test the immunogenicity of plastid-derived L1 and LTB-L1 proteins in animal models. Our group has research collaboration with German Cancer Research Centre (DKFZ), Heidelberg, Germany. In future, we are planning to investigate the immunogenic properties of L1 and LTB-L1 proteins in mice.

7.2 Inducible expression of LTB-L1 in chloroplasts

The pleiotropic effects observed in tobacco plants by the expression of LTB-L1 protein can be overcome by inducible expression. For this purpose, an ethanol inducible system, already established for tobacco by Lössl *et al*. (2005) can be used. This approach could help to investigate the hypothesis that the expression of LTB-L1 protein is specifically responsible for the detrimental effects on plants.

7.3 Transformation of edible plant species

Transplastomic plants can be potentially used as edible material either in unprocessed or partially processed form. Recently, there have been promising advancements in establishing the protocols for the transformation of edible plants such as lettuce, spinach, cabbage and tomato (Lössl and Waheed 2011). Due to high contents of nicotine and other alkaloids, tobacco is not an optimal candidate for edible vaccine production. Therefore, the research work performed in the present study is planned to further expand to edible plant species.

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