Chloroplast based expression of recombinant Dengue virus envelope proteins in plants

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by

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For my beloved family
and
my wonderful friends

"Bewahre mich vor der Angst, ich könnte das Leben versäumen.
Gib mir nichts, was ich mir wünsche, sondern was ich brauche.
Lehre mich die Kunst der kleinen Schritte."
(Antoine de Saint-Exupéry)

Preliminary remarks

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This is to certify that this PhD dissertation entitled "**Chloroplast based expression of recombinant Dengue virus envelope proteins in plants**" submitted to obtain the degree of Dr. nat. techn. at the University of Natural Resources and Applied Life Sciences, Vienna, is a record of bona fide work carried out by Dipl.-Ing. Johanna Anneliese Maria Gottschamel, Bakk.techn., University Enrolment no. 0240190 at the Department for Crop Sciences and no part of the project work has been submitted for any other degree or diploma.
Her dissertation is satisfactory for submission in partial fulfillment of the requirement for Dr. nat. techn of University of Natural Resources and Applied Life Sciences, Vienna.

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Abstract

Plants are economic, safe and clean expression systems with the potential to serve as production platforms for recombinant proteins with almost unlimited scalability. Integration of transgenes into the chloroplast genome offers the advantage of targeted and site specific integration and the obtainable recombinant protein yields are mostly higher compared to nuclear transformation. Furthermore, the maternal inheritance of chloroplasts in most plant species strongly limits transmission of transgenes via pollen. The time consuming construction of plastid transformation vectors can be shortened by implementing the Gateway® recombinant cloning technology which provides a rapid and highly efficient way to transfer DNA fragments between vectors carrying compatible recombination sites. This accelerates the development of pharmaceutical products from plastids. Dengue Fever is the most rapidly spreading mosquito transmitted viral disease threatening more than 40 % of the world's population. The Dengue virus is typically transmitted by the bite of the blood feeding, day active mosquito *Aedes aegypti*. The virus occurs in four closely related but antigenically and genetically distinct serotypes (DEN 1, DEN 2, DEN 3 and DEN 4) for which currently no approved vaccine is available.

The present work demonstrates that chloroplasts are a suitable and cost-effective production platform for a recombinant protein vaccine candidate based on the Dengue virus envelope protein domain III (EDIII). The functionality of the constructed Gateway® compatible plastid transformation vector was first proven by the expression of GFP using this vector in tobacco chloroplasts. Transplastomic plants were generated with the biolistic method, the homoplastomic state of the regenerated plants was verified by Southern blot and protein expression levels were analysed by Western Blot. The recombinant proteins EDIII 1 and EDIII 3 were obtained through constitutive expression in tobacco chloroplasts and expression of EDIII 1, EDIII 2, EDIII 4 and EDIII 1-4 was achieved with the inducible expression system upon ethanol induction. Furthermore, the Dengue antigenes EDIII 1 and EDIII 1-4 were also constitutively expressed in lettuce chloroplasts in order to facilitate the development of an oral vaccine candidate.

The data obtained here demonstrate the feasibility of expressing EDIII based antigens in chloroplasts and they contribute to the development of a safe, efficacious and affordable vaccine against Dengue fever.

Kurzfassung

Pflanzen sind wirtschaftliche, sichere und saubere Expressionssysteme und können als Produktionsplattformen für rekombinante Proteine mit nahezu unbegrenzter Erweiterbarkeit dienen. Die Integration des Transgens in das Chloroplastengenom bietet den Vorteil einer gezielten und ortsspezifischen Integration und die erzielten Ausbeuten für rekombinante Proteine sind meistens höher als bei Kerntransformationen. Außerdem verhindert die maternale Vererbung von Chloroplasten in den meisten Pflanzen die Verbreitung der Transgene durch Pollen. Die zeitraubende Konstruktion von Plastiden-Transformationsvektoren kann durch die Gateway® recombinant cloning technology verkürzt werden. Die Adaption dieser Technologie für Plastiden ermöglicht eine schnelle und sehr effiziente Übertragung von DNA-Fragmenten zwischen Vektoren mit kompatiblen Rekombinationstellen. Dies beschleunigt die Entwicklung von pharmazeutischen Produkten aus Plastiden. Dengue-Fieber bedroht mehr als 40% der Weltbevölkerung und ist die am raschesten grassierende Viruserkrankung die durch Stechmücken übertragen wird. Das Dengue-Virus wird typischerweise durch die Blut saugende, tagaktive Mücke *Aedes aegypti* übertragen. Das Virus tritt in vier eng verwandten, aber antigenisch und genetisch unterschiedlichen Serotypen auf (DEN 1, DEN-2, DEN DEN 3 und 4) gegen die derzeit kein Impfstoff erhältlich ist.

Die vorliegende Arbeit zeigt, dass Chloroplasten eine geeignete und kostengünstige Produktionsplattform für einen auf dem Dengue Virus Hüllprotein Domäne III (ED III) basierenden Impfstoffkandidaten sind. Die Funktionalität des konstruierten Gateway® Plastiden-Transformationsvektor wurde zuerst durch die Expression von GFP unter Verwendung dieses Vektors in Tabak Chloroplasten überprüft. Die rekombinanten Proteine EDIII 1 und EDIII 3 wurden durch konstitutive Expression in Tabak-Chloroplasten erzeugt, während die Expression von EDIII 1, EDIII 2, EDIII 4 und EDIII 1-4 mit dem Ethanol- induzierbaren Expressionssystem erreicht wurde. Um die Entwicklung eines oralen Impfstoff-Kandidaten zu erleichtern, wurden die Dengue Antigene EDIII 1 und EDIII 1-4 zusätzlich konstitutiv in Salat Chloroplasten produziert.

Die hier präsentierten Daten beweisen, dass die Expression der EDIII basierten Antigene in Chloroplasten möglich ist und sie tragen zur Entwicklung eines sicheren, wirksamen und erschwinglichen Impfstoffes gegen Dengue-Fieber bei.

1 Introduction

1.1 Plants as green factories for pharmaceutical proteins

Throughout history plants have been gathered, cultivated and processed by mankind not only for food and feed, but also as a valuable source for medicinal products. During the last three decades, advances in biotechnology have extended the utilization of plants well beyond these traditional applications by converting plants into factories for diagnostic reagents, pharmaceutical proteins and industrial enzymes (Ma & Wang, 2012). Although, most of the available recombinant protein drugs are mainly produced in mammalian cells, in *E.coli*, in yeast or insect cells, the recent advances in genetic engineering and molecular biology have created the possibility of producing biopharmaceutical proteins like antibodies, vaccines, growth factors, enzymes and hormones using genetically modified plants (Ma & Wang, 2012). Moreover, the conventional fermenter-based approaches are difficult to scale up and the risk of contamination with endotoxins or human pathogens results in the need of complex purification systems and downstream processing (Daniell et al, 2009; Fischer & Emans, 2000). Plant based expression systems on the other hand offer an inexpensive, safe and clean production platform for recombinant proteins. They are more economic compared to mammalian cell culture, due to simple growth requirements and an almost unlimited scalability. There is no risk of contamination from potential human pathogens as plants are not hosts for human infectious agents and plants possess the ability to carry out a couple of post-translational modifications, which are often required for the biological function of therapeutic proteins (Bock, 2014; Clarke et al, 2011; Scharff & Bock, 2013). For the production of a particular target protein, based on its specific characteristics, one of the following expression approaches can be chosen: a) stable expression from the nuclear genome achieved either *via* biolistic transformation or *via* *Agrobacterium*-mediated transformation; b) expression from the plastid genome; c) *Agrobacterium*-mediated transient expression or; d) expression from plant tissues carrying recombinant plant viral sequences (Streatfield, 2007).

One of the crucial steps in plant based recombinant protein expression is the selection of a suitable plant species, which is amenable for transformation, has a high protein expression potential, allows the correct folding of the recombinant protein including post-translational modifications and has the possibility of extensive accumulation (Tiwari et al, 2009). Several plant species have been examined as platforms for the safe

production of pharmaceutical proteins providing advantages such as scalability, environmental containment and lower overall costs than conventional manufacturing processes (Melnik & Stöger, 2013). Tobacco is an easily transformed plant and it has been the model system for plant transformation for decades (Bevan et al, 1983; Horsch et al, 1985), but the technology has vastly expanded and the list of plant expression hosts now includes leafy crops (alfalfa, lettuce, clover), legumes (soybean, pea, pigeon pea), simple plants (*Physicomitrella patens*, *Chlamydomonas reinhardtii*, Lemna), cereals (rice, wheat, barley, maize), fruits and vegetables (tomato, potato, carrot, cabbage, banana), oil crops (oilseed rape and safflower) and the model plant *Arabidopsis thaliana* (Fischer et al, 2004).

Since the first report on expression of a monoclonal antibody in transgenic tobacco (Hiatt et al, 1989) and the expression of the antigenically functional hepatitis B surface antigen (HBsAg) as the first vaccine protein ever produced in plants (Mason et al, 1992), a great number of economically interesting molecules have been obtained from plant based expression (Basaran & Rodriguez-Cerezo, 2008; Rybicki, 2010). Many plant derived therapeutics are undergoing clinical trials (Yusibov et al, 2011) and extensive research in molecular farming lead to the successful production and commercialization of several biopharmaceuticals (Faye & Gomord, 2010).

Three plant-made pharmaceuticals have already been approved for human use:

1) a secretory IgA used for the prevention of tooth decay produced in transgenic tobacco (CaroRx™ from Planet Biotechnology Inc, USA, www.planetbiotechnology.com/products.html);
2) a human intrinsic factor used as a dietary supplement for the treatment of vitamin B-12 deficiency produced in *A. thaliana* leaves (Cobento Biotech AS, Denmark, (Fedosov et al, 2003));
3) the enzyme taliglucerase alfa, a recombinant glucocerebrosidase for treatment of Gaucher's disease, produced in carrot cell suspension culture (Elelyso from Pfizer Inc., USA and Protalix BioTherapeutics Inc., Israel, www.elelyso.com).

In addition, the vaccine against poultry's Newcastle disease produced in tobacco suspension culture by Dow AgroSciences has obtained USDA approval and Medicago Inc. (Canada) has reported positive final results from its H5N1 Avian Influenza VLP vaccine Phase II trial.

Since the WHO stated in 2005 that plant derived vaccines are a promising strategy to increase the availability of affordable vaccines in resource poor countries (van der Laan et al, 2006), plants have become an attractive alternative vaccine production system (Arntzen et al, 2005; Daniell et al, 2009; Hefferon, 2013; Streatfield, 2007). These novel efforts have led to the production of various antigens in plants and these plant made vaccines can be administered orally, intramuscularly or as intravenous injections after isolation and purification from the plant tissues (Kwon et al, 2013; Roy et al, 2010a). So far, several plant-derived vaccine candidates and antibodies against infectious diseases predominantly occurring in developing countries, like hepatitis B virus (Chen et al, 2011; Huang et al, 2008; Thanavala et al, 2005), rabies (Roy et al, 2010a; Roy et al, 2010b; Yusibov et al, 1997) and human papillomavirus (Maclean et al, 2007; Regnard et al, 2010; Waheed et al, 2010; Waheed et al, 2011b) have been developed.

1.2 Chloroplast based expression of recombinant proteins

Despite the recent developments and improvements, there are some limitations and safety concerns about the use of nuclear transformed plants for vaccine production including low transgene expression levels (Daniell et al, 2001b) and the serious problem of transgene out-crossing via pollen mediated gene flow (Pilson & Prendeville, 2004). Transgene spread can be prevented by the use of plants with male sterility, by plants that are infertile and clonally propagated or by transient expression systems for use in greenhouse containment.

Another, effective way to prevent transgene transmission is integration of the transgenes into the plastid genome. Valuable improvements can be provided to the current state of transgenic plants by application of the transplastome technology (Koop et al, 1996; Svab et al, 1990). Remarkable increases in antigen yield have been reported for chloroplast based expression systems when compared to nuclear approaches (Daniell et al, 2001a; Rigano et al, 2009; Scotti et al, 2009).

1.2.1 Chloroplast transformation

Chloroplasts are subcellular compartments confined by a double membrane and specialized in photosynthesis. These plant cellular organelles contain their own DNA (ptDNA) and transcription and translation machinery. The circular double stranded DNA, with a size range of 120 to 180 kb depending on the plant species, is highly polyploid and in most higher plants organized in a quadripartite way with 2 inverted repeat regions (IR) spanning between the long-single-copy and the short-single copy region (Shaw et al, 2007). The IR region is characterized by the presence of the ribosomal RNA genes and a variable number of additional genes depending on the plant species (Staub, 2002). The site specific integration of transgenes into these spacer regions avoids position effects and gene silencing (Daniell, 2006). The presence of up to 10.000 copies of ptDNA in photosynthetic cells (Bendich, 1987) is beneficial to obtain very high recombinant protein expression levels (Bock & Warzecha, 2010; Koop et al, 2007; Maliga, 2002) with the current reported maximum above 70% of total soluble protein (Oey et al, 2009). Chloroplast transformation has emerged as a very precise alternative genetic engineering technique to prevent transgene spread via pollen as chloroplasts are maternally inherited in most plants (Daniell, 2007) and the absence of plastids in pollen eliminates the risk of out-crossing. Furthermore, the possibility of expressing several different transgenes simultaneously using operons (Bock, 2013), the ability of

the plastid translation machinery to produce recombinant proteins with proper folding, disulfide bond formation and lipidation (New et al, 2012) and the absence of epigenetic gene silencing mechanisms (Daniell et al, 2001a; Rigano et al, 2009; Verma et al, 2010) makes chloroplasts a very attractive expression platform for biopharmaceutical proteins. Chloroplast transformation is mainly obtained via the biolistic approach (Svab et al, 1990) and integration of the transgene expression cassette into the plastid genome occurs site-directed via homologous recombination of flanking sequences (Maliga, 2004). The vector DNA, carrying the transgene expression cassette containing the gene of interest (goi) and an antibiotic resistance gene, is coated onto small gold particles used as micro-projectiles that transport the foreign DNA into the plant cell (Sanford, 1990). Integration of the transgene expression cassette is based on the site specific recombination between homologous sequences in the plastid genome and the transformation vector (Figure 1). The *aad*A gene (aminoglycoside 3′ adenyltransferase), conferring resistance against both antibiotics spectinomycin and streptomycin, is included for selection of transformed plant cells (Svab & Maliga, 1993). The transformed tissue is regenerated into complete plants on revised medium for organogenesis of plants (RMOP) containing the respective antibiotic. To achieve homoplasmic plants, i.e. plants containing only transformed plastids, regenerated shoots are subjected to an additional round of selection on antibiotic containing regeneration medium (Singh et al, 2009). Plastid transformation is regularly used in tobacco but has rapidly expanded to other crops including potato (Sidorov et al, 1999), tomato (Zhou et al, 2008), lettuce (Kanamoto et al, 2006; Lelivelt et al, 2005), soybean (Dufourmantel et al, 2004; Moravec et al, 2007), cauliflower (Nugent et al, 2006).

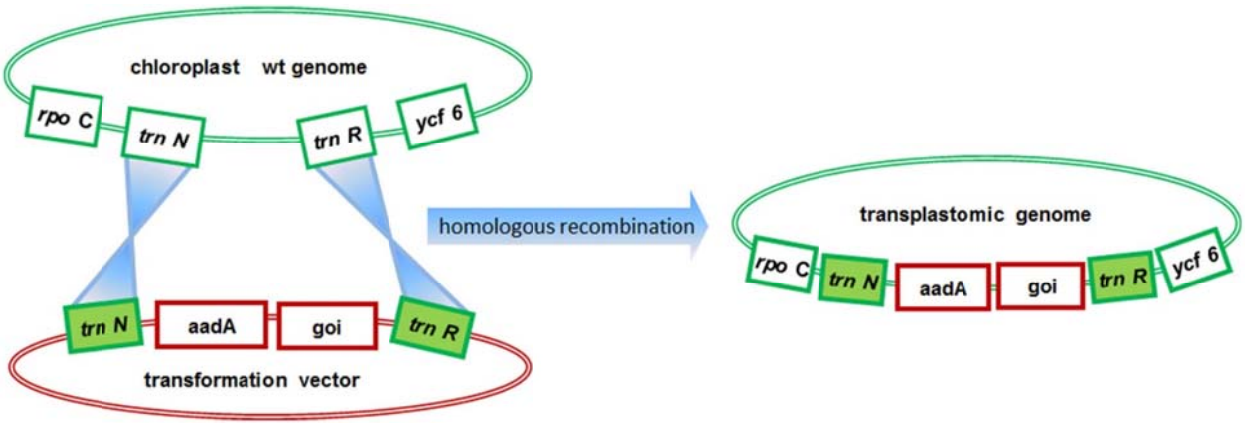


Figure 1 Homologous Recombination. Site-directed integration of the transgene expression cassette into the intergenic spacer region of the chloroplast genome occurs via homologous recombination facilitated by the homologous regions (*trnN* and *trnR*) in the plastid genome and the transformation vector.

1.2.2 Vaccine candidates expressed in the chloroplast

Most attempts to produce chloroplast derived antigenes have been made for bacterial diseases, but also viral antigens and vaccines targeting protozoan parasites have been expressed. Vaccines against enterogenic *E.coli (Kang et al, 2004; Kang et al, 2003; Rosales-Mendoza et al, 2009; Sim et al, 2009)*, *Clostridium tetani* (Tregoning et al, 2005; Tregoning et al, 2003), *Bacillus anthracis (Gorantala et al, 2011; Koya et al, 2005; Watson et al, 2004)*, *Yersinia pestis* (Arlen et al, 2008), *Borrelia burgdorferi* (Glenz et al, 2006) and a multi-epitope vaccine against diphtheria, pertussis and tetanus (Soria-Guerra et al, 2009) have been produced through the chloroplast. Several viral epitopes have been expressed including L1 protein of HPV-16 (Fernandez-San Millan et al, 2008; Waheed et al, 2010), Vaccinia virus envelope protein (Rigano et al, 2009), Hepatitis E E2 (Zhou et al, 2006), Hepatitis C core protein (Madesis et al, 2010) and Epstein-Barr viral capsid antigen complex (Lee et al, 2006). Functional evaluation by inhibition of proliferation of the malarial parasite have been reported for apical membrane antigen-1 and merozoite surface protein-1 expressed in tobacco and lettuce chloroplasts (Davoodi-Semiromi et al, 2010).

1.2.3 Inducible transgene expression

Although, most foreign proteins are non-toxic in chloroplasts in some cases, abnormal phenotypes like chlorosis of the leaves, male sterility and growth retardation have been reported (Lössl et al, 2003; Oey et al, 2009; Ruf et al, 2007; Tregoning et al, 2003; Waheed et al, 2011b). Inducible expression systems could provide a tool to overcome these detrimental effects by controlling the transgene expression and production of foreign protein at any developmental stage or even post-harvest (Lössl & Waheed, 2011). Several regulatory systems achieving inducibility through chemicals (Caddick et al, 1998; Gatz, 1997; Padidam, 2003), light conditions (Boetti et al, 1999), pathogen infection (Johnson et al, 2003; Lebel et al, 1998) or growth stage dependent-conditions (Hennig et al, 1993; Hoff et al, 2001) have been investigated. More recently, a synthetic plastid encoded riboswitch allowing inducible expression after exogenous theophylline application has been reported (Verhounig et al, 2010).

The very first trans-activation system for chloroplast expression was developed using ethanol as an inductor and transforming both the nucleus and the plastid genome (Lössl et al, 2005). The nuclear encoded, plastid targeted RNA polymerase from λ bacteriophage T7 (McBride et al, 1994) is expressed using the ethanol inducible *alcA*

promoter and the transcription factor AlcR derived from *Aspergillus nidulans*' alcohol dehydrogenase regulon (Caddick et al, 1998; Roslan et al, 2001; Salter et al, 1998). The T7 RNA polymerase (T7RNAP) is targeted to the chloroplast due to the N-terminally fused transit peptide of the small RuBisCo subunit from *Pisum sativum* (Dasgupta et al, 1998; Nawrath et al, 1994). Upon ethanol spraying of the plants, the T7RNAP is produced and transported into the chloroplast where it recognizes the T7 promoter controlling the expression of the desired transgene (Figure 2). This system has been successfully implemented to overcome the growth retardation and male sterility observed in plants constitutively expressing polyhydroxybutyric acid (PHB) in plastids (Lössl et al, 2003). Upon ethanol induction the transplastomic plants, transformed with the inducible expression system of the *phb* operon, produced PHB, and in the absence of ethanol flowers and fertile seeds developed (Lössl et al, 2005). Comparison of inducible and constitutive expression showed that the expression of PHB synthase reached a level equal to that obtained from constitutive expression after seven days of ethanol induction (Lössl & Waheed, 2011).

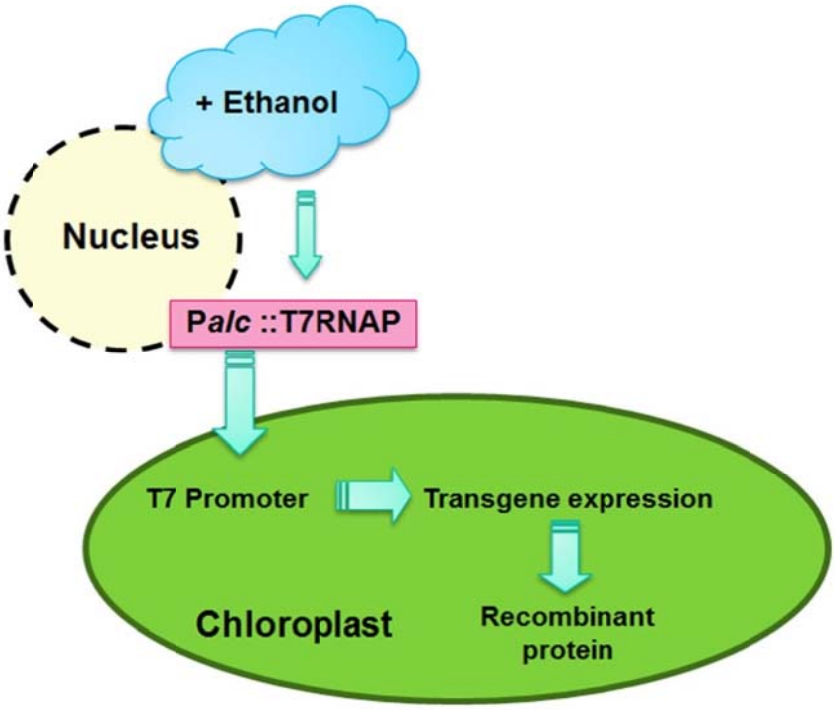


Figure 2 Ethanol induced recombinant protein expression. Upon ethanol spray of the plants, the T7RNAP is transcribed and translated in the nucleus and then transported into the chloroplast where it recognizes the T7 promoter and switches on the expression of the transgene (Lössl et al, 2005).

1.3 Gateway® recombinant cloning technology

The Gateway® recombinant cloning technology is a very accurate cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) providing a rapid and highly efficient way to transfer DNA fragments between vectors carrying compatible recombination sites (**Error! Reference source not found.**). This system carries out two reactions:

(1) the BP-reaction: *att*B x *att*P → *att*L + *att*R mediated by the integrase (Int) and integration host factor (IHF) proteins and

(2) the LR reaction: *att*L x *att*R → *att*B + *att*P mediated by Int, IHF and excisionase (Xis) (Hartley et al, 2000).

The main limitation of cloning with traditional restriction enzymes that might also cut inside the gene of interest's sequence is circumvented by using the Clonase® enzyme mixes (Invitrogen^{TM}) which contains the necessary enzymes for each reaction. The Entry clone is created by the Gateway® BP Clonase® enzyme mix mediated transfer of the *att*B sites flanked gene of interest (goi) into the *att*P site bearing pDONR221^{TM}. The fragment containing the goi flanked by *att*B1 and *att*B2 can either be produced by PCR or by linearizing a suitable plasmid. Subsequently, the fragment in the Entry clone can be transferred to any Destination vector containing *att*R sites using the Gateway® LR Clonase® enzyme mix (Karimi et al, 2002). The *att* recombination sites in the Donor and the Destination vectors flank a *ccd*B gene (control of cell death) and a chloramphenicol-resistance gene (Bernard, 1995), thus can only be propagated in *ccd*B survival^{TM} *E.coli* that contain a *gyrA462* mutation providing resistance to the lethal effects of the *ccd*B gene. The presence of the counterselectable *ccd*B gene and the chloramphenicol resistance gene in both the Donor and the Destination vector provides a unique system of negative selection that eliminates all unwanted by-product plasmids after recombination resulting in maximum cloning efficiency.

The system has been successfully used for the creation of expression vectors for different bacterial-, yeast-, mammalian- and insect cell culture- expression systems (Katzen, 2007) which are commercially available and marketed by Life Technologies (Carlsbad, CA, USA, http://www.lifetechnologies.com).

Several plant Destination vectors have been constructed for aims including protein localization, promoter functional analysis, gene overexpression, gene knockdown by RNA interference, production of epitope-tagged proteins for affinity purification, or analysis of protein/protein interactions using fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) or bimolecular

fluorescence complementation (BiFC) (Earley et al, 2006). Plants have been transformed with Gateway® compatible vectors via several methods including *Agrobacterium*-mediated delivery, PEG transformation, particle bombardment and electroporation, although only vectors for transient expression or stable nuclear transformation have been reported up to now (reviewed by Dubin et al, 2008; Karimi et al, 2007).

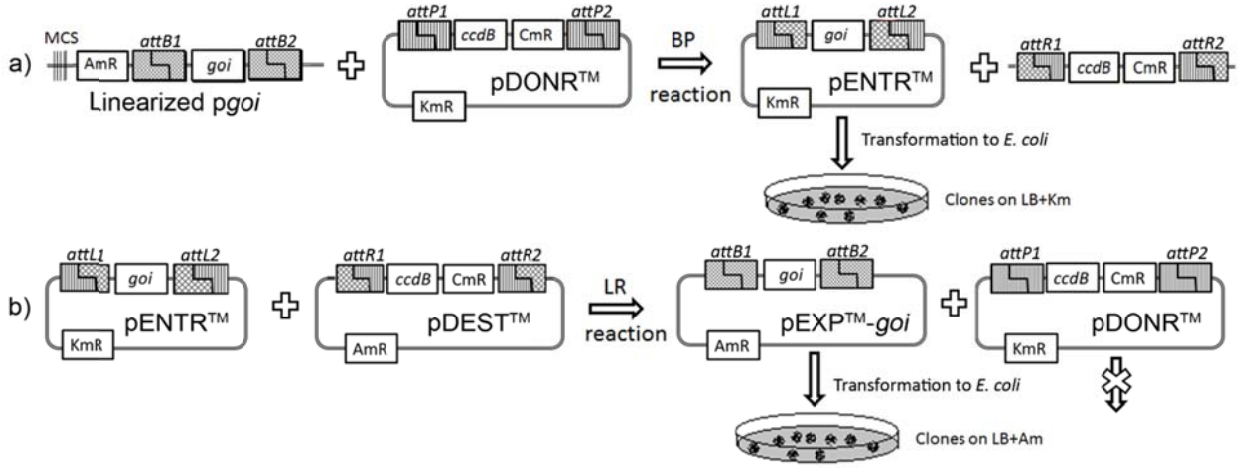


Figure 3 Scheme of Gateway® recombinant cloning technology (Gottschamel et al, 2013). a) In the BP reaction the Integrase, mediates the insertion of the goi sequence into the donor vector (pDONR^{TM}) yielding the Entry clone (pENTR^{TM}). (b) In the LR reaction the goi is then integrated into the Destination vector (pDEST^{TM}) mediated by Integrase and Excisionase yielding the final Expression clone (pEXP^{TM}-goi) which is used for chloroplast transformation and protein expression. Ordinary *E.coli* cells transformed with the pDONR^{TM}, which is the by-product of the LR reaction, cannot grow due to the lethal effects of the *ccd*B gene. Am: ampicillin; AmR: ampicillin resistance gene; Km: kanamycin; KmR: kanamycin resistance gene; LB: LB medium; *att*B1/B2/P1/P2/L1/L2/R1/R2: Gateway® recombination sites.

1.4 Dengue Fever

Dengue Fever is the most rapidly spreading mosquito-transmitted viral disease threatening more than 40 % of the world's population. This febrile disease is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific. Dengue transcends international boundaries and is emerging rapidly as the consequence of globalization, rapid unplanned and unregulated urban development, improper water storage, unsatisfactory sanitary conditions, climate change and global warming (Chaturvedi & Nagar, 2008). In 2010 the first European local transmissions of Dengue were reported in France and Croatia and in 2012 an outbreak of Dengue on Portugal's island Madeira resulted in over 2 000 cases (WHO, 2013). Infection with the Dengue viruses can cause Dengue fever (DF), Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Dengue fever is a flu-like illness accompanied by symptoms like headache, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands or rash. The severe forms DHF and DSS are potentially deadly complications due to plasma leaking, fluid accumulation, respiratory distress, severe bleeding or organ impairment (WHO, 2013). Dengue infections are a significant cause of morbidity and mortality and lead to adverse social and economic impacts in many developing tropical countries (Gubler, 2002). The WHO estimates 50 - 100 million new infections occurring each year (WHO, 2013), an additional 500 000 cases of DHF/DSS and over 20 000 Dengue related deaths each year (WHO, 2006).

The Dengue virus belongs to the genus *Flavivirus*, family *Flaviviridae* (Calisher et al, 1989) and its genome is a ~11 kb long positive single stranded RNA (Schlesinger, 1977). The RNA is transcribed as polycistrons and the polyprotein undergoes post-translational cleavage by viral and host proteases generating three structural and 7 non-structural proteins (Clyde et al, 2006; Lindenbach & Rice, 2003). The enveloped virus particles are of icosahedral shape with a diameter of 500 Å corresponding to 50 nm (Kuhn et al, 2002). The virus is typically transmitted by the bite of the blood feeding, day active mosquito *Aedes aegypti* (WHO, 2009) and occurs in four closely related but antigenically and genetically distinct serotypes DEN-1, DEN-2, DEN-3 and DEN-4 (Weaver & Vasilakis, 2009). Infection with any one serotype usually causes the mild form of the disease (Dengue fever) and provides lifelong homologous immunity to that serotype with only transient cross protection against the others (Kurane & Ennis, 1992). However, subsequent infection with a different serotype leads to the life-threatening forms of the disease: DHF and DSS. Dengue pathogenesis appears to

be the result of complex interactions of host and viral factors, with the two most evident contributors being antibody dependent enhancement and inherent virulence of the Dengue viruses (Swaminathan & Khanna, 2009). One hypothesis for the increased severity seen in secondary infections is a mechanism called “antibody dependent enhancement” (ADE) (Halstead, 2003). It has been reported that antibodies produced during a primary infection are highly cross-reactive and non-neutralizing among the Dengue virus serotypes and therefore can complex with and facilitate the uptake of heterotypic viruses into Fc-receptor bearing cells which leads to their increased replication and potently promotes ADE (Dejnirattisai et al, 2010; Halstead, 1988). Phylogenetic analysis identifying two different genotypes of DEN-2: the “Asian” genotype associated with DHF and the “American” genotype associated with DF (Rico-Hesse et al, 1997) go along with the suggestion that certain strains have inherent virulence making them capable of causing the severe disease form while other strains are associated only with the mild form (Swaminathan & Khanna, 2009). General consensus regarding DHF pathogenesis is that a massive cytokine storm triggered directly or indirectly by various factors is responsible for endothelial cell damage leading to plasma leakage (Pang et al, 2007).

Currently there is no approved vaccine to protect against Dengue fever (WHO, 2013). The treatment of acute Dengue is supportive, using either oral or intravenous rehydration for mild or moderate disease, and intravenous fluids and blood transfusions for more severe cases. Prevention of Dengue transmission is currently only possible through vector control and protection from the bites of infected mosquitos through various strategies:

- the use of personal household protection such as window screens, long-sleeved clothes, insecticide treated materials, coils and vaporizers;
- preventing mosquitoes from accessing egg-laying habitats by environmental management, applying appropriate insecticides to water storage outdoor containers and removing artificial man-made habitats;
- application of insecticides as space spraying during outbreaks as one of the emergency vector control measures (WHO, 2013).

1.5 Vaccine development against Dengue Fever Virus

The most effective way to reduce disease and mortality rates of infectious diseases is to vaccinate susceptible populations at risk. The development of a vaccine against Dengue fever offers the potential for effective prevention and long-term control of the virus infection. Despite this, more than 60 years after the discovery of the virus and the start of systematic research into Dengue vaccines, no such vaccine has been brought to the market yet (WHO, 2013). The development of the vaccine has been hindered and delayed by the complex pathogenesis of the disease, by the need to control four viruses simultaneously and by the lack of a suitable animal model. However the increasing spread and intensity of the disease over the past years has triggered new interest and investment in Dengue vaccine research.

1.5.1 Current status of vaccine development

Infection with any one of the Dengue serotypes provides lifelong immunity to that serotype, but secondary infection with a different serotype can predispose an individual to potentially fatal DHF and DSS**.** Anti-dengue antibodies specific to one serotype cross-react with the remaining serotypes, but do not cross-protect against them (Halstead, 1988). This has prompted the view that an efficient Dengue vaccine must be tetravalent, procuring protection against all four virus serotypes at once (Hombach et al, 2005). The main strategies applied for vaccine production against Dengue fever consist in traditionally and molecularly attenuated live viruses, chimeric live virus vaccines, vector based vaccines, DNA vaccines and recombinant subunit vaccines (Guzman et al, 2009).

The most advanced Dengue vaccine candidates have been developed as single serotype-specific vaccine formulations (monovalent vaccines) and are being evaluated as physical four-in-one mixtures for their capacity to elicit protective immunity against the four serotypes (Swaminathan et al, 2010). Empirically attenuated vaccine strains for all four Dengue serotypes have been obtained by repeated serial passage in primary dog kidney cells (Halstead & Marchette, 2003) by two independent research groups. The Mahidol University in Thailand (Bhamarapravati & Yoksan, 2001) licensed their vaccine candidate strains to Sanofi Pasteur (France, Mahidol vaccine), while the Walter Reed Army Institute of Research (Eckels et al, 2000b) licensed their candidate strains to GlaxoSmithKline (Belgium, WRAIR vaccine) for large scale production and further evaluation. Repeated reports of unbalanced immune responses in human trials using

the attenuated tetravalent vaccine formulations of both, the Mahidol (Edelman et al, 2003; Kanesa-Thasan et al, 2001) and the WRAIR vaccine (Eckels et al, 2000a; Sun et al, 2003) have stalled further development and commercialization of these vaccine candidates.

An alternate strategy has been adopted by Sanofi-Pasteur where the structural genes of the empirically attenuated Yellow fever virus strain 17D (YF17D, (Monath, 1997)) were replaced with the premembrane (prM) and envelope (E) gene of the Dengue viruses to create four monovalent chimeric yellow fever Dengue vaccine strains (CYD strains, (Guirakhoo et al, 2001)). Immunization of monkeys with a tetravalent vaccine formulation resulted again in an unbalanced immune response with the highest response being directed against DEN-2 (Guirakhoo et al, 2001). However, after several dose adjustments and promising results of an administration study in healthy adult volunteers (Morrison et al, 2010), the tetravalent CYD formulation entered Phase II trials. The results reported by the pediatric phase 2b trial in Thailand showed good protection against DEN-1, DEN-3 and DEN-4, but not against DEN-2 (Sabchareon et al, 2012). Currently, the tetravalent vaccine candidate CYD15 is undergoing a phase III clinical trial in Dengue-endemic areas in Latin America with the scope of evaluating the efficacy and safety of protection in healthy children and adolescents aged 9 to 16 years (Sanofi-Pasteur, 2014).

Other approaches include a deletion of 30 nucleotides in the viral 3'UTR (Δ30 vaccines, (Durbin et al, 2001)), intertypic chimeric Dengue vaccines (Bhamarapravati et al, 1996) self-destructing virus mutants with a furin protease cleavage site in the membrane glycoprotein (Brown, 2004), RepliVax vectors that undergo only one cycle of infection in the vaccinated host (Frolov et al, 2007) and the utilization of the live attenuated Schwarz measles virus vaccine as a carrier for Dengue antigenes (Brandler et al, 2007).

1.5.2 EDIII based antigene expression in chloroplasts

The current tetravalent Dengue vaccine candidates, which are in advanced stages of development, are based on live-attenuated virus strains or genetically manipulated chimeric *Flavivirus*. However, the continuous difficulties associated with these vaccine candidates have necessitated the exploration of alternative non-replicating subunit vaccines. The majority of attempts to produce a recombinant protein based vaccine focus on the envelope (E) protein of Dengue viruses.

The E protein consists of three domains (Modis et al, 2003): the envelope domain I (EDI), flanked by a dimerization domain (EDII) containing the fusion peptide and an immunoglobulin-like domain (EDIII) which contains the host cell surface receptor binding motif (Chen et al, 1997) and several serotype specific neutralizing epitopes (Chin et al, 2007; Megret et al, 1992). The EDIII protrudes from the virus surface to facilitate binding to the host cell surface receptor (Crill & Roehrig, 2001) and mediates host membrane fusion (Allison et al, 2001). The EDIII domain, spanning amino acids 300 - 400 of the E protein, appears to have only very low intrinsic potential for eliciting cross-reactive antibodies against heterologous serotypes (Hombach et al, 2005) and therefore has emerged as the most promising region for vaccine development (Guzman et al, 2010).

Recombinant antigens based on the E protein or the EDIII have been produced using bacteria (McDonald et al, 2009; Simmons et al, 1999; Srivastava et al, 2000), yeast (Cardoso et al, 2013; Etemad et al, 2008), insect cells (Ivy et al, 2000), and plant expression systems (Martinez et al, 2010; Saejung et al, 2007). In order to avoid the unbalanced immune response elicited by tetravalent formulations consisting of stoichiometrically mixed monovalent vaccines, a recombinant fusion protein linking the EDIII domain of Dengue virus serotypes 1, 2, 3 and 4 has been developed. This fusion protein was able to elicit neutralizing antibodies against all four serotypes (Batra et al, 2007; Etemad et al, 2008).

For the present study we have opted for the expression of the tetravalent fusion protein (EDIII 1-4) and the corresponding monovalent forms (EDIII-1, -2, -3 and -4) in tobacco and lettuce chloroplasts, because plant based expression is cheap, easy to up-scale and safe regarding contamination with human pathogens. Furthermore, localization of the transgene in the maternally inherited chloroplast genome prevents transgene spread via pollen, thus increasing the biosafety of the genetically modified plants. Tobacco has been used because it is a non-food/non-feed crop, has a relatively good tractability to genetic manipulation and is an excellent biomass and seed producer (Svab & Maliga, 1993). Unfortunately, the high content of nicotine and alkaloids make tobacco a rather unsuitable material for edible vaccines. For the purpose of oral administration of the Dengue vaccine, which would eliminate expensive requirements of sterile injectables and maintaining a cold-chain (Streatfield, 2006), lettuce has been chosen as a more digestible and non-toxic alternative.

1.6 Objectives of the work

This PhD work has a strong focus on utilization of the cost-effective plant production platform for the development of a Dengue vaccine at low costs for the benefit of millions of people, in particular children in low income countries. To date, there is no vaccine against Dengue fever available on the market and therefore the development of an efficient, safe and affordable vaccine is of great importance. Chloroplast based expression of pharmaceutical proteins offers several advantages. These include high expression levels, easy containment due to maternal inheritance of plastids and the possibility to express multiple transgenes with one successful transformation event.

The presented study therefore has the following three main objectives.

1.6.1 Development of a plastid transformation vector introducing the Gateway® recombinant cloning technology

With three expression strategies, two plant species and four different Dengue virus serotypes a large number of plastid transformation vectors had to be constructed. Conventionally this is done by enzymatic restriction digestions and ligation reactions, which are the most time-consuming steps in chloroplast based expression of recombinant proteins. This tedious cloning procedure could be shortened and simplified by the use of the Gateway® recombinant cloning technology. Gateway® cloning introduces the *att*B1 sequence at the 5´ and the *att*B2 sequence at the 3´ regions of the expression cassette. However, insertion of a novel sequence like attB1 between promoter and 5´UTR includes the risk to damage this very sensitive region and to affect the critical RNA interactions required for protein expression.

Therefore the aim of this preparatory part of the work was

a) to investigate if the presence of a novel sequence like *att*B1 between the rrn16 PEP+NEP promoter and the 5´ UTR T7g10 has an impact on the transgene expression and
b) to demonstrate the feasibility of the Gateway® cloning system for plastid transformation by GFP expression.

1.6.2 Expression of EDIII antigens in tobacco using a constitutive and an inducible expression system

Up to now no vaccine against Dengue fever has been brought to the market, mainly due to the fact that successful vaccination against all four virus serotypes must be achieved at once (Hombach et al, 2005). So far, yeast-expressed monovalent EDIIIs and a fusion protein consisting of the EDIIIs of all four serotypes have shown immunogenicity in mice (Etemad et al, 2008).

In order to demonstrate that tobacco plastids are a suitable and cost-effective production platform for such putative vaccine candidates, two strategies were envisaged:

a) the constitutive expression approach with a strong plastid derived promoter to obtain high yields of recombinant protein and
b) the ethanol-inducible expression system allowing the controlled expression of EDIII to circumvent putative detrimental effects of recombinant protein expression on plant growth.

1.6.3 Expression of EDIII antigens in lettuce for the development of oral vaccines

Although tobacco is still the most preferred species in chloroplast transformation, it is less suitable for the expression of proteins with intended oral administration due to its high alkaloid content. For subsequent application of the EDIII proteins as an oral vaccine it is necessary to express the antigenic proteins in an edible crop like lettuce that can be consumed in raw form.

In order to obtain constitutive expression of EDIII proteins in lettuce, the following steps were necessary:

a) design and construction of lettuce specific plastid transformation vectors and
b) plant transformation, regeneration of homoplastomic lettuce plants and analysis of recombinant protein expression levels.

2 Materials and Methods

Materials

2.1 Bacterial strains

- One Shot® OmniMAX™ 2 T1^{R} chemically competent *E.coli* (Cat. No. C8540-03, Invitrogen^{TM}, Thermo Fisher Scientific Inc., USA)
- One Shot® *ccdB* Survival™ 2 T1^{R} chemically competent *E.coli* (Cat. No. A10460, Invitrogen^{TM}, Thermo Fisher Scientific Inc., USA)

2.2 Plant materials

Tobacco:

1) Seeds of *Nicotiana tabacum* cv. Petit Havana: wild-type (wt)
2) Seeds of *Nicotiana tabacum* transformed with the T7 RNA Polymerase in the nucleus: 285-78-T7
3) Seeds of *Nicotiana tabacum* expressing EDIII 1 constitutive: N.t.-EDIII 1
4) Seeds of *Nicotiana tabacum* expressing EDIII 2 constitutive: N.t.-EDIII 2
5) Seeds of *Nicotiana tabacum* expressing EDIII 3 constitutive: N.t.-EDIII 3

Lettuce:

6) Seeds of *Lactuca sativa L.* cv. Barkley: wild-type (wt)

Constructed transplastomic plant lines:

Tobacco:

1) *Nicotiana tabacum* line expressing GFP constitutive: N.t.-PN-GFP
2) *Nicotiana tabacum* line expressing EDIII 1 inducible: 285-78-T7-EDIII 1
3) *Nicotiana tabacum* line expressing EDIII 2 inducible: 285-78-T7-EDIII 2
4) *Nicotiana tabacum* line expressing EDIII 4 inducible: 285-78-T7-EDIII 4
5) *Nicotiana tabacum* line containing T7-EDIII 1-4 in the plastid: N.t.-T7-EDIII 1-4
6) *Nicotiana tabacum* line expressing EDIII 1-4 inducible: 285-78-T7-EDIII 1-4

Lettuce:

7) *Lactuca sativa* line expressing EDIII 1-4 constitutive: S12-PN-EDIII 1-4
8) *Lactuca sativa* line expressing EDIII 1 constitutive: S16-PN-EDIII 1

2.3 Chemicals and reagents

Table 1 Ready to use reagents and Kits.

Kits	Company
AP-conjugate substrate Kit, Cat. No. 170-6432	Bio-Rad, USA
DIG-High Prime DNA Labeling and Detection Starter Kit II; Kit for chemiluminescent detection with CSPD, Cat. No. 11585614910	Roche Applied Science, USA
Gateway® BP Clonase® Enzyme Mix, Cat. No. 11789-013	Invitrogen, USA
Gateway® LR Clonase® Enzyme Mix, Cat. No. 11791019	Invitrogen, USA
Gateway® Vector Conversion System with One Shot® *ccdB* Survival Cells, Cat. No. 11828-029	Invitrogen, USA
Roti®-Nylon plus, pore size 0.45 µm, Cat. No. K058.1	Carl Roth GmbH, Germany
HisPur™ Cobalt Purification Kit, Cat. No. 90090	Thermo Scientific, USA
iBlot®Gel Transfer Stacks, Cat. No. IB301001	Life technologies, USA
Pierce™ BCA Protein Assay Kit, Cat. No. 23227	Thermo Scientific, USA
Qiagen® Plasmid Maxi Kit, Cat. No. 122637	Qiagen GmbH, Germany
Qiagen® Plasmid Midi Kit, Cat.No. 12145	Qiagen GmbH, Germany
QIAprep® Spin Miniprep Kit, Cat. No. 27106	Qiagen GmbH, Germany
QIAquick® Gel Extraction Kit, Cat. No. 28706	Qiagen GmbH, Germany
Rapid DNA ligation Kit, Cat.No. 1422	Thermo Scientific, USA
Reagents	
0.6 µm Gold Microcarriers	Bio-Rad, USA
1 kb DNA ladder	New England Biolabs, USA
100 bp DNA ladder	New England Biolabs, USA
100 mM dNTP Set, PCR Grade	Invitrogen, USA
6X Loading Dye	Thermo Scientific, USA
Anti-Rabbit IgG (Fc), AP conjugated, Cat. No. S3731	Promega, USA
Ethidium bromide 1% in water	Carl Roth, Germany
GeneAmp® 10X PCR Buffer	Applied Biosystems, USA
Lambda DNA/Eco130I (StyI) Marker 16,	Fermentas, Lithuania
Polyclonal rabbit anti-dengue antibody produced against amino acid sequence: KFKVVKEIAETQHGT	Davids Biotechnology, Germany
Polyclonal rabbit anti-GFP antibody, Cat. No. ABIN398856	antibodies-online GmbH, Germany
Protein marker IV	Peqlab GmbH, Germany
Quick Start™ Bovine Serum Albumin Standard	Bio-Rad, USA
Quick Start™ Bradford 1x Dye Reagent	Bio-Rad, USA
Sigmafast™ BCIP®/NBT	Sigma-Aldrich, USA
Spectra Multicolor Broad Range Protein Ladder	Thermo Scientific, USA

Table 2 Primers and probes. Primers were custom-synthesized by Eurofins MWG Operon (Germany) and reconstituted with sterile ddH2O to a stock concentration of 100 mM, further diluted to a working concentration of 10 mM and stored at -20°C in aliquots of 500 µl.

Primer	**Sequence 5'->3'**
p1	ACCCATGGCTTCTAAAGGAG
p2	AGACAGCGACGGGTTCTCTG
p3	GATCCGAGCCATAGAATTTC
p4	TGCTGGCCGTACATTTGTACG
p5	TACCCGGGAATTGTGACCTC
p6	AGAGTCCGACCACAACGACC
p7	GCTGAAACTCAACATGGAACTG
p8	ATGCTTTTTCACCAGCACCT
p9	TTGCTGAAACTCAACATGGA
p10	CCAAAAGGAGGTTCAGCTTC
p11	TGAAGATGGACAAGGAAAAGC
p12	CTCCACCACCTCCTTTACCA
p13	ACTACTCAAGCTGCATTATATACC
p14	GCACCTTTTACTAAGATCAATG
p15	GGAGGTAGGATGGGCAGTTG
p16	GGACTCGAACCGCTGACATC
p17	GGACTCGAACCGCTGACATC
p18	AACGACCTTTTGGAAACTTC
p19	TCTGTGAGCGTGACGGTGGT
p20	TTACGCGAACGCGAAGTCCG
pM13F	GTAAAACGACGGCCAG
pM13R	CAGGAAACAGCTATGACC
p296	TGACTTATATACTCGTGTCAAC
p297	CTGCTAATGTCTACTGTTTGT

Probe	aadA	den1	den2	den3	insl	psaB	trnA

Table 3 Enzymes.

Name/Description	**Company**
AmpliTaq® DNA Polymerase (5U/µl)	Applied Biosystems, USA
ApaI, BglII, SmaI, NcoI, SacII, PstI, EcoRV, XbaI, NheI, KpnI	New England Biolabs, USA

Table 4 Chemicals.

Name/ Description	Company
Acetic acid (CH_3COOH)	Sigma-Aldrich, USA
Acrylamide/bis-acrylamide (19:1) 40%	Sigma-Aldrich, USA
Agarose	Sigma-Aldrich, USA
Ammonium acetate (NH_4Ac)	Sigma-Aldrich, USA
Ammoniumpersulfate (APS)	Sigma-Aldrich, USA
Ampicillin	Sigma-Aldrich, USA
Bacto Agar	Carl Roth, Germany
Bacto Tryptone	Carl Roth, Germany
BAP (6-benzylaminopurine)	Sigma-Aldrich, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA
Brilliant Blue G	Sigma-Aldrich, USA
Calcium Chloride Dihydrate	Merck, Germany
Chloramphenicol	Duchefa Biochemie, Netherlands
Chloroform	Sigma-Aldrich, USA
cOmplete Protease Inhibitor Cocktail	Roche Applied Science, USA
Developer	AGFA, Belgium
Dithiothreitol (DTT)	Sigma-Aldrich, USA
EDTA dinatriumsalt	Fluka, Switzerland
Ethanol 96 %	Sigma-Aldrich, USA
Fixer	AGFA, Belgium
Gelzan™ CM Gelrite®	Sigma-Aldrich, USA
Glycerol	Carl Roth, Germany
Glycine	Carl Roth, Germany
Hexadecyl-trimethyl-ammonium bromide (CTAB)	Duchefa Biochemie, Netherlands
Hydrochloric Acid (HCl)	J.T.Baker, Netherlands
Isopropanol	Merck, Germany
Kanamycin sulfate	Sigma-Aldrich, USA
Magnesium acetate tetrahydrate	Sigma-Aldrich, USA
Magnesium chloride hexahydrate	Carl Roth, Germany
Maleic acid	Fluka, Switzerland
Methanol	Fluka, Switzerland
MS (Murashig&Skoog) incl. vitamins	Duchefa Biochemie, Netherlands
N,N,N',N'-tetramethylethylenediamine (Temed)	Sigma-Aldrich, USA
Naphtalene acetic acid (NAA)	Duchefa Biochemie, Netherlands
Polyvinylpyrrolidone (PVP) 40	Sigma-Aldrich, USA
Potassium acetate (KAc)	Carl Roth, Germany
Potassium chloride (KCl)	Sigma-Aldrich, USA
Potassium hydroxide (KOH)	Merck, Darmstadt, Germany

Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich, USA
SDS (Sodium lauryl sulfate)	Sigma-Aldrich, USA
Sodium acetate ($NaC_2H_3O_2$)	Merck, Germany
Sodium Chloride (NaCl)	Carl Roth, Germany
Sodium Citrate tribasic dihydrate	Sigma-Aldrich, USA
Sodium hydroxide (NaOH)	Carl Roth, Germany
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich, USA
Spectinomycin dihydrochloride	Sigma-Aldrich, USA
Spermidine	Duchefa Biochemie, Netherlands
ß-Mercaptoethanol	Sigma-Aldrich, USA
Sucrose	Duchefa Biochemie, Netherlands
Tris	Duchefa Biochemie, Netherlands
Tween 20	Sigma-Aldrich, USA
Yeast extract	Carl Roth, Germany

Table 5 Buffers, solutions and media.

Name/ Description	**Recipe**
Buffers	
Alkali-Transfer Buffer 5X, 1 L	Concentration 175,5 g NaCl 3 M 80 g NaOH 2 M 1 L H_2O
AP color development Buffer 1X	AP color development Buffer 25X was diluted 1:25 in distilled water and stored at +4°C.
Blotting Buffer, 10X, pH 8.3, 1 L	Concentration 3.02 g Tris 25 mM 14.4 g Glycine 192 mM 200 ml Methanol 20 % 1 L H_2O
CTAB Buffer 1X, 200 ml	Concentration 4 g CTAB 2 % 40 ml 1 M Tris-HCl 200 mM 8 ml 0.5 M EDTA 20 mM 16.36 g NaCl 1.4 M 2 g PVP 40 1 % filled to 200 ml with distilled water, sterilized by autoclaving and stored at +4°C.
Detection Buffer 1X, pH 9.5, 30 ml	Concentration 3 ml 1 M Tris-HCL 0.1 M 60 µl 5 M NaCl 0.1M 27 ml H_2O

Laemmli Buffer (LB) 5X, pH 6.8, 50 ml			Concentration
	5 g	SDS	10 %
	5 ml	13 M ß-MercaptoEtOH	10 %
	25 ml	100 % Glycerol	50 %
	45 µl	100 % Bromphenol Blue	0,09 %
	10.42 ml	1.5 M Tris-HCl, pH 6.8	312.5 mM
	10 ml	H_2O	
Maleic acid Buffer 1X pH 7.5, 1 L			Concentration
	100 ml	Maleic acid 10X	0.1 M
	100 ml	1 M NaCl	0.1 M
	800 ml	H_2O	
PBS 10X, pH 7.4, 1L			Concentration
	80 g	NaCl	137 mM
	2 g	KCl	2.7 mM
	14.4 g	Na_2HPO_4	100 mM
	2.4 g	KH_2PO_4	2 mM
	Filled to 1 L with distilled water and 22utoclaved.		
PBS-T 1X	PBS 10X was diluted 1:10 in distilled water; 1 ml Tween-20 was added per 1 L Buffer.		
Plant Extraction Buffer I (PEB I), 1X, 10 ml			Concentration
	200 µl 5 M NaCl		100 mM
	200 µl 0.5 M EDTA, pH 8		10 mM
	2 ml 1 M Tris-HCl, pH 8		200 mM
	5 µl Tween-20		0.05 %
	100 µl 10 % SDS		0.1 %
	11 µl 13 M ß-MercaptoEtOH		14 mM
	2 ml 1 M Sucrose		200 mM
	200 µl 100 mM PMSF		2 mM
	5.3 ml H_2O		
Plant Extraction Buffer II (PEB II), 1X, 10 ml			Concentration
	714 µl 1 M Sucrose		0.7 M
	5 ml 1 M Tris-HCl pH 9.5		0.5 M
	1 ml 0.5 M EDTA		50 mM
	833 µl 1.2 M KCl		0.1 M
	20 µl 13 M ß-MercaptoEtOH		0.2 %
	1 cOmplete mini tablet		1X
	8.7 ml H_2O		
Running Buffer, 10X, pH 8.3, 1L			Concentration
	30,2 g	Tris	0.25 M
	144 g	Glycine	1.92 M
	10 g	SDS	1 %
	1 L	H_2O	
Separating Gel-Buffer 4X, pH 8.8, 1 L			Concentration
	181.5 g	Tris	1.5 M
	2 g	SDS	0.2 %
	1 L	H_2O	
	Set pH with concentrated HCl.		

Stacking Gel-Buffer 4X, pH 6.8, 1 L	Concentration 60.5 g Tris 0.5 M 2 g SDS 0.2 % 1 L H_2O Set pH with concentrated HCl
TAE Buffer 1X, 1 L	TAE 50X was diluted 1:50 in distilled water.
TAE Buffer 50X, 1 L	Concentration 242 g Tris 2 M 7.1 ml Acetic acid 0.7 % 100 ml 0.5 M EDTA 0.05 M Filled to 1 L with distilled water and autoclaved.
TBS 10X, pH 7.6, 1 L	Concentration 80 g NaCl 1370 mM 24.2 g Tris 200 mM Filled to 1L with distilled water and autoclaved.
TBS-T 1X	TBS 10X was diluted 1:10 in distilled water; 1 ml Tween 20 was added per 1 L Buffer
TE Buffer 1X, pH 5.6, 100 ml	Concentration 1 ml 1 M Tris-HCl 10 mM 200 µl 0.5 M EDTA 1 mM Filled to 100 ml with distilled water and autoclaved.
Wash Buffer (WB) 1X, pH 7.5, 1 L	Concentration Maleic acid 750 ml Buffer 1X 1x 2 ml Tween- 20 0.3 %
SSC Buffer 20X, 1 L	Concentration 175,32 g NaCl 3 M 88,2 g Sodium citrate 300 mM
Solutions	
Acryl-amide 30 %	37 ml of Acryl-amide (40%) were filled up to 50 ml with distilled water and stored at +4°C.
Ampicillin 25 mg/ml, 10 ml	250 mg ampicillin were dissolved in 10 ml distilled water, filter sterilized and stored at -20 °C.
APS 10 %, 10 ml	1 g Ammonium persulfate was dissolved in 10 ml distilled water, aliquoted and store at -20°C.
BAP 1 mg/ml, 100 ml	100 mg 6-benzylaminopurine were dissolved in 0.5 ml 1 M NaOH, filled up to 100 ml with distilled water, filter sterilized and stored at -20°C.
Blocking solution 1X for Southern Blot	10X Blocking solution was diluted 1:10 in 1X Maleic acid Buffer.
Blocking solution for Western Blot	0.5 % BSA in 1X TBS-T or 1X PBS-T
$CaCl_2$ 2.5 M, 20 ml	10.8 g $CaCl_2$ x 6 H_2O were dissolved in 20 ml distilled water, filter sterilized, aliquoted and stored at -20°C.
Chloramphenicol 3 mg/ml, 50 ml	150 mg chloramphenicol were dissolved in 5 ml ethanol, filled up to 50 ml with distilled water, filter sterilized and stored at +4°C.
DTT 1 M, 50 ml	7.7 g DTT were dissolved in 50 ml water and aliquots were stored at -20°C.

dNTPs 25 mM each, 100µl	25 µl dATP 25 µl dCTP 25 µl dGTP 25 µl dTTP mixed and stored in aliquots at -20°C.
EDTA 0.5 M, pH 8.0, 500 ml	84.05 g EDTA 11.25 g NaOH dissolved in 250 ml distilled water and autoclaved.
Kanamycin 25 mg/ml, 100 ml	250 mg kanamycin were dissolved in 100 ml distilled water, filter sterilized and stored at -20 °C.
Maleic acid 10X, pH 7.5, 1 L	116 g Maleic acid were dissolved in 1 L of distilled water, the pH adjusted with solid NaOH and autoclaved.
HCl 0.25 M, 1 L	25 ml 37% HCl were diluted with 975 ml distilled water.
MgAc 0.1 M, 500 ml	10.7 g Magnesium acetate tetrahydrate were dissolved in 500 ml distilled water and autoclaved.
NAA 1mg/ml, 50 ml	50 mg Naphtalene acetic acid were dissolved 50 ml distilled water, filter sterilized and stored at -20°C.
NaCl 1 M, 1 L	58.44 g NaCl were dissolved in 1 L distilled water and autoclaved.
NaCl 5 M, 500 ml	146.4 g NaCl were dissolved in 1 L distilled water and autoclaved.
NaOH 0.4 M, 1 L	16 g NaOH were dissolved in 1 L distilled water.
NaAc 3 M, pH 5.2, 200 ml	49.2 g Sodium acetate were dissolved in 200 ml distilled water.
0.1 M NH_4OAc, 50 ml	0.38 g Ammonium acetate were dissolved in 50 ml Methanol and autoclaved.
KCl 250 mM, 100 ml	1.86 g KCl were dissolved in 100 ml distilled water.
KAc 3 M, 100 ml pH 5.2	29.44 g KAc were dissolved in 100 ml distilled water.
SDS 10 %, 100 ml	10 g SDS were added to 100 ml distilled water and heated to 68 °C. The pH was adjusted to 7.2 and the solution sterilized by autoclaving.
Spectinomycin (Spec) 100 mg/ml, 100 ml	10 g Spectinomycin were dissolved in 100 ml distilled water, filter sterilized and stored at -20°C.
Spermidine 0.1 M, 10 ml	0.255 g Spermidine were dissolved in 10 ml distilled water, aliquots were stored at -20°C.
SSC 0.5X + 0.1 % SDS, 500 ml	12.5 ml 20X SSC and 5 ml 10% SDS were filled up to 500 ml with distilled water.
SSC 2X + 0.1 % SDS, 500 ml	50 ml 20X SSC and 5 ml 10% SDS were filled up to 500 ml with distilled water.
Sucrose 1 M, 250 ml	85.5 g Sucrose were dissolved in 250 ml distilled water, autoclaved and stored at +4°C.
Tris-HCl 1 M, 1 L	121.1 g Tris were dissolved in 1000 ml distilled water; pH adjusted with concentrated HCl and autoclaved.
PMSF 100 mM, 50 ml	0.87 g PMSF were dissolved in 50 ml DMSO, aliquots of 1 ml stored at -20°C.

Media	
LB-Medium, pH 7.0, liquid, 1 L	10 g Bacto Tryptone 5 g Yeast extract 10 g NaCl Mixed and dissolved in 1 L distilled water, pH adjusted to 7.0 and sterilized by autoclaving.
LB-Medium, pH 7.0, solid, 1 L	10 g Bacto Tryptone 5 g Yeast extract 10 g NaCl 10 g Bacto-Agar Mixed and dissolved in 1 L distilled water, pH adjusted to 7.0 and sterilized by autoclaving.
MS-medium, pH 5.8, solid, 1 L	4.4 g MS-Salt including vitamins 10 g Sucrose 3.1 g Gelzan™ Mixed and dissolved in 1 L distilled water, pH adjusted to 5.8 and sterilized by autoclaving.
RMOP medium, pH 5.8, solid, 1 L	4.4 g MS-Salt including vitamins 30 g Sucrose 3.1 g Gelzan™ Filled up to 1L with distilled water, pH adjusted to 5.8 and sterilized by autoclaving. After autoclaving the following is added: 1 ml BAP (1mg/ml) 100 µl NAA (1mg/ml) 5 ml Spec (100 mg/ml).

Table 6 Laboratory equipment and material.

Name/Description	**Company**
Appliances	
Bombardment chamber PDS1000He	Bio-Rad, USA
SP5 II confocal microscope system	Leica Microsystems, Germany
Autoclave	Matachana, Spain
Table Top Centrifuge	Thermo Scientific, USA
Clean bench Herasafe™	Thermo Scientific, USA
Vortex	VWR, USA
pH meter	WTW, Germany
Ultracentrifuge	Eppendorf, Germany
MJ Mini™ Personal Thermo Cycler	Bio-Rad, USA
Shaker Titertek®	Flow Laboratories, USA
Orbital Shaker SSL3	Stuart, UK
Nanodrop	Thermo Scientific, USA
Retsch Mill	Retsch, Germany
Water bath	Grant, UK
Heat block	Techne Inc, USA
Hot plate stirrer	IKA-Works, USA
Precision Balance	Mettler-Toledo, Switzerland
Gel electrophorese apparatus	Bio-Rad, USA
Power supply	Bio-Rad, USA
iBlot Blotting device	Thermo Scientific, USA
PAA Electrophorese chamber	Bio-Rad, USA
Gel doc	Bio-Rad, USA
Minitron Incubator	Infors HT, Switzerland
Vacuum pump	Edwards Limited, UK
Fridge	Whirlpool, USA
Freezer -20°C	Whirlpool, USA
Freezer -80°C	Sanyo, Japan
Micro-pipettes	Eppendorf, Germany
Glass plates for PAA Gels	Bio-Rad, USA
Spacers for PAA Gels	Bio-Rad, USA
Gel casting device for PAA Gels	Bio-Rad, USA
Macrocarrier	BioRad, USA
UV-VIS Spectrophotometer	Shimadzu, Japan
Multiplate Reader	Biochrom, UK
Milli-Q-water purification system	Merck-Millipore, Germany
HM-4000 Multidizer™ Hybridization Oven	UVP,USA

Consumables	
glasware	Schott-Duran, Germany
25/50 ml plastic tubes	Greiner Bio One, USA
Finntips 10µl, 200µl, 1000µl	Thermo Scientifiy, USA
1.5/2 ml reaction tubes	Eppendorf, Germany
PCR tubes	Thermo Scientific, USA
petridishes	VWR, USA
Magenta-boxes	Sigma-Aldrich, USA
Retsch mill steel beads	Retsch, Germany
Lumi Film for chemiluminescent detection	Roche Applied Science, USA
gloves	VWR, USA
Whatmann filter paper	GE Healthcare, UK

Methods

2.4 Bacterial growth conditions

Overnight cultivation of *E.coli* was done either on LB plates with 10 g/l Bacto-Agar or in 5 ml liquid LB medium containing the appropriate antibiotics (Table 7) for maintaining the selection pressure at 37°C and shaking at 200 rpm.

33 % glycerol stocks were made of every liquid culture by mixing 0.5 mL of 100 % Glycerol and 1 mL liquid cell culture. The glycerol stocks were shock frozen in liquid N_2 and stored at -80°C.

Table 7 Final concentration of antibiotics in LB medium used for plates and liquid culture of transformed *E. coli*.

Antibiotic	Final concentration
ampicillin	100 [mg/L]
chloramphenicol	30 [mg/L]
kanamycin	100 [mg/L]
spectinomycin	500 [mg/L] for tobacco 30 [mg/L] for lettuce

2.5 Plant growth conditions

2.5.1 Seed sterilization

Seeds of *Nicotiana tabacum* and *Lactuca sativa* were soaked in 6 % bleach along with a few drops of washing liquid for 1 minute. The solution was pipetted out and the seeds were then washed in 70 % ethanol for 1 minute, washed three times in distilled water and air dried at room temperature in the clean bench. The seeds were stored at 4 °C.

2.5.2 In vitro plant tissue culture and regeneration

Seeds were germinated on solid MS-Medium containing the appropriate antibiotic (Table 8); young seedlings were transferred to Magenta-Boxes containing the same medium.

Tissue culture of bombarded leave discs was carried out on RMOP-Medium containing the appropriate antibiotics (Table 8); young shoots developing from the callus tissue were transferred into Magenta-Boxes containing MS-medium incl. antibiotics for rooting and further growth.

All in vitro cultures were incubated at 25°C, at a 16 h light - 8 h dark cycle in growth chambers equipped with Universal lamps with white fluorescence, light intensity; 0.5–1 W/m^2 Osram L85 W/25.

Table 8 Antibiotic concentrations used in media for seed germination, *in vitro* culture of plants and tissue culture of bombarded leave discs.

Seeds/plant	**Medium + Antibiotic**
N. tabacum *L. sativa*	MS
285-78-T7	MS + 100 mg/L kanamycin
N.t.-EDIII 1 N.t.-EDIII 2 N.t.-EDIII 3 N.t.-PN-GFP	MS + 500 mg/L spectinomycin
285-78-T7-EDIII 1 285-78-T7-EDIII 2 285-78-T7-EDIII 4 285-78-T7-EDIII 1-4	MS + 100 mg/L kanamycin + 500 mg/L spectinomycin
S12-PN-EDIII 1-4 S16-PN-EDIII 1	MS + 30 mg/L spectinomycin
Bombarded *N. tabacum* wt leave discs	RMOP + 500 mg/L spectinomycin
Bombarded 285-78-T7 leave discs	RMOP + 100 mg/L kanamycin + 500 mg/L spectinomycin
Bombarded *L. sativa* leave discs	RMOP + 30 mg/L spectinomycin

2.5.3 Greenhouse growth conditions

Rooted plants were transferred to soil, acclimatized gently and grown in the greenhouse with additional light for 16 h and light intensity of 300 µE $s^{-1}m^{-2}$ at 25°C and relative humidity of 60 %.

2.5.4 Ethanol spray experiments

Five weeks old plants growing in Magenta boxes on MS medium containing spectinomycin were sprayed with ~ 0.5 ml 5 % ethanol on seven consecutive days and a whole plant for every plant line was taken as a sample before the spraying (day 0) and then every following day before the next spraying (day 1 to day 7). Plant lines sprayed: 285-78-T7-EDIII 1, 285-78-T7-EDIII 2, 285-78-T7-EDIII 4, 285-78-T7-EDIII 1-4, 285-78-T7.

2.6 DNA preparation

2.6.1 Plasmid DNA isolation

Plasmid DNA was isolated using the Kits purchased from Qiagen (Qiagen® Plasmid Maxi Kit, Qiagen® Plasmid Midi Kit and QIAprep® Spin Miniprep Kit) and following the instructions supplied with the Kits.

Briefly, *E.coli* overnight culture in liquid LB medium was harvested by centrifugation at 8000 g for 3 min and the pellet was re-suspended in Buffer P1. The bacterial cell pellet was lysed by alkaline lysis, the cell debris and other contaminants were precipitated and the cleared lysate was applied to the silica membrane to allow DNA binding. After several washing steps the DNA was eluted from the column with sterile distilled water and stored at +4°C.

2.6.2 Ethanol-Precipitation of DNA

Plasmid DNA was precipitated by incubation with 1/10 volume 3 M NaAc and 3 volumes 96 % ethanol overnight at –20°C. The solution was centrifuged at 14000 rpm at +4°C for 15 minutes, and the pellet was washed twice with 70 % ethanol, air dried for 20°C at room temperature (RT), dissolved in 1X TE Buffer and stored at -20°C.

2.6.3 Plant DNA isolation

Total plant DNA is isolated using a modified CTAB – procedure (Murray & Thompson, 1980).

Plant leaves were collected and frozen in liquid nitrogen and ground to fine powder either using pestle and mortar or a Retsch mill. 500 µl of pre-warmed CTAB Buffer (65°C) was added to 200 mg frozen sample material and incubated for 1 hour at 65°C in a re-circulating water bath, gently mixed by inverting from time to time. Samples were allowed to cool down for 5 minutes at RT before addition of 500 µl chloroform. The tubes were shaken for 30 minutes at RT and then centrifuged for 10 minutes at 10000 rpm at +4°C. The upper watery phase was transferred into a new tube and chilled Isopropanol was used to precipitate the DNA. After centrifugation for 10 minutes at 10000 rpm at +4°C, the pellet was washed twice with 70% ethanol, centrifuged for 1 minute at 10000 rpm at +4°C and then air dried for 30 minutes. DNA was re-suspended in sterile distilled water and stored for later use at -20°C.

2.6.4 Quantification of DNA concentration

The DNA concentration is measured using the Nanodrop (Thermo Scientific) device or by visual comparison to the DNA ladder on an agarose gel.

2.6.5 Agarose gel electrophoresis

Standard 0.8 % Agarose gels were prepared with 1X TAE Buffer and used to check the quality of DNA isolations and to separate PCR products and restriction fragments. 2 µl 6X LD per 10 µl sample were loaded and the DNA was separated at 80 V.

2.6.6 Gel extraction

Gel extraction and purification of restriction fragments or PCR products was carried out according to the Qiagen protocol of the Gel Extraction Kit after electrophoretic separation of the samples in a 0.8 % Agarose gel. DNA was eluted with 30 µL Elution Buffer.

2.7 Cloning and *E.coli* heat shock transformation

2.7.1 DNA digestion with restriction enzymes

Restriction digestion of plasmid DNA or total plant DNA with appropriate restriction endonucleases were performed in the corresponding buffer systems provided by the manufacturer at 37°C overnight in a reaction volume of 30 µl. Restriction digests were heat inactivated by incubation at 65°C for 20 minutes prior to any further utilization.

2.7.2 Ligation of vector backbone with DNA fragments

The Rapid DNA Ligation Kit was used to ligate DNA fragments according to the protocol provided by the manufacturer and the formula given below was used to determine the required amount of insert based on a 3:1 molar ratio of insert to backbone DNA.

$$ng[insert] = \frac{3}{1} * \left(\frac{size\ of\ insert\ in\ bp}{size\ of\ backbone\ in\ bp}\right) * ng[backbone]$$

The following reaction mixture was prepared in a reaction tube and incubated at 16 °C overnight (Table 9).

Table 9 Standard Ligation reaction set-up.

Substance	**Amount**
Linear backbone DNA	~ 50 ng
Insert DNA	3:1 molar ratio of insert to vector DNA
10X ligation Buffer	1.5 µl
Ligase enzyme	1.0 µl
H_2O	add to 15.0 µl

2.7.3 *E. coli* transformation

All Plasmids (1 µl) and ligation products (5 µl) are transformed into chemically competent One Shot® *E.coli* by heat shock according to the manufacturer's protocol (Invitrogen™).

The cells were thawed on ice and after addition of the DNA they were incubated for 30 minutes on ice, followed by a heat shock treatment at 42°C for 30 seconds and two minutes incubation on ice. 250 µl of SOC medium were added to 50 µl of initial cell culture and incubated at 37°C for 1 hour with gentle shaking. 10 µl and 50 µl of the culture were plated on LB plates containing the suitable antibiotics for selection and grown at 37°C overnight. The rest of the transformation culture was stored at +4°C and if necessary, cells were pelleted, re-suspended in a smaller volume of liquid LB medium and plated out again.

Single colonies were picked and grown overnight in liquid LB-medium containing antibiotics, harvested and used for glycerol-stock and plasmid isolation.

2.7.4 Gene synthesis and sequencing

The nucleotide sequences encoding the transgenes (EDIII 1-4, EDIII 1, EDIII 2, EDIII 3, EDIII 4 and GFP) were codon usage optimized for the tobacco plastid and gene synthesis of these custom designed sequences was carried out by GeneArt (Germany) resulting in the vectors: pEDIII 1-4, pEDIII 1, pEDIII 2, pEDIII 3, pEDIII 4 and pGFP. In these vectors the transgenes already have a C-terminal 6xHis tag and are flanked by the *att*B1 and *att*B2 sites for Gateway® cloning.

Plasmid DNA samples were sent to LGC Genomics (Germany) for sequencing and the sequencing data were analyzed using the software: Vector NTI advanced® 11.5 sequence alignment AlignX tool.

2.7.5 Gateway® cloning procedure

All Gateway® cloning steps were carried out according to the protocol provided from Invitrogen™.

The BP-reaction is performed with the *PstI* linearized vector including the gene of interest (goi) and the Donor vector pDONR221™ and the BP enzyme mix at 25°C for 1 hour. The LR-reaction is performed with the previously produced Entry- and Destination- vectors and the LR enzyme mix for 1 hour at 25°C. The resulting Entry vectors and Expression vectors are transformed into One Shot® OmniMAX™ *E. coli* cells by heat shock method. The formula given below is used to calculate the correct amounts of goi and Donor vector with X for goi and Donor vector and N for the size in bp of the goi and the Donor vector, respectively. The standard set up for the carried out BP- and LR-reactions are given in Table 10. The BP- and the LR-reactions were stopped by adding 1 µL Proteinase K and incubation at 37°C for 10 minutes.

$$ng[X] = (50\ fmol) * N * \left(660\ \left[\frac{fg}{fmol}\right]\right) * 1\ ng/10^6 fg$$

Table 10 Standard Set-up of BP- and LR-reaction.

BP-reaction	**10µL**
goi	x µL
pDONR221™	160 ng
TE Buffer, pH 8.0	3µL
BP Clonase II	2µL

LR-reaction	**10µL**
Destination vector	160 ng
Entry vector	120 ng
LR Clonase II	2 µL

2.8 Vector construction

2.8.1 Destination vectors pDEST-PN-T, pDEST-T7-T and pDEST-PN-L

In order to obtain the tobacco specific plastid transformation vectors, the Gateway® reading frame cassette RfA (Figure 4 (a)) was first inserted into pT7PN-T (Figure 4 (b)) to produce pDEST-PN-T (Figure 4 (c)) and subsequently the P*rrn*16 was cut-out to produce pDEST-T7-T (Figure 4 (d)). The lettuce specific plastid transformation vector pDEST-PN-L (Figure 4 (e)) was obtained by insertion of the complete *aad*A expression cassette and the Gateway® cassette into a backbone vector containing the regions homologous to the *trn*I/*trn*A sequence from *L. sativum (Ruhlman et al, 2007)*. Insertion of the custom designed fragment psbAT7PN into pT7PHB-NF (Lössl et al, 2005) yielded the intermediary vector pT7PN-T. Both, the backbone donating vector pT7PHB-NF and the insert containing vector pMA-psbAT7PN (obtained from GeneArt) were double digested with *SacII* and *NcoI,* the digested DNA was separated by gel-electrophoresis and the 6233 bp backbone and the 685 bp insert fragments were cut out from the gel, purified and ligated. The ligation product was transformed into chemically competent *E.coli* by heat shock method. Since this cloning step changes also the promoter of the *aad*A cassette, positive clones were selected on LB-plates containing ampicillin and spectinomycin. Single colonies were picked and plasmid DNA isolated from liquid overnight cultures of these positive clones was sent for sequencing to verify the correct nucleotide sequence and orientation of the insert.

Conversion of pT7PN-T into the Gateway® vector was done according to the protocol provided by Invitrogen™ using the Gateway® Vector Conversion System. pT7PN-T was linearized with *EcoRV* and after gel-purification blunt-end ligation was used to introduce the RfA containing *att*R recombination sites flanking a *ccdB* gene and a chloramphenicol-resistance gene. After transformation of *ccd*B survival™ *E. coli* with the ligation product, positive clones were selected on LB plates containing both ampicillin and chloramphenicol and the correct orientation of the RfA was verified by sequencing. pDEST-T7-T was achieved by double-digestion of the pDEST-PN-T with *XbaI* and *NheI*, gel extraction of the restricted fragment and subsequent religation of the vector. Putative pDEST-T7-T clones are selected on LB plates containing ampicillin after transformation of *ccd*B survival™ *E.coli* with the ligation product. The presence of only the T7 promoter in the new vector was confirmed by sequencing.

Re-digest of pDEST-PN-T and the backbone vector pLettuce-MA (GeneArt, Germany) containing the lettuce specific sequences for homologous integration into the plastid genome with *KpnI* and *SacII* yielded the 3432 bp insert and the 4337 bp backbone which were separated by gel electrophoresis and the correct fragments were cut out and purified from the gel. The ligation product was transformed into *ccd*B Survival™ 2 $T1^R$ chemically competent *E. coli* and positive clones were selected on ampicillin and spectinomycin containing solid LB medium. The correct sequence of the pDEST-PN-L was verified by sequencing.

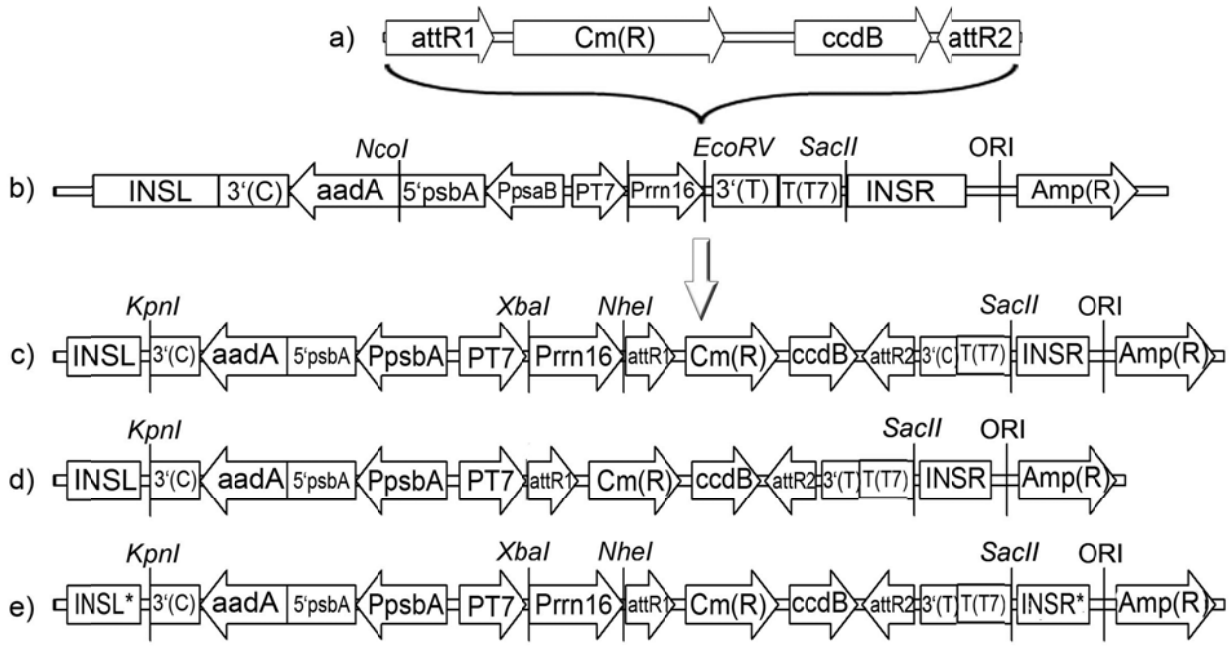


Figure 4 Schematic representation of Destination vector construction. a) The Gateway® RfA cassette consisting of *att*R1-Cm(R)-ccdB-*att*R2; b) pT7PN-T was constructed by cloning the psbAT7PN 685 bp fragment into pT7PHB-NF using *NcoI* and *SacII*; c) pDEST-PN-T was obtained by blunt end ligation of the RfA into pT7PN-T via the *EcoRV* site; d) pDEST-T7-T was obtained by double digest of pDEST-PN-T with *NheI* and *XbaI* and religation of the resulting backbone; e) pDEST-PN-L was obtained by transferring the whole *aad*A expression cassette together with the Gateway® RfA into pLettuce-MA using *KpnI* and *SacII*. *aad*A: spectinomycin resistance gene; Amp(R): ampicillin resistance gene; *att*R1/*att*R2: Gateway® recombination sites; *ccd*B: control of cell death gene; Cm(R): chloramphenicol resistance gene; INSL/INSR: tobacco specific left/right insertion site; INSL*/INSR*: lettuce specific left/right insertion site; P*psb*A: tobacco *psb*A promoter (Staub & Maliga, 1993); P*rrn*16: tobacco *rrn*16 PEP+NEP promoter (Ye et al, 2001); PT7: phage λ derived T7 promoter (Tabor & Richardson, 1985); 3'(C): 3'UTR of chlamydomonas *rbc*L gene; 3'(T): 3'UTR of tobacco *rbc*L gene; 5'*psb*A: 5'UTR of tobacco *psb*A gene; T(T7): T7 terminator; ORI: bacterial origin of replication.

2.8.2 Entry vectors and Expression vectors

The Entry vectors were created by the Gateway® BP Clonase® enzyme mix mediated transfer of the *att*B sites flanked gene of interest into the *att*P site bearing pDONR221™. The BP reactions were done according to the protocol provided by Invitrogen™. The vectors donating the *att*B site flanked gene of interest were linearized by *PstI* digest in order to maximize the recombination efficiency. The BP reaction products were transformed into OmniMax™ *E.coli* via heat shock method, cells were plated on solid LB medium containing kanamycin and positive clones were identified by colony PCR (PCR 8, Table 15) with primers pM13F/pM13R (Figure 6 (a)). The goi sequence in the Entry vectors was then transferred into the respective Destination vector containing *att*R sites using the Invitrogen® LR Clonase® enzyme mix. The LR reactions were done according to the protocol provided by Invitrogen™. The LR reaction products were transformed into OmniMax™ *E.coli* via heat shock method, cells were plated on solid LB medium containing ampicillin and spectinomycin and positive clones were identified by colony PCR (PCR 9, Table 15) with primers p296/p297 (Figure 6 (b)). Correctness of all performed cloning steps was verified by sequencing of plasmid DNA isolated from PCR positive clones. Figure 5 shows an example of an Entry vector and an Expression vector. Table 11 summarizes the performed BP and LR reactions and the resulting Entry- and Expression vectors.

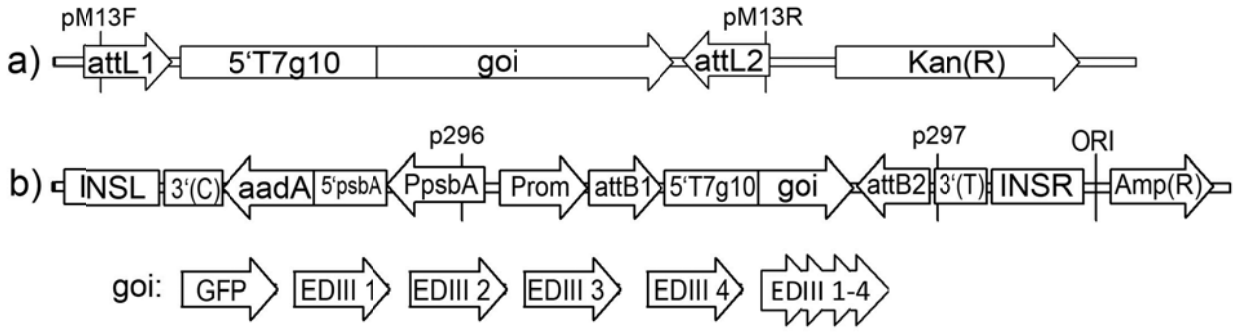


Figure 5 Schematic representation of an example of a) an Entry vector and b) an Expression vector. *aad*A: spectinomycin resistance gene; *att*B1/B2/L1/L2: Gateway® recombination sites; INSR/INSL: right/left insertion site; P*psb*A: tobacco *psb*A promoter; Prom: promoter for transgene expression; 3'(C): 3'UTR of chlamydomonas *rbc*L gene; 3'(T): 3'UTR of tobacco *rbc*L gene; 5'*psb*A: 5'UTR of tobacco *psb*A gene; ORI: bacterial origin of replication; pM13F/pM13R and p296/p297: primers used for colony PCR; goi: gene of interest.

Table 11 Summary of all performed BP and LR reactions and the constructed Entry vectors and Expression vectors. –T indicates Expression vectors containing tobacco specific INSL/INSR and –L indicates vectors containing lettuce specific INSL/INSR. The *att*B1-goi-*att*B2 fragment was contained in the respective vectors which were obtained from GeneArt (Germany) and linearized by *PstI* digest.

BP reaction	***att*B1-goi-*att*B2**	**accepting vector**	**resulting Entry vector**
1	pEDIII 1	pDONR221™	pENTR-EDIII 1
2	pEDIII 2	pDONR221™	pENTR-EDIII 2
3	pEDIII 3	pDONR221™	pENTR-EDIII 3
4	pEDIII 4	pDONR221™	pENTR-EDIII 4
5	pEDIII 1-4	pDONR221™	pENTR-EDIII 1-4
6	pGFP	pDONR221™	pENTR-GFP
LR reaction	**Entry vector**	**Destination vector**	**resulting Expression vector**
1	pENTR-GFP	pDEST-PN-T	pEXP-PN-GFP-T
2	pENTR-EDIII 1-4	pDEST-T7-T	pEXP-T7-EDIII 1-4-T
3	pENTR-EDIII 1	pDEST-T7-T	pEXP-T7-EDIII 1-T
4	pENTR-EDIII 2	pDEST-T7-T	pEXP-T7-EDIII 2-T
5	pENTR-EDIII 3	pDEST-T7-T	pEXP-T7-EDIII 3-T
6	pENTR-EDIII 4	pDEST-T7-T	pEXP-T7-EDIII 4-T
7	pENTR-EDIII 1-4	pDEST-PN-L	pEXP-PN-EDIII 1-4-L
8	pENTR-EDIII 1	pDEST-PN-L	pEXP-PN-EDIII 1-L

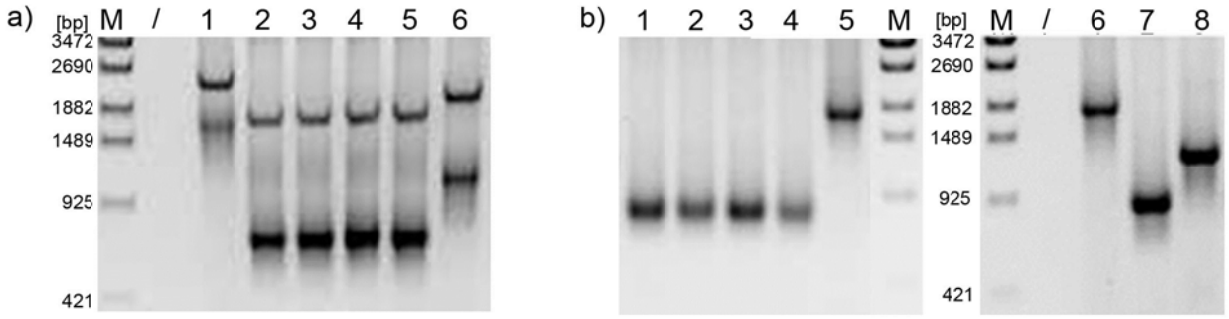


Figure 6 Gel Electrophoreses of colony PCR reactions. a) PCR product obtained with pM13F/pM13R on putative Entry vectors; Lanes: 1) pENTR-EDIII 1-4, 1701 bp; 2) pENTR-EDIII 1, 713 bp; 3) pENTR-EDIII 2, 723 bp; 4) pENTR-EDIII 3, 723 bp; 5) pENTR-EDIII 4, 720 bp; 6) pENTR-GFP, 1171 bp.
b) PCR product obtained with p296/p297 on putative Expression vectors.; Lanes: 1) pEXP-T7-EDIII 1-T, 698 bp; 2) pEXP-T7-EDIII 2-T, 718 bp; 3) pEXP-T7-EDIII 3-T, 718 bp; 4) pEXP-T7-EDIII 4-T, 715 bp; 5) pEXP-T7-EDIII 1-4, 1696 bp; 6) pEXP-PN-EDIII 1-4-L, 1841 bp; 7) pEXP-PN-EDIII 1-L, 853 bp; 8) pEXP-PN-GFP-T, 1232 bp; M: Fermentas Marker 16. The expected PCR product size is stated for every vector.

2.9 Plastid transformation by biolistic bombardment method

Transformation of chloroplasts and regeneration of transplastomic plants was achieved with the biolistic transformation method using a PDS-1000/He Particle Delivery System and following the modified protocol from Verma et al, 2008. Table 12 lists the performed transformations and the generated transplastomic plant lines.

2.9.1 Preparation of plant material

Leaves of six weeks old plants grown under sterile conditions were harvested, placed on RMOP-medium facing the abaxial side up and incubated at 25°C in the dark overnight.

2.9.2 Sterilization of microcarriers

30 mg of gold particles were accurately weighed and transferred to 1.5 ml Eppendorf tube. 1 ml of 70 % ethanol was added to the tube, vortexed for 15 minutes at +4°C and centrifuged for 30 seconds at maximum speed. The supernatant was removed and the gold pellet was washed again with 70 % ethanol, washing was repeated for two more times. After the third wash the supernatant was discarded and the gold particles were re-suspended in 500 µl of 50 % glycerol resulting in a final concentration of 60 mg/ml.

2.9.3 DNA coating of microcarriers

5 µl DNA (1µg/µl), 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine were added while vortexing to 50 µl of sterile microcarriers re-suspended in glycerol. The mixture was incubated on ice for 10 minutes and then centrifuged for 1 minute at 8 000 rpm. The supernatant was carefully removed and the pellet was first washed with 140 µl 70 % ethanol and centrifuged 1 minute at 10000 rpm; second the pellet was washed with 140 µl of 100 % ethanol for 1 minute and centrifuged at 10000 rpm. The supernatant was removed and the DNA-coated microcarriers were carefully re-suspended in 48 µl 100 % ethanol and kept on ice until used.

2.9.4 Bombardment

All the equipment and the bombardment chamber were sterilized with 70 % ethanol. 6 µl of freshly prepared DNA coated gold particles were loaded on macro carries in the macro carrier holder. The sterile rupture disk of 1100 psi was placed in the retaining cap and secured to the gas acceleration tube. Plant tissues were bombarded with DNA coated gold microcarriers in the vacuum chamber at a pressure of 1100 psi.

2.9.5 Selection and regeneration of transformed plants

The bombarded leaf discs were placed on RMOP medium and incubated in the dark at 25°C for two days. Then the leaves were cut into small pieces (~ 5 mm^2), transferred to RMOP medium containing spectinomycin (500 mg/L for tobacco and 30 mg/L for lettuce) and kept at 25°C under standard light conditions. Three to four weeks after the transformation the resistant shoots started to regenerate and were transferred to fresh medium. In order to obtain homoplasmic plants, transplastomic shoots were subjected to 2 additional rounds of regeneration on RMOP medium containing spectinomycin. Integration of the transgene expression cassette into the tobacco plastid genome was verified by a 2552 bp PCR product with primers p3/p4. PCR positive plantlets were used for further analysis. Presence of the transgene expression cassette in the lettuce plastid genome was verified by a 836 bp PCR product for S16-PN-EDIII 1 and a 1841 bp PCR product for S12-PN-EDIII 1-4 with primers p296/p297.

Table 12 Summary of performed transformation events.

bombarded leaves	transformation vector	transplastomic plant lines
wt tobacco	pEXP-PN-GFP-T	N.t.-PN-GFP
285-78-T7	pEXP-T7-EDIII 1-T	285-78-T7-EDIII 1
285-78-T7	pEXP-T7-EDIII 2-T	285-78-T7-EDIII 2
285-78-T7	pEXP-T7-EDIII 4-T	285-78-T7-EDIII 4
285-78-T7	pEXP-T7-EDIII 1-4-T	no plants regenerated
wt tobacco	pEXP-T7-EDIII 1-4-T	N.t.-T7-EDIII 1-4
wt lettuce	pEXP-PN-EDIII 1-L	S16-PN-EDIII 1
wt lettuce	pEXP-PN-EDIII 1-4-L	S12-PN-EDIII 1-4

2.9.6 Pollination

Plant line 285-78-T7-EDIII 1-4 was obtained by manually pollinating plant line N.t.-T7-EDIII 1-4 with pollen collected from plant line 285-78-T7. Seeds obtained from this pollination were germinated on spectinomycin and kanamycin containing MS medium and the presence of the T7 RNA polymerase in the nuclear genome of green seedlings was verified by a 603 bp PCR product using primers p19/p20.

2.10 Molecular analysis to verify the transformants

2.10.1 PCR

PCR reactions were carried out according to the standard protocol (Table 13 and Table 14). The specific conditions for every reaction are given in Table 15 and the respective primers are described in Table 2. Each 10 µl reaction contained 9 µl master-mix and 1 µl template DNA or one picked colony. Table 15 gives an overview of the performed PCR reactions and corresponding conditions.

Table 13 Standard PCR conditions. Ta and te depend on the specific PCR reaction.

Step		T [°C]	t [min:sec]	
1	initial denaturation	95	03:00	
2	denaturation	95	00:40	30 cycles
3	annealing	Ta	00:40	
4	elongation	72	te	
5	final elongation	72	10:00	
6	hold	16	∞	

Table 14 Standard Master-mix composition for PCR reactions.

Substance	[µl] for 10 µl reaction	Concentration
H_2O	5.65	-
Taq Buffer 10X	1	1x
primer 1 10 µM	1	1 µM
primer 2 10 µM	1	1 µM
dNTPs 2.5 mM/each	0.25	250 µM/each
Taq [5U/µl]	0.1	0.025 U/µl

Table 15 Summary of performed PCR reactions. Ta: annealing temperature in °C; te: elongation time in minutes; template: plasmid DNA or total plant DNA.

No.	Primer pair	Template	Ta [°C]	te [min]	Product [bp]
1	p17/p18	pKP9	54	1	488
2a	p7/p8	pKP9-EDIII 1	49	0.5	223
2b	p9/p10	pKP9-EDIII 2	49	0.5	187
2c	p11/p12	pKP9-EDIII 3	49	0.5	197
3	p5/p6	pDEST-T7-T	54	1	773
4	p13/p14	pKP9	51	1	518
5	p15/p16	pDEST-PN-L	57	1	665
6	p1/p2	N.t.-PN-GFP	53	2,5	2230
7	p3/p4	N.t.-PN-GFP 285-78-T7-EDIII 1 285-78-T7-EDIII 2 285-78-T7-EDIII 4 N.t.-T7-EDIII 1-4 285-78-T7-EDIII 1-4	53	3	2552
8	pM13F/pM13R	pEntr-EDIII 1-4 pEntr-EDIII 1 pEntr-EDIII 2 pEntr-EDIII 3 pEntr-EDIII 4 pEntr-GFP	50	3	1701 713 723 723 720 1171
9	p296/p297	pEXP-PN-GFP-T pEXP-T7-EDIII 1-T pEXP-T7-EDIII 2-T pEXP-T7-EDIII 3-T pEXP-T7-EDIII 4-T pEXP-T7-EDIII 1-4-T pEXP-PN-EDIII 1-L pEXP-PN-EDIII 1-4-L S12-PN-EDIII 1-4 S16-PN-EDIII 1	50	3	1232 698 718 718 715 1696 853 1841 1841 853
10	p19/p20	285-78-T7-EDIII 1-4	57	1	603

2.10.2 Southern blot analysis

The Southern Blot analyses were carried out according to the protocol provided with the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). Plant DNA was isolated from transplastomic and wild-type plants after three consecutive rounds of selection and subculture on spectinomycin containing RMOP medium and analyzed using DIG labeled probes (Table 16) that bind inside the transgene expression cassettes and the plastid genome. 10 µg of plant DNA was cut with *ApaI* (for N.t.-PN-GFP, 285-78-T-EDIII 1, 285-78-T7-EDIII 2, 285-78-T7-EDIII 4, N.t.-T7-EDIII 1-4 and 285-78-T7-EDIII 1-4), with *SmaI* (for S12-PN-EDIII 1-4 and S16-PN-EDIII 1) or with *BglII* (for N.t-EDIII 1, N.t.-EDIII 2 and N.t.-EDIII 3), separated by electrophoresis in a 1 % Agarose gel at 50 V overnight and transferred onto a positively charged nylon membrane by capillary action using either the alkali-transfer method with 1X Alkali-Transfer Buffer (for N.t.-GFP) or the semi-dry transfer method, overnight. After immobilization of the DNA by baking the membrane at 80°C for 2 hours, the DNA was pre-hybridized for 3 hours at 45°C and hybridized with the specific labeled probe (Table 17) at 45°C overnight to visualize the sequence of interest. Stringency washes were performed with 2X SSC + 1 % SDS at RT and 0.5X SSC + 1 % SDS at 65°C. After incubation in blocking solution for 30 minutes at RT and incubation in antibody solution for 30 minutes at RT, the membrane was washed twice with 1X Wash Buffer, 1 ml of CSPD ready to use solution was applied to the membrane and incubated at 37°C for 10 minutes. The signal was detected by exposure to X-ray film and developer and fixer solution were used to develop the X-ray film.

Table 16 Probes and their binding region. PCR indicates the PCR reaction performed to produce the probe (Table 15) for the hybridization with den probe equal amounts of den 1, den 2 and den 3 probes were mixed and applied to the membrane.

Probe	Size [bp]	PCR	Binding in
aadA	488	1	*aad*A gene inside the transgene expression cassette
den 1 den 2 den 3	223 187 197	2a 2b 2c	dengue gene inside the transgene expression cassette
insl	773	3	tobacco INSL region near *trn*N
psaB	518	4	tobacco *psa*B gene
trnA	665	5	lettuce INSL region near *trn*A

Table 17 Overview of plant samples and the respective probes used for hybridization.

Plant line	Hybridized with probe
N.t.-GFP	insl
wt tobacco N.t.-EDIII 1 N.t.-EDIII 2 N.t.-EDIII 3	psaB, aadA, den
285-78-T7 285-78-T7-EDIII 1 285-78-T7-EDIII 2 258-78-T7-EDIII 4 285-78-T7-EDIII 1-4	insl
wt lettuce S12-PN-EDIII 1-4 S16-PN-EDIII 1	trnA

2.10.3 Western blot analysis

To extract total soluble protein (TSP), 200 mg of frozen leaf sample were ground into fine powder using liquid nitrogen and homogenized in 500 µl PEB I by vortexing for 3 minutes at RT. The supernatant was collected after centrifugation for 10 minutes at 13000 rpm at +4°, aliquoted and stored at -20°C.

Alternatively, 200 mg of frozen leaf sample were ground into fine powder using liquid nitrogen and homogenized in 500 µl PEB II by vortexing for 1 minute at RT in order to extract total protein (TP). 500 µl of Phenol were added to the plant cell extract, vortexed briefly and centrifuged at 13000 rpm for 10 minutes at +4°C. 200 µl of the upper green supernatant were transferred into a new tube and 1 ml of 0.1 M NH_4OAc in Methanol was added and the proteins were precipitated for 3 hours at -20°C. After centrifugation at 13000 rpm at +4°C for 10 minutes the pellet was washed twice with 500 µl 0.1 M NH_4OAc in Methanol and then air dried at RT. Finally the protein pellet was dissolved in 100 µl 1 % SDS and stored at -20°C.

20 µl of the sample were mixed with 5 µl Laemmli Buffer, denatured at 95°C for 10 minutes, spun down and loaded onto a 12 % PAA gel. Proteins were separated by electrophoresis first at 80 V until the sample had completely entered the separating gel, followed by 110 V until the dye front reached the end of the gel, and then transferred onto the nitrocellulose membrane and blocked with 0.5 % BSA in TBS-T for 1 hour. The membrane was briefly rinsed with TBS-T and then incubated with the primary antibody 1:1000 diluted in TBS-T overnight at +4°C. The membrane was washed three times with TBS-T at RT and incubated for 1 hour with alkaline phosphatase conjugated goat anti-mouse IgG (Promega) as a secondary antibody diluted 1:10000 in TBS-T at RT. Proteins were detected by colorimetric reaction using either the AP color development Kit (Bio-Rad, USA) or with Sigmafast™ BCIP®/NBT (Sigma).

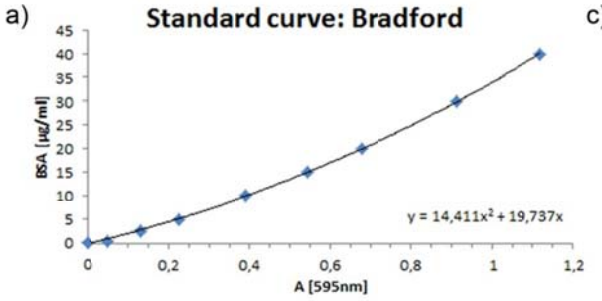
Coomassie staining with Brilliant Blue G was carried out in order to verify equal loading amounts of proteins. The PAA gels were stained for 1 hour at RT with the Coomassie staining solution and de-stained overnight in 10 % Acetic acid.

2.10.4 Quantification of extracted protein

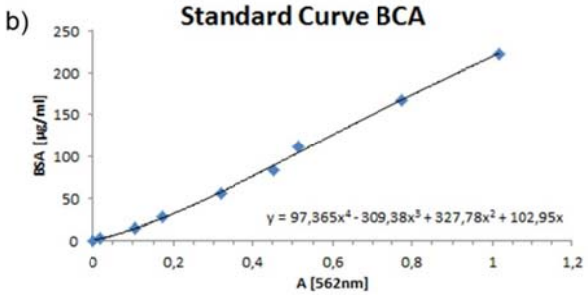
Quantification of isolated total soluble protein (TSP) was done using the Bradford assay and following the instructions provided in the manufacturer's protocol. The BSA standards were prepared by diluting the provided 2 mg/ml Stock in PEB I (Figure 7 (c)).The standards and samples were measured using the standard procedure where 1 ml ready to use Bradford Reagent is added to 20 µl sample, incubated at RT for 10 minutes and then measured at 595 nm. The standard curve was obtained as a regression equation (Figure 7 (a)) and was used to calculate the concentration of TSP in the unknown samples. All measurements were carried out in technical duplicates.

Quantification of total protein (TP) was done using the BCA protein assay Kit (Pierce). The BSA standard dilution series was prepared by diluting the provided 2 mg/ml Stock in PEB II to the final concentrations given in (Figure 7 (c)). Assays were carried out according to the protocol provided with the Kit and the microplate procedure was used where 25 µl of sample are mixed with 200 µl 1X Working reagent, incubated at 37°C for 30 minutes and then measured at 652 nm. The standard curve was obtained by regression (Figure 7 (b)) and the concentration of the unknown samples was calculated. All measurements were carried out in technical duplicates.

a)



b)



c)

No.	BSA Stock [µg/ml]	Bradford [µg/ml]	BCA [µg/ml]
1	2000	40	222
2	1500	30	167
3	1000	20	111
4	750	15	83
5	500	10	56
6	250	5	28
7	125	2,5	14
8	25	0,5	3
9	0	0	0

Figure 7 BSA standard curves. a) Standard curve and regression equation obtained for the Bradford assay using the BSA Stock solution prepared in PEB I with the final concentrations in the reaction as listed in c) under Bradford; b) Standard curve and regression equation obtained for the BCA assay using the BSA Stock solution prepared in PEB II with the final concentrations in the reaction as listed in c) under BCA; c) Table of prepared BSA Stock solutions and the final concentrations of BSA in the reaction for the Bradford assay and BCA assay, respectively. The sample is 1:50 diluted in the Bradford assay, while it is 1:9 diluted in the BCA assay.

2.11 Fluorescence confocal microscopy

GFP fluorescence in the plastids of transplastomic plants was observed on a SP5 II confocal system (Leica Microsystems, Germany) equipped with a HCX PL APO CS 63.0x1.20 water objective. The 405-nm line of an argon ion laser was used for excitation. Images were taken at the emission bandwidth of 500 – 540 nm for GFP and 688 – 757 nm for autofluorescence of the chloroplasts and processed using the Leica LAS AF software.

2.12 Mass spectrometric analysis

The MS analyses were kindly carried out at the UMB MS/Proteomics core facility (Norwegian University of Life Sciences, IKBM, Ås, Norway).

Samples bands were cut out from a Coomassie stained gel, digested with Trypsin following the protocol by Shevchenko et al, 2006 and peptides were purified and concentrated with modified STAGE microcolumns as described in Rappsilber et al, 2003.

The mass spectrometer (Q Exactive™ Hybrid Quadrupole - Orbitrap Mass Spectrometer) was set up as follows (Top10 method): a full scan (300-1600 m/z) at R=70.000 was followed by (up to) 10 MS2 scans at R=35000, using an NCE setting of 28. Singly charged precursors were excluded for MSMS, as were precursors with z>5. Dynamic exclusion was set to 20 seconds.

Raw files were converted to mgf format using the msconvert module of ProteoWizard (http://proteowizard.sourceforge.net/), and submitted to database search (either Dengue virus type 1 and type 3, or NCBInr using taxonomy other green plants) on an in-house Mascot (v.2.4) server using 10 ppm/20mamu tolerance for MS and MS/MS, respectively, and allowing for up to 2 miscleavages. Carbamidomethylated cysteine and oxidized methionine were selected as fixed and variable modifications, respectively.

Data was analyzed with the Scaffold 4.0 Proteomics Software.

3 Results

The findings and outcomes of this work can be summarized into three main results. First, this study reports the construction of a plastid transformation vector compatible with the Gateway® cloning technique and the successful expression of GFP using this vector in tobacco chloroplasts with an expression level of 3 % TSP (Gottschamel et al, 2013).

Second, the expression of antigen-encoding genes based on the Dengue virus envelope protein domain III (EDIII) has been accomplished by engineering the tobacco chloroplasts with two different vector systems. The recombinant EDIII 1 and EDIII 3 proteins were constitutively expressed in tobacco chloroplasts, while expression of EDIII 1, EDIII 2, EDIII 4 and EDIII 1-4 was obtained with the inducible expression system upon ethanol induction. Third, the constitutive expression of the Dengue antigenes EDIII 1 and EDIII 1-4 in lettuce chloroplasts was achieved in order to facilitate the development of an oral vaccine candidate.

3.1 Development of a plastid transformation vector introducing the Gateway® recombinant cloning technology

This study has presented a novel Destination vector (pDEST-PN-T) that can be converted into a plastid transformation vector in one single step using the Gateway® recombinant cloning technology. Furthermore, expression of GFP in transplastomic tobacco plants obtained using this vector, demonstrated the feasibility of the Gateway® recombinant cloning technology for chloroplast transformation.

3.1.1 Construction of pDEST-PN-T and pEXP-PN-GFP-T

The plastid transformation vector compatible with the Gateway® recombinant cloning system, was constructed by inserting the Gateway® reading frame cassette A (RfA) into a tobacco specific plastid transformation vector. The resulting Destination vector pDEST-PN-T (Figure 8 (a)) contains the *aad*A gene expression cassette, the constitutive *rrn16* PEP+NEP promoter (Ye et al, 2001) for transgene expression and the RfA flanked by tobacco specific INSL and INSR. BP reaction of the linearized vector containing the *att*B site flanked *gfp* and the *att*P site bearing pDONR221^{TM} resulted in pENTR-GFP (Figure 8 (b)). LR reaction of pENTR-GFP and pDEST-PN-T yielded the final plastid transformation vector pEXP-PN-GFP-T (Figure 8 (c)).

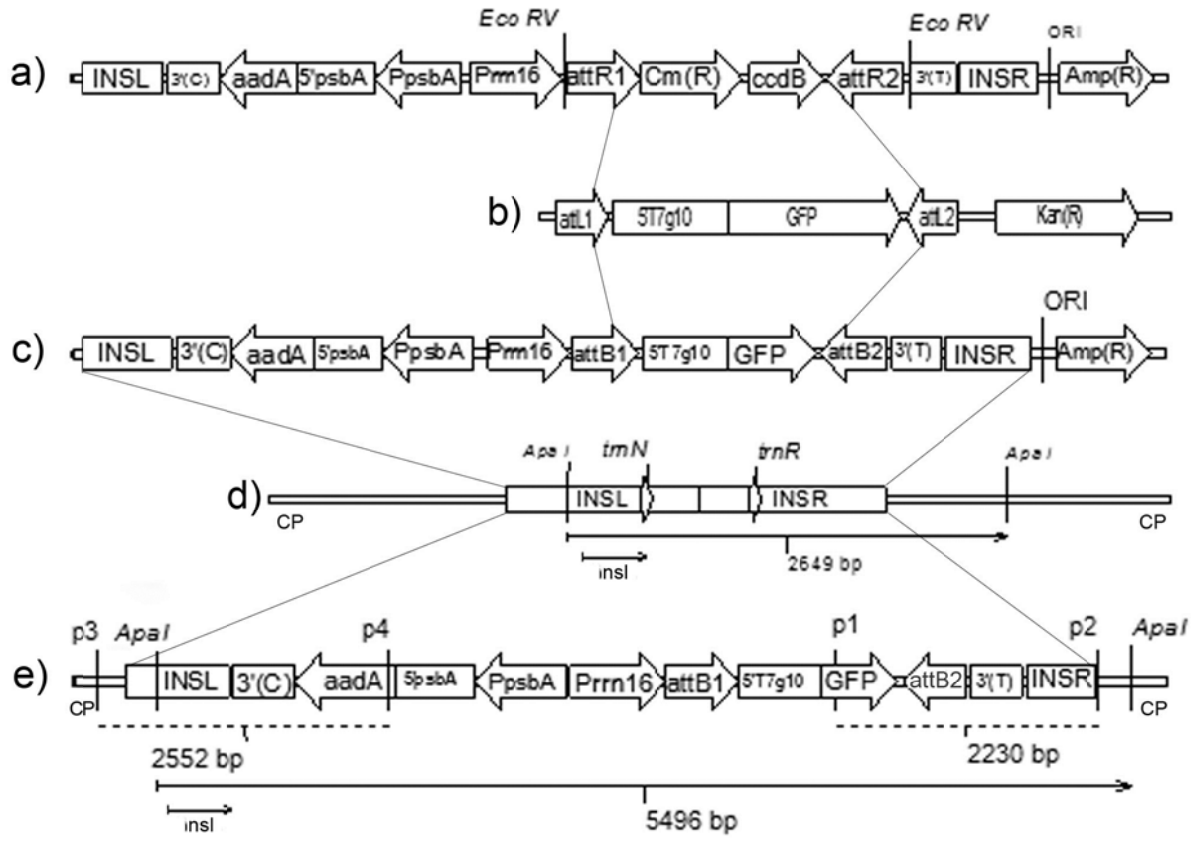


Figure 8 Schematic representation of the generation of transplastomic tobacco plants expressing GFP with the Gateway® Expression vector. a) pDEST-PN-T was constructed by insertion of the Gateway® RfA cassette (*att*R1-Cm(R)-*ccd*B-*att*R2) into the *EcoRV* site of a plastid transformation vector. b) pENTR-GFP contains the *gfp* sequence and the 5'UTR of bacteriophage T7 gene 10 (5'T7g10) flanked by *att*L1 and *att*L2. c) pEXP-PN-GFP-T was created by the LR reaction of pENTR-GFP and pDEST-PN-T and contains *gfp* under control of the *rrn*16 PEP+NEP promoter (P*rrn*16), the *aad*A gene under control of the *psb*A promoter (P*psb*A) and 5'UTR (5'*psb*A) and the 3'UTR of tobacco *rbc*L (3'T) and chlamydomonas *rbc*L (3'C), respectively. d) Wild-type tobacco plastid genome (CP). e) Tobacco plastid genome with integrated transgene expression cassette. The 773 bp Southern blot probe (insl) is shown as an arrow and the expected *ApaI* fragments are shown as arrows marked with their molecular weight. INSL/INSR: left/right insertion site; Amp(R): ampicillin resistance gene; Kan(R): kanamycin resistance gene; Cm(R): chloramphenicol resistance gene; *ccd*B: control of cell death gene; *aad*A: spectinomycin resistance gene; *trn*N/*trn*R: sequences coding for tRNA-N/tRNA-R; *att*B1/B2/R1/R2/L1/L2: Gateway® recombination sites; p1/p2/p3/p4: primers used for PCR and the corresponding PCR products are shown as dotted lines; ORI: bacterial origin of replication.

3.1.2 Regeneration of transplastomic plants

GFP expressing plants were obtained by chloroplast transformation of *N. tabacum* cv. Petit Havana with pEXP-PN-GFP-T using the biolistic approach. The site specific integration of the transgene into the wild-type chloroplast genome (Figure 8 (d)) due to homologous recombination generated transplastomic plants (Figure 8 (e)). Transgenic shoots developing from callus tissue on RMOP medium containing spectinomycin were tested for transgene integration by PCR. Correct integration of the transgenic sequence into the plastid genome was shown by a PCR product of 2230 bp with primers p1/p2 and 2552 bp with primers p3/p4 (Figure 9 (a)). Three independently transformed and regenerated plant lines (N.t.-PN-GFP 1, 2 and 3) were further characterized. Southern blot analysis verified the homoplastomic state of all three plant lines by the presence of only the 5496 bp fragment in GFP transformed plants, compared to the 2649 bp fragment in wild-type (Figure 9 (b)).

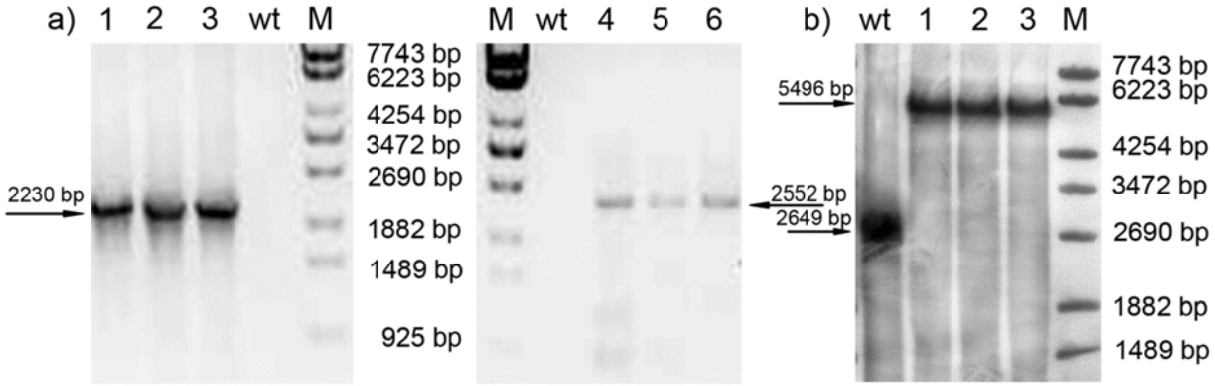


Figure 9 PCR and Southern blot analysis of transplastomic tobacco plants. a) PCR using primer pairs with one primer binding inside the transgene expression cassette (p1 in the *gfp* sequence, p4 in the *aad*A sequence) and the second primer binding inside the plastid genome (p2 downstream of INSR, p3 upstream of INSL). The expected PCR product showing correct integration of the transgenic sequence into the plastid genome is 2230 bp with p1/p2 (lane 1-3) and 2552 bp with p3/p4 (lane 4-6). b) Southern blot analysis of DNA isolated from three independently regenerated plant lines (lane 1-3) and wild-type (wt) was performed using a 773 bp DIG labeled probe that binds inside the *trnN* region (INSL) of the plastid genome. The expected fragment after *ApaI* digest is 5496 bp for transgenic plants and 2649 bp for wt plants. The positions of primers (p1, p2, p3, p4) and the corresponding expected size of PCR products, as well as restriction sites, probe position and the size of expected Southern blot bands are indicated in Figure 8. M: λ DNA/Eco130I (StyI) Marker 16, Fermentas.

3.1.3 Analysis of GFP expression

Expression of GFP was visually monitored using confocal laser scanning microscopy and co-localization of the GFP signal and the plastid autofluorescence was well observable in leaves of homoplastomic plants (Figure 10 (a)), thus confirming the expression of GFP in the chloroplasts. The total soluble protein content of plant extracts was determined using the Bradford assay and immunoblot analysis performed with the soluble fraction of protein extract and an anti-GFP primary antibody showed the expected 28 kDa band (Figure 10 (b)). The quantification of GFP expression by comparison of signal intensity to the GFP standard on the Western Blot determined an expression level of 3 % TSP.

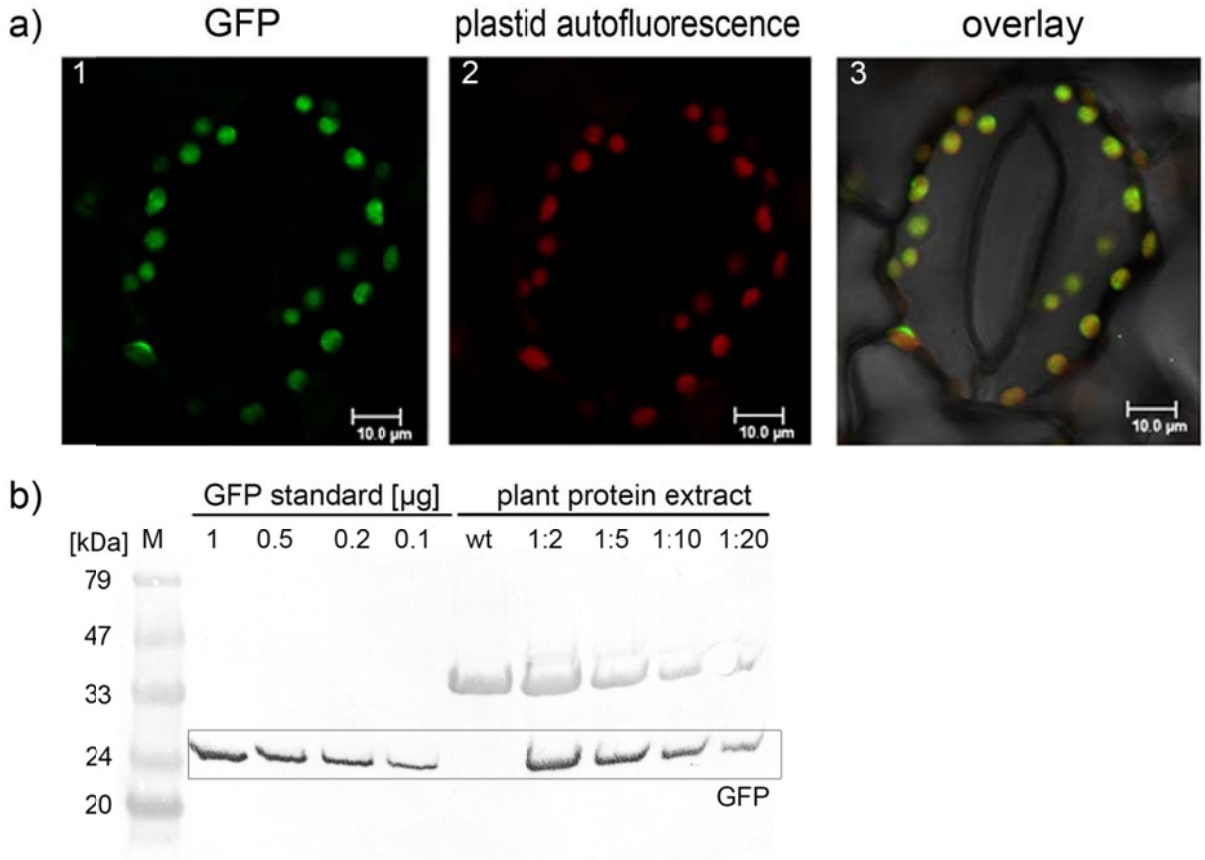


Figure 10 Detection and Quantitation of GFP expression. a) GFP fluorescence in the plastids of transplastomic plants observed with a confocal laser scanning microscope. 1) GFP fluorescence; 2) auto fluorescence of chloroplasts and 3) the overlay of pictures 1 and 2 shows the localization of GFP in the plastids. b) Western blot. The extract from the transplastomic plant was loaded in several dilutions and the GFP standard series was used as a reference. The ~ 28kDa protein bands of GFP were detected using the colorimetric detection method. Unspecific binding of the antibody to the crude plant extract results in the band migrating at 33 kDa which is present also in the wild-type sample. The dilution loaded of the plant protein extract is indicated above the lanes.

3.2 Expression of EDIII antigens in tobacco using a constitutive and an inducible expression vector

The results obtained in this study demonstrate that the production of monovalent envelope domain III proteins (EDIII) and a fusion protein consisting of the EDIIIs of all four virus serotypes in tobacco plastids is feasible. Homoplastomic plants were obtained and the proteins were expressed either in a constitutive or an inducible way.

3.2.1 Constitutive expression of EDIII 1, EDIII 2 and EDIII 3 in tobacco chloroplasts

3.2.1.1 Vector construction and plant regeneration

Vector construction was done at the Norwegian Institute of Agricultural and Environmental Research (Bioforsk, Ås, Norway). The vectors used for chloroplast transformation are derivatives of pKP9 (Zhou et al, 2008) and contain the EDIII domain of the Dengue virus serotypes 1, 2 and 3 under control of the constitutive promoter of the plastid ribosomal RNA operon. Plant transformation and regeneration of transformed plants have been carried out at the Max-Plank-Institute of Molecular Plant Physiology (MPI-MP, Golm, Germany).

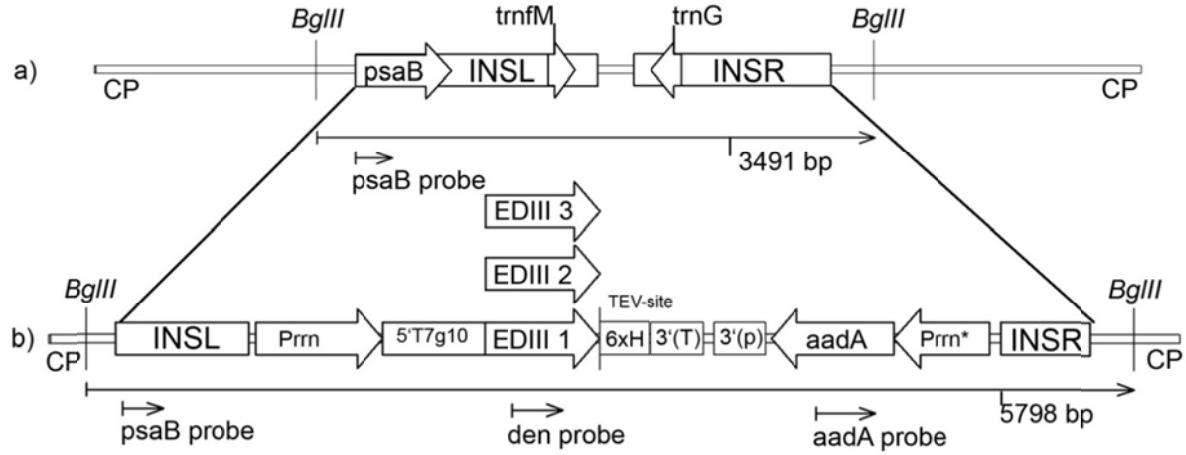


Figure 11 Schematic representation of the generation of transplastomic tobacco plants constitutively expressing the Dengue antigenes. a) Wild-type tobacco plastid genome (CP). b) Tobacco plastid genome with integrated transgene expression cassette for EDIII 1, EDIII 2 and EDIII 3, respectively. The Southern blot probes binding in the *psa*B region, the dengue gene and the *aad*A gene, respectively, are shown as arrows and the expected *BglII* fragments are shown as arrows marked with their molecular weight. INSL/INSR: left/right insertion site; *psa*B: 3' end of the *psa*B gene; *trn*fM/*trn*G: coding sequence for tRNA-fM/tRNA-G; P*rrn*: promoter of the tobacco plastid ribosomal RNA operon (Ruf et al, 2001); P*rrn**: chimeric rRNA operon promoter (Svab & Maliga, 1993); 5'T7g10: 5'UTR of bacteriophage T7 gene 10; EDIII 1/2/3: coding sequence for Dengue envelope protein domain III; 6xH: Hexa-his-tag; 3'(T): 3'UTR of tobacco *rbc*L gene; 3'(p): 3'UTR of tobacco *psb*A gene; *aad*A: spectinomycin resistance gene.

3.2.1.2 Charaterization of transplastomic tobacco plants

Seeds harvested from selfed transgenic plants transformed with the vectors pKP9-EDIII 1, pKP9-EDIII 2 and pKP9-EDIII 3, respectively, have been kindly provided by the MPI-MP. The homogenous green phenotype of the transplastomic seedlings, germinated on spectinomycin containing medium, proved the absence of segregation of the antibiotic resistance gene in the F1 generation, thus indicating homoplasmy of the transplastomic plant lines (Figure 12).

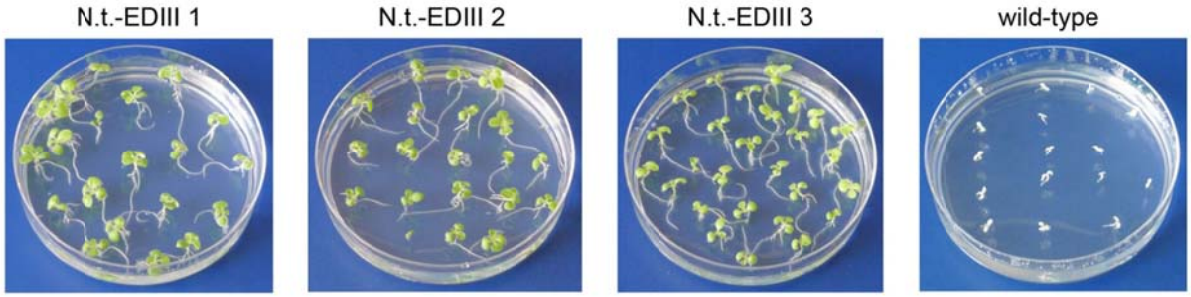


Figure 12 Germination test of transgenic tobacco seeds. Seeds obtained from three selfed transplastomic plants and wild-type seeds were germinated on 500mg/L spectinomycin containing medium.

Southern blot analyses were performed to verify the homoplastomic state of the transplastomic plant lines. Site specific integration between *trn*fM and *trn*G in the LSC region of the tobacco plastid genome (Figure 11 (a)) resulted in transplastomic plants (Figure 11 (b)). Each plant was analysed using three probes, targeted to three different sequences in the transgene expression cassette: “aadA” to confirm the presence of the *aad*A gene; “den” to confirm the presence of the EDIII coding sequence and “psaB” to verify integration into the chloroplasts genome. Presence of the *aad*A and the Dengue gene in the chloroplast genome was confirmed by the 5798 bp *BglII* restriction fragment detected when hybridized with the aadA probe (Figure 14 (a)) and the den probe (Figure 14 (b)). Homoplasmy of the analysed transplastomic plant lines was confirmed by only the presence of the 5798 bp fragment compared to the 3491 bp fragment in the wild-type plant when hybridized with the psaB probe (Figure 14 (c)).

Plants growing in soil under standard greenhouse conditions showed phenotypic alterations compared to the wild-type plants regarding growth rate, leaf size and coloration of the leaves (Figure 13). No morphological differences were observable for flower development and fertile seeds were obtained from all transgenic plant lines through self-fertilization.

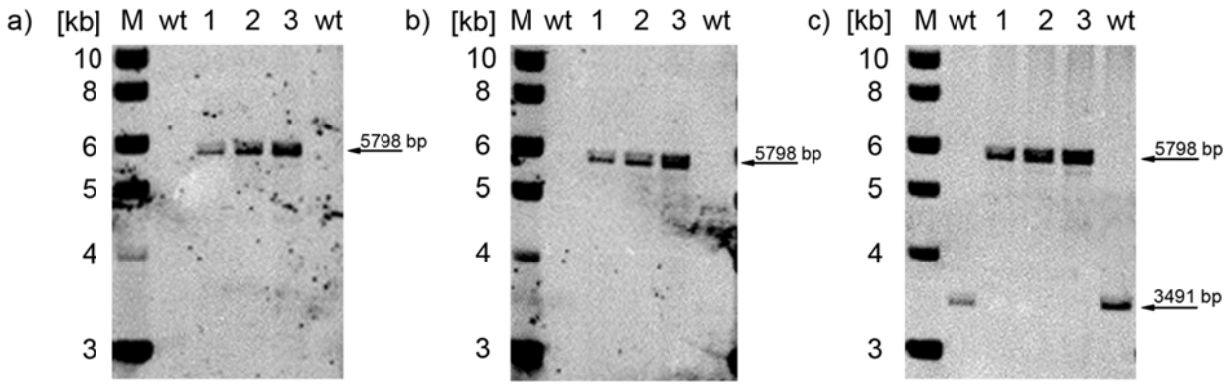


Figure 14 Southern blot analysis of transplastomic tobacco plant lines. *BglII* digested DNA from transplastomic and wild-type (wt) plants was hybridised to a DIG labeled probe. a) Hybridization signal obtained with the aadA probe; b) Hybridization signal obtained with the den probe; c) Hybridization signal obtained with the psaB probe. The expected fragment is 5798 bp for transplastomic plants and 3491 bp for the wild-type. Lane 1: N.t.-EDIII 1; Lane 2: N.t.-EDIII 2; Lane 3: N.t.-EDIII 3; M: *BglII* sites, probe position and the size of expected Southern blot bands are indicated in Figure 11; M: 1kb DNA ladder, NEB. The membrane was first hybridized with psaB probe, the striped and hybridized with den probe, striped again and hybridized with aadA probe.

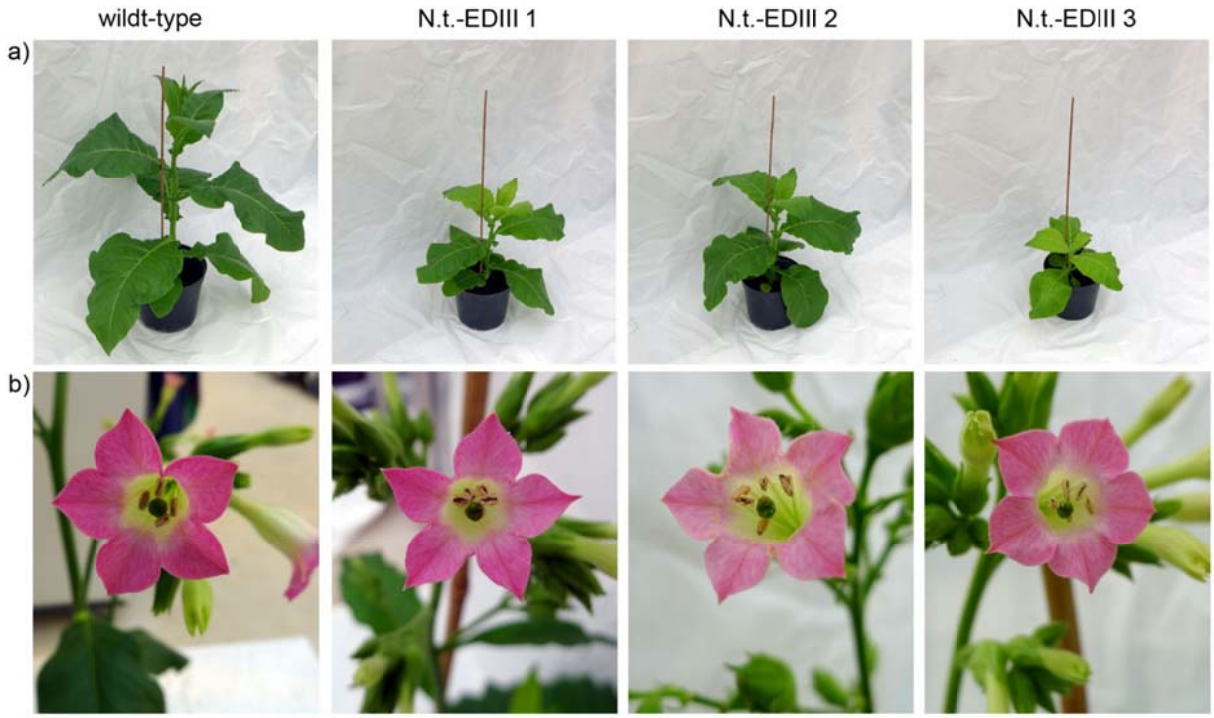


Figure 13 Phenotypic characterisation of plants constitutively expressing EDIII 1, EDIII 2 and EDIII 3. a) Seven weeks old plants; e) Flowers on mature plants.

3.2.1.3 Analysis of recombinant protein expression

Leaves of six weeks old homoplastomic plants growing in the greenhouse were labeled L1 (oldest) to L6 (youngest), harvested and both total soluble protein (TSP) and total protein (TP) were extracted from each leaf separately. Immunoblot analyses performed with TSP extracts detected the EDIII 1 protein, but failed to detect EDIII 2 and EDIII 3 (Figure 15 (a) – (c)). Furthermore the expression level of EDIII 1 increased from old to young leaves.

Immunoblot analyses performed with the TP extract detected EDIII 1 and EDIII 3, but with various signal intensities for the different leaf stages (Figure 15 (d) – (f)). In addition to the expected 13 kDa band corresponding to the monovalent Dengue antigen, also bands at higher molecular masses: ~28 kDa, ~40 kDa and ~50 kDa were detected. The absence of these bands in the wild-type sample indicates that these bands are not due to unspecific binding of the antibody to plant protein.

Mass spectrometric analysis of the additional protein bands (marked *1 - *8 in Figure 15) showed the presence of the same peptides in all the bands (Table 18), therefore confirming the specific binding of the anti-dengue antibody and strengthening the hypothesis of dimer/trimer formation of the recombinant EDIII 1 and EDIII 3 proteins.

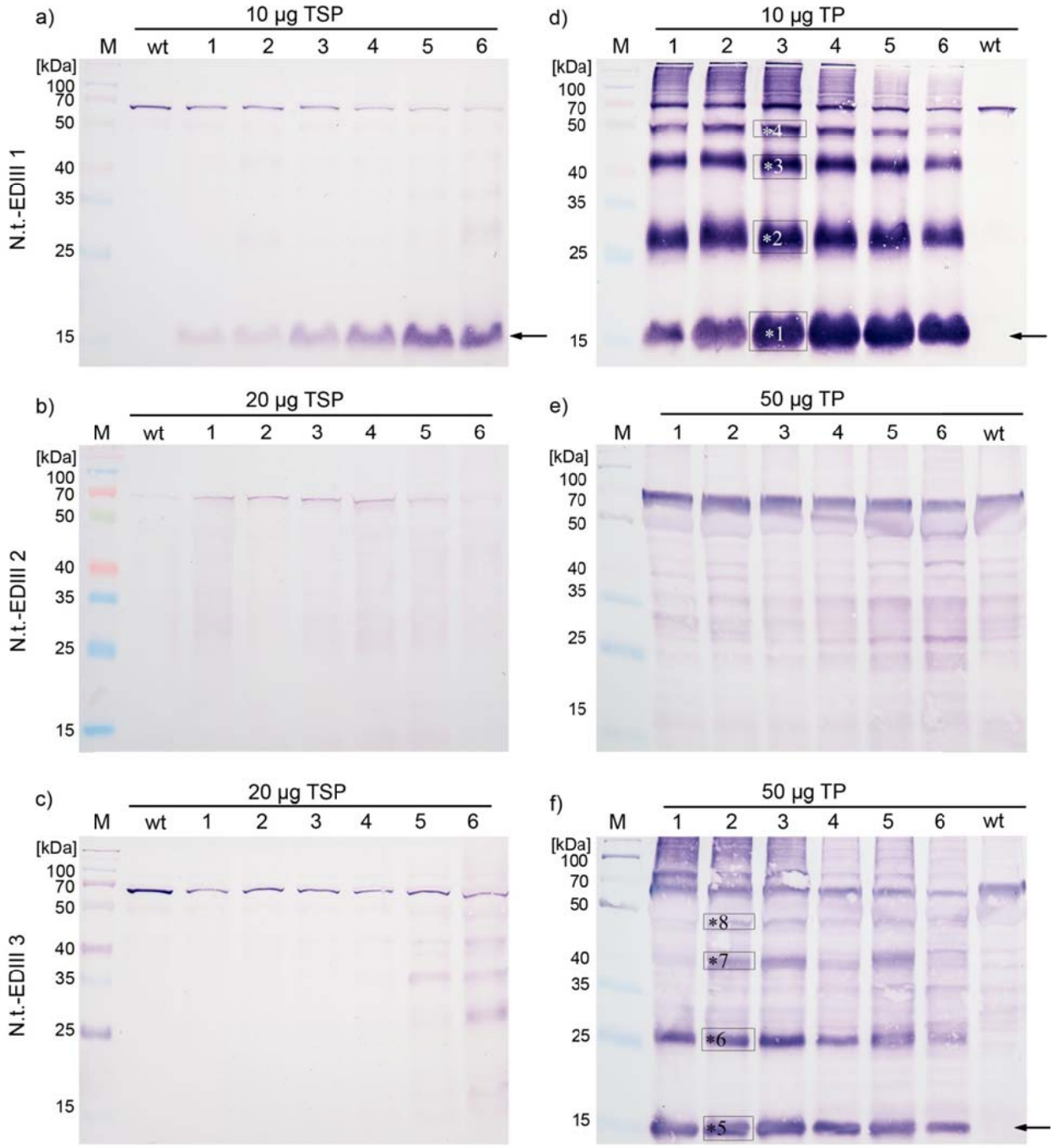


Figure 15 Western blot analysis of constitutively expressed Dengue antigens. Immunoblot analysis performed with TSP and TP extracted from tobacco plant lines N.t.-EDIII 1, N.t.-EDIII 2 and N.t.-EDIII 3. a) - c): Total soluble protein (TSP); d) - f) Total protein (TP); Lane 1-6: protein extract from leaf one (lane 1, oldest leaf) to leaf six (lane 6, youngest leaf); wt: wild-type; molecular size of EDIII 1, EDIII 2 and EDIII 3: ~13 kDa; The arrows mark the 13 kDa band of the recombinant protein in (a), (d) and (f); The boxed bands marked with an asterisk in (d) and (f) were cut out from an identical coomassie stained gel and subjected to mass spectrometric analysis. The amount of TSP/TP loaded for each sample is stated above the gel and the size of the marker bands are indicated in kDa. M: spectra multicolour broad range protein ladder, Thermo Scientific.

Table 18 Mass spectrometric analysis. All possible peptides obtained after trypsin digest of EDIII 1 or EDIII 3, respectively are listed in the first column. The bands cut out from the coomassie stained gel were digested with trypsin, and the purified and concentrated peptides were analysed by mass spectrometry. *1 - *8 represent the bands in Figure 15 and sample 9 was an *E.coli* derived EDIII 3 as a positive control. + indicates the presence of the peptide in the respective sample, - indicates that the peptide was not found in this sample. Peptides marked with # were only found as parts of bigger peptides due to only partial trypsin digest.

Peptides for EDIII 1	***1**	***2**	***3**	***4**	
1) MSYVMCTGSFK	+	-	-	-	
2) #LEK	+	+	+	+	
3) EVAETQHGTVLVQVK	+	+	+	+	
4) #YEGTDAPCK	+	+	+	+	
5) IPFSSQDEK	+	+	+	+	
6) #GVTQNGR	+	+	+	+	
7) LITANPIVTDK	+	+	+	+	
8) EKPVNIEAEPPFGESYIVVGAGEK	+	+	+	+	
9) ALK	+	+	-	-	
10) #LSWFK	+	+	-	-	
11) K	-	-	-	-	
12) GSSIGK	-	-	-	-	
13) GGGGGENLYFQGHHHHHH*	+	+	-	-	
Peptides for EDIII 3	***5**	***6**	***7**	***8**	**9**
1) MSYAMCLNTFVLK	-	-	-	-	-
2) K	-	-	-	-	-
3) EVSETQHGTILIK	+	+	+	+	+
4) #VEYK	+	-	+	+	+
5) #GEDAPCK	+	-	+	+	+
6) IPFSTEDGQGK	+	-	+	+	+
7) #AHNGR	+	-	-	+	+
8) LITANPVVTK	+	+	+	+	+
9) EEPVNIEAEPPFGESNIVIGIGDK	+	-	+	+	+
10) ALK	+	-	-	-	-
11) #INWYR	+	-	-	-	-
12) GSSIGK	-	-	-	-	-
13) GGGGGENLYFQGHHHHHH*	+	-	+	-	-

3.2.2 Inducible expression of EDIII 1, EDIII 2, EDIII 4 and EDIII 1-4 in tobacco chloroplasts

3.2.2.1 Construction of plastid transformation vectors

Gateway® cloning of the EDIII sequences into the tobacco specific pDEST-T7-T yielded the plastid transformation vectors pEXP-T7-EDIII 1-T, pEXP-T7-EDIII 2-T, pEXP-T7-EDIII 4-T and pEXP-T7-EDIII 1-4-T (Figure 16 (a)). Site specific integration between *trn*N and *trn*R in the IR region of the tobacco plastid genome (Figure 16 (b)) via the homologous recombination mechanism resulted in transplastomic plants carrying the corresponding transgene expression cassettes (Figure 16 (c)).

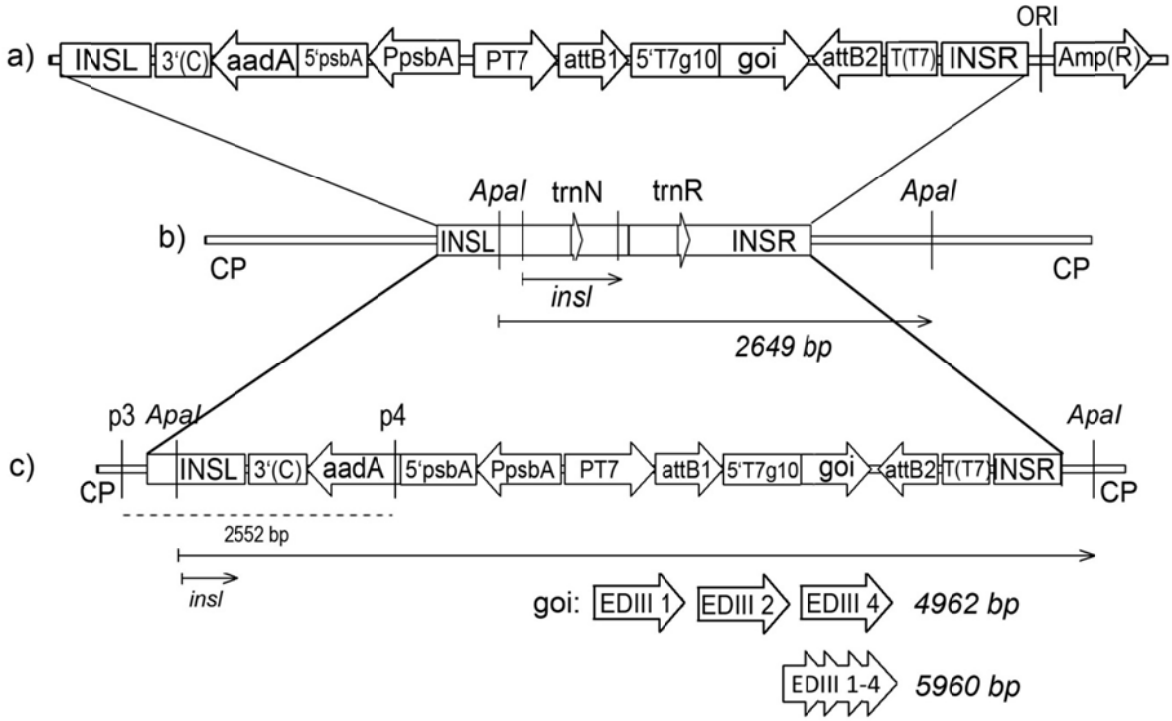


Figure 16 Schematic representation of the generation of transplastomic tobacco plants expressing the Dengue antigens upon ethanol induction. a) Final plastid transformation vector pEXP-T7-goi-T; b) Wild-type tobacco plastid genome (CP); c) Tobacco plastid genome with integrated transgene expression cassette for EDIII 1, EDIII 2, EDIII 4 and EDIII 1-4, respectively. The 773 bp Southern blot probe (insl) is shown as an arrow and the expected *ApaI* fragments are shown as arrows and their size is indicated next to the respective goi. *aad*A: spectinomycin resistance gene; Amp(R): ampicillin resistance gene; *att*B1/*att*B2: Gateway® recombination sites; INSL/INSR: tobacco specific left/right insertion site; *trn*N/*trn*R: sequences coding for tRNA-N/tRNA-R; EDIII 1/2/4/1-4: coding sequence for transgene including a Hexa-his-tag; P*psb*A: tobacco *psb*A promoter (Staub & Maliga, 1993); PT7: phage λ derived T7 promoter (Tabor & Richardson, 1985); 3'(C): 3'UTR of chlamydomonas *rbc*L gene; 5'*psb*A: 5'UTR of tobacco *psb*A gene; T(T7): T7 terminator; ORI: bacterial origin of replication. p3/p4: primers used for PCR and the corresponding PCR product is shown as a dotted line marked with its molecular weight.

3.2.2.2 Transplastomic plant regeneration and characterization

Transgenic shoots developing from callus tissue on RMOP medium containing spectinomycin and kanamycin were tested for transgene integration by PCR. Correct integration of the transgenic sequence into the plastid genome was shown by a PCR product of 2552 bp with primers p3/p4 (Figure 17 (a)). Bombardment of 285-78-T7 leaves resulted in the direct generation of 285-78-T7-EDIII 1, 285-T7-EDIII 2 and 285-T7-EDIII 4, while 285-78-T7-EDIII 1-4 was only obtained after back-crossing of N.t.-T7-EDIII 1-4 with pollen derived from 285-78-T7. The transplastomic plant lines (285-78-T7-EDIII 1, 285-78-T7-EDIII 2, 285-78-T7-EDIII 4 and 285-78-T7-EDIII 1-4) were further characterized by Southern blot analysis using *ApaI* to produce restriction fragments and a DIG labeled probe binding inside the INSL sequence. The homoplastomic state of all four transgenic plant lines was verified by the presence of only the 4962 bp fragment (for 285-78-T7-EDIII 1, 285-78-T7-EDIII 2, 285-78-T7-EDIII 4) or the 5960 bp fragment (for 285-78-T7-EDIII 1-4) in transformed plants, compared to the 2649 bp fragment for 285-78-T7 (Figure 17 (b)).

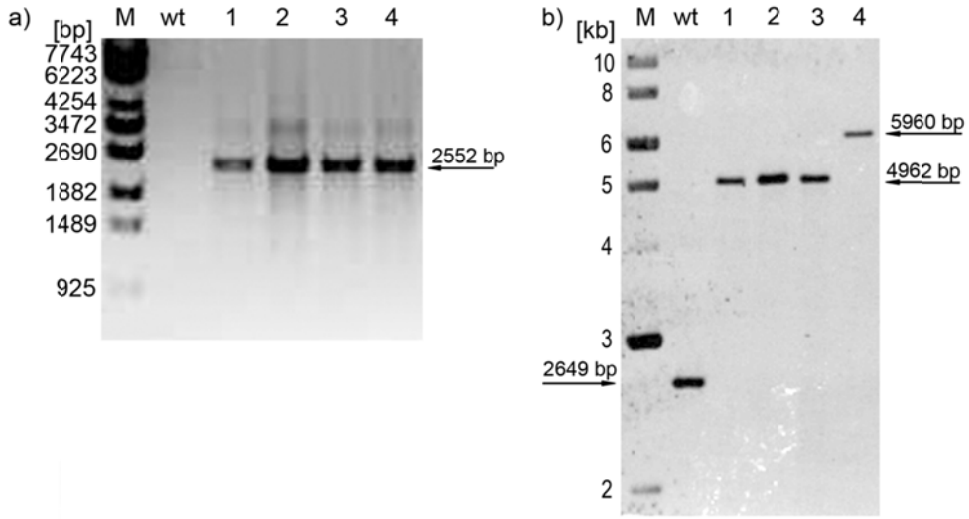


Figure 17 PCR and Southern blot analysis of transplastomic tobacco plants. a) PCR using primer pair with one primer binding inside the transgene expression cassette (p4) and the second primer binding inside the plastid genome (p3) upstream of INSL. The expected PCR product showing correct integration of the transgenic sequence into the plastid genome is 2552 bp. b) Southern blot analysis of DNA isolated from regenerated plant lines and 285-78-T7 was performed using a 773 bp DIG labeled probe that binds inside the *trn*N region (INSL) of the plastid genome. The expected fragment size after *ApaI* digest is 4962 bp (for lane 1-3) or 5960 bp (for lane 4) and 2649 bp for wt lane. Lane 1: 285-78-T7-EDIII 1; lane 2: 285-78-T7-EDIII 2; lane 3: 285-78-T7-EDIII 4; lane 4: 285-78-T7-EDIII 1-4; wt: 285-78-T7. The positions of primers (p3, p4) and the corresponding expected size of the PCR product, as well as restriction sites, probe position and the size of expected Southern blot bands are indicated in Figure 16. M in a): λ DNA Marker 16, Fermentas; M in b): 1kb DNA ladder, NEB.

Slight growth retardations were observable for plant lines 285-78-T7-EDIII 4 and 285-78-T7-EDIII 1-4 when compared to the growth of plant line 285-78-T7 in soil under standard greenhouse conditions (Figure 18 (a)), but flower set and development occurred normally and fertile seeds were obtained from all transplastomic plant lines (Figure 18 (b)). Seeds harvested from selfed transgenic plants were germinated on spectinomycin and kanamycin containing medium. The seedlings demonstrated a homogenous green phenotype, thus proving the absence of segregation of the antibiotic resistance gene in the F1 generation (Figure 18 (c)).

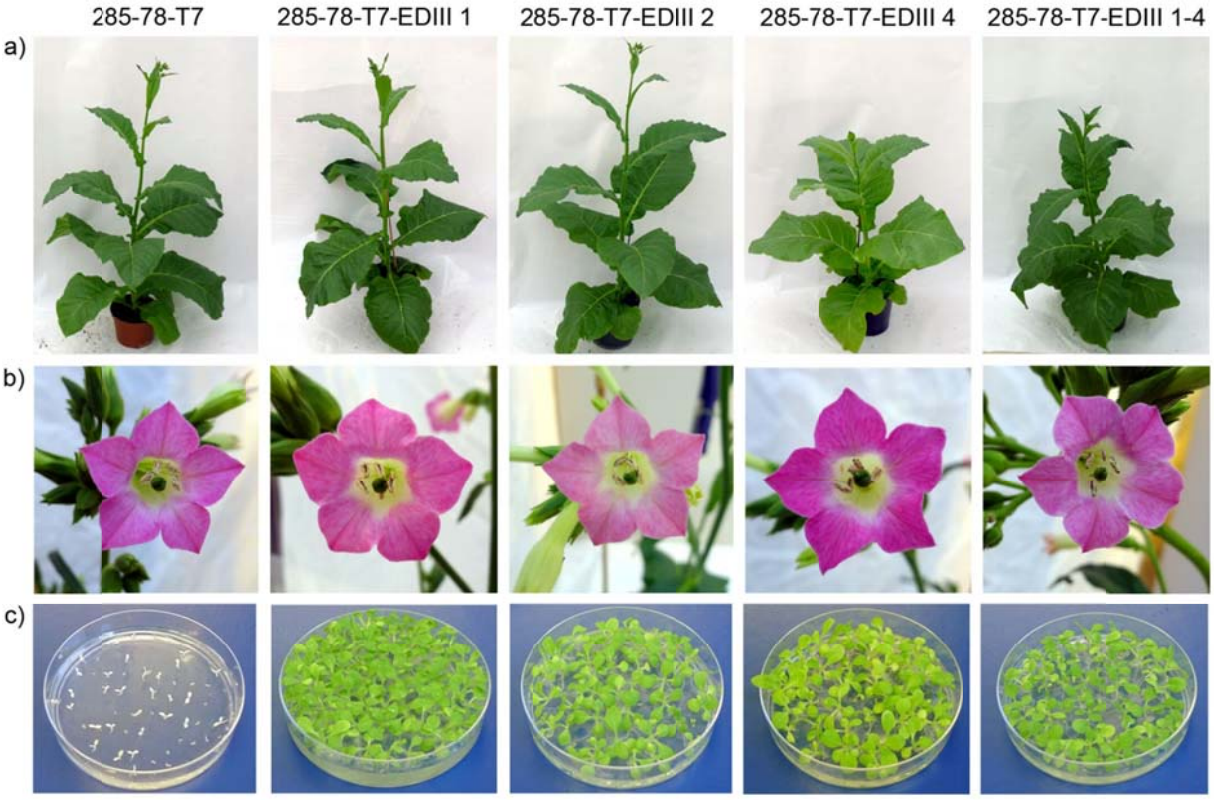


Figure 18 Phenotypic characterisation of transplastomic plants. a) Nine weeks old plants; b) Flowers on mature plants; c) Germination test with seeds obtained from selfed transplastomic plants on spectinomycin and kanamycin containing medium.

3.2.2.3 Induction of recombinant protein expression by ethanol treatment

Five weeks old *in vitro* plants were sprayed with 5 % ethanol on seven consecutive days to induce recombinant protein expression. No negative effect of the ethanol treatment on the plants was observable during the treatment (Figure 19). Total protein (TP) was extracted from plant lines 285-78-T7-EDIII 1, 285-78-T7-EDIII 2, 285-78-T7-EDIII 4 and 285-78-T7-EDIII 1-4 for every day of the ethanol spray experiment and immunoblot analysis detected the recombinant EDIII proteins in the respective plant lines, though with differences in the expression level (Figure 20). EDIII 1 and EDIII 2 were detected with a steady expression level in all the samples, while EDIII 4 and EDIII 1-4 expression levels showed an increase following repeated ethanol exposure.

Figure 19 Ethanol treatment of transplastomic plants for the induction of Dengue antigen expression. a) Five weeks old plants in Magenta boxes before the first ethanol treatment (day 0); b) The same plants as in a) after treatment with 5 % ethanol on seven consecutive days (day 7).

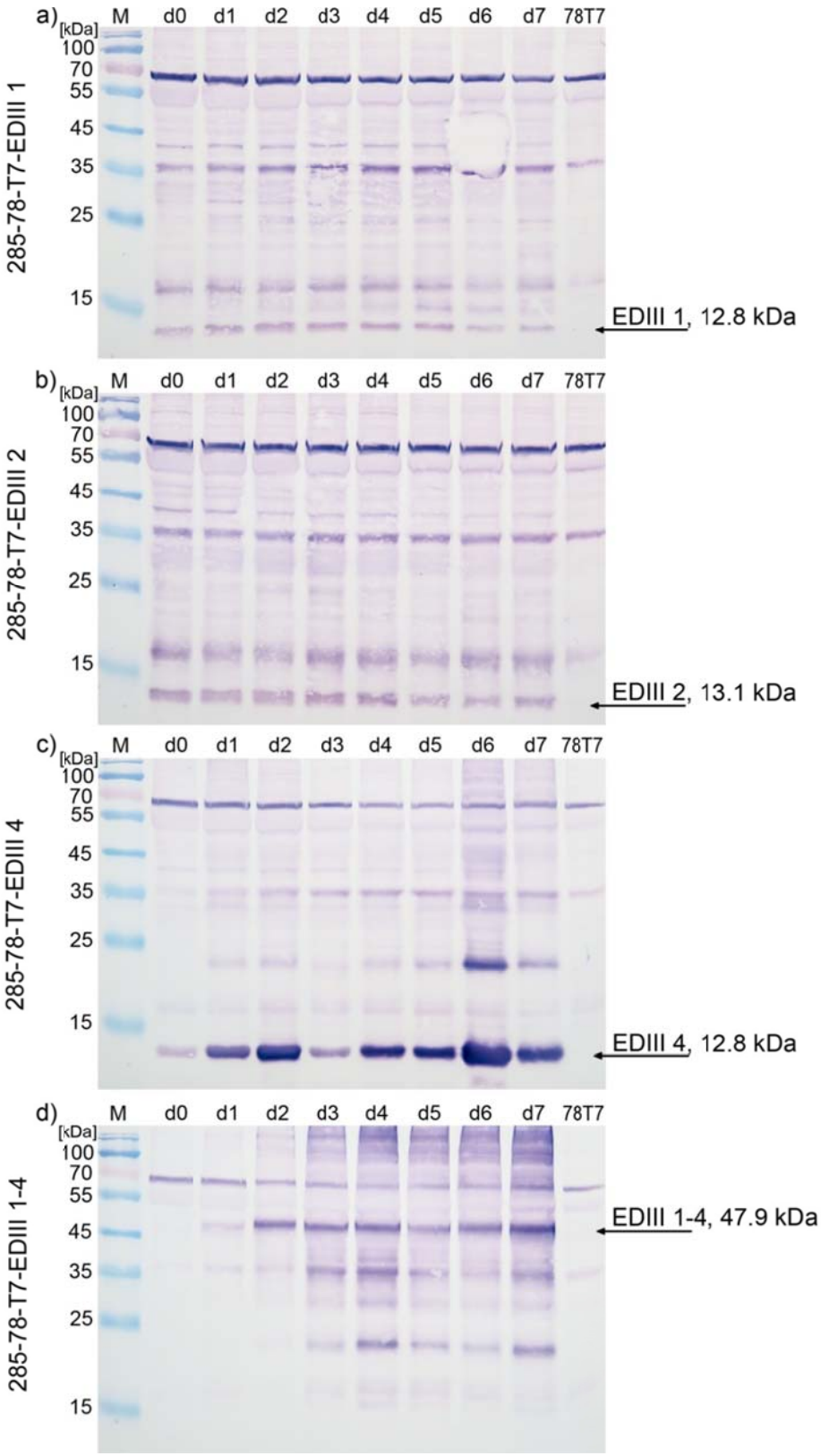


Figure 20 Western blot analysis of inducibly expressed Dengue antigens. Immunoblots performed with 50 µg TP from ethanol induced transplastomic plants. a) 285-78-T7-EDIII 1; b) 285-78-T7-EDIII 2; c) 285-78-T7-EDIII 4; d) 285-78-T7-EDIII 1-4; d0: before ethanol treatment; d1: one day after the first ethanol treatment; d2: two days after the first ethanol treatment; … d7: sample taken seven days after the first ethanol treatment; 78T7: 285-78-T7 on day 7 of ethanol treatment. The arrow indicates the respective recombinant protein and its estimated size is stated.

3.3 Expression of monovalent EDIII 1 and tetravalent EDIII 1-4 antigens in lettuce plastids

3.3.1 Vector construction

The lettuce plastid transformation vector pDEST-PN-L was constructed by insertion of the *aad*A expression cassette and the Gateway® RfA between lettuce specific sites for homologous recombination. The vectors pEXP-PN-EDIII 1-L and pEXP-PN-EDIII 1-4-L (Figure 21 (a)) used for lettuce plastid transformation were obtained by Gateway® cloning of the sequences for EDII 1 and EDIII 1-4 into the lettuce specific pDEST-PN-L. Homologous recombination into the intergenic spacer region between *trn*I and *trn*A in the IR region of the lettuce plastid genome (Figure 21 (b)), resulted in transplastomic plants carrying the corresponding transgene expression cassettes (Figure 21 (c)).

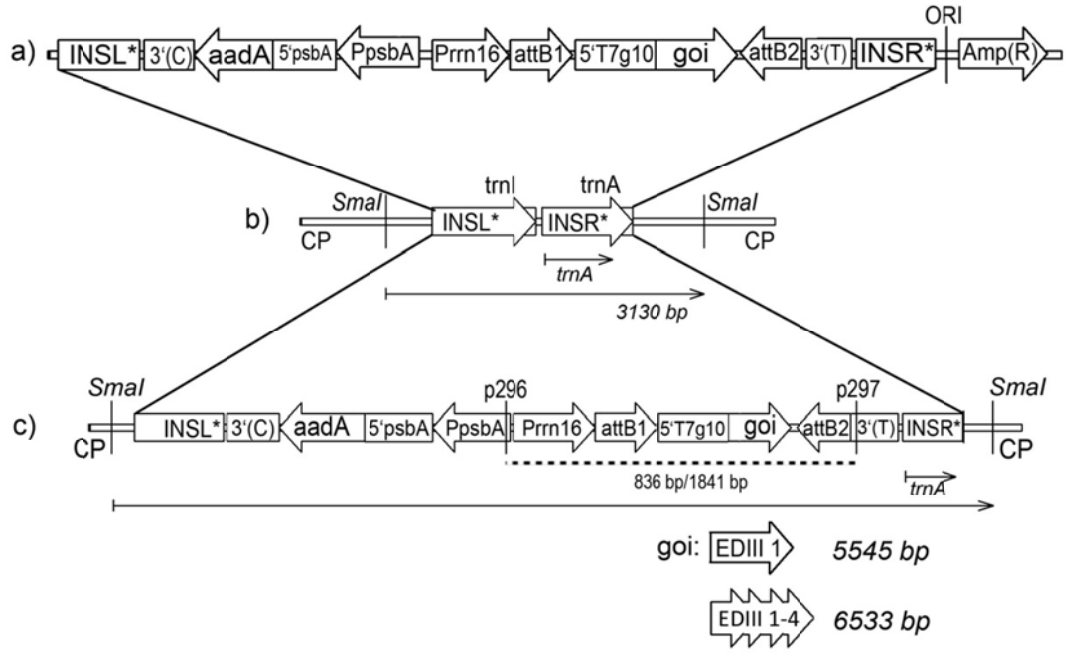


Figure 21 Schematic representation of the Expression vectors for the generation of transplastomic lettuce plants. a) The final lettuce specific plastid transformation vector pEXP-PN-goi-L; b) Wild-type lettuce plastid genome (CP); c) Lettuce plastid genome with integrated transgene expression cassette for EDIII 1 and EDIII 1-4, respectively. The Southern blot probe (trnA) is shown as an arrow and the expected *SmaI* fragments are shown as arrows and their size is indicated next to the respective goi. *aad*A: spectinomycin resistance gene; Amp(R): ampicillin resistance gene; *att*B1/*att*B2: Gateway® recombination sites; INSL*/INSR*: lettuce specific left/right insertion site; *trn*I/*trn*A: sequences coding for tRNA-I/tRNA-A; EDIII 1/1-4: coding sequence for transgene including a Hexa-his-tag; P*psb*A: tobacco *psb*A promoter (Staub & Maliga, 1993); P*rrn*16: tobacco *rrn*16 PEP+NEP promoter (Ye et al, 2001); 3'(C): 3'UTR of chlamydomonas *rbc*L gene; 5'*psb*A: 5'UTR of tobacco *psb*A gene; 3'(T): 3'UTR of tobacco *rbc*L gene; ORI: bacterial origin of replication. p296/p297: primers used for PCR and the corresponding PCR product is shown as a dotted line marked with its molecular weight for both transgenes.

3.3.2 Transplastomic plant regeneration and characterization

Transgenic shoots developing from callus tissue on RMOP medium containing spectinomycin were tested for transgene integration by PCR. Presence of the transgenic sequence in the plastid genome was shown by a PCR product of 1841 bp for EDIII 1-4 and 836 bp for EDIII 1 with primers p296/p297 (Figure 22 (a)). The transplastomic plant lines (S12-PN-EDIII 1-4 and S16-PN-EDIII 1) were further characterized by Southern blot analysis. The homoplastomic state of both plant lines was verified by the presence of only the 5545 bp fragment (for S16-PN-EDIII 1) or the 6533 bp fragment (for S12-PN-EDIII 1-4) in transformed plants, compared to the 3130 bp fragment in wild-type (Figure 22 (b)) after digest of total plant DNA with *SmaI*. No phenotypic alterations were visible on transplastomic plants growing in the greenhouse (Figure 23 (a)) and flower set and seed development was normal. Plants were grown to full maturity (Figure 23 (b)) and seeds harvested from transgenic plants were germinated on spectinomycin containing medium. The homogenous green phenotype of the seedlings proved the absence of segregation of the antibiotic resistance gene in the F1 generation (Figure 23 (c)).

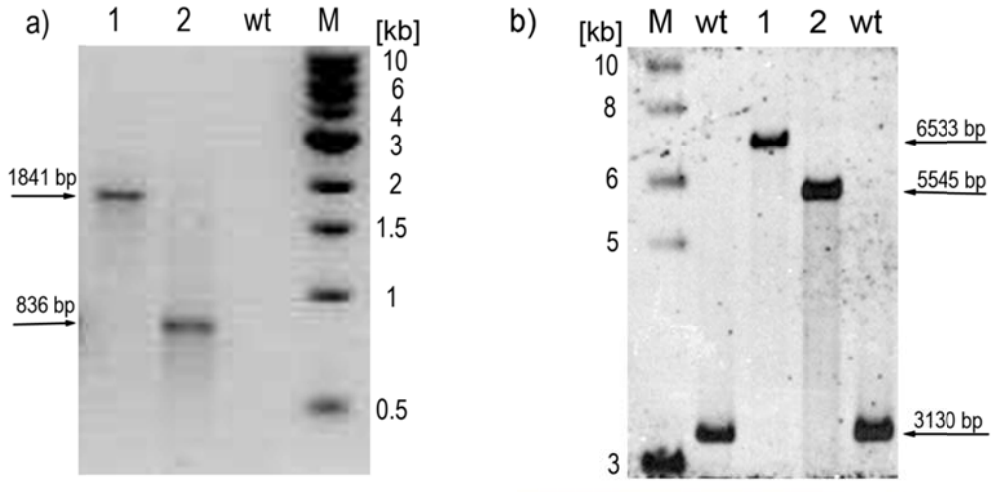


Figure 22 PCR and Southern blot analyses of regenerated lettuce plants. a) PCR using primers p296/p297 binding inside the transgene expression cassette. The expected PCR product is 1841 bp for S12-PN-EDIII 1-4 (lane 1) or 836 bp for S16-PN-EDIII 1 (lane 2). b) Southern blot analysis of DNA isolated from regenerated plant lines and wild-type (wt) was performed using a 665 bp DIG labeled probe that binds inside the *trn*A region (INSR) of the plastid genome. The expected fragment size after *SmaI* digest is 6533 bp for S12-PN-EDIII 1-4 (lane 1) or 5545 bp for S16-PN-EDIII 1 (lane 2) and 3130 bp for wt plants. The positions of primers and the corresponding expected size of PCR product, as well as restriction sites, probe position and the size of expected Southern blot bands are indicated in Figure 21. M: 1kb DNA ladder, NEB.

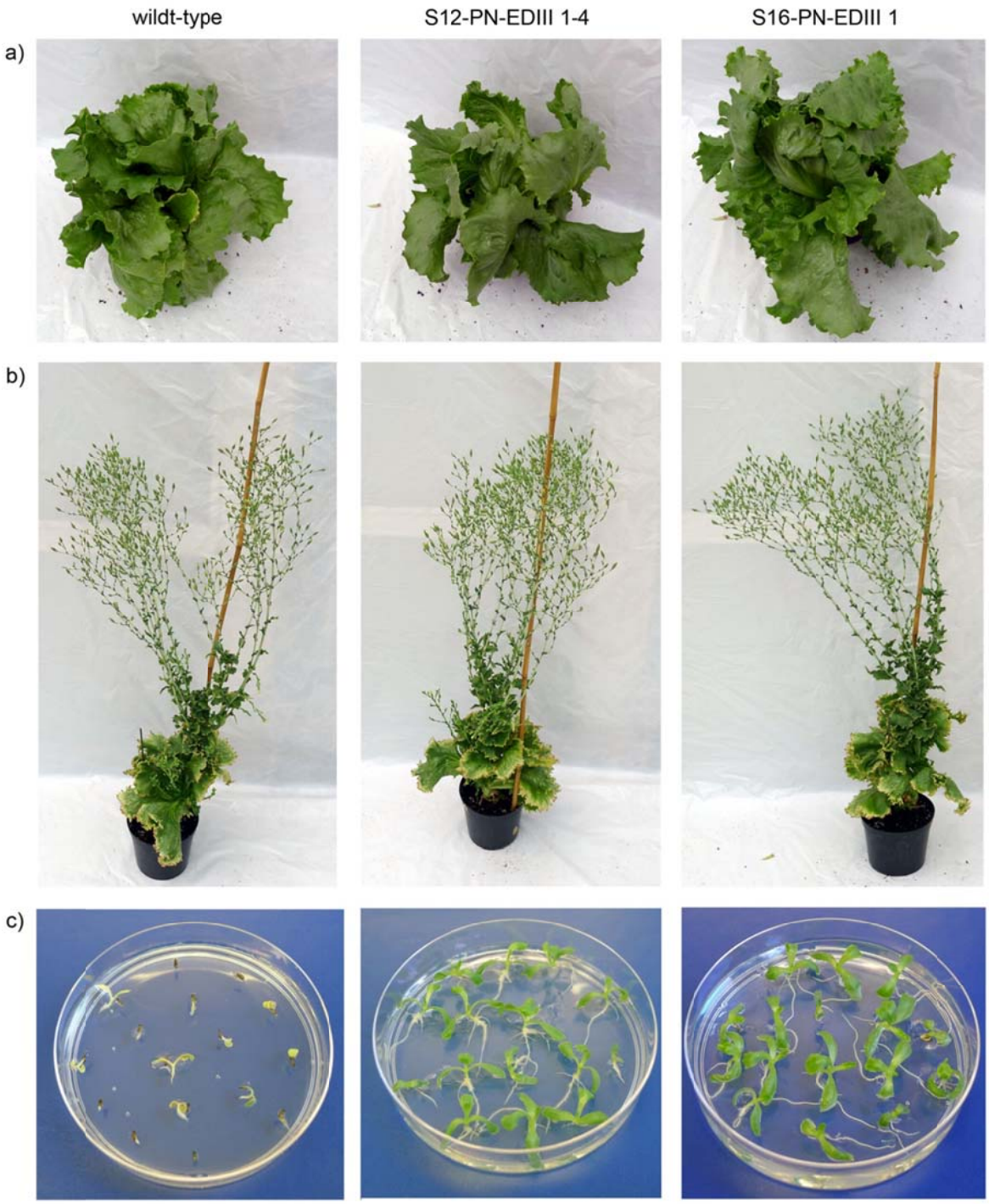


Figure 23 Phenotype and Germination assay of transplastomic lettuce plants. a) Plants growing in the greenhouse; b) Flowering plants; c) One week old seedlings obtained from transplastomic plants and wild-type seeds germinated on spectinomycin (30 mg/L) containing medium.

3.3.3 Analysis of the expression of EDIII 1-4 and EDIII 1 antigens

Total protein (TP) and total soluble protein (TSP) were isolated from plants growing in the greenhouse, quantified by BCA and Bradford assay and immunoblot analysis performed with an anti-dengue antibody detected both the 13 kDa EDIII 1 and the 47 kDa EDIII 1-4 in the respective plants (Figure 24).

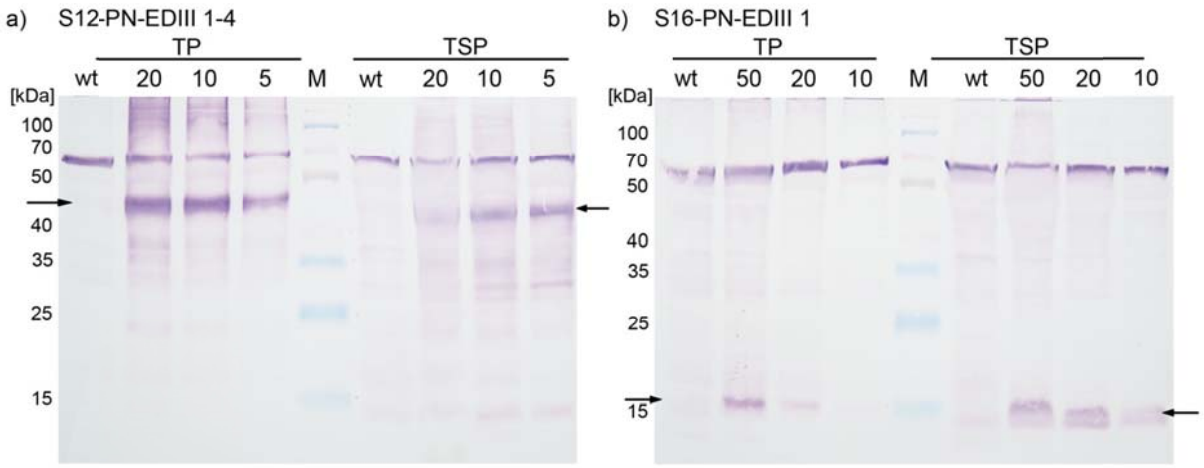


Figure 24 Western Blot analysis of Dengue antigens expressed in lettuce. a) TP and TSP isolated from plant line S12-PN-EDIII 1-4; b) TP and TSP isolated from plant line S16-PN-EDIII 1; the amount of TSP/TP loaded is given above the respective lane in µg; in a) 20 µg TP/TSP were loaded for the wild-type, while in b) 50 µg TP/TSP were loaded for the wild-type. The arrows indicate the 47 kDa EDIII 1-4 and the 13 kDa EDIII 1; M: spectra multicolour broad range protein ladder (Thermo Scientific), molecular size is indicated in kDa.

4 Discussion

The present study has described the production of vaccine candidates against Dengue fever in green plants. Chloroplast genetic engineering has been employed to develop an economic and safe production platform for the recombinant protein vaccine candidates. The expression of the tetravalent EDIII fusion protein and the four monovalent EDIII forms has been achieved by using chloroplast engineering in tobacco and lettuce, thus proving the feasibility of the chosen approaches.

Dengue fever (DF) and Dengue haemorrhagic fever (DHF) are caused by the Dengue viruses consisting of four antigenically related but distinct virus serotypes. With an estimated 50 – 100 million new infections every year (WHO, 2013), this viral disease is a significant cause of morbidity and mortality in developing tropical and subtropical countries (Gubler, 2012). Due to its occurrence in four serotypes and its complex pathogenesis, so far there is no vaccine available against Dengue fever. The serotype specific antibodies, produced in the course of the first infection, cross-react with the remaining serotypes during secondary infection, causing the severe forms of the disease: Dengue shock syndrome and DHF (Halstead, 1988). Therefore, an effective Dengue vaccine must be tetravalent and confer protection against all four virus serotypes (Hombach et al, 2005).

Currently, the most promising tetravalent vaccine candidate (CYD15) containing four monovalent chimeric yellow fever Dengue vaccine strains is undergoing a phase III clinical trial (Sanofi-Pasteur, 2014). However, previous difficulties associated with tetravalent Dengue vaccine candidates consisting of the four live-attenuated virus strains for the Mahidol- (Edelman et al, 2003; Kanesa-Thasan et al, 2001) and the WRAIR-vaccine (Eckels et al, 2000a; Sun et al, 2003) have put an increased focus on developing a recombinant protein vaccine. The most promising attempts focus on the envelope protein domain III (EDIII) (Guzman et al, 2010). This domain contains the main serotype specific epitopes (Chin et al, 2007; Megret et al, 1992) and has only very low intrinsic potential for eliciting cross-reactive antibodies against heterologous serotypes (Hombach et al, 2005). A recombinant fusion protein linking the EDIII domains of Dengue virus serotypes 1, 2, 3 and 4 has elicited neutralizing antibodies against all four serotypes (Batra et al, 2007; Etemad et al, 2008) and indicates a way to avoid unbalanced immune responses reported for tetravalent formulations consisting of stoichiometrically mixed monovalent vaccines (Sabchareon et al, 2012).

In order to reduce the workload and time necessary for constructing the required plastid transformation vectors, the first part of this work consisted in implementing the Gateway® recombination cloning technology. Cloning with traditional restriction enzymes is laborious and time consuming, because the choice of suitable restriction enzymes is frequently limited by the transgene's sequence and often considerable adaptations of the transgene's sequence are required to eliminate unwanted cutting sites. These major limitations are circumvented by the Gateway® cloning system allowing the rapid and efficient insertion of any transgene into the Expression vector without considerations regarding interference of internal restriction sites of a candidate sequence. Since the construction of the first series of plant Destination vectors (Dubin et al, 2008; Earley et al, 2006; Karimi et al, 2007) continual improvements and modifications have led to an increasing number of vectors available for different purposes (Buntru et al, 2013; Dubin et al, 2010; Karimi et al, 2013; Lyska et al, 2013). However, only vectors for transient expression or stable nuclear transformation have been reported until we started the attempt of implementing the Gateway® cloning system in plastid genome engineering.

A set of novel Destination vectors, that can be converted into a final plastid transformation vector in one single step using Gateway® recombinant cloning technology, has been developed. The Gateway® cloning introduces the *att*B1 sequence at the 5´ and the *att*B2 sequence at the 3´ regions of the expression cassette and insertions of a novel sequence between promoter and 5´UTR includes the risk of damaging this very sensitive region. However, GFP expression achieved in tobacco chloroplasts transformed with the Gateway® compatible plastid transformation vector proved that the *att*B1 sequence has no negative effect on the critical RNA interactions required for protein expression. The obtained GFP accumulation level of 3% TSP was furthermore considerably higher than the results previously generated using the same regulatory elements and integration sites in the plastid genome (Waheed et al, 2011a; Waheed et al, 2011b). No negative impact of the Gateway® cloning procedure was detectable during the whole process of plastid transformation and regeneration of transplastomic plants. Thus, taking advantage of the unique properties of the Gateway® cloning system, the first Gateway® plastid transformation vector has been created, providing a straightforward and streamlined cloning strategy.

The transplastomic plant lines containing the transgene expression cassette controlled by a strong constitutive *rrn*16 promoter showed growth retardations and leaf chlorosis. According to previous reports, plastid based expression of recombinant proteins mostly does not result in abnormal phenotypes. However, there are an increasing number of studies where phenotypic alterations of transplastomic plants have been reported. The two main reasons underlying pigment deficiency or delay in plant development are either toxicity of the transgene product due to interference of the recombinant protein with essential processes in the chloroplast or severe metabolic burden due to hyper recombinant protein expression levels (Scotti & Cardi, 2014). These detrimental effects include male sterility (Lössl et al, 2003), chlorotic leaves (Tregoning et al, 2003), stunted growth (Magee et al, 2004; Tissot et al, 2008) or a combination thereof (Waheed et al, 2011b). Although, expression of up to 72 % of total leaf protein of a CTB-Pins fusion protein without negative effects on plant growth has been reported (Ruhlman et al, 2010), over-expression of lysine to ~70 % TSP caused phenotypic alterations in transformed plants (Oey et al, 2009). In several cases, the aberrant phenotype can be directly connected to the foreign protein (Hennig et al, 2007; Ruiz & Daniell, 2005). Nevertheless, it could also be the result of exhaustion of the chloroplast's gene expression capacity resulting in Rubisco depletion and a general decrease in plastid-encoded proteins (Bally et al, 2009; Zhou et al, 2008). The fact that the growth retardation and mild chlorosis observed in our transplastomic plants could be overcome by expressing the transgene upon ethanol induction, suggests that in our case these detrimental effects are more likely an effect of recombinant protein toxicity than overload of the chloroplast's metabolism.

The detected levels of constitutively expressed EDIII 1 were varying depending on the leaf age with the highest protein accumulation being detected in the youngest leaves. Similar findings have been reported for the VP6 protein, suggesting that the protein is produced during early leaf development, but then subjected to degradation during plant growth (Birch-Machin et al, 2004). In contrast to that, the expression levels of EDIII 3 remained nearly unchanged in all leaf ages, indicating that this protein might be more stable than EDIII 1.

Although, the Southern blot pattern confirmed the homoplastomic state of N.t.-EDIII 2, Western blot analyses were not able to detect the EDIII 2 protein. Previously, a plastid protein with a half-life less than one hour has been reported (Whitney & Andrews, 2001) and the high turnover of plastidial protein synthesis can lead to rapid degradation of proteins (Kim et al, 1994). A N-end rule for plastid proteins has been proposed where the protein stability is influenced by the penultimate N-terminal amino acid residue (Apel et al, 2010; De Marchis et al, 2012), but in our case all three EDIII proteins have the same penultimate amino acid.

Unexpectedly, the immunoblot analyses revealed the presence of additional protein bands with higher molecular masses than the predicted 13 kDa for EDIII 1 and EDIII 3, respectively. The size of these bands (~28, ~40 and ~50 kDa) would correlate nicely with dimer or trimer formation of the EDIII proteins. The absence of these bands in the wild-type sample clearly indicates that they result from specific binding of the anti-dengue antibody. To proof our hypothesis, mass spectrometric analyses were performed and the data obtained by analyzing the contents of the expected band together with the additional bands confirmed that all analyzed samples contain the Dengue-peptides. The mature Dengue virus particles are formed by E protein dimers organized in a herringbone configuration on the viral surface (Kuhn et al, 2002) and the main dimerization properties are associated with domain II (Modis et al, 2003), while our recombinant protein consists only of the domain III of the envelope protein. However, the homodimers of the E protein interact closely with each other during the virus life cycle (Mukhopadhyay et al, 2005) and the post fusion structure at low pH is characterized by a trimeric arrangement of E protein monomers (Modis et al, 2004). Therefor it cannot be excluded, that the domain III retains some characteristics that favor protein aggregation even when expressed in an isolated way. Such information is of importance for our future work and for other studies about the production of recombinant proteins in chloroplasts.

Although this phenomenon has not been reported for similar proteins expressed in *E.coli* (Khanam et al, 2006) or yeast cells (Batra et al, 2010; Cardoso et al, 2013), a putative dimeric species has been detected in solubilized inclusion bodies of EDIII 2 (Jaiswal et al, 2004). The ability to form strong aggregates of the chloroplast produced EDIII proteins may be an advantage for the stimulation of the immune system and induction of intestinal secretory IgA following oral immunization.

The activation of transgene expression after the highly sensitive regeneration phase could be a way to avoid phenotypic alterations caused by the recombinant protein. Inducible expression systems allow the plants to grow to maturity and then the full biomass capacity can be exploited to produce the recombinant protein upon induction. Several different induction systems for plastids have been reported (Buhot et al, 2006; Mühlbauer & Koop, 2005; Tungsuchat et al, 2006; Verhounig et al, 2010). In this work, the ethanol inducible expression system based on the nuclear encoded plastid exported T7 RNA Polymerase has been employed for the expression of EDIII 1, EDIII 2, EDIII 4 and EDIII 1-4. The functionality of this system to overcome growth reduction and male sterility caused by the expression of the phb operon has been previously demonstrated (Lössl et al, 2005). Plants growing in the greenhouse without ethanol treatment showed only a minimal growth delay compared to the wild-type plants and fertile seeds were obtained from all plant lines. Protein expression was achieved in all *in vitro* plant lines after repeated ethanol exposure and in accordance with previously reported leakiness of the system (Lössl et al, 2005) a low level of recombinant protein was also detectable in un-induced plants, most likely causing the slightly slower growth of the transplastomic plants.

Interestingly, no protein aggregates were visible on the immunoblots performed with protein extracted from the ethanol induced plants, except for EDIII 4. However, EDIII 4 has also the highest expression level and the aggregates appear with increasing protein concentration, suggesting that they might not be distinguishable from the background on the other Western blots due to sensitivity reasons.

The expression level obtained from constitutive expression of EDIII 1 is higher than the one obtained after ethanol induction. Since in both plant lines the identical protein is expressed in the same tobacco background, this cannot be due to protein instability or protein degradation by plant proteases. More likely, the availability of the T7RNA Polymerase in the plastid constitutes a bottle neck for the recombinant protein expression (Lössl et al, 2005). On the other hand, in the case of EDIII 2, where the expression level remained below the detection limit with the constitutive approach, the ethanol inducible system allowed the expression of detectable EDIII 2 quantities.
Importantly, the successful expression of the EDIII 2 protein and the hardly notable growth alterations in the transplastomic plants demonstrate the usefulness of this trans-activation system to overcome hurdles in transgene expression and recombinant protein related phenotypic alterations.

Since the main target group for a Dengue vaccine are the relatively poor people in developing countries, an ideal Dengue vaccine should be affordable, effective, heat-stable, and easily administered. Orally delivered plant produced antigens are bio-encapsulated by the plant cell wall (Daniell et al, 2009) and stimulated protective immune responses (Arlen et al, 2008; Davoodi-Semiromi et al, 2010; Ruhlman et al, 2007; Verma et al, 2010). Furthermore, recombinant protein-based vaccines expressed in edible plants can be produced and delivered using the same cells, completely eliminating the cost of purification and formulation (Gregory & Mayfield, 2014).

Homoplastomic lettuce plants expressing the EDIII 1 and the EDIII 1-4 protein were obtained by transformation with lettuce specific Gateway® plastid transformation vectors and as described in our recent study (Gottschamel et al, 2013), no negative effects were observable during plant transformation and regeneration, proving the applicability of the Gateway® cloning system also for lettuce.

The detected expression level for EDIII 1 is considerably lower in lettuce than the one obtained with constitutive expression in tobacco. This has been observed for other recombinant proteins expressed in both tobacco and lettuce chloroplasts (Davoodi-Semiromi et al, 2010; Ruhlman et al, 2007) and in addition, the different regulatory elements used in this work, presumably also contribute to the variable expression levels. In general, it is well documented that the recombinant protein yield depends on the regulatory elements used for transgene expression (Cardi et al, 2010; Inka Borchers et al, 2012; Koop et al, 2007; Maliga, 2003; Yang et al, 2013), but protein stability plays an even more important role in protein accumulation (Apel et al, 2010; Birch-Machin et al, 2004; De Marchis et al, 2012; Elghabi et al, 2011).

As already noticed with the constitutively expressed EDIII 1 and EDIII 3 proteins in tobacco, also in lettuce the EDIII 1-4 seems to be more abundant in the insoluble fraction than in the total soluble protein extract. A comparison of signal intensities on the Western Blots performed with total protein extracted from lettuce and ethanol induced tobacco, indicates that not only the EDIII 1-4 concentration is higher in lettuce, but also that less degradation is visible in lettuce than in tobacco.

Several other groups have reported the expression of antigens in lettuce as a more digestible and non-toxic alternative to tobacco (Davoodi-Semiromi et al, 2010; Ruhlman et al, 2007; Ruhlman et al, 2010; Verma et al, 2010). The data obtained from our experiments will help to further develop a low-cost and thermo-stable vaccine candidate against Dengue fever with the possibility of oral delivery.

Recombinant tetravalent and monovalent EDIII antigens have been previously expressed in *E.coli* (Khanam et al, 2006; McDonald et al, 2009; Simmons et al, 1999; Srivastava et al, 2000; Tripathi et al, 2008; Tripathi et al, 2011; Zhao et al, 2014), in yeast (Arora et al, 2013; Batra et al, 2010; Cardoso et al, 2013; Etemad et al, 2008; Nguyen et al, 2013), in insect cells (Ivy et al, 2000) and also in plants (Kim et al, 2009; Martinez et al, 2010; Saejung et al, 2007). So far only the expression of a Dengue virus serotype-3 premembrane and envelope polyprotein has been reported in plastids (Kanagaraj et al, 2011) and the present study provides new insights and detailed information on chloroplast based expression of recombinant EDIII proteins. In addition, the utilization of three different expression strategies and two plant species confers comparative properties to this study that provide valuable information for further research in this field.

Although no absolute quantification of the obtained recombinant protein expression levels is possible, comparison and rough estimations lead to the conclusion that EDIII 1 and EDIII 3 are best expressed constitutively in tobacco, while the more problematic proteins EDIII 2, EDIII 4 and EDIII 1-4, still can be expressed to a reasonable level in tobacco via the ethanol inducible approach. Furthermore, lettuce appears to be an especially convenient expression system for EDIII 1-4, with a considerable yield, hardly any degradation and an edible background facilitating the processing of the pharmaceutical protein for oral administration.

5 Conclusion

In the present study the tetravalent fusion protein (EDIII 1-4) and the corresponding monovalent forms (EDIII 1, -2, -3 and -4) have been expressed in tobacco and lettuce chloroplasts. The plastid expression system offers transgene confinement, high levels of protein expression, absence of gene silencing and other epigenetic mechanisms, the possibility of stacking transgenes into operons and highly precise, site-specific transgene integration (Bock, 2014). Tobacco is a non-food/non-feed crop, has a relatively good tractability to genetic manipulation and is an excellent biomass and seed producer (Svab & Maliga, 1993). Furthermore, lettuce has been chosen as an edible crop to facilitate the oral administration of the Dengue vaccine.

The constitutive expression levels of EDIII 1 and EDIII 3 clearly show that chloroplasts are a suitable production platform for these proteins. Though, the failure to detect EDIII 2 and the phenotypic alterations observed in all the constitutively expressing tobacco plants indicate that even closely related proteins may behave differently and may require different expression conditions. However, the successful expression of EDIII 2 alongside with EDIII 1, EDIII 4 and EDIII 1-4 achieved with the ethanol inducible expression system, demonstrates that these hurdles can be overcome by choosing a suitable expression system. Furthermore, the successful expression of EDIII 1 and EDIII 1-4 in lettuce promotes and facilitates the development of orally administrable medicament formulations.

Taken together, this work shows (i) for the first time that the Gateway® cloning system is adaptable for the construction of plastid transformation vectors allowing the rapid, easy and accurate cloning of various different transgenes in parallel reactions; (ii) that the expression of the Dengue virus envelope protein domain III is feasible in tobacco and lettuce chloroplasts. However since every recombinant protein is unique, this work also points out that it is crucial to apply the appropriate expression system for every individual recombinant protein in order to achieve satisfying expression levels. Expressing challenging proteins via the ethanol inducible expression system offers an effective possibility to overcome detrimental effects caused by the recombinant protein on plant growth and fertility. The production of the recombinant EDIII proteins that are aimed for pharmaceutical purposes in an edible plant, paves the way for a vaccine with oral administration.

6 Further Perspectives

- ## Transcription level analysis

In order to further investigate the EDIII 2 protein expression in homoplastomic tobacco plants, RNA expression and stability of EDIII 1-4 encoding genes will be investigated. The data gained from these experiments may help to reveal the cause of the absent EDIII 2 protein accumulation in the constitutive expressing transgenic plant lines.

- ## Quantification of the expression level

A task that still has to be completed is the quantification of the recombinant protein expression level. The production of a purified *E.coli* derived protein standard has been delayed, due to unexpected difficulties with the expression and purification of the recombinant EDIII proteins in *E.coli*. Strategies to overcome these hurdles have been developed and the work is in progress.

- ## Immunological studies

The next important step will be to prove the immunogenicity of the produced recombinant proteins and to show their ability to confer protection against the Dengue virus. Immunological studies have been designed to determine the immune system's response in mouse feeding experiments. This assay is ongoing and furthermore, Plaque reduction neutralization tests (PRNT) have been scheduled to evaluate the protecting capabilities of the EDIII proteins. The outcomes of these studies are expected to provide useful information for further Dengue vaccine development.

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8 Appendix

8.1 List of Figures

8.2 List of Tables

8.3 Curriculum vitae

Name: Johanna Gottschamel, Dipl. Ing. Bakk. techn.

Date of birth: 06.03.1984 in Friesach/Carinthia

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School education

09/1994 – 06/2002	Bundesgymnasium Tanzenberg, Carinthia
09/1999 – 06/2000	Exchange Student at the Liceo Classico Europeo „Uccellis“, Udine, Italy

University

10/2002 – 02/2006	**Bachelor course “Food science and Biotechnology”** at the University of Natural Resources and Applied Life Sciences, Vienna Bachelor course finished with distinction
11/2005 – 01/2006	**Bachelor thesis:** „Chemical inducible promoter systems in plants” at the Department of Applied Genetics and Cell Biology of the University of Natural Resources and Applied Life Sciences, Vienna
03/2006 – 10/2008	**Master course „Biotechnology“** at the University of Natural Resources and Applied Life Sciences, Vienna main focus: Plant Biotechnology Master course finished with distinction
09/2006 – 07/2007	**Erasmus – stay** at the Università degli Studi di Bari, Italy **Master thesis:** „Detection of VPLs by ISEM in transgenic grapevines expressing different GFLV CP constructs“ at the Facoltà d’Agraria, Bari, Italy in cooperation with the Plant Biotechnology research group of the University of Natural Resources and Applied Life sciences, Vienna, Austria
05/2010 –	**Dr. Lössl Group,** Department of crop sciences, University of Natural Resources and Applied Life Sciences, Vienna **PhD: “**Chloroplast based expression of recombinant Dengue-virus envelope proteins in plants”
04/2012 –	**Research - stay** at Norwegian Institute of Agricultural and Environmental Research (Bioforsk), Ås, Norway

Work experience

2002 – 2006	**Treibacher Industrie AG** periodical summer internship Chemical analysis on industry waste material
10/2007 – 12/2007	**Plant Biotechnology Group**, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences Internship in Plant Biotechnology
04/2008 – 12/2008	**Austrian Institute of Technology**, Research Division: Nano-System-Technologies Mammalian cell culture and lab-on-a-chip measurements with *C. albicans*
02/2009 – 01/2010	**Group Touraev, MFPL, University of Vienna** "Dissecting the mechanism of microspore reprogramming and embryogenesis in *Arabidopsis thaliana*"

Hobbies

Sport:	Snowboarding, Yoga, Climbing
Other Interests:	Travelling and pets

Publications

Gottschamel J, Richter L, Mak A, Jungreuthmayer C, Birnbaumer G, Milnera M, Brückl H, Ertl P (2009) "Development of a Disposable Microfluidic Biochip for Multiparameter Cell Population Measurements" Anal Chem. Oct 15;81(20):8503-12.

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TECHNICAL REPORT

A novel chloroplast transformation vector compatible with the Gateway® recombination cloning technology

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Abstract To analyze the suitability of Gateway® vectors for transformation of chloroplasts, we converted a standard plastid transformation vector into a Gateway® destination vector containing the necessary recombination sites *att*R1 and *att*R2. Insertion of the green fluorescent protein (GFP) coding sequence with associated T7g10 ribosome binding site into this destination vector created the expression vector for transformation of tobacco chloroplasts with the biolistic method. Correct integration of the transgene into the plastid genome was verified by PCR and the homoplasmic nature of the transformed plants was confirmed by Southern Blot analysis. Expression of the GFP reporter protein was monitored by confocal laser scanning microscopy (CLSM) and quantification by western blot analysis showed a GFP accumulation level of 3 % total soluble protein (TSP). The presented results clearly demonstrate that the Gateway® recombination sites are compatible with all steps of plastid transformation, from generation of transplastomic plants to expression of GFP. This is the first report of a plastid transformation vector made by the Gateway® recombinant cloning technology, which proves the suitability of this system for use in chloroplasts.

Keywords Plastid transformation · Gateway® recombinant cloning technology · GFP

Introduction

Plastid derived recombinant protein expression offers several advantages including high levels of transgene expression, absence of epigenetic effects and transgene containment via maternal inheritance (Bock 2007). Plastid transformation is a targeted integration based on the mechanism of homologous recombination. Delivery of the plasmid DNA into the plant cell and the chloroplast is mainly achieved by the biolistic approach (Svab et al. 1990), but alternatively also PEG transformation has been used (Golds et al. 1993). Construction of transformation vectors must combine a selection marker with the gene of interest flanked by two insertion sequences for the left and the right flank (INSL and INSR) which confer homologous

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recombination into the specified region of the plastome (Maliga 2004).

Gateway® recombinant cloning technology is a very accurate cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy 1989) providing a rapid and highly efficient way to transfer DNA fragments between vectors carrying compatible recombination sites. The entry clone is created by the BP Clonase® enzyme mix mediated transfer of the *att*B sites flanked gene of interest into the *att*P site bearing pDONR221™ (Fig. 1a). Subsequently, the fragment in the entry clone can be transferred to any destination vector containing *att*R sites using the LR Clonase® enzyme mix (Fig. 1b) (Karimi et al. 2002). The presence of the counterselectable *ccdB* gene and the chloramphenicol resistance gene in both the donor and the destination vector provides a unique system of negative selection that eliminates all unwanted by-product plasmids after recombination resulting in maximum cloning efficiency. Plants have been transformed with Gateway® compatible vectors via several methods including *Agrobacterium*-mediated delivery, PEG transformation, particle bombardment and electroporation, although only vectors for transient expression or stable nuclear transformation have been reported up to now (Dubin et al. 2008; Karimi et al. 2007).

Up to now no research group on chloroplast transformation has ventured to replace the tedious cloning procedure based on restriction enzymes by the Gateway® system. Gateway® cloning introduces the *att*B1 sequence at the 5′ and *att*B2 at the 3′ regions of the expression cassette. The motifs of the 5′ region however are known to be very sensitive for sequence alterations, as shown in studies on 5′UTR (Eibl et al. 1999; Zou et al. 2003). Functioning of 5′UTR with its ribosomal binding site is very dependent on its proper interaction with the flanking transcript in the coding region downstream of the start codon (Kuroda and Maliga 2001). Insertion of a novel sequence like *att*B1 between promoter and 5′UTR includes the risk to damage or even destroy these critical RNA interactions required for protein expression.

The aim of this work was first to investigate if the presence of a novel sequence like *att*B1 between the P*rrn*PEP + NEP promoter (Ye et al. 2001) and the 5′ UTR T7g10 (Studier et al. 1990) has an impact on

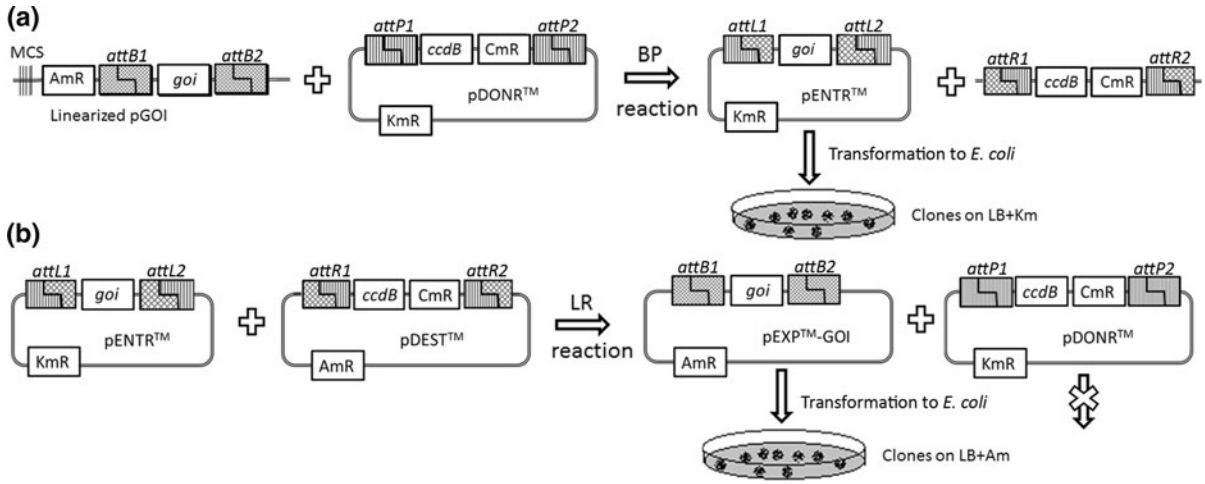

Fig. 1 Scheme of Gateway® recombinant cloning technology. **a** In the BP reaction (attB x attP → attL + attR) the Integrase, mediates the insertion of the *goi* sequence into the donor vector (pDONR™) yielding the entry clone (pENTR™). The fragment containing the *goi* flanked by *att*B1 and *att*B2 can either be produced by PCR or by linearizing a suitable plasmid (pGOI) with a corresponding restriction enzyme. **b** In the LR reaction (attL x attR → attB + attP) the *goi* is then integrated into the destination vector (pDEST™) mediated by Integrase and Excisionase yielding the final Expression clone (pEXP™-GOI) which is used for transformation and protein expression (Hartley et al. 2000). The att recombination sites in the donor and the destination vectors flank a *ccdB* gene (control of cell death) and a CmR (chloramphenicol-resistance) gene (Bernard 1995), thus this vectors can only be propagated in *ccdB* survival™ *E. coli* that contain a gyrA462 mutation providing resistance to the lethal effects of *ccdB*. Am: ampicillin; AmR: ampicillin resistance gene; Km: kanamycine; KmR: kanamycin resistance gene; *att*B1/B2/P1/P2/L1/L2/R1/R2: Gateway® recombination sites; MCS: multiple cloning site

gene of interest (*goi*) expression and second to demonstrate the feasibility of the Gateway® cloning system for plastid transformation.

Results and discussion

Construction of plastid transformation vector

In order to construct the plastid transformation vector compatible with the Gateway® recombinant cloning system, the Gateway® reading frame cassette A (RfA) consisting of the *ccdB* gene and the chloramphenicol resistance gene flanked by *attR1* and *attR2* was cloned into a standard plastid transformation vector. The resulting destination vector pDEST-PN-T (Fig. 2a) contains the *aadA* gene cassette, the constitutive P*rrn*PEP + NEP promoter for transgene expression and the RfA flanked by tobacco specific INSR and INSL. The P*rrn*PEP + NEP promoter consists of the nuclear encoded polymerase (Prrn^{-62}NEP) promoter (Hajdukiewicz et al. 1997) fused downstream to the plastid-encoded polymerase (PEP) promoter P*rrn16* (Svab and Maliga 1993). BP reaction of the linearized vector containing the *att*B site flanked green fluorescent protein (GFP) and the *att*P site bearing pDONR221™ resulted in pENTR-GFP (Fig. 2b). LR reaction of pENTR-GFP and pDEST-PN-T yielded the final plastid transformation vector pEXP-PN-GFP-T (Fig. 2c).

Generation of transplastomic plants

To obtain GFP expressing plants, leaves of *Nicotiana tabacum* 'Petite Havana' were bombarded with

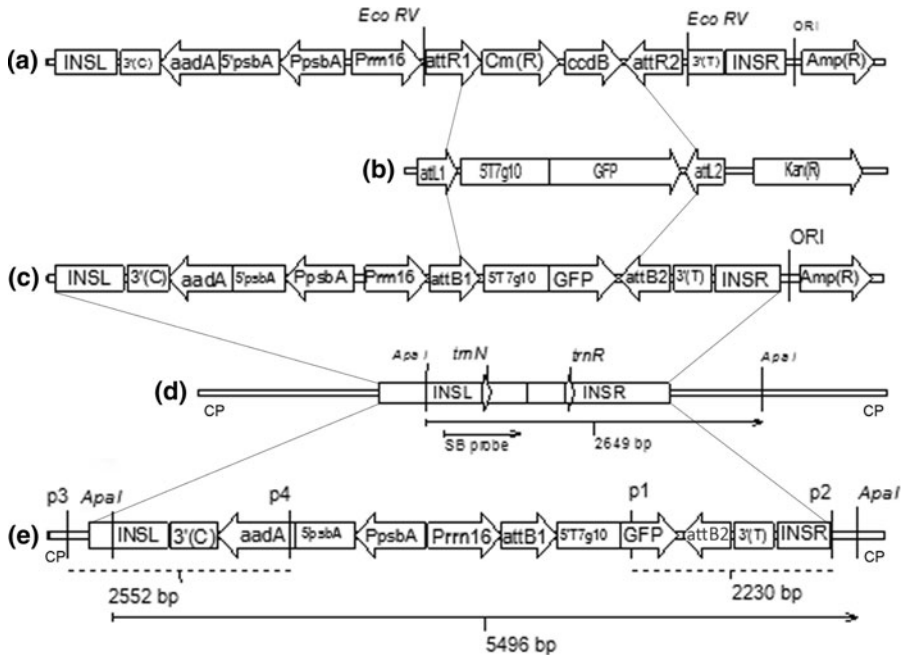

Fig. 2 Schematic representation of vector construction. **a** pDEST-PN-T (8,629 bp) is constructed by blunt end ligation of the Gateway® RfA cassette (*att*R1-Cm(R)-*ccdB*-*att*R2) into the *EcoRV* site of a plastid transformation vector. **b** pENTR-GFP (3,347 bp) contains the GFP sequence and the 5′UTR of bacteriophage T7 gene 10 (5′T7g10) flanked by *att*L1 and *att*L2. **c** pEXP-PN-GFP-T (7,777 bp) is created by the LR reaction of pENTR-GFP and pDEST-PN-T and contains GFP under control of the P*rrn*PEP + NEP promoter (PPN), the *aadA* gene under control of the psbA promoter (PpsbA) and 5′UTR (5′psbA) and the 3′UTR of tobacco *rbcL* (3′T) and chlamydomonas *rbcL* (3′C), respectively. **d** Wild type tobacco plastid genome (CP). **e** Tobacco plastid genome with integrated transgene expression cassette. Correct insertion is verified with primers p1/p2 at INSR and p3/p4 at INSL and the corresponding PCR products are shown as dotted lines; the 773 bp Southern Blot probe located in INSL is shown as an *arrow* and the expected fragments are shown as *arrows* marked with their molecular weight. INSR: right insertion site (containing *trnR*); INSL: left insertion site (containing *trnN*); Amp(R): ampicillin resistance gene; Kan(R): kanamycin resistance gene; Cm(R): chloramphenicol resistance gene; *ccdB*: control of cell death gene; *aadA*: spectinomycin resistance gene; *att*B1/B2/R1/R2/L1/L2: Gateway® recombination sites

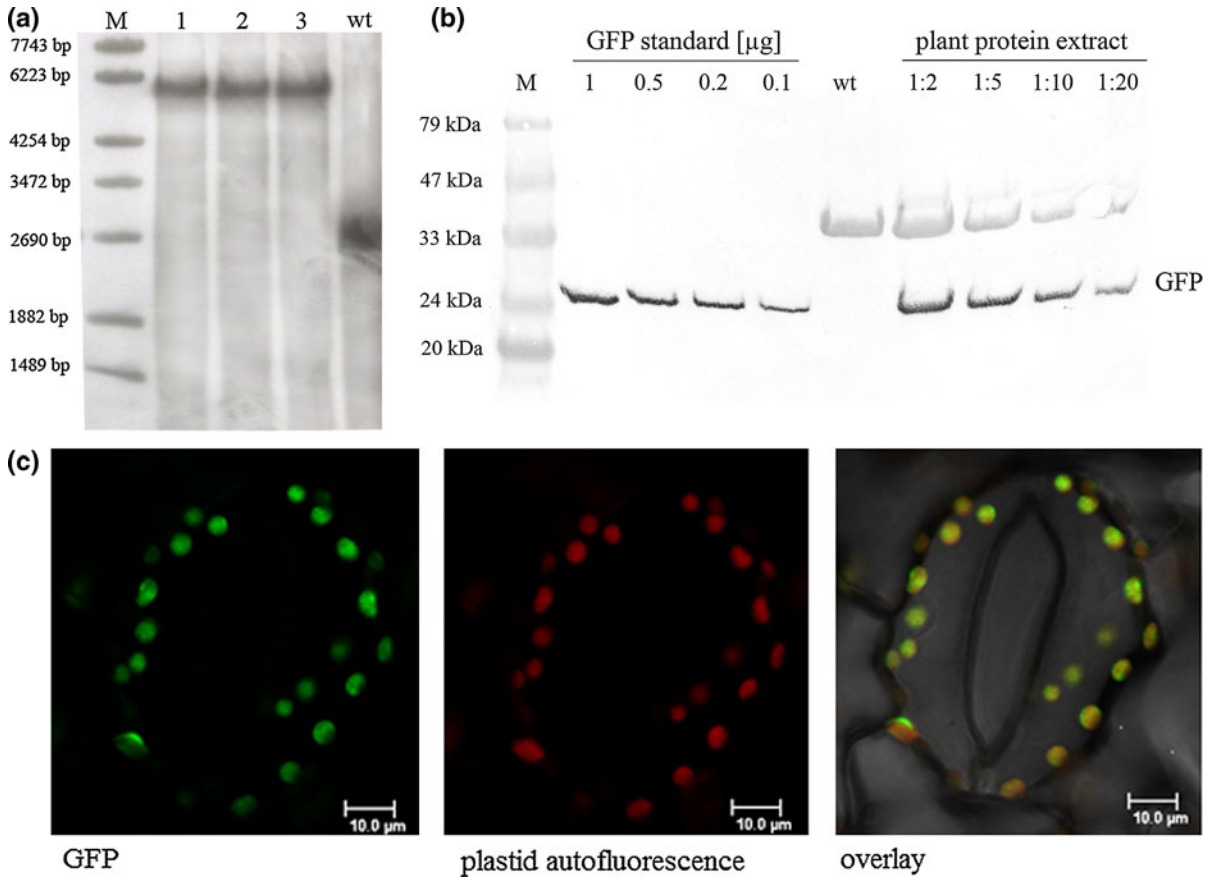

Fig. 3 Characterization of transplastomic plants and detection of GFP expression. **a** Southern Blot analysis of three transplastomic plant lines (*lane* 1, 2 and 3) was performed using a 773 bp DIG labeled probe that binds inside the *trnN* region (INSL) of the plastid genome after three consecutive rounds of selection and subculture on spectinomycin containing RMOP medium. Plant DNA was cut with *ApaI*, separated by electrophoresis and transferred onto a positively charged nylon membrane by alkali-transfer. **b** For Immunoblot analysis soluble proteins were extracted, separated by SDS-PAGE, transferred onto nitrocellulose membrane and western blot analysis with a rabbit-anti-GFP primary antibody (ABIN398856, antibodies-online.com) and an AP conjugated anti-rabbit-IgG secondary antibody (S3731, Promega) was performed. The extract from the transplastomic plant was loaded in several dilutions and the GFP standard series was used as a reference. Protein bands were detected by colorimetric reaction with Sigmafast™ BCIP®/NBT (Sigma). **c** GFP fluorescence in the plastids of transplastomic plants was observed on a SP5 II confocal system (Leica Microsystems, Heidelberg, Germany) equipped with a HCX PL APO CS 63.0 × 1.20 water objective. The 405-nm line of an argon ion laser was used for excitation. Images were taken at the emission bandwidth of 500–540 nm for GFP and 688–757 nm for autofluorescence of the chloroplasts and processed using the Leica LAS AF software

0.6 µm gold-microcarriers coated with plasmid DNA using a Bio-Rad Biolistic PDS-1000/He gun (Daniell 1997; Svab and Maliga 1993) and transgenic shoots were regenerated on RMOP medium containing spectinomycin (Verma et al. 2008). The site specific integration of the transgene into the wild type chloroplast genome (Fig. 2d) due to homologous recombination generated transplastomic plants (Fig. 2e). DNA was extracted from in vitro material of regenerated plant lines by CTAB procedure (Murray and Thompson 1980) and correct integration of the transgenic sequence into the plastid genome was shown by a PCR product of 2,230 bp with p1/p2 and 2,552 bp with p3/p4 (supplementary material). Three independently transformed and regenerated plant lines (wt-PN-GFP 1, 2 and 3) were further characterized. Southern Blot analysis verified the homoplastomic state by the presence of only the 5,496 bp fragment in GFP transformed plants, compared to the 2,656 bp fragment in wild type (Fig. 3a).

Expression and quantification of GFP

For the detection of GFP expression level, protein was extracted from leaves of transplastomic and wild type plants (Verma et al. 2008) and total soluble protein (TSP) was quantified by Bradford assay (Sigma). Immunoblot analysis performed with the soluble fraction of protein extract and an anti-GFP primary antibody showed the expected 28 kDa band (Fig. 3b), thus proving plastid derived recombinant GFP expression. Unspecific binding of the antibody to the crude plant extract results in the band migrating at 33 kDa which is present also in the wild-type sample. The quantification of GFP expression by comparison of band intensity to the GFP standard on the western blot determined an expression level of 3 % TSP. Visual detection of GFP expression in leaves was monitored using confocal laser scanning microscopy and co-localization of the GFP signal and the plastid autofluorescence is well observable in leaves of homoplastomic plants (Fig. 3c).

Conclusion

This study presents a novel destination vector that can be converted into a plastid transformation vector in one single step using Gateway® recombinant cloning technology. GFP expression in tobacco chloroplasts has been achieved by chloroplast transformation with a Gateway® compatible transformation vector containing the reporter gene *gfp* under control of the constitutive P*rrn*PEP + NEP promoter. The homoplastomic state of the regenerated plants was proven by Southern Blot hybridization and western analysis showed a GFP accumulation of 3 % TSP. In this study no negative impact of the Gateway® cloning procedure was detectable during the whole process of plastid transformation and regeneration of transplastomic plants. The presence of the *att*B1 Gateway® recombination site between the promoter and the 5′UTR did not affect the recombinant protein expression in the chloroplast genome. The obtained protein accumulation reported in this work represents an increase compared to the expression levels of 1, 5 and 2 %TSP previously achieved using the same regulatory elements and integration sites *trn*N and *trn*R in the plastid genome (Waheed et al. 2011a, b). We clearly demonstrate that this system is adaptable for the construction of plastid transformation vectors allowing the rapid, easy and accurate cloning of various different transgenes in parallel reactions. Moreover, the simultaneous assembly of multiple DNA fragments into one single destination vector is allowed by the MultiSite Gateway® Technology. This will further facilitate constructing plastid transformation vectors and thereby considerably reduce the timeframe required for recombinant protein expression in chloroplasts.

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