Expression of Dengue Antigens in Tobacco Green Factory

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Ås, December 2012

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Abstract

Dengue fever is a viral disease primarily transmitted by the mosquito *Aedes aegypti*, though other species have been known to transmit this disease. Dengue fever is endemic to tropical and subtropical regions, and this puts about 40% of the worlds population at risk of infection. In the last 30 years there has been an increasing number of dengue infections and in May 2012 PAHO/WHO issued an epidemiological alert warning countries at risk to prepare for dengue fever outbreaks.

There are four different serotypes of dengue virus (DENV1-4), and infection by one serotype may cause dengue fever, a mild disease that is rarely fatal. A secondary infection by another serotype can cause a severe dengue infection such as dengue hemorrhage fever and dengue shock syndrome, which, in worst-case scenarios, can be fatal. Currently there are no vaccines or specific treatments available on the market and the only treatment is treatment of the symptoms.

The reason why a secondary dengue infection by another serotype may cause severe disease is due to antibody dependent enhancement (ADE). This is a challenge in the development of a good dengue vaccine, as it would need to protect against all four serotypes. This might be obtained by combining antigens representing each serotype in the vaccine as a formulation of monovalent peptides or as a tetravalent protein. A vaccine based on the envelope domain III (EDIII) of the dengue virus has been shown to have great potential as this protein contains several epitopes that bind antibodies that are able to neutralize all four serotypes, whereas it does not give rise to enhancing antibodies.

The use of plants to produce pharmaceuticals is a relatively new field with potential for both cheap and safe production. Unlike cell based systems, plants cannot contain human or animal pathogens. Tobacco can be used for this purpose and is advantageous because it is a non-feed, non-food plant. In this project the gene sequences encoding EDIII of serotypes 1 and 3 carrying a C-terminal His-tag were introduced separately into nuclear genomes of tobacco using *Agrobacterium* transformation. The expression of the monovalent EDIII proteins were controlled by an ethanol inducible promotor, T7 RNA P. EDIII of serotype 2 with several fusion partners, to aid stability and solubility, was attempted expressed transiently in tobacco

leaves using agroinfiltration. Molecular DNA techniques were used to confirm the presence of the genes introduced in the nuclear transformants, and protein analyses were conducted to detect heterologously expressed protein from both expression systems. The results are presented in this thesis.

Sammendrag

Dengue feber er en virus sykdom som primært overføres av myggen *Aedes aegypti*, men kan også overføres av andre myggarter. Dengue er endemisk til tropiske og subtropiske områder, noe som fører til at ca. 40 % av verdens befolking er i risiko for infeksjon. I de siste 30 årene har det vært en drastisk økning i antall dengue tilfeller, og i mai 2012 utstedte PAHO/WHO en epidemiologisk advarsel om at land i faresonen burde forberede seg på dengue utbrudd.

Det finnes fire ulike serotyper av dengue virus (DENV1-4) og infeksjon med en av disse kan forårsake dengue feber, en mild sykdom med sjeldent dødelig utfall. En sekundær infeksjon av en annen serotype derimot kan føre til alvorlig dengue infeksjon som dengue hemoragisk feber og denguesjokk, som i verste tilfelle kan være dødelig. Foreløpig finnes ingen vaksiner eller spesifikke behandlinger på markedet og den eneste behandlingen er behandling av symptomene.

Sekundære dengue infeksjoner av ulik serotype kan forårsake alvorlig sykdom på grunn av antistoff avhengig forsterkning (ADE). Dette er en utfordring i utviklingen av en god dengue vaksine siden den må beskytte mot alle fire serotypene. En løsning for dette er å utvikle en vaksine som inneholder antistoff for hver serotype, enten som en blanding av fire monovalente peptider eller som et tetravalent protein. En vaksine basert på envelope domain III (EDIII) til dengue virus har vist seg å ha stort potensial. Dette proteinet inneholder flere epitoper som binder antistoffer som kan nøytralisere alle fire serotypene og som ikke induserer forsterkende antistoffer.

Produksjonen av legemidler i planter har dukket opp som et relativt nytt område som er både økonomisk og trygt. I forhold til andre produksjonssystemer er ikke planter vert for humane eller animalske patogener. Tobakk blir brukt i dette prosjektet og er fordelaktig siden den ikke er en mat eller fôr plante. I denne oppgaven skal gen sekvensen som koder for EDIII serotype

1 og 3 med C-terminal His-tag bli introdusert separat i nukleære genomer av tobakk med bruk av *Agrobacterium* transformering. Uttrykket av de monovalente EDIII proteinene ble kontrollert av en etanol induserbar promotor, T7 RNA P. EDIII av serotype 2 med flere fusjonspartnere, for forbedre stabilitet og løselighet, ble prøvd uttrykt transient i tobakks blader med bruk av agroinfiltrering. Molekylære DNA teknikker ble brukt for å bekrefte tilstedeværelse av gener introdusert i kjernetransformater. Protein analyser ble utført for å detektere heterologt uttrykt protein fra begge ekspresjonssystemene. Resultatene er presentert i denne avhandlingen.

Contents

Chapter 1 Introduction

1.1 Dengue

Dengue is a viral disease transmitted primarily by the mosquito *Aedes aegypti*, but can also be transmitted by *Aedes albopictus* (Gibbons 2010). Dengue is a single stranded RNA virus that belongs to the family *Flaviviridae* and consists of four distinct serotypes; DENV1-4, which all can cause dengue fever (Guzman & Isturiz 2010).

Figure 1: The dengue vector Aedes aegypti.

Dengue fever (DF) can be characterized by sudden high fever, joint, muscle and bone aches, headaches, nausea, skin rashes and skin hemorrhages. DF is rarely fatal with mortality rates less than 1% (WHO 1997). Infection by any of the four serotypes can cause DF and immunity towards that serotype with temporary immunity to the other three serotypes (Guzman & Vazquez 2010; Raviprakash et al. 2009).

Dengue hemorrhage fever (DHF) and dengue shock syndrome (DSS) are caused by reinfection of a serotype in humans that have previously been infected by another serotype. DHF can be characterized by high fever, enlarged liver (hepatomegaly), circulatory failure and low blood platelet count (thrombocytopenia), in addition with many of the symptoms from DF (WHO 1997). The most characteristic symptom for DHF is the hemorrhagic tendencies. Examples of this can be bleeding in the gastrointestinal tract, minor skin hemorrhaging (petechiae) or larger skin hemorrhaging (ecchymosis) (Tantawichien 2012; WHO 1997). DHF can progress to DSS by a rapid deterioration of health. This usually happens with patients after a 2-7 day fever period that have experienced an intermediate break

in fever. A common effect of DSS is circulatory failure and if left untreated can evolve to metabolic acidosis and severe hemorrhaging of internal organs. In severe cases there can be development of intracranial hemorrhaging (WHO 1997). The mortality for DHF/DSS, if left untreated, can be as high as 20%, but this number can be greatly reduced by proper treatment (Mongkolsapaya et al. 2003).

On May 28th the Pan American Health Organization (PAHO) issued an epidemiological alert for dengue. PAHO stated that: "The Pan American Health Organization / World Health Organization (PAHO/WHO) recommends Member States (especially those located in the regions of Central America and the Caribbean), that could have a greater risk of dengue outbreaks during the second half of 2012, to begin preparing and establish integrated response mechanisms for the prevention of dengue caused deaths." (PAHO 2012).

1.1.1 Dengue vaccine development

Dengue is endemic to tropical and subtropical regions of the world, as illustrated in Figure 2. However, a large part of the worlds population reside in these areas, which puts about 40% of the worlds population at risk (WHO 2012).

Dengue is an increasing global health risk and the World Health Organization (WHO) estimates that between 50-100 million dengue infections occur each year with 500 000 hospitalizations. Before 1970 only 9 countries had experienced dengue outbreaks, whereas today it has spread to 100 countries and is endemic to all continents (WHO 2012). In recent years there has also been re-emergence of dengue in several areas that have been dengue free for decades. In 2001 there was an outbreak of dengue in Hawaii, caused by the mosquito *Aedes albopictus*, there had not been a dengue outbreak in Hawaii since 1944 (Effler et al. 2005). The same mosquito also caused an outbreak of dengue in Ningbo, China, something that had not occurred since 1929 (Xu et al. 2007). In both cases, travellers were discovered to be the cause of the outbreaks. The spread of dengue can partially be explained by an increase in global trade and air travel, in addition to the changing climate (Maantay & Becker 2012; Rezza 2012).

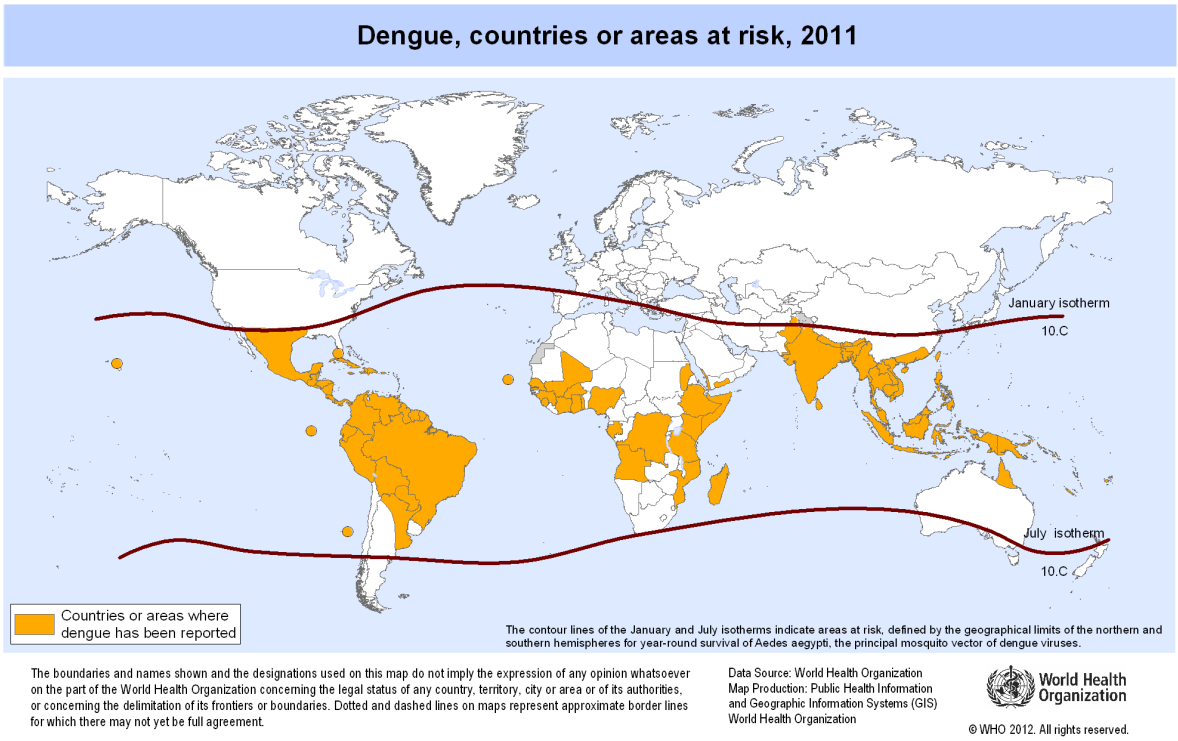

Figure 2: Countries at risk of dengue outbreaks in 2011 (WHO 2012).

Currently there are no cures or vaccines against dengue and the only treatment is to treat the symptoms or vector control. There have been efforts to develop a dengue vaccine for several decades and with the increasing global dengue threat there is a great need for a vaccine. Dengue belongs to the same family as the yellow fever virus (YFV), *Flaviviridae*, and YFV is of the same size (40-50 nm) as dengue virus. Despite the similarities a vaccine for yellow fever was already developed in 1937 (Theiler & Smith 1937). In contrast to YFV, dengue virus has four different serotypes and infection by two different serotypes at separate occasions can enhance virulence. This is due to an immunological mechanism called antibody-dependent enhancement (ADE) (Halstead 1979; Heinz & Stiasny 2012).

After a primary infection of dengue, the body creates neutralizing and non-neutralizing antibodies. When a second infection occurs by a different serotype, the antibodies bind to the virions, but do not neutralize them. The virion-antibody complex can bind to Fc receptors of immune cells, such as macrophages, allowing the non-neutralized virion to infect the cell, where it can proliferate. This mechanism increases the number of target cells and therefore enhances the infection. The mechanism for ADE is still not fully understood (Racaniello 2009).

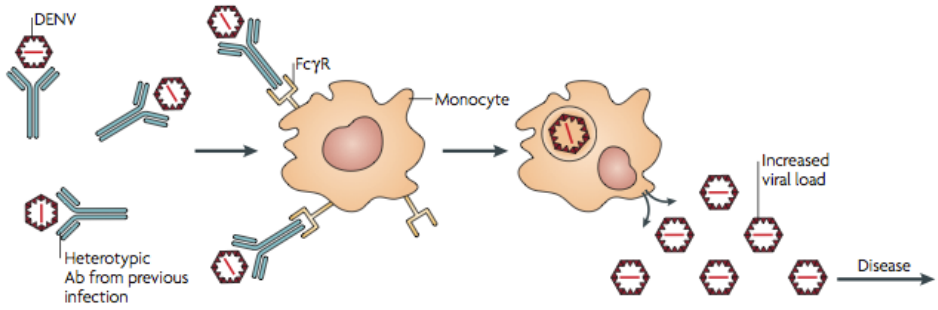

Figure 3: Antibody dependent enhancement of a secondary dengue infection.

To avoid ADE and the possible risk of DHF and DSS, a vaccine has to have the ability to protect against all serotypes collectively. This can be done by developing a tetravalent vaccine containing antigens for all four serotypes (Whitehead et al. 2007).

1.1.2 Envelope domain III

The dengue virus consists of three structural proteins; the capsid protein (C), the envelope protein (E) and the membrane protein (M), in addition to several non-structural proteins as seen in Figure 4.

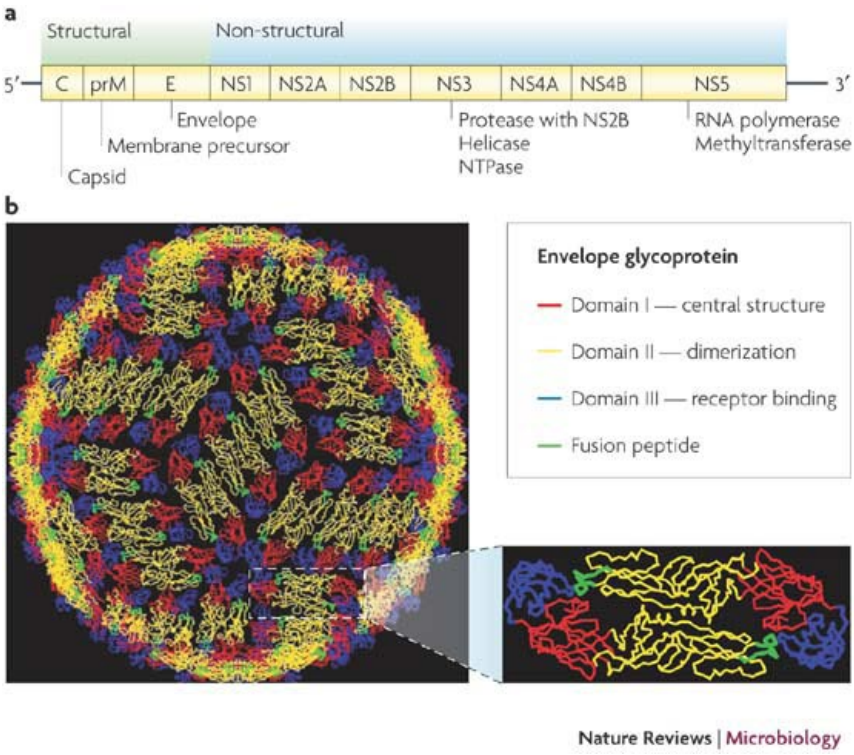

Figure 4: The dengue genome (a) and dengue envelope domains (b) (Whitehead et al. 2007)

The envelope consist of three domains (I, II and III), which have different roles (Guzman & Vazquez 2010). Envelope domain III (EDIII) binds to receptors in the cell membranes and contains antigenic determinants that are serotype specific and dengue specific (Batra et al. 2011; Crill & Roehrig 2001). There are differences in the amino acid sequences in the EDIII between the four serotypes, but the folding is identical. Because of this there is little chance of developing cross-reactive antibodies, something that greatly reduces the risk of ADE (Guzman et al. 2010). EDIII antibodies have also been shown to be effective in blocking absorption of viruses in cells and is therefore an potential vaccine candidate (Crill & Roehrig 2001).

1.2 Molecular farming

Molecular farming can be defined as the production of recombinant proteins in plants (Fischer et al. 1999). Molecular farming has opened for several new approaches in vaccine production. Traditional viral vaccines are usually attenuated (contain weakened viable virus) or inactivated ("killed" virus). Gene technology has opened doors for production of vaccines that are engineered virus-like particles or monoclonal antibodies. Vaccines are usually produced in yeast, bacteria, insects or mammalian cell cultures. These production systems are often costly and contain risks such as allergic reactions and human pathogens. Plants however do not contain any such pathogens and are relatively cost-effective with low production costs (Yusibov et al. 2011). Downstream processing, such as purification, is responsible for most of the cost associated with molecular farming (Shih & Doran 2009).

One process to develop transgenic plants is *Agrobacterium*-mediated transformation. *Agrobacterium* is a genus of gram-negative soil bacteria in which some species are plant pathogens. One such specie is *Agrobacterium tumefaciens*, known to cause crown gall disease in dicotyledonous plants (Gelvin 2003).

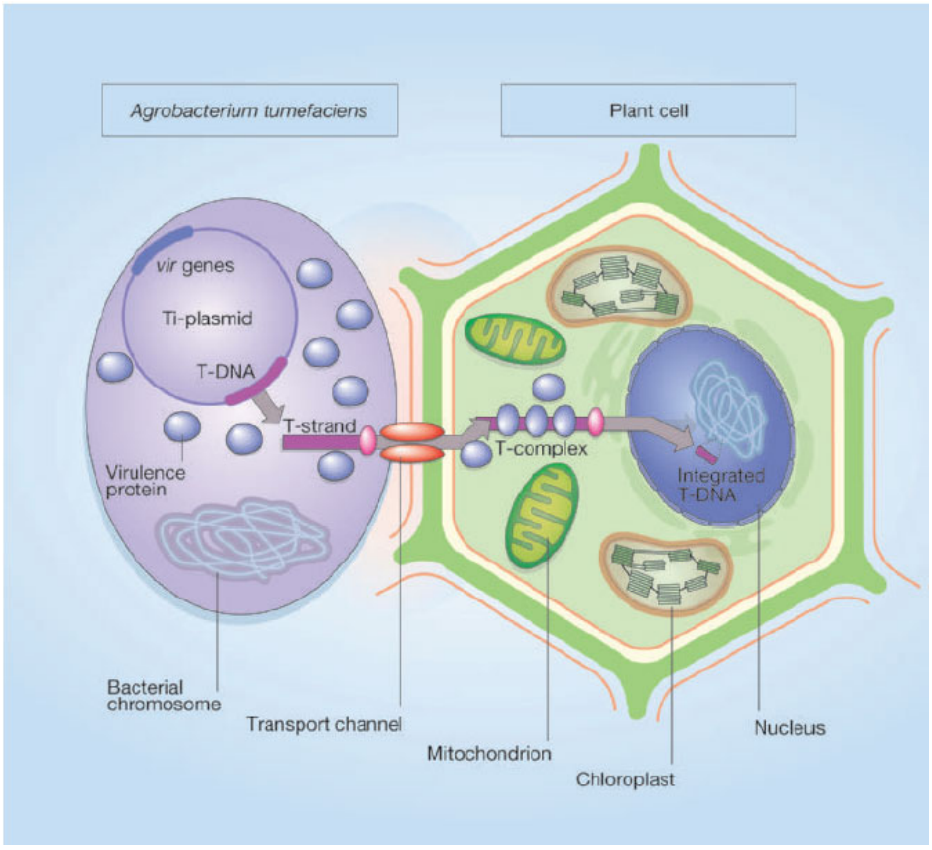

Figure 5: Transformation of a plant cell by Agrobacterium tumefaciens (Gelvin 2005).

A. tumefaciens contains naturally a unique plasmid called the tumor inducing (Ti) plasmid. A segment of the Ti plasmid can be transferred by the bacteria to plant nuclear genomes (Chilton et al. 1980). When located on the Ti plasmid, this region is called the transfer (T) region, but is most commonly known as the T-DNA. The start and stop of the T region is marked by two short repetitive border sequences known as the left border (LB) and right border (RB). The Ti plasmid can contain several T regions, all separately defined with their own LB and RB (Gelvin 2003). The Ti plasmid also contains several virulence (*vir*) genes that are induced by phenolic compounds emitted from plant wound sites (Lee & Gelvin 2008). These *vir* genes code for several enzymes that have different properties. Some *vir* enzymes nick the T-DNA region in the LB and the RB, while others transport this single stranded T-DNA to plant cells. Some *vir* enzymes are probably also transported with the T-DNA to plant cells and help the single-stranded T-DNA become double-stranded before integration to the plant nuclear genome (Gelvin 2003).

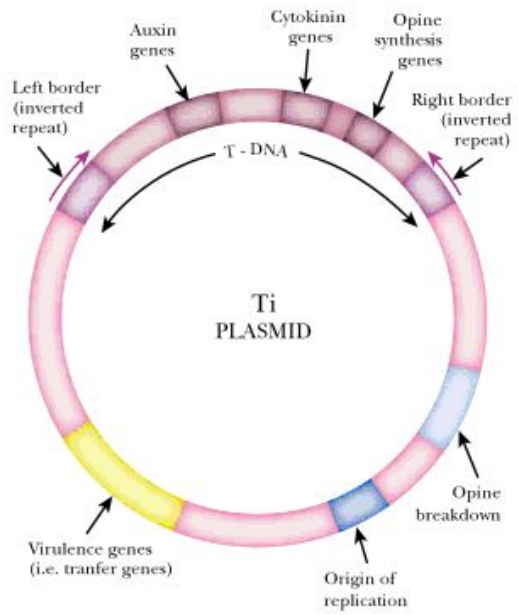

Figure 6: The Ti plasmid. Here shown with the T-DNA, the virulence genes and the left and right border of the T-DNA (Clark 2005).

Agrobacterium's natural ability to genetically transform plants is commonly exploited in plant biotechnology. *A. tumefaciens* ability is not limited to dicotyledonous plants, but can also be used to transform monocotyledons, yeast and fungi (Gelvin 2003).

Development of the binary vector system has been of significant importance in the field of biotechnology. Previously, genes had been inserted directly into the T-region of the Ti Plasmid, but this method has disadvantages. The Ti plasmids are large and have low copy numbers in *Agrobacterium*. In addition they are difficult to isolate and manipulate genetically, and they do not replicate in *Escherichia coli*. Separating the *vir* genes and the T-DNA on two plasmids opened a possibility to create smaller plasmids with unique restriction sites. These plasmids could be replicated in *E. coli* and were selection specific (Lee & Gelvin 2008).

1.2.1 Nicotiana benthamiana and Nicotiana tabacum

Tobacco is a commonly used plant in the production of recombinant proteins. Tobacco has a high biomass yield of 100 tons of leaf biomass per hectare per year, making it possible to produce large quantities of protein. There is also a wide range of expression systems available, such as transient expression, transgenic plants and virus-infected plants. Another very positive aspect of using tobacco is that it is non-feed, non-food and therefore the chances

of contaminating food and feed chains with transgenic material are considerably lower than with other plant species. A negative aspect of using tobacco to produce pharmaceuticals is that it contains high levels of nicotine, which is toxic and therefore has to be removed. Two species of tobacco were used in this study, *N. tabacum*, a cultivated tobacco, and *N. benthamiana*, a non-cultivate. *N. benthamiana* is a specie of tobacco that is very useful to produce recombinant proteins using viral vectors and that supports the systemic replication of several different viruses (Fisher & Schillberg 2004).

1.2.2 Transient expression

Transient expression is a fast and efficient way to temporarily achieve high expression of recombinant proteins and to study gene functions. The basis for transient expression is that not all T-DNA transferred to the plant cell will be integrated to the plant genome. Some T-DNA remains in the nucleus and can be transcribed there. In plants the most commonly used method is *Agrobacterium*-mediated transient expression, which has several advantages. Infiltration will ensure good contact between plant and bacterial cells, a necessity in the transfer of DNA (Kapila et al. 1997). *Agrobacterium* has the ability to transfer several transgenes to the same cell, which can be essential in the development of multimeric antibodies (Vaquero et al. 1999).

A vector developed for efficient transient expression is the pEAQ vector. There are several pEAQ vectors, some developed for gateway cloning and some developed for regular molecular cloning. These are viral vectors based on the larger Cowpea Mosaic Virus (CPMV) RNA-2 vectors. The pEAQ vectors contain a modified 5' untranslated region (UTR) from CPMV RNA-2 and a 35S promoter from the Cauliflower Mosaic virus (CaMV) to enhance expression levels (Sainsbury et al. 2009). The vectors also contain a p19 gene for suppression of post-transcriptional gene silencing (Voinnet et al. 2003).

In case the gene of interest does not express as desired, several strategies can be attempted to increase yield, stability and solubility of the protein. One strategy is the addition of fusion proteins.

Hydrophobins can be used as fusion proteins to increase solubility and in contrast to their name they are not very hydrophobic. Hydrophobins are rather amphiphilic and soluble in

water. They are very effective in altering the solubility of their fusion partners and simplify protein extraction. They are small proteins ranging in size from 25 to 100 amino acids and are natural surface proteins in filamentous fungi. HFBI belongs to class II hydrophobins and originates from *Trichoderma reesei* and is about 7.5 kDa in size (Lahtinen et al. 2008; Linder et al. 2004)

GFP is a widely used reporter gene in molecular biology and is native to the jellyfish *Aequorea victoria*. GFP is about 27 kDa in size and has many advantages as a reporter gene. It is highly stable and resistant to most proteases. In addition GFP can be expressed in several species ranging from bacteria such as *E. coli*, fungi and in several plants such as tobacco (Chalfie & Kain 2006). GFP as a fusion partner is commonly used as a reporter gene to e.g. observe protein function and movement in tissue, but has shown to be able to enhance yield and expression of recombinant protein in *E. coli* in addition to protect its fusion partner from intracellular degradation (Skosyrev et al. 2003).

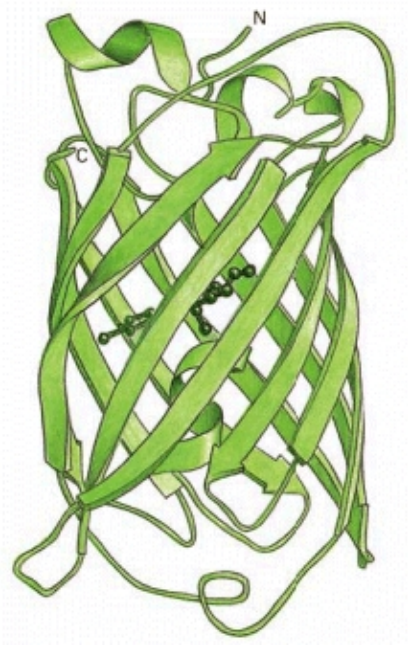

Figure 7: Structure of green fluorescent protein (GFP) (Alberts et al. 2002).

Another option to increase yield can be to target the protein to other cell organelles, such as the endoplasmic reticulum (ER). The cell cytosol contains proteases and other destabilizing conditions while the ER offers a more protected environment (Rivard et al. 2006). By adding a signal peptide (SP) and KDEL it is possible to direct the protein to the ER and retain it there (Fiedler et al. 1997).

1.2.3 Nuclear transformation

As mentioned, *Agrobacterium* integrates T-DNA into the plant nuclear genome, making possible the regeneration of excised plant tissue (explant) to fully transgenic plants (Chilton et al. 1980; Deblock et al. 1984). This is due to the fact that all plant cells are totipotent. Totipotency is the ability for a cell to differentiate to all cells in an organism. This is exploited in the lab e.g. by growing explants inoculated by *Agrobacterium* on media containing a balance of plant hormones auxin and cytokinin (Slater et al. 2008). By using antibiotic in addition as a selection marker it is possible to distinguish between transformed and untransformed cells. The transformed cells will start developing sprouts, while the untransformed cells will wither and die.

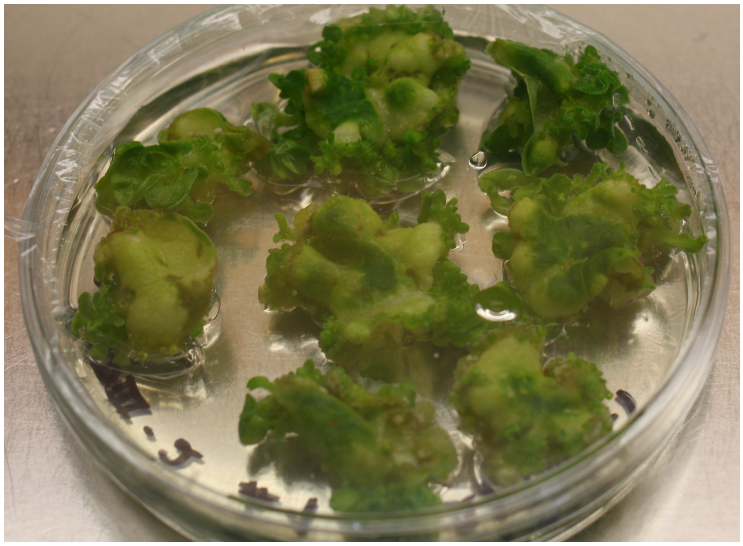

Figure 8: Transformed tobacco leaf disks showing the sprouts forming at the edges and the necrosis in the center.

In this research the binary vectors pSRN-TPSS-T7 was used for stable nuclear transformation. These vectors are designed for transport and integration of genes to plant chloroplasts. This is due to the T7 RNA polymerase, which enables this process (Lossl et al. 2005). The T7 RNA polymerase was removed by restriction digestion since the aim was nuclear transformation and not chloroplast transformation. The pSRN vector contains an alcR transcription factor and an alcA promoter. This is a two-part expression system, which is inducible by ethanol. The alcR reacts to an exogenous source of ethanol and, in response, binds to the target promoter alcA which initiates gene expression (Roberts et al. 2005). By spraying the transformed tobacco plants with ethanol it is possible to facilitate expression of the cloned genes.

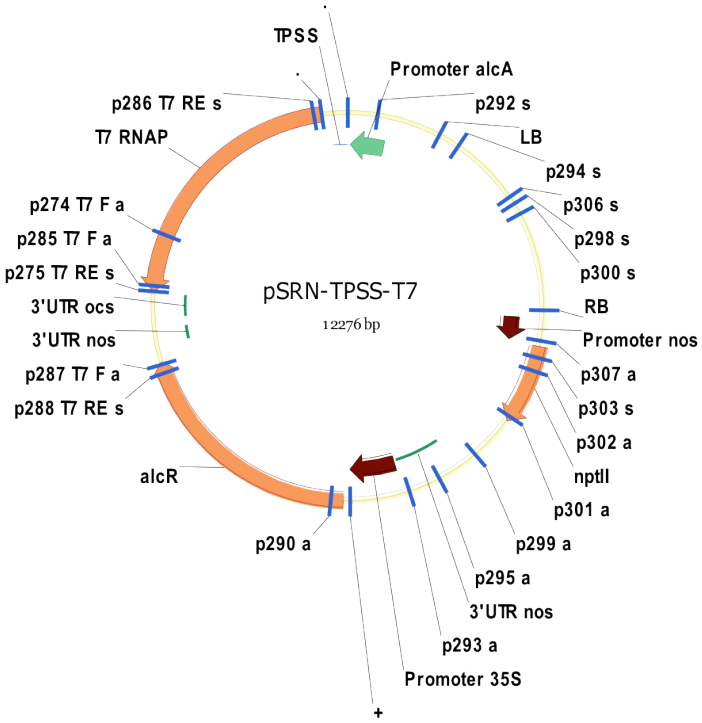

Figure 9: The pSRN-TPSS-T7 vector.

The alcR/alcA system is used to avoid possible deleterious effects of the transgenes to the plant development. With an externally controlled gene expression it is also possible to separate the immediate gene effects from the long-term (Deveaux et al. 2003).

1.3 Aim

The main aim of the project was to express candidate antigens in tobacco as a first step in the development of an affordable, plant derived dengue vaccine.

Previously there has been little success in developing dengue vaccines, as there are complex immunological processes associated with dengue disease. Since most countries affected by dengue disease are developing countries, the vaccine needs to be inexpensive and easily available. The best chance for developing such a vaccine is using a plant based method.

In this thesis the aim was to express dengue antigens in tobacco, either by transient expression or nuclear transformation, and in the process acquire knowledge of molecular and biochemical techniques.

Chapter 2 Materials

2.1 Chemicals

Table 1: List of chemicals and their suppliers.

Name	Supplier
1-Naphthaleneacetic acid (NAA)	Sigma-Aldrich, St. Louis, MO, USA
2-propanol	Merck, Whitehouse Station, NJ, USA
4'-Hydroxy-3',5'-dimethoxyacetophenone (Acetosyringone)	Sigma-Aldrich, St. Louis, MO, USA
40% Acrylamide	Sigma-Aldrich, St. Louis, MO, USA
5-bromo-4-chloro-indolyl-β-D- galactopyranoside (X-gal)	Sigma-Aldrich, St. Louis, MO, USA
6-Benzylaminopurine (BAP)	Sigma-Aldrich, St. Louis, MO, USA
Acetic acid	Sigma-Aldrich, St. Louis, MO, USA
Agarose	Sigma-Aldrich, St. Louis, MO, USA
Ammonium persulfate (APS)	Promega, Madison, WI, USA
Bacto Agar	Becton, Dickinson and company, Franklin Lakes, NJ, USA
Bacto Yeast Extract	Becton, Dickinson and company, Franklin Lakes, NJ, USA
Broad Range Protein Molecular Weight Markers	Promega, Fitchburg, WI, USA
Bromophenol blue	Sigma-Aldrich, St. Louis, MO, USA
Calcium chloride ($CaCl_2$)	Merck, Whitehouse Station, NJ, USA
Cefotaxime	ACS Dobfar Generics, Luxembourg, Luxembourg
Chloramphenicol	Sigma-Aldrich, St. Louis, MO, USA
Coomassie Brilliant Blue R-250	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Dithiothreitol (DTT)	Sigma-Aldrich, St. Louis, MO, USA
DNA ladder 1 kb	New England Biolabs, Ipswich, MA, USA
DNA ladder 100 bp	New England Biolabs, Ipswich, MA, USA
dNTP	New England Biolabs, Ipswich, MA, USA
Ethanol	Arcus, Humelstown, PA, USA
Ethidium bromide	VWR, West Chester, PA, USA
Ethyl(2-mercaptobenzoato-(2-)-O,S) mercurate(1-) sodium (Thiomersal)	Sigma-Aldrich, St. Louis, MO, USA
Ethylenediamineteraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA
Gelzan	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
Glycin	Merck, Whitehouse Station, NJ, USA

Hydrochloric acid (HCl)	Merck, Whitehouse Station, NJ, USA
Kanamycin	Sigma-Aldrich, St. Louis, MO, USA
Magnesium chloride ($MgCl_2$)	Merck, Whitehouse Station, NJ, USA
Methanol	Merck, Whitehouse Station, NJ, USA
morpholine-4-ethanesulfonic acid hydrate (MES hydrate)	Sigma-Aldrich, St. Louis, MO, USA
Murashige and Skoog medium	Duchefa, Haarlem, The Netherlands
Natrium hydroxide (NaOH)	Merck, Whitehouse Station, NJ, USA
Skim milk powder	Fluka, St. Louis, MO, USA
Sodium chloride (NaCl)	Merck, Whitehouse Station, NJ, USA
Sodium dodecyl sulfate (SDS)	Duchefa, Haarlem, The Netherlands
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas(Thermo Fisher Scientific), Waltham, MA, USA
Sucrose Crystallized	Duchefa, Haarlem, The Netherlands
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, St. Louis, MO, USA
Tris Ultrapure	Duchefa, Haarlem, The Netherlands
Tryptone	Duchefa, Haarlem, The Netherlands
Tween20	Sigma-Aldrich, St. Louis, MO, USA
β-mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA

2.2 Kits, enzymes and buffers

Table 2: List of kits, enzymes, buffers and their suppliers.

Name	Supplier
100X BSA	New England Biolabs, Ipswich, MA, USA
10X Buffer 4	New England Biolabs, Ipswich, MA, USA
10X PCR buffer with 15mM MgCl2	Applied Biosystems, Carlsbad, CA, USA
10X T4 DNA Ligase Reaction buffer	New England Biolabs, Ipswich, MA, USA
AmpliTaq® DNA Polymerase	Applied Biosystems, Carlsbad, CA, USA
AP Conjugate Substrate Kit	Bio-Rad, Hercules, CA, USA
DNeasy Plant Mini Kit	Qiagen, Venlo, The Netherlands
ExtrAvidin® –Alkaline Phosphatase	Sigma-Aldrich, St. Louis, MO, USA
iBlot® Transfer Stack, Regular	Invitrogen, Carlsbad, CA, USA
NdeI	New England Biolabs, Ipswich, MA, USA
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel, Dueren, Germany
Platinum® *Pfx* DNA Polymerase	Invitrogen, Carlsbad, CA, USA
Quantum Prep® Plasmid Miniprep	Bio-Rad, Hercules, CA, USA
SmaI	New England Biolabs, Ipswich, MA, USA
SpeI	New England Biolabs, Ipswich, MA, USA
T4 DNA Ligase	New England Biolabs, Ipswich, MA, USA

2.3 Vectors and plasmids

Table 3: List of plasmids and vectors used in this thesis and their suppliers.

	Name	Supplier
Cloning	pPCR-Script Cam SK(+)	Stratagene, Santa Clara, CA, USA
Transient expression	pEAQ-HT-GFP	George Lomonossoff, John Innes Centre
	pEAQ-HT-Dest1/denv2 HFBI	Ingrid Holtsmark, Bioforsk
	pEAQ-HT-Dest1/SP denv2 HFBI	Ingrid Holtsmark, Bioforsk
	pEAQ-HT-Dest1/GFP denv2	Ingrid Holtsmark, Bioforsk
	pEAQ-HT-Dest1/SP GFP denv2	Ingrid Holtsmark, Bioforsk
Nuclear transformation	pSRN-TPSS-T7	Andreas Lössl, BOKU

Table 4: List of vectors constructed for nuclear transformation of *N. tabacum*.

Constructed vectors	Transformed *N. tabacum*
pSRN-EDIII1	pSRN-EDIII1
PSRN-EDIII3	pSRN-EDIII3

2.4 Antibodies

Table 5: List of antibodies and their suppliers

Name	Supplier
Anti-dengue, raised in rabbit	Ingrid Holtsmark, Bioforsk
Anti-Rabbit IgG, raised in Goat	Sigma-Aldrich, St. Louis, MO, USA
Living Colors® A.v. Peptide Antibody, raised in rabbit	Clontech, Mountain View, CA, USA

2.5 Solutions

Table 6: List of solutions and their recipes.

Name	Recipe
10X TBS	24.22g Tris base and 292.2g NaCl was dissolved in distilled H_2O and pH adjusted to 7.5 to a final volume of 1 liter.
1X TTBS	1X TTBS was diluted from 10X TBS with 500 µl Tween20 to a final volume of 1 liter.
5X Running buffer	15.1 g Tris buffer, 25 ml 20% SDS and Glycine was dissolved in distilled H_2O and volume adjusted to 1 litre
4X Loading Buffer	The loading buffer was made with: 4 mg Bromophenol blue, 0.8 g SDS, 4 ml 100% Glycerol, 4.8 ml 0.5M Tris buffer (pH=6.8), 0.4 ml β-mercaptoethanol and 0.7 ml distilled H_2O.
Staining buffer	20 ml acetic acid, 50 ml methanol and 0.1 g Coomassie Blue was mixed with 30 ml distilled H_2O.
Destaining buffer	25 ml methanol, 10 ml acetic acid and 65 ml distilled H_2O was mixed together.

2.6 Growth medium

Table 7: List of growth medium and recipes.

Name	Recipe
MS-II solution	In 1 liter 4.71 g Murashige and Skoog salt, 20 g sucrose, 0.5 g MES salt were dissolved in dH_2O and pH adjusted to 5.7. The solution was autoclaved and stored at 4°C.
MS-II medium	MS-II solution was prepared and 3.3 g gelzan added to the solution before autoclaving. The medium was poured to 9 cm petri dishes, left to set and stored at 4°C.
Co-cultivation medium	MS-II medium was prepared, autoclaved and cooled to 55°C before adding 0.1 µg/ml NAA and 1µg/ml BAP per liter. The medium was poured to 9 cm petri dishes, left to set and stored at 4°C.
MS-II selection medium	Co-cultivation medium was prepared, autoclaved and cooled to 55°C with the addition of 100 µg/ml kanamycin and 500 µg/ml cefotaxim to NAA and BAP. The medium was poured to 9 cm petri dishes, left to set and stored at 4°C.
Hormone free MS-medium for root induction	MS-II solution was prepared with only half Murashige and Skoog salt (2.36 g) per liter. The medium was autoclaved and poured to magenta boxes. The boxes were left to set and stored at 4°C.
LA (Lysogeny Agar)	LB was prepared and 15 g agar per liter added before autoclaving. The medium was cooled to 50-55°C before antibiotic was added. The medium was poured onto 9 cm petri dishes, left to set and stored at 4°C.
LB (Lysogeny Broth)	10 g tryptone, 5 g yeast extract, 10 g NaCl were dissolved in distilled H_2O. The pH was adjusted to 7.5 and the final volume to 1 liter. The solution was autoclaved, cooled and stored at 4 °C.

2.7 Primers

Table 8: List of primers and primer sequences. All primes were supplied by Invitrogen.

Template	Application	Primer Name	Primer sequence (5'-3')
pUC57 EDIII 1	Adding restriction sites	EDIII1 SpeI FW	TGATCTACTAGTATGTCTTATGTAATGTGTACTGGTTCT
		EDIII1 NdeI Rev	GATGTACATATGTTAATGATGATGATGATGATGACCTTG
pUC57 EDIII 3	Adding restriction sites	EDIII 3 SpeI FW	TCGATGACTAGTATGTCTTATGCTATGTGTTTAAATACTTTTGT
		EDIII 3 NdeI Rev	GTGAACCATATGTTAATGATGATGATGATGATGACCTTGA
pSRN vectors	Sequencing	p292 Forward AS	TGAATTAAGCTTGGCCGCCG
		p275 T7 RE SP	AATGCGCTTGCGCTTCAGGC
pSRN EDIII 1	PCR Screening	EDIII1 Scr FW	GCTGAAACTCAACATGGAACTG
		EDIII1 Scr Rev	ATGCTTTTTCACCAGCACCT
pSRN EDIII 3	PCR Screening	EDIII 3 Scr FW	TGAAGATGGACAAGGAAAAGC
		EDIII1 Scr Rev	CTCCTCCACCACCTCCTTTA
pPCR-Script	Screening	M13 Forward	GTAAAACGACGGCCAGT
		M13 Reverse	CATGGTCATAGCTGTTTCC
pEAQ vectors	Screening	C1 Forward	AACGGTTGTCAGATCGTGCTTCGGCACC
		C3 Reverse	CTGAAGGGACGACCTGCTAAACAGGAG

2.8 Laboratory equipment

Table 9: List of laboratory equipment and their supplier.

Technique	Instrument	Supplier
Centrifugation	Biofuge Pico 5810 R Galaxy Mini Heraeus Fresco21	Heraeus Laboratories, Hanau, Germany Eppendorf, Hamburg, Germany VWR, West Chester, PA, USA Thermo Scientific, Waltham, MA, USA
Gel imaging	Molecular Imager Gel Doc XR System	Bio-Rad Laboratories, Hercules, CA, USA
Incubation	DB-2A Techne Dri-Block Heater GD120 Water	Sigma-Aldrich, St. Louis, MO, USA Grant Instruments, Cambridge, UK
Vortexing	MS2 Minishaker Vortexer	IKA, Staufen, Germany VWR, West Chester, PA, USA
Spectrofotometer	Nanodrop 2000 UV-1601	Thermo Scientific, Waltham, MA, USA Shimadzu, Kyoto, Japan
Gyro rocker	Stuart SSL3 Gyro-Rocker	Sigma-Aldrich, St. Louis, MO, USA
Western blotting	iBlot® Gel Transfer Device	Invitrogen, Carlsbad, CA, USA
Standard PCR	T100 Thermal Cycler C1000 Thermal Cycler S1000 Thermal Cycler	Bio-Rad Laboratories, Hercules, CA, USA Bio-Rad Laboratories, Hercules, CA, USA Bio-Rad Laboratories, Hercules, CA, USA
Electroporation	BTX ECM 630 Electro Cell Manipulator	Harvard Apparatus, Holliston, MA, USA
Magnetic stirrer	RCT Classic Magnetic Stirrer	IKA, Staufen, Germany
pH meter	WTW InoLabR 7110 pH meter	Xylem, White Plains, NY, USA

2.9 Software

Table 10: List of software and their source.

Name	Source
Quantity One v. 4.5.0	Bio-Rad Laboratories, Hercules, CA, USA
Vector NTI 11	Invitrogen, Carlsbad, CA, USA
Primer3Plus	(Rozen & Skaletsky 2000) (available at: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)
CLC Main Workbench 6.6.2	CLC Bio, Aarhus, Denmark
Standard Nucleotide BLAST	The National Center for Biotechnology Information (available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Chapter 3 Methods

3.1 Transformation of *E.coli*, *Agrobacterium* and construction of dengue-expression vectors

For the purpose of this thesis, vectors with the desired genes EDIII1 and EDIII3 were constructed and introduced to *A. tumefaciens* by electroporation for nuclear transformation. Already constructed vectors containing EDIII of serotype 2 (denv2) with fusion partners were also introduced to *A. tumefaciens* for transient expression.

To construct the vectors, specific primers with restriction sites were designed and were used in PCR to amplify the genes with the added restriction sites. pPCR-Script is a high-copy number vector which was used as an intermediary stage to amplify the genes in *E. coli*. The PCR-Script vector with the genes could then be isolated from the cells at high concentration, and the insert confirmed by sequencing. The restriction enzymes *SpeI* and *NdeI* were used to cleave out the gene of interest at the specific cleavage sites introduced during the PCR. The obtained restriction fragments were isolated by gel electrophoresis. Restriction enzyme digestion with the same enzymes was also used on the destination vector pSRN-TPSS-T7 to open the vector and prepare for use as a backbone in a ligation reaction. Using the fragment sizes of the insert and vector, and their respective concentrations, the amount needed for a T4 ligation reaction could be calculated. After ligation the new constructed vector was transformed into *E. coli* for amplification. The transformed cells were grown on selection medium and colonies screened using PCR. Positive colonies could be used to amplify the vector, extracting it for sequencing and transforming *A. tumefaciens* by using electroporation.

3.1.1 Preparation of competent *E. coli* cells (chemocompetent)

For transformation, competent *E. coli* cells needed to be prepared. *E. coli* does not take up DNA naturally for genetic transformations and have to be made competent through a process involving $CaCl_2$ (Aich et al. 2012). For preparation of the competent *E. coli* cells the following protocol was followed:

The desired strain of *E.coli* (JM109) was grown overnight in 5 ml LB and 1 ml of the culture was transferred to 100 ml LB in a 1L Erlenmeyer flask. The culture was incubated at 37 °C

for 2-4 hours at 180 rpm. The culture was monitored by measuring OD_{600} every 30 minutes and when the OD_{600} reached 0.35, the cells were harvested.

The culture was transferred to sterile, ice-cold 50 ml falcon tubes and kept on ice for 10 minutes. The falcon tubes were then centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was decanted and the pellet dried by inverting the tubes on sterile paper towels for 1 minute. The pellet was resuspended in 30 ml ice-cold $MgCl_2$-$CaCl_2$ solution by gently pipetting with a 5 ml pipette. The suspension was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant decanted. The pellet was then resuspended in 2 ml ice-cold $CaCl_2$ by gently pipetting before adding 355µl 98% glycerol and 82µl DMSO. The tubes were stored on ice for 15 minutes before adding an additional 82µl DMSO. The suspension was dispensed in ice-cold 1.5ml eppendorf tubes in 50µl aliquots. The competent cells were frozen immediately in liquid nitrogen and stored at -80°C.

3.1.2 Transformation of competent JM109 *E. coli* cells using heat shock

The competent *E. coli* cells were enabled to take in external plasmid DNA by using a transformation process known as "heat shock". In this process the competent cell solution is added plasmid DNA and stressed by exposing them to heat for a very short period of time.

The competent *E. coli* cells were first defrosted on ice before 50 ng of plasmid DNA (usually between 2-4 µl) was added to 50 µl competent cells. The tube was flicked lightly to mix carefully, and left on ice for 30 minutes. The tube was then put in a water bath at 42°C for 45 seconds before being put back on ice for 2 minutes and adding 400 µl of LB (without antibiotic). The tube was incubated at 37°C for 1 hour at ~150 rpm shaking. 100 µl of the cell suspension was spread on LB plates with appropriate antibiotic and incubated overnight at 37°C.

3.1.3 Electroporation of *Agrobacterium tumefaciens* (LBA4404)

Competent *A. tumefaciens* cells were transformed using electroporation. In this process the competent *Agrobacterium* cells are exposed to a short high voltage electrical pulse. This electric pulse creates pores in the membrane of the cells and enable plasmid DNA to be absorbed (Mahmood et al. 2008).

Electrocomepetent *A. tumefaciens* cells were collected from the -80°C freezer. The eppendorf tubes and cuvettes needed for this transformation were kept on ice. Depending on the concentration, 1-2µl DNA was added to 21 µl competent cells and mixed carefully before immediately adding 20 µl to an electro cuvette. The cuvette was tapped lightly to distribute the suspension in the cuvette and to remove potential air bubbles. The moisture on the surface of the cuvette was removed and the cuvette was placed in the electroporator. Setting the electroporator to 2000V, 200 Ω and 25 µF the cell suspension was exposed to the short electric pulse before removing the cuvette and adding 1ml of LB (without antibiotic). The suspension was pipetted over to a 15 ml falcon tube and incubated at 28°C with ~160 rpm shaking for 2 hours. 100 µl of the suspension was spread on LB plates with appropriate antibiotic and the plates incubated for 2-3 days at 28°C.

3.1.4 Long-term storage of bacterial cultures

Transformed bacterial cells were stored long term for future use by mixing 500 µl of a liquid culture thoroughly with 500 µl of 50% glycerol. The suspensions where frozen in liquid nitrogen and stored at -80°C.

3.1.5 PCR cloning

The amplified gene fragments with the restrictions sites were cloned pPCR-Script Cam SK(+). This was done due to the enzymes *NdeI* and *SpeI* seeming unable to efficiently cut the purified PCR fragments directly.

The following components were mixed in a PCR tube:

6.6 µl	dH_2O
2 µl	pPCR-Script vector
2 µl	10X T4 DNA Ligase Reaction Buffer
1 µl	rATP
0.4 µl	SmaI
2 µl	T4 DNA Ligase
6 µl	Cleaned PCR product
20 µl	Total volume

The reaction was incubated at room temperature for 1-2 hours before being transformed into competent *E. coli* JM109 cells by heat shock transformation.

LB plates with chloramphenicol (34 µg/ml) was prepared by spreading 40 µl X-gal (40 mg/ml) to the plates and air drying for about 20 minutes before adding 100 µl of the cell suspension. X-gal was added to the plates to facilitate blue/white selection. pPCR-Scriptc contains a *LacZ* gene that codes for β-galactosidase, an enzyme that hydrolyzes X-gal to 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue substance. The vector is designed to place the insert within the *LacZ* gene and disrupt the transcription of β-galactosidase. The cells with the gene placed successfully within the vector will give white colonies, whereas cells with the original PCR-Script plasmid will give blue colonies (Wilson & Walker 2010).

3.1.6 Antibiotic selection

To selectively grow transformed bacteria, antibiotics were added to lysogeny media. The type antibiotic added and the concentrations were dependent on the vectors as they contained marker genes for an antibiotic and were able to grow on media containing this. Non-transformed bacteria did not contain the marker gene, and would also not grow, though some random mutations due occur.

Table 11: Overview of antibiotic concentrations in selection medium.

Vector	**Antibiotic**	**Concentration**
pSRN	Kanamycin	50 µg/ml
pPCR-Script Cam SK (+)	Chloramphenicol	34 µg/ml
pEAQ	Kanamycin	50 µg/ml

3.1.6 Restriction enzyme digestion

pPCR-Script vectors containing the gene of interest were double digested to release the gene fragment. The destination vector, pSRN-TPSS-T7, was also opened by double digestion. In restriction enzyme digestion the enzymes recognizes specific short nucleotide sequences that are called restriction sites, and cleave the DNA at these sites. The enzymes used here, *NdeI* and *SpeI*, left an overhang of a few nucleotides, creating sticky ends.

The following components were mixed in a PCR tube:

0 µl	H_2O
5 µl	10X Buffer 4
0.5 µl	100X BSA
2 µl	SpeI (10,000 units/ml)
1 µl	NdeI (20,000 units/ml)
41.5 µl	Vector
50 µl	Total volume

The reaction was incubated on a PCR machine for 4 hours, before being heat inactivated. The conditions were as described in Figure 10.

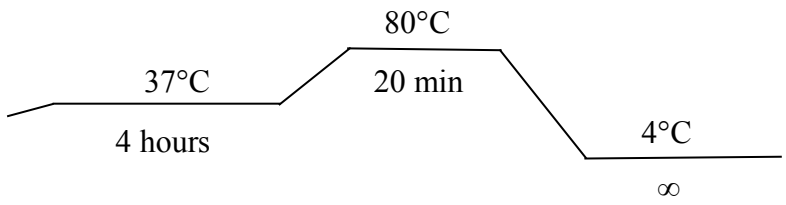

Figure 10: Conditions for restriction enzyme digest.

3.1.7 Gel extraction

Restriction digest reactions were run on an agarose gel by gel electrophoresis to separate the fragments. The desired DNA fragments were collected from gels and extracted using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) kit. All centrifugation were done at 11,000 x g.

DNA extraction

Desired fragments on the gels were cut out by a scalpel, placed in an eppendorf tube and weighed before adding 200 µl of Buffer NTI per 100 mg of agarose gel. The fragments and buffer were incubated for 5-10 minutes at 50°C until the gel was completely dissolved. The sample was vortexed every 2-3 minutes during incubation.

Binding DNA to silica membrane

The sample was transferred to a NucleoSpin® Gel and PCR Clean-up Column in a 2 ml collection tube and centrifuged for 30 seconds discarding flow-through.

Washing silica membrane of residue

The column was added 700 µl of Buffer NT3 and the sample centrifuged for 30 seconds. Flow-through was discarded and this step repeated. The sample was then centrifuged for 1 minute to remove all residues of Buffer NT3.

Eluting DNA

The column was placed in a 1.5 ml eppendorf tube, 25-30 µl Buffer NE added, and the sample incubated for 1 minute at room temperature. Buffer NE was heated to 70°C before eluting DNA to improve yield. The sample was centrifuged for 1 minute and the extracted DNA was stored at -20°C for future use.

3.1.8 Ligation

T4 DNA ligation is process where two DNA strands are joined. This is catalyzed by T4 DNA ligase which creates a phosphodiester bond between the 5' and 3' ends of the DNA strands (Glick & Pasternak 2010). Due to the use of the same restriction sites, the vector and the insert had complementary sticky ends.

$$insert\ mass\ in\ ng = 3\times \frac{insert\ length\ in\ bp}{vector\ length\ in\ bp} \times vector\ mass\ in\ ng$$

The amount of insert needed to perform a ligation was calculated using the equation above. For this ligation reaction, it was calculated with a 3:1 ratio between insert and vector.

$$amount\ in\ \mu l = \frac{mass\ in\ ng}{concentration\ in\ ng/\mu l}$$

The equation above was used to calculate how many µl was required in a reaction, both for insert and vector.

The following were combined for the ligation reaction:

to 10 µl	dH_2O
1 µl	10X T4 DNA Ligase Buffer
50 - 100 ng	Vector DNA
50 – 100 ng	Insert DNA
1 µl	rATP
1 µl	T4 DNA Ligase
10 µl	Total volume

The reaction was mixed carefully together since T4 DNA Ligase is sensitive to shearing. The ligase reaction was incubated overnight at 16°C on a PCR machine. The ligase was then inactivated by heating the reaction to 65°C for 10 minutes.

3.1.9 Verification of constructed vectors by sequencing

For sequencing, samples were sent to GATC. This was done by sending in a 20 µl sample of extracted plasmid with a concentration of 30-100 ng/µl. Primers were also sent along with the samples. The volume of each primers was 20 µl and the concentration 10 µM.

3.2 Transient expression in *Nicotiana benthamiana* by Agroinfiltration

Agroinfiltration is a process where a suspension of *Agrobacterium* is directly injected into plant leaves a facilitate transient expression (Van der Hoorn et al. 2000). In this case *A. tumefaciens* was injected into *N. benthamiana* leaves by using a syringe without a needle. The *Agrobacterium* suspension contains acetosyringone, a phenolic signal compound that activates the virulence genes in *Agrobacterium* and improves transient expression (Faizal & Geelen 2012; Kapila et al. 1997). For transient expression, pEAQ vectors containing constructs for denv2 HFBI, SP denv2 HFBI, GFP denv2 and SP GFP denv2 were used. In addition, pEAQ-HT-GFP was used as a positive control while agroinfiltration buffer, without *A. tumefaciens*, was used as a negative control.

3.2.1 Preparation of cells for Agroinfiltration

Agroinfiltration buffer:

500 µl	1M MES-KOH pH 5.7
500 µl	1M $MgCl_2$
100 µl	100mM Acetosyringone (dissolved in DMSO)
48.9 ml	dH_2O
50 ml	Total volume

Transformed *A. tumefaciens* cells containing the gene of interest were grown overnight in 5 ml LB with selection. LB was prepared by adding 500 µl 1M $MgCl_2$ and 500 µl 1M MES-KOH (pH 5.7) to 50 ml LB. Directly before transferring 1 ml of the overnight culture, 10 µl 100mM Acetosyringone was added to the LB solution. Acetosyringone was dissolved in DMSO and prepared fresh for every infiltration. The 50 ml culture was grown overnight at 28°C.

The culture was transferred to a 50 ml falcon tube and spun at 4000rpm for 20 minutes, after which the supernatant was discarded. The pellet was resuspended in 5ml Agroinfiltration buffer and OD_{600} measured. The suspension was diluted further in agroinfiltration buffer until the OD_{600} was 0.5. The suspensions were then incubated in room temperature for 2 to 4 hours before infiltration.

3.2.2 Agroinfiltration

An estimated 5 ml of the suspensions were needed for infiltration for one plant. Plants 6-8 weeks of age were chosen. All leaves that were not going to be infiltrated were removed until 5 leaves remained. These were marked infiltrated with a 10 ml syringe without a needle. This was done by placing the opening of the syringe to the leaf and adding counter pressure by holding a finger on the opposite side of the leaf. Two plants, with 5 leaves each, were infiltrated for each construct as a replicate.

The leaves were collected 0, 3, 6, 9 and 12 days post infiltration (dpi). Day 0 was leaves collected straight after infiltration.

3.3 Agrobacterium mediated nuclear transformation of *Nicotiana tabacum*.

Agrobacterium mediated nuclear transformation was used for stable expression of dengue antigens in tobacco. The explant was soaked in an agrobacterium suspension to facilitate insertion of the genes EDIII1 and EDIII3 into the plant genome through wounded sites on the plant tissue.

3.3.1 Nuclear transformation

A culture of *A. tumefaciens* containing the desired vector and gene were grown overnight in 5 ml LB with kanamycin (50 µg/ml). In a 250 ml Erlenmeyer flask, 50 ml LB with kanamycin (50 µg/ml) was added and 1 ml of the overnight culture was transferred. The culture was incubated at 28°C at ~170rpm overnight for 16-20 hours.

Leaf disks were excised from sterile leaves of *N. tabacum* and stored in petri dishes with filter paper soaked in MS-II. For each vector construct to be transformed, 110 leaf disks per vector construct were estimated, 100 for the transformation, and 10 left untransformed for negative control of the media. The overnight culture was centrifuged at 4000 rpm for 15 minutes and supernatant decanted. The pellet was washed with 5 ml MS-II before being resuspended in 10 ml MS-II.

In a sterile bench the *Agrobacterium* suspension was filled in a petri dish. Another petri dish was also filled with MS-II and a third had clean filter paper. The leaf disks were placed in the bacteria suspension, 10 at a time, and left for 1 minute. The disks were turned once and made sure that the whole surface made contact with the suspension. They were moved to the MS-II to be cleaned and transferred immediately to the filter paper to dry somewhat. The disks were then placed on a solid MS-II media without any selection and covered in aluminum foil to avoid contact with light, before they were placed in a light room for 3 days.

After 3 days the disks were moved to new petri dishes with MS-II containing kanamycin, BAP, NAA and cefotaxime. They were placed back into the light room and incubated for 10 days before being transferred to fresh media. The disks were moved to fresh media every 10 days until sprouts formed. The sprouts were cut and placed on fresh media and left to grow an

additional 10 days before being recut and placed in boxes with hormone free MS-medium for root induction. The plants were left to grow and form roots.

Plants were screened by collecting plant tissue, extracting DNA and using PCR to confirm presence of the transgene. For each construct, 10 confirmed positive plants were placed on soil and left to grow to an appropriate height.

3.3.2 Ethanol induction of the T7 promotor in the pSRN vectors.

Plants transformed by nuclear transformation were sprayed with a 5% ethanol solution. Leaves were collected day 0, 1, 4 and 7 and frozen in liquid nitrogen. Day 0 was leaves collected before spraying with ethanol. The leaves were ground in liquid nitrogen and saved for further analyses.

3.4 Polymerase Chain Reaction (PCR) analysis

PCR is a process for rapid amplification of DNA fragments and was developed by Kary Mullis. By using primers to recognize specific areas of DNA and amplify these with the use of dNTPs and *Taq* polymerase, it is possible to increase the amount of desired DNA. *Taq* polymerase is a DNA polymerase that elongates the primers using the dNTPs in the solution, and it can withstand high temperatures without degradation.

In the PCR reaction, the double stranded template DNA is heated to 95°C to separate the strands. The temperature is lowered to about 55°C, depending on the primers, for attachment of the primers to the template strands. The temperature is raised to 72°C for elongation of the primers by addition of dNTPs with the use of *Taq* polymerase. This process is repeated several times until a great concentration of DNA is achieved (McPherson & Møller 2006).

3.4.1 Primer design

For analyzing samples using PCR or adding restriction sites to the dengue antigens, primers needed to be designed. When designing primers, there are several aspects to consider. A primer should be between 18-22 bases in length so that it's long enough to bind specifically, but still short enough to bind to the template at the proper annealing temperature. One should also consider the melting temperature (Tm), which is ideally between 52-58 °C. It is also

important that corresponding primer pairs have similar Tm. The Tm is partially determined by the percentage of guanine or cysteine in the primer and should be between 40-60 % so that the primer will bind well, but not so much that the Tm will be too high.

Repeats should also be avoided in the primer as to not create secondary structures such as primer dimers or hairpin loops. Primer dimers occur when the primer binds to another identical primer or to the corresponding primer from the primer pair. Hairpin loops occur when the primer binds to itself and all these secondary structures impair primer effectivity.

Designing primers with restriction sites

Primers were designed using the Vector NT1 software. They were designed to amplify specific genes and add restriction sites to the amplified fragments for molecular cloning.

Designing primers for screening of nuclear transformed plants

Primers were designed using the Primer3Plus software available online. The sequences were pasted into the 'sequence window' and primers over 200 bp were chosen. These primers amplified areas inside the gene fragments.

3.4.2 Pfx PCR

For correct amplification by PCR of the gene of interest, Platinum® *Pfx* DNA Polymerase (Invitrogen) was used. Platinum® *Pfx* DNA Polymerase possesses proofreading activity and yields high fidelity amplification.

Pfx PCR reaction volumes:

17.55 µl	dH_2O
2.5 µl	10 *Pfx* Amplification Buffer
3 µl	dNTP (2.5 mM)
0.5 µl	MgSO4 (50 mM)
0.375 µl	Forward primer (20 mM)
0.375 µl	Reverse primer (20 mM)
0.2 µl	Platinum® *Pfx* DNA Polymerase
0.5 µl	DNA template
25 µl	Total volume

The reaction was run on a PCR machine with conditions as described in Figure 11.

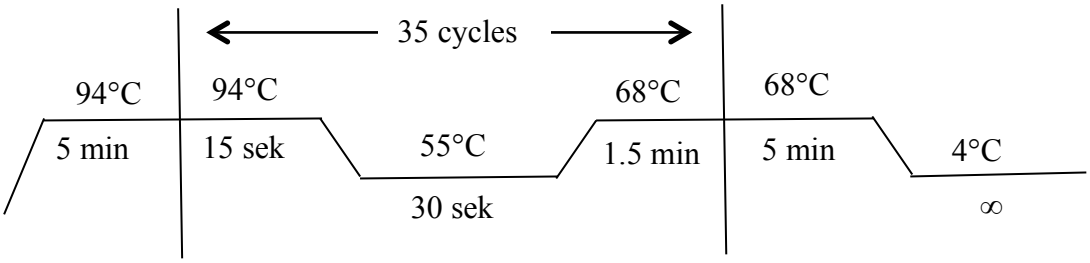

Figure 11: Conditions for Pfx PCR.

3.4.3 Standard PCR

Most screening analysis were done by using a standard PCR.

Standard PCR reaction volumes:

12.9 µl	dH_2O
2.5 µl	10X PCR buffer with 15 mM $MgCl_2$
2.5 µl	dNTP (2.5 mM)
2.5 µl	Forward primer (2 mM)
2.5 µl	Reverse primer (2 mM)
0.1 µl	Taq DNA polymerase
2 µl	Template DNA
25 µl	Total volume

The PCR reactions were run in a PCR machine with conditions as described in Figure 12.

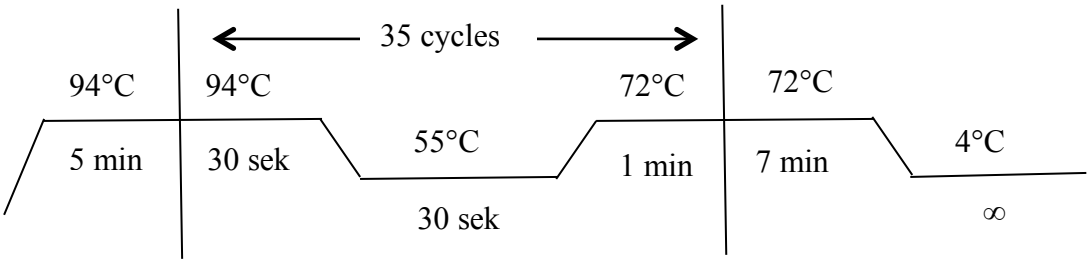

Figure 12: Conditions for standard PCR.

3.4.4 Colony PCR

Colony PCR was used to screen bacterial colonies for possible positives.

Colony PCR reaction volumes:

12.9 µl	dH_2O
2.5 µl	10X PCR buffer with 15 mM $MgCl_2$
2.5 µl	dNTP (2.5 mM)
2.5 µl	Forward primer (2 mM)
2.5 µl	Reverse primer (2 mM)
0.1 µl	Taq DNA polymerase
2 µl	Bacterial suspension
25 µl	Total volume

Bacterial colonies on an agar plate were picked up with a pipette tip. The tip was then placed in an eppendorf tube containing 10 µl dH_2O. 2 µl of this suspension was used for colony PCR. The reaction was run on a PCR machine with conditions as described in Figure 13.

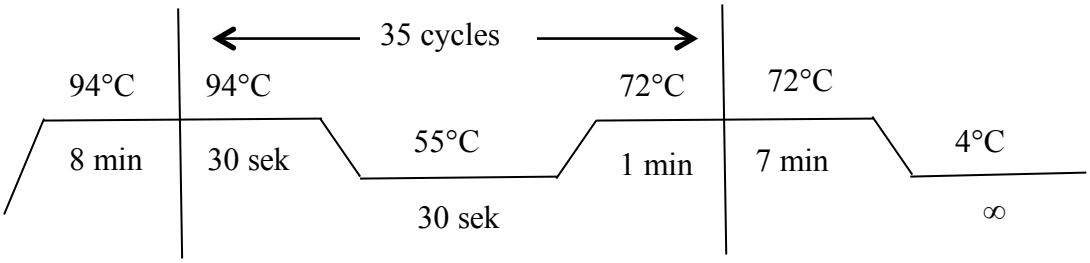

Figure 13: Conditions for colony PCR.

3.4.5 PCR clean-up

Gene fragments amplified by *Pfx* PCR were cleaned using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) kit. All centrifugation was done at 11,000 x g.

Binding DNA to silica membrane

Buffer NTI was added at 2X the volume of the PCR sample and mixed. The sample was transferred to the NucleoSpin® Gel and PCR Clean-up column in a 2 ml collection tube and centrifuged for 30 seconds.

Washing silica membrane to remove residue

The column was added 700 µl Buffer NT3 and the sample centrifuged for 30 seconds. Flow-through was discarded and the sample centrifuged an additional 1 minute to remove residues of Buffer NT3.

Eluting DNA

The column was placed in a 1.5 eppendorf tube and 20-25 µl Buffer NE added to the membrane, before incubating at room temperature for 1 minute. The sample was then centrifuged for 1 minute. The cleaned PCR product was stored at -20°C until further use.

3.4.6 Plasmid DNA extraction

Plasmids from *E.coli* were extracted using the Quantum Prep® Plasmid Miniprep (Bio-Rad) kit for use in transformations of *A. tumefaciens* or *E. coli*, or for restriction enzyme digests. All the centrifuging was done at 14,000 x g.

Cell lysis

An overnight culture was transferred to a 1.5 ml eppendorf tube and centrifuged 30 seconds. An additional 1.5 ml of the overnight culture was added to the tube and the process repeated, giving a pellet from a total of 3 ml overnight culture. The pellet was resuspended in 200 µl Cell Resuspension Solution by vortexing thoroughly. The solution was added 250 µl of the Cell Lysis Solution and mixed by inverting the tube 10 times. The sample was then added 250 µl Neutralization Solution and mixed by inverting the tube 10 times.

Binding DNA to Spin Filter membrane

The sample was centrifuged for 5 minutes and the supernatant transferred to a Spin Filter in a 2 ml wash tube. The debris pellet was discarded. The Quantum Prep matrix was prepared by shaking the solution thoroughly to mix and adding 200 µl to the sample. The sample was centrifuged for 30 seconds and flow-through discarded.

Washing Spin Filter membrane

The Spin Filter was added 500 µl Wash Buffer and the sample centrifuged for 30 seconds. Flow-through was discarded. Another 500 µl Wash Buffer was added to the Spin Filter and the sample centrifuged for 2 minutes. The flow-through was discarded and the sample centrifuged for an additional 2 minutes to remove residual Wash Buffer.

Eluting DNA

The Spin Filter was transferred to a 1.5 ml eppendorf tube and 50 µl H_2O added before centrifuging for 1 minute. The extracted plasmid was stored at -20°C until further use.

3.4.7 Extraction of genomic DNA from plant tissue

Genomic DNA from *N. tabacum* leaves was extracted using DNeasy Plant Mini Kit (QIAGEN) for screening of positive transformants using PCR.

Lysing plant cells

Samples of 100 mg ground plant tissue was added 400 µl of Buffer AP1 and 4 µl RNase A and mixed by vortexing. The samples were then placed in a water bath at 65°C for 10 minutes and inverted every 3 minutes to mix.

Precipitating detergents, proteins and polysaccharides

The samples were added 130 µl of Buffer AP2 and they were left to incubate on ice for 5 minutes. The samples were centrifuged for 5 minutes at 20,000 x g and the lysate pipetted to a QIAshredder Mini spin column and centrifuges again for 2 minutes at 20,000 x g.

Binding DNA to Spin Filter membane

The flow-through, usually about 510 µl, was transferred to a new 1.5 ml eppendorf tube containing 765 µl (1.5 x flow-through) Buffer AP3/E and mixed by pipetting. The lysate was

transferred to a DNeasy Mini spin column, 650 µl at a time, and centrifuged at 6000 x g for 1 minute. Flow-through was discarded and the process repeated for the rest of the lysate.

Washing Spin Filter membrane

The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW added. The column was centrifuged for 1 minute at 6000 x g and flow-through discarded. Another 500 µl Buffer AW was added to the spin column and it was centrifuged for 2 minutes at 20,000 x g.

Eluting DNA

The DNeasy Mini spin column was moved to a 1.5 ml eppendorf tube and 50 µl Buffer AE added directly to the membrane. The spin columns were left to incubate in room temperature for 5 minutes before centrifuging for 1 minute at 6000 x g. Another 50 µl of Buffer AE was added to the spin column and the process repeated. The DNA was stored at -20°C for further use.

3.4.8 Gel electrophoresis

Gel electrophoris is the separation of DNA fragments on a gel. In agarose gel electrophoresis the agarose is dissolved in 1X TBE buffer by heat. When cooled, the agarose solution will form a porous gel. The gel is placed in a chamber with electrodes forming negative and positive poles that are activated using electricity. DNA is negatively charged and will therefore travel toward the positive pole of the gel. Larger fragments will be subject to more friction in the gel than smaller fragments and will not travel as fast (Calladine et al. 2004).

By using a DNA ladders that contains bands in known size the fragments can be compared with these to indicate fragment size. The ladders used here are shown in Figure 14.

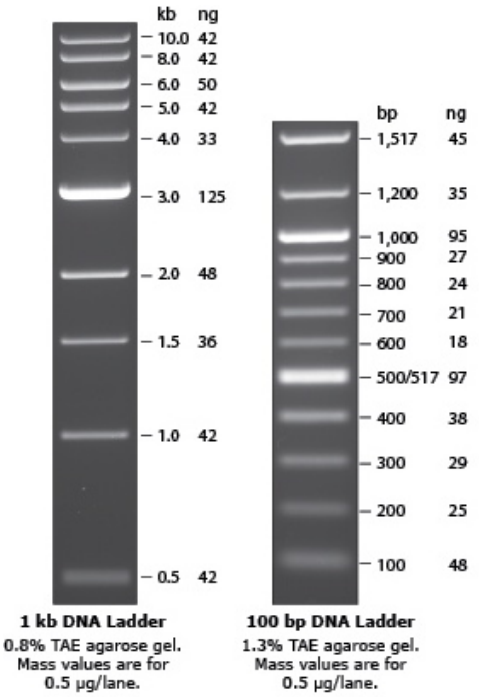

Figure 14: DNA ladders used for identification of fragment size

The gel contains Ethidium Bromide, a carcinogen that binds to the DNA between nucleotide pairs and will illuminate under an UV light, making the DNA on the gel visible (Karcher 1995).

Agarose gel electrophoresis

Gels containing 1% agarose (1g/100 ml) were prepared by adding agarose to 1xTBE buffer and then heated in a microwave until the agarose was completely dissolved. The solution was cooled to about 60°C before 0.07% Ethidium Bromide was added (1 drop/50 ml) and mixed. The gel solution was then poured into a prepared gel mold with a loading comb and left to set. Loading buffer was added to the samples with 1:5 ratio and mixed before being loaded to the gel wells. For reference, 3 µl 100 bp and 1 kb were loaded to the outer wells. The gels were then run at 80-100V and 400mA for approximately 40-60 minutes. The gels were visualized and photographed under an UV light using the Gel Doc system (Bio Rad) and Quantity One 1-D software (Bio Rad).

3.5 Protein analysis

To investigate the protein accumulation of the expressed dengue antigens, protein analysis was conducted. This was done by extracting soluble protein from plant tissue and analyzing these with polyacrylamide gel electrophoresis and Western blotting.

3.5.1 Extraction of soluble protein from plant tissue

Extraction buffer:

1 ml	1M NaCl
200 µl	0.5M EDTA
2 ml	1M Tris-HCl pH 8
5 µl	Tween20
50 µl	20% SDS
2 ml	1M sucrose
100 µl	1M DTT
4.645 ml	dH_2O
10 ml	Total volume

N. benthamiana tissue was ground in liquid N_2 and homogenized with a pestle in 100 µl extraction buffer. The samples were kept on ice to avoid degradation of proteins and vortexed periodically during homogenization. The samples were centrifuged for 3 minutes at 4°C and 24,000 x g. The pellet was then rehomogenised in the supernatant. This process was repeated until the supernatant had a strong green color after centrifugation. The samples were then spun for 10 minutes at 4°C and 24,000 x g. The supernatant, now containing the soluble protein, was pipetted over in a new eppendorf tube and heated for 5 minutes at 95°C. If a precipitate was formed, SDS up to 2% was added. The samples were dispensed in aliquots with 30 µl required for Western blotting and 10 µl for coomassie staining. The replicates were combined in equal volumes in the aliquots for gel application. Aliquoting was done to avoid multiple freeze-thaw cycles, which would degrade protein each time.

3.5.2 Polyacrylamide gel electrophoresis (SDS-Page) and Western blotting

SDS-Page is another form of gel electrophoresis with the same principle as agarose gel electrophoresis; the proteins are separated due to size. Acrylamide is the basis for this gel and is highly toxic in its original form. Acrylamide is polymerized by adding APS and TEMED to form polyacrylamide. Polyacrylamide crosslink and form evenly sized pores for the proteins to pass through. The percentage of acrylamide can be adjusted to suit the protein size with higher percentages for smaller proteins and vice versa (Rosenberg 2005).

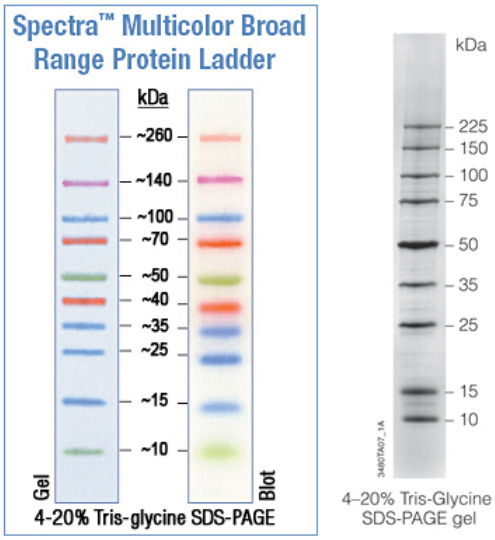

Figure 14: Protein ladders from Fermentas and Promega. The Spectra Multicolor ladder (Fermentas) is a pre-stain ladder is therefore visible on the gel without detection.

To identify protein size, ladders with bands of known sizes were used for reference. Both ladders are broad range protein ladders, but the ladder from Fermentas is visible on Western blot membranes without binding antibodies or detection solutions.

Polyacrylamide gel

12% Resolving gel

	Reagents	Final concentrations
2.1975 ml	dH_2O	
1.25 ml	1.5M Tris-HCl pH 8.8	375 mM
25 µl	20% SDS	0.1 %
1.5 ml	40% Acrylamide	12 %
25 µl	10% APS	0.05 %
2.5 µl	TEMED	
5 ml	Total volume	

4% Stacking gel

	Reagents	Final concentration
3.225 ml	dH_2O	
1.25 ml	0.5M Tris-HCl pH 6.8	≈ 375 mM
25 µl	20% SDS	≈ 0.1 %
0.5 ml	40% Acrylamide	4 %
25 µl	10% APS	≈ 0.05 %
5 µl	TEMED	
5.03 ml	Total volume	

The materials needed for casting the gel were prepared before making the gel solutions. The glass plates were cleaned with 96% ethanol and tissue paper (dust free) before placing them in the gel casting apparatus in the fume hood and making sure they were sealed. The solutions for the 12% resolving gel were mixed together, but acrylamide, APS and TEMED were added in the fume hood. The gel solution was pipetted between the glass plates, leaving space for the stacking gel. The top of the resolving gel was protected by adding 1 ml 2-propanol to even the surface and prevent contact with oxygen. The gel was left to set for about 30-40 minutes. The top of the gel was cleaned with dH_2O and excess water removed with tissue paper.

Back in the fume hood, the gel solution for the 4% stacking gel was added on top of the resolving gel and a gel comb inserted tightly to prevent contact with oxygen. The gel was left to set for about 20-30 minutes and the gel comb was removed when the gel started to recede from the comb. It was important to be observant of this so the gel would not dry out. If the gels were not used immediately, they were wrapped in wet paper and plastic and stored at 4 °C.

Polyacrylamide gel electrophoresis (SDS-Page)

Prepared gels were placed in an electrode assembly, making sure it was sealed and placed in a gel tank. The electrode assembly formed an inner chamber, which was filled with 1X running buffer. Samples were prepared by adding 10 µl 4X loading buffer and heated at 95°C for 4 minutes, before adding them to the gel. For size reference, 10 µl of Spectra™ Multicolor Broad Range Protein Ladder (10-260 kDa) was added to the outer well. Running buffer (1X) was filled in the gel tank, covering the lower part of the inner chamber. The gel was first run at 66V for about 30 minutes, until the loaded samples had reached the resolving gel and aligned. The voltage was then turned up to 120V and the gels were left running for another 30-40 minutes.

3.5.3 Western Blotting

Western blotting is a process for detection of specific antigens. The proteins in the SDS gel are transferred to a nitrocellulose membrane. This membrane is first blocked with a milk solution to avoid unspecific binding of the antibodies to the membrane and eliminate background noise (Rosenberg 2005). After blocking the membrane is treated with a primary antibody. This antibody binds to antigens transferred to the membrane. The primary antibody is chosen to be specific for the antigens to be examined. All excess antibody is washed off before addition of the secondary antibody. The secondary antibody binds to the primary and is specific to the animal the primary antibody was developed in. In this case the secondary antibody is anti-rabbit. Alkaline phosphatase binds to the secondary antibody and is necessary for the detection step, as the detection solution reacts with this and will visualize the antigens transferred to the membrane.

Protein transfer

For the blotting process the iBlot® Device and iBlot® Transfer Stacks from Invitrogen were used. First the anode stack was placed in the device. After running the gels they were removed from the glass plates and the stacking gel cut away. They were then placed on to the anode stack. The iBlot filter paper was soaked in dH_2O and placed on top of the gels. The blotting roller was then used to remove air bubbles. The cathode stack was then placed on top of the filter paper and the blotting roller used to remove air bubbles. Lastly the sponge was placed in the in lid of the iBlot Device. For my blotting purposes, program P3 (6 minutes) was chosen.

Blocking

After the blotting process the sponge, cathode and filter paper were removed. A scalpel was used to cut around the gels into the top layer of the anode stack, the membrane. The gels were discarded and the membrane placed in a petri dish with 2.5-7% skimmed milk in TTBS. The membrane was left blocking for a minimum of 30 minutes in room temperature.

Primary antibody binding

The blocking solution was poured off the membrane and primary antibody, diluted to 400X in TTBS with skimmed milk (2.5-7%), added to the membrane. This was left to incubate with shaking in room temperature for 2 hours. After incubation, the primary antibody solution was poured off and the membrane washed with TTBS. The membrane was washed 6 times, first after 2 minutes, and then every 5 minutes. It was made sure that between every washing step, most of TTBS was poured off.

Secondary antibody binding

The secondary antibody diluted in TTBS with skimmed milk (2.5-7%) was added, in this case this was anti-rabbit diluted 30,000X. The membrane was incubated with shaking in room temperature for 1 hour. The membrane was washed 6 times, first after 2 minutes, and then every 5 minutes. It was made sure that between every washing step, most of TTBS was poured off.

Detection

After incubation with the secondary antibody, streptavidin-conjugated alkaline phosphatase diluted 40,000X in TTBS was added to the membrane. This was left to incubate in room temperature for 2 hours. The membrane was washed 6 times, first after 2 minutes, and then every 5 minutes. It was made sure that between every washing step, most of TTBS was poured off.

For detection, the kit AP Conjugation Detection Kit (Bio-Rad) was used. The two solutions in the kit, A and B were diluted 100X in 1X AP buffer and added to the membrane. The detection solution was left on the membranes until bands were visual and at the desired strength.

3.5.4 Coomassie staining

After gel electrophoresis, the glass plates were separated from the SDS gel and stacking gel removed. The gel was placed in a container and covered with staining buffer. The stain contains coomassie, a substance that binds to proteins but not to the gel (Rosenberg 2005). The gel was left to soak for 2-3 hours. After the soaking period, staining buffer was poured off and destaining buffer added. A piece of paper tissue was also placed with the gel in the container to soak up the remaining stain as coomassie will temporarily stain the SDS gel. To remove the excess stain, a destain step is needed. The excess dye will go out of the gel to the destain solution in an effort to obtain equilibrium. By addition of a paper tissue, the stain will be absorbed by it and equilibrium cannot be obtained, so more dye diffuses from the gel. The destaining buffer was left on the gels until they were desirably destained, about 7-8 hours.

Chapter 4 Results

4.1 Agroinfiltration of *Agrobacterium tumefaciens* in *Nicotiana benthamiana.*

N. benthamiana plants were grown in the greenhouse and were infiltrated with *A. tumefaciens* when they were between 6-8 weeks of age. Plants were also infiltrated with only the infiltration buffer as a negative control. *A. tumefaciens* was transformed with the following pEAQ vectors: pEAQ-HT-GFP (positive control), Dest1/denv2 HFBI, Dest1/SP denv2 HFBI, Dest1/GFP denv2, Dest1/SP GFP denv2. The constructs for infiltration contained fusion proteins for increased solubility, stability and yield. SP was used for retention in the ER for protein protection.

For this experiment, five time intervals were planned, at 0, 3, 6, 9 and 12 days post inoculation/infiltration (dpi). Leaves harvested straight after infiltration represents 0 dpi. To avoid contamination, the negative control was the first infiltration while the positive control was the last. In addition, gloves were changed and workbench was cleaned between each infiltration.

4.1.1 Morphological investigations

The leaves were inspected to evaluate any morphological changes. One leaf was then collected from each plant, at each of the harvest days, and photographed for documentation.

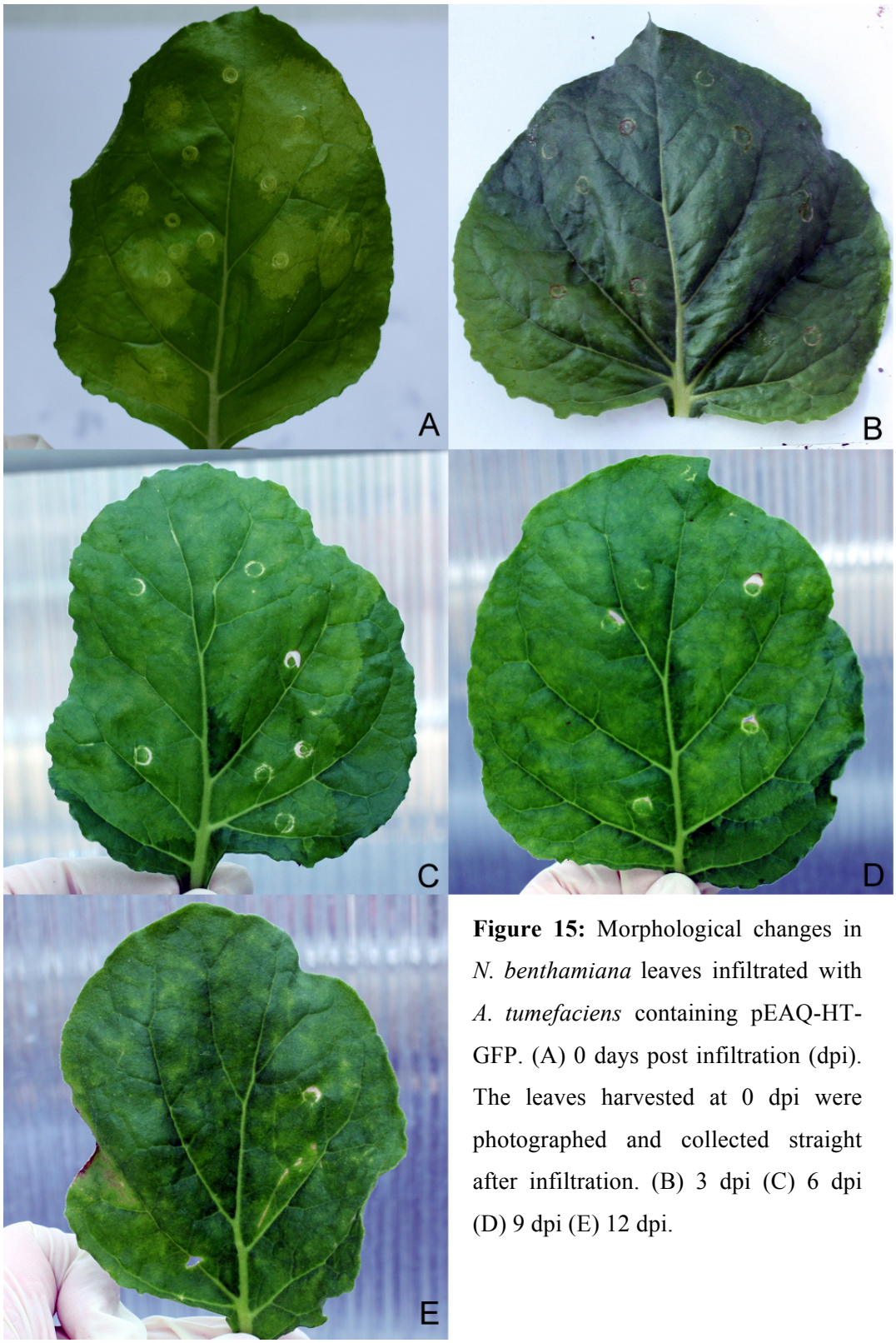

Figure 15: Morphological changes in *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing pEAQ-HT-GFP. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 3 dpi (C) 6 dpi (D) 9 dpi (E) 12 dpi.

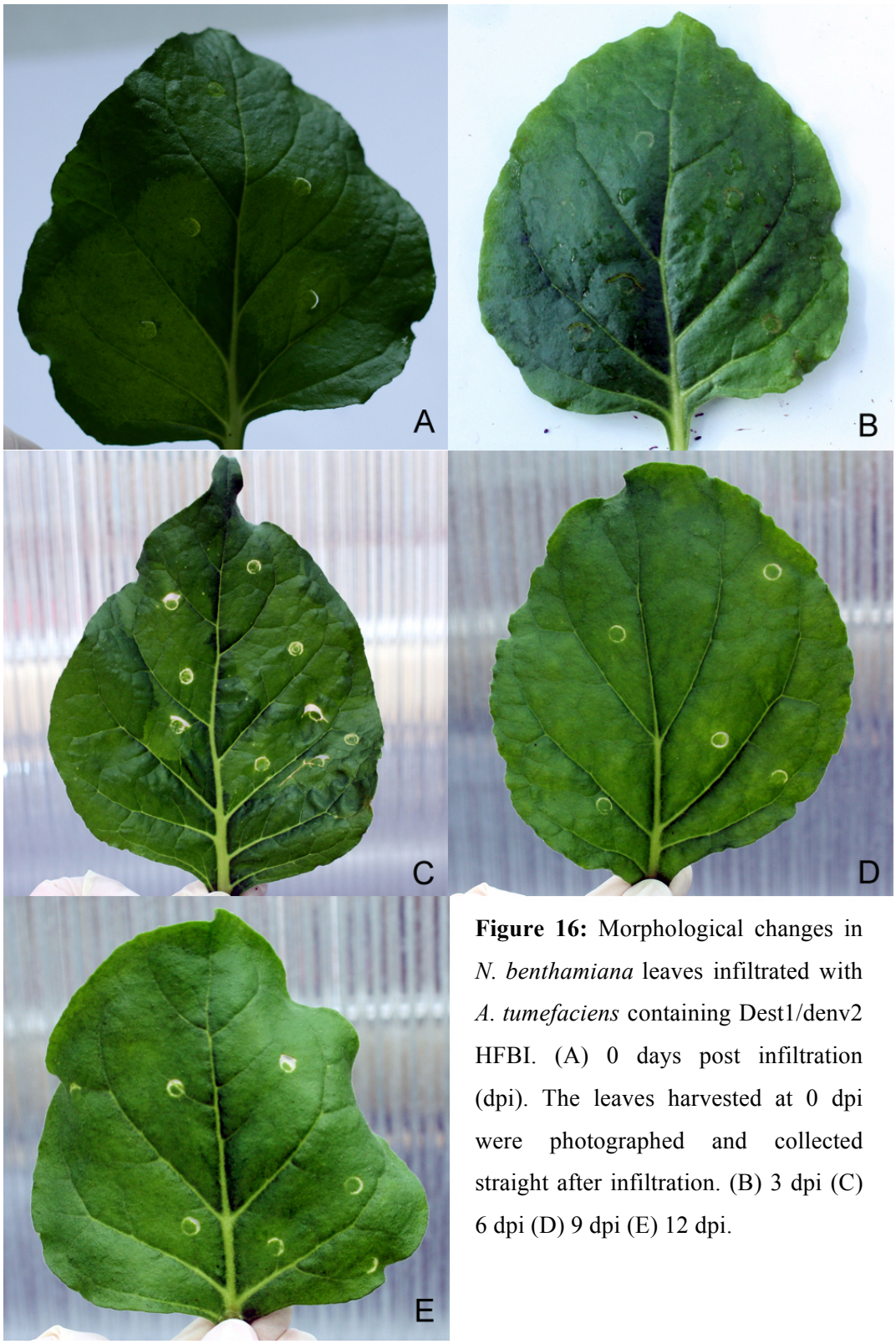

Figure 16: Morphological changes in *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing Dest1/denv2 HFBI. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 3 dpi (C) 6 dpi (D) 9 dpi (E) 12 dpi.

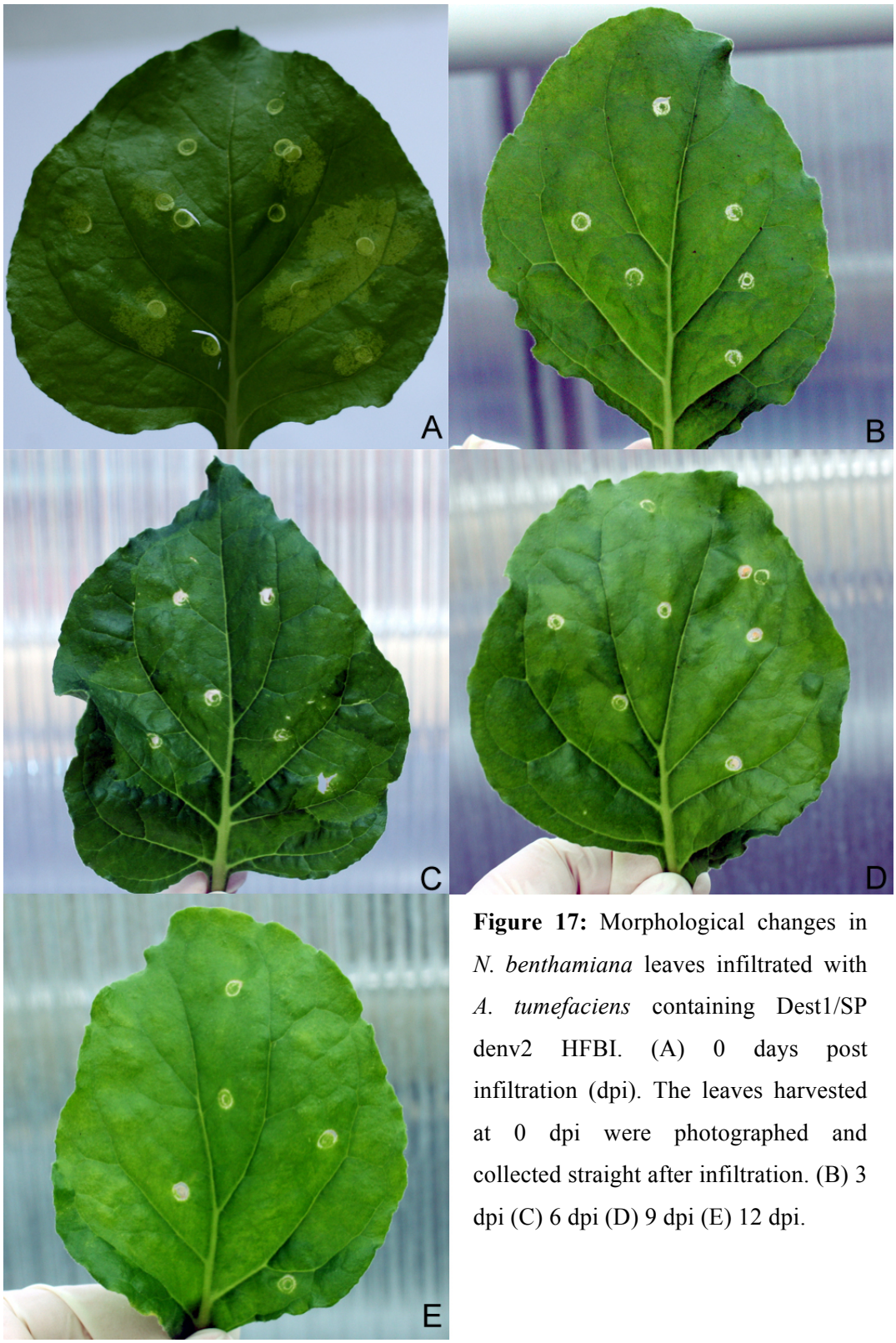

Figure 17: Morphological changes in *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing Dest1/SP denv2 HFBI. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 3 dpi (C) 6 dpi (D) 9 dpi (E) 12 dpi.

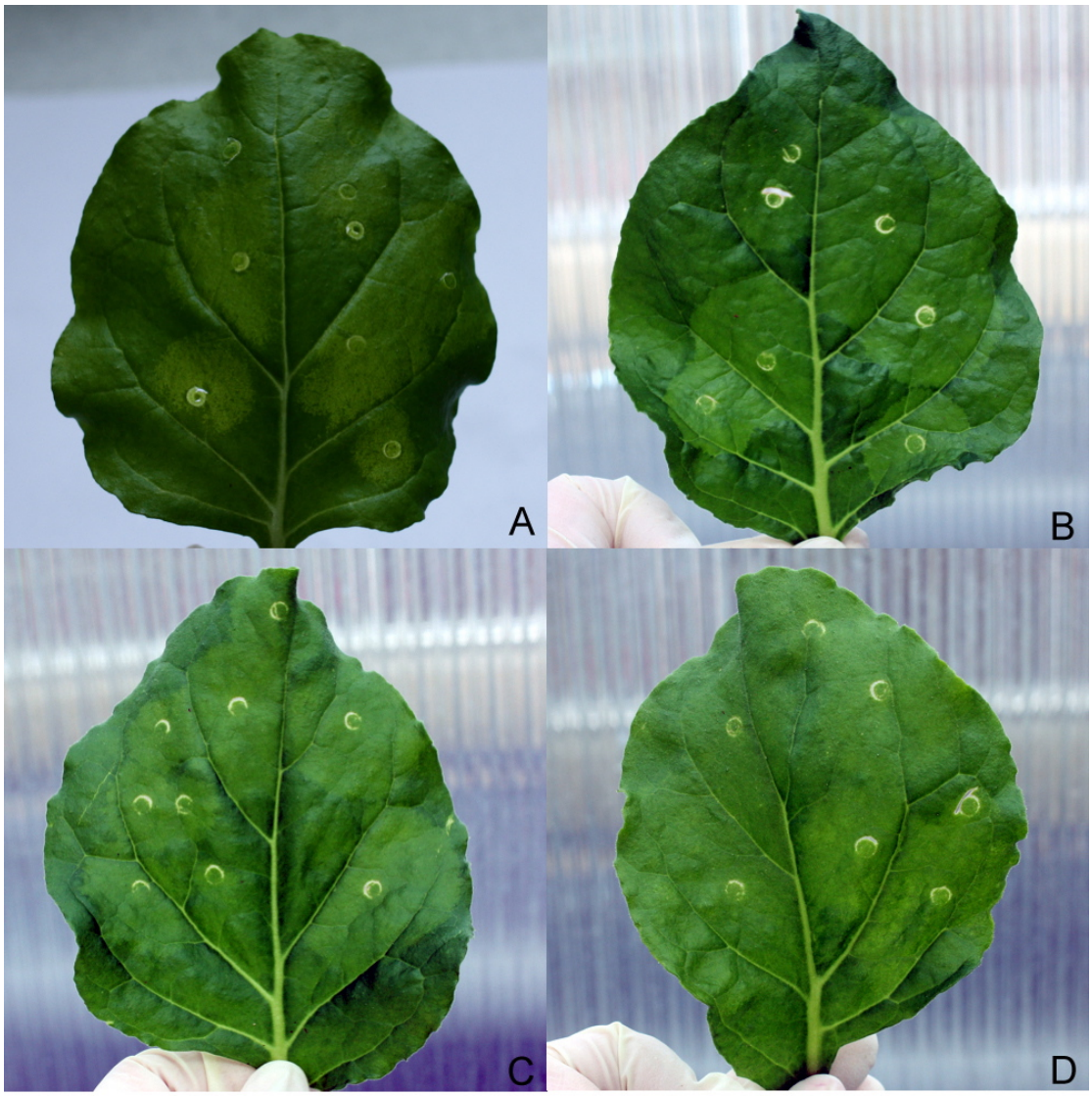

Figure 18: Morphological changes in *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing Dest1/GFP denv2. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 6 dpi (C) 9 dpi (D) 12 dpi

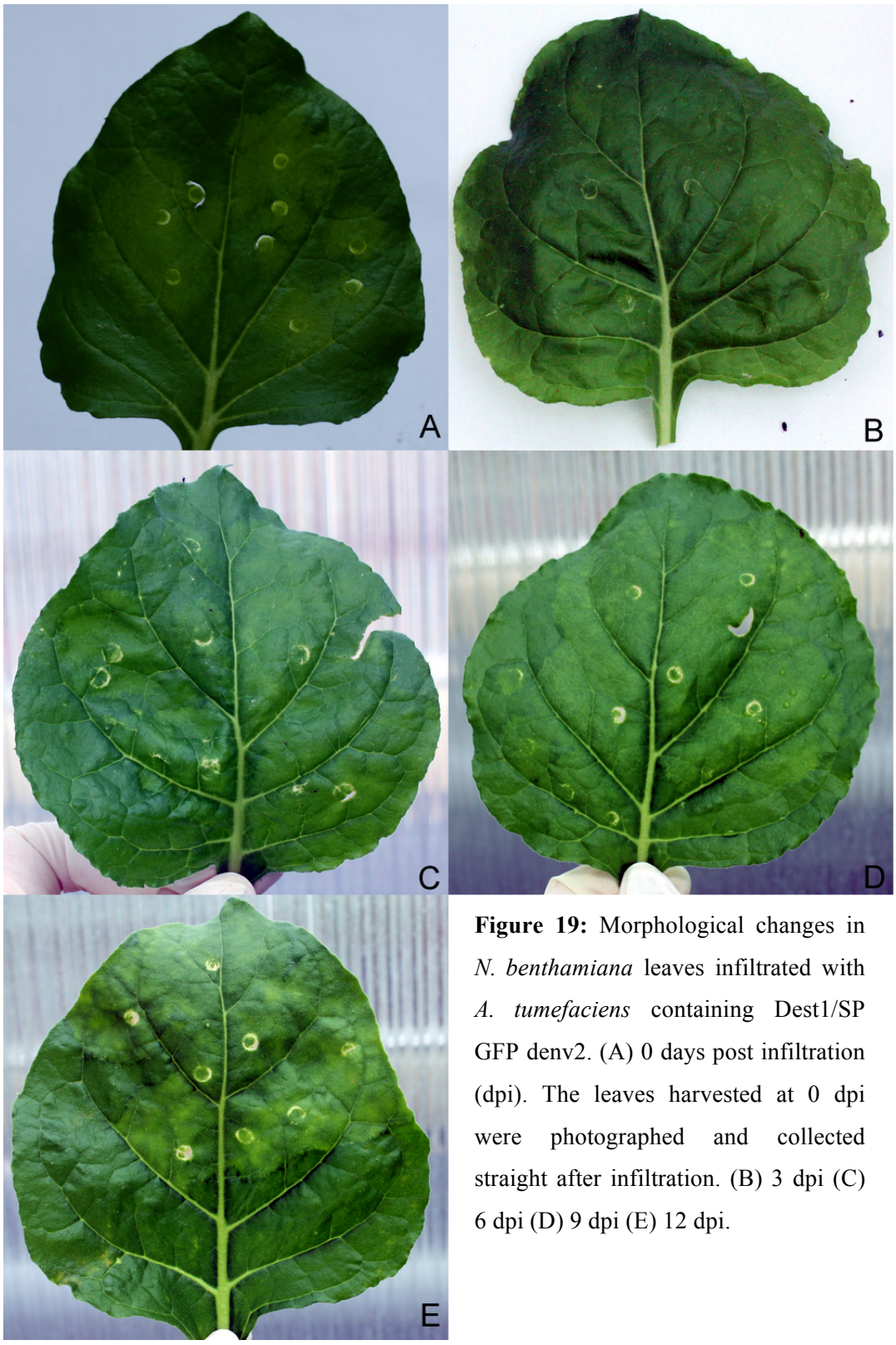

Figure 19: Morphological changes in *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing Dest1/SP GFP denv2. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 3 dpi (C) 6 dpi (D) 9 dpi (E) 12 dpi.

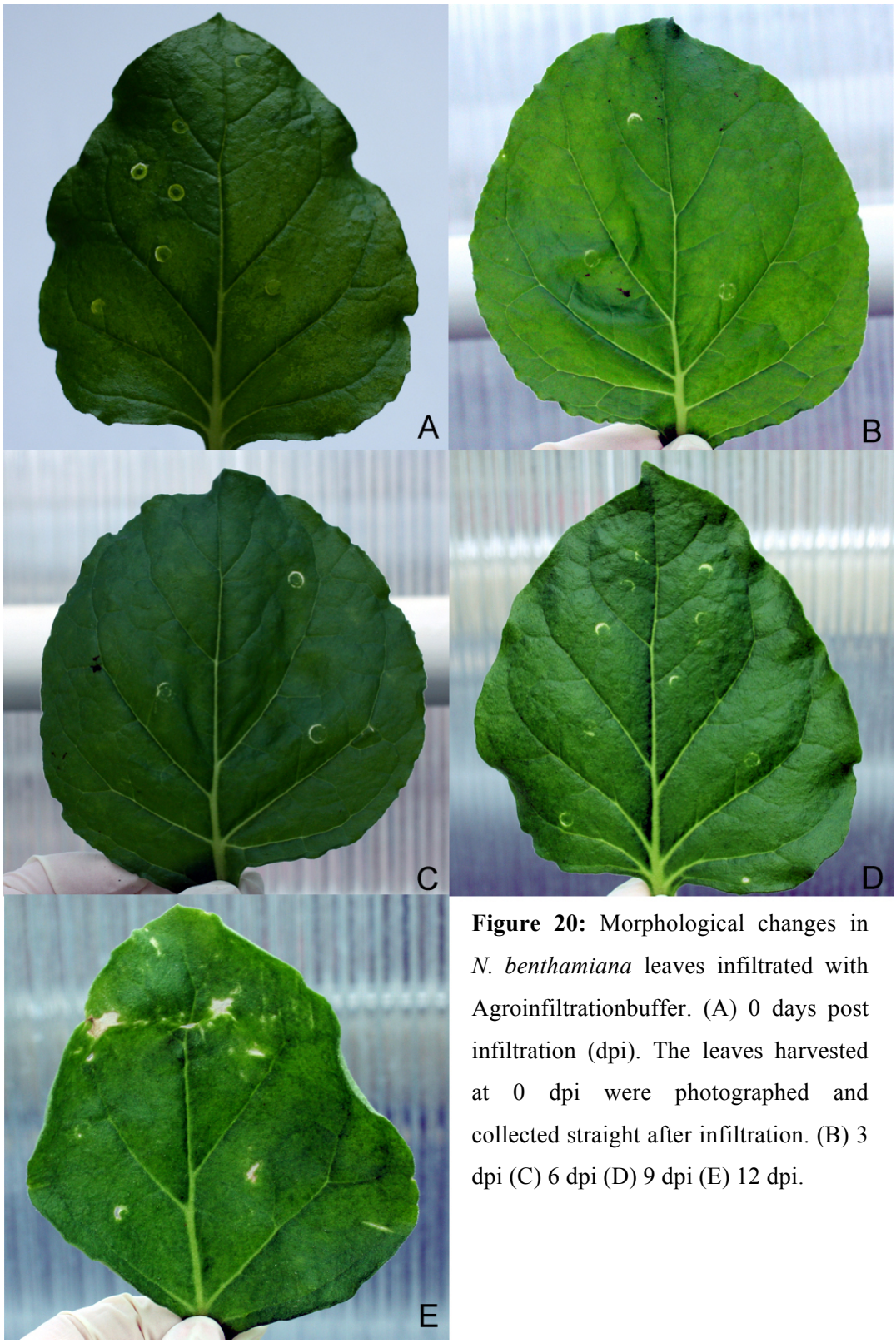

Figure 20: Morphological changes in *N. benthamiana* leaves infiltrated with Agroinfiltrationbuffer. (A) 0 days post infiltration (dpi). The leaves harvested at 0 dpi were photographed and collected straight after infiltration. (B) 3 dpi (C) 6 dpi (D) 9 dpi (E) 12 dpi.

No morphological changes could be observed immediately upon infiltration. Infiltration fluid could only be seen in the leaves when using back lighting as seen in Figure 15-20. At 3 dpi the leaves all had necrosis at the injection sites, but no other morphological change was

visible. As seen in C in Figure 15, 16, 17 and 19, and B in Figure 18, all leaves had clear lighter green areas that originated from the injection sites at 6 dpi. Figure 15 and 17 show that these areas are most pronounced with the positive control and the SP denv2 HFBI construct. No such area was visible with the negative control. At 9 dpi the light areas had spread further throughout the leaves and covered most of the leaf area. For the positive control and the denv2 HFBI construct, the whole leaf was covered. Again, these observations were not made with the negative control. At 12 dpi the color difference in the leaves was less pronounced, and the leaves did become coarser and somewhat rubbery. Overall the morphological changes were most visible at 6 and 9 dpi, as seen in Figure 15-20, for all infiltrations except the negative control.

4.1.2 Western blot and coomassie staining of soluble protein isolated from infiltrated *N. benthamiana* leaves.

The harvested infiltrated *N. benthamiana* leaves were frozen in liquid nitrogen and stored at -80 °C. They were pulverized using liquid nitrogen and the soluble protein extracted using the protocol described in the methods chapter. The samples were added to a SDS gel with the Spectra™ Multicolor Broad Range Protein Ladder and run by gel electrophoresis. A sample from wild type *N. benthamiana* was added to the gels for comparison and the gels were later blotted using the Western protocol described in Methods.

For the positive control (Figure 21A) anti-GFP was used as a primary antibody. The rest of the samples were probed with anti-dengue as a primary antibody.

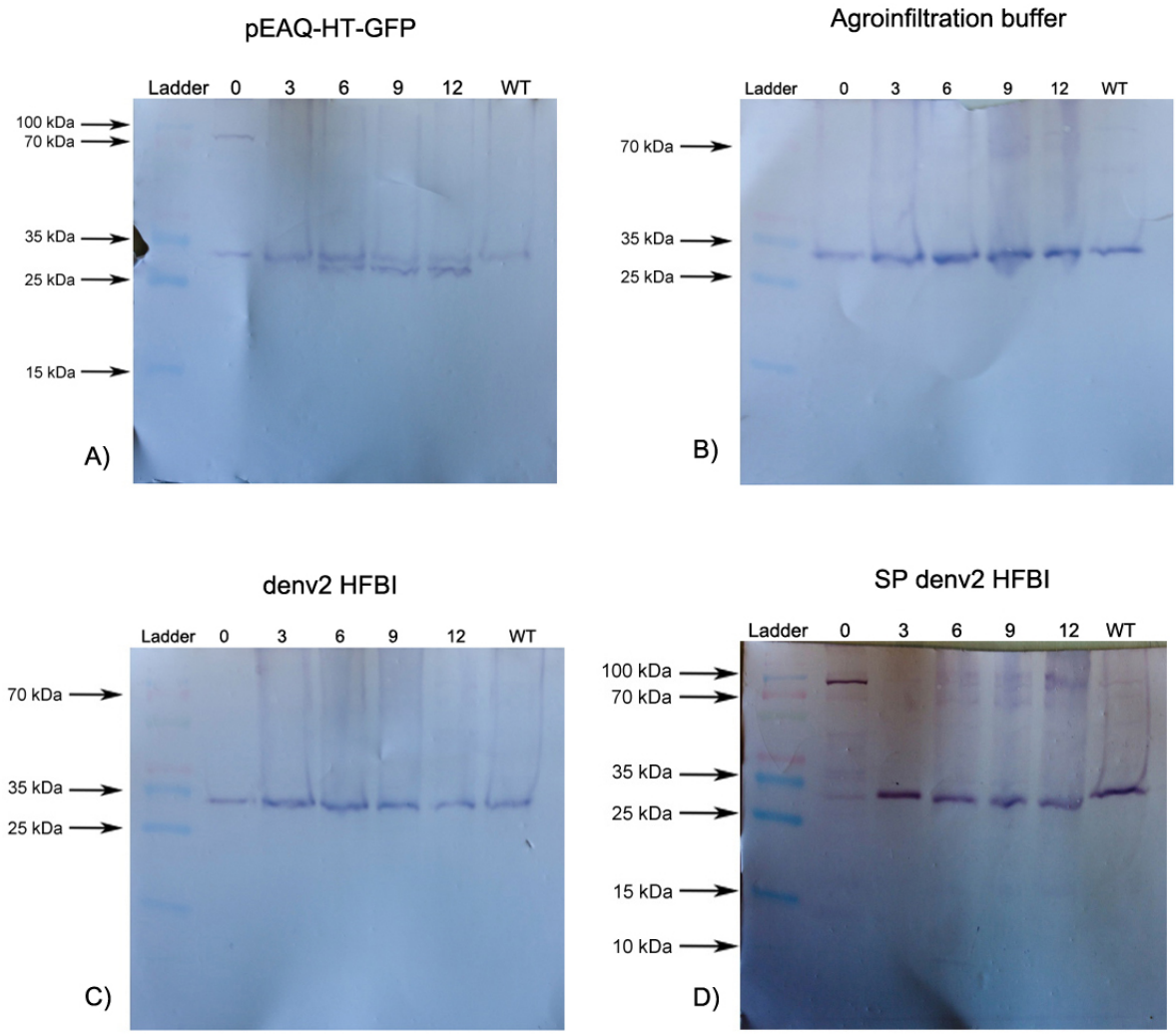

Figure 21: Western blot of extracted soluble protein from infiltrated *N. benthamiana* leaves with *A. tumefaciens* containing pEAQ-HT-GFP (A), agroinfiltration buffer (B), denv2 HFBI (C) and SP denv2 HFBI (D). The membranes were probed with anti-GFP (A) or anti-dengue (B, C, D) antibodies and the Spectra™ Multicolor Broad Range Protein Ladder was used for size comparison. Proteins were extracted from leaves harvested at 0, 3, 6, 9 and 12 days post infiltration (dpi). Lane WT contains protein extracted from uninfiltrated wild type *N. benthamiana* leaves.

Figure 21 shows the Western blot of extracted soluble protein from infiltrated *N. benthamiana* leaves. In samples extracted from pEAQ-HT-GFP infiltrated leaves (Figure 21A) a band at about 27-30 kDa can be seen for the samples harvested at 6, 9, 12 dpi. It is not visible in the sample containing the soluble proteins extracted from wild type *N. benthamiana*, or in any of the other Western blots. The estimated protein size for GFP is about 27 kDa and this band is probably the GFP expression.

The Western blot for SP denv2 HFBI samples (Figure 17D) contains bands at 16-18 kDa and in samples extracted from leaves harvested at 0, 3, 6, 9 and 12 dpi. This band is not visible in the wild type sample. This could indicate the expression of SP denv2 HFBI, but the estimated mass is approximately 23 kDa, 7-5 kDa larger than the visible band.

The Western blot for infiltrated *N. benthamiana* leaves with Dest1/denv2 HFBI (Figure 21C), shows only a single band in all the lanes of about 30-34 kDa. This is the same band that is visible in all the Western blots (Figure 21A-D). It is also seen in the samples with proteins from wild type tobacco and is most likely an unspecific band. There is no band indicating the presence of the denv2 HFBI protein.

In Figure 21D several larger bands can be observed in the sample from 0 dpi. One of the bands, about 70-90 kDa is also visible in the 0 dpi sample from pEAQ-HT-GFP infiltrated leaves (Figure 21A). A smaller band (less than 15 kDa), barely visible, can also be seen in Figure 21D. It seems to be present in all the lanes, also in the last lane, which contains the soluble proteins extracted from wild type tobacco.

Unfortunately, the electrodes in one of the gel tanks did not work and therefore no proteins migrated through the gel. The gels with Dest1/GFP den2 and Dest1/SP GFP denv2 were run anew and stained using coomassie.

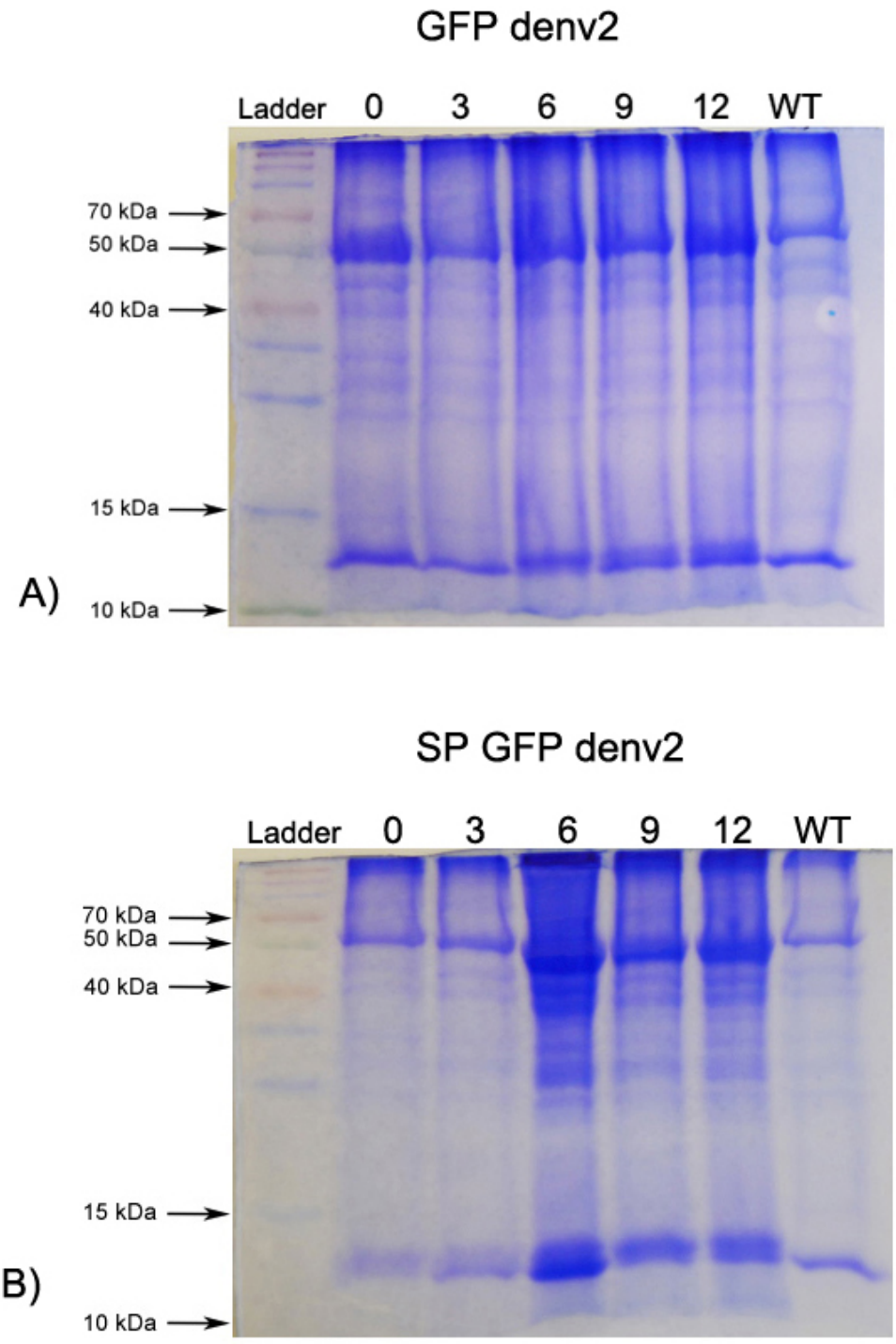

Figure 22: Coomassie stained SDS-Page gel of extracted soluble protein from infiltrated *N. benthamiana* leaves with GFP denv2 (A) and SP GFP denv2 (B). The Spectra™ Multicolor Broad Range Protein Ladder was used for size comparison. Proteins were extracted from leaves harvested 0, 3, 6, 9 and 12 days post infiltration (dpi). Lane WT contains protein extracted from uninfiltrated wild type *N. benthamiana* leaves

Figure 22 shows the stained SDS gel with the extracted soluble protein from leaves infiltrated with GFP denv2 (A) and SP GFP denv2 (B). The estimated protein size for GFP denv2 is about 40 kDa and 43 kDa for SP GFP denv2. However, there does not seem to be any specific bands of that size that are only present in the samples extracted from infiltrated leaves, and that are not present in uninfiltrated leaves in either of the gels.

There does seem to be some bands at about 12-13 kDa that are only present in lanes 6, 9 and 12, giving an appearance of a double band in that area (Figure 22A). The same band is visible on the gel with SP GFP denv2 samples, and in lanes containing proteins from 3, 6, 9 and 12 dpi (Figure 22B). This is most likely degraded protein.

4.2 *Agrobacterium* mediated nuclear transformation of *Nicotiana tabacum*

A pSRN vector was constructed with EDIII1 and EDIII3 genes separately and transformed to *E. coli*. Positive colonies were detected using colony PCR and from two positive colonies the plasmid was extracted and transformed to *A. tumefaciens*. *Agrobacterium* colonies were screened with PCR and positives were used for tobacco transformation.

Tobacco plants of the species *Nicotiana tabacum* were transformed using *A. tumefaciens*. Explants were co-cultured with *A. tumefaciens* suspension for 10 min with gentle shaking and subsequently incubated in the dark for 3 days on the medium without antibiotic selection. The explants were then transferred to new media with kanamycin (100 µg/ml) and incubated in a light room. The explants were transferred to new media every 10 days. When sprouts formed, these where cut and placed in Magenta boxes.

4.2.1 PCR screening of possible nuclear transformants

Samples of all the explants were collected and genomic DNA extracted. Specific primers were designed using Primer3Plus to amplify small areas in the gene enabling shorter PCR programs. The primers were also specified to give PCR products of 200 bp or larger. This was to avoid confusion with primer dimers.

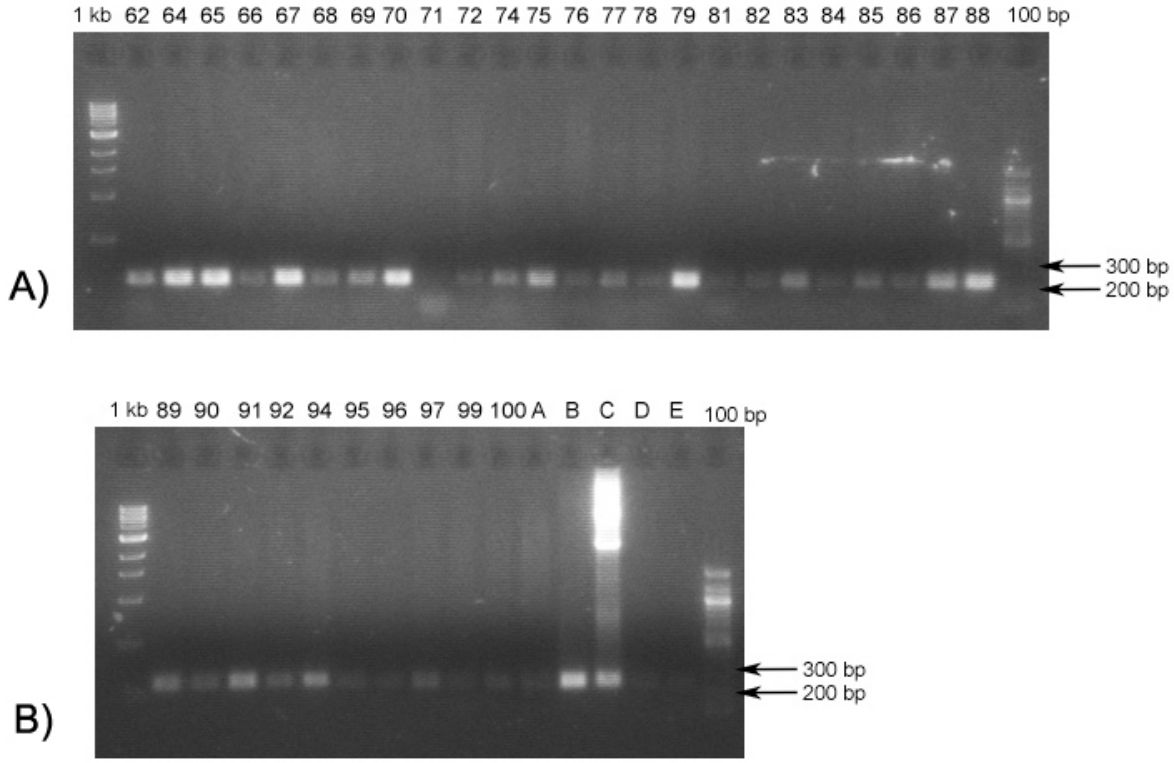

Figure 23: PCR screening of transformants of EDIII1. Genomic DNA was isolated from the plants and screened using the gene specific primers EDIII1 Scr Forward and EDIII1 Scr Reverse. Positive transformants would be identified by a product of 223 bp. Samples 62-100 were run on the same agarose gel, so the controls in gel B are applicable to gel A. The controls in lane A to E contain PCR of genomic DNA extracted from wild type *N. tabacum* (A), pSRN-EDIII1 (B), PCR of pUC57-EDIII1 (C), pSRN-TPSS-T7 (D) and H_2O (E).

Figure 23 shows the transgenic plants screened for the EDIII1 gene with PCR. As seen in the gels, many of the transgenic plants (marked by number) have PCR products of approximately 223 bp, indicating that they are positive. The samples 62-100 (Figure 23A and B) were analyzed together with a single PCR reaction, and run on the same gel, but on different rows. The controls shown in Figure 23B are also applicable as controls to samples in 23A. Unfortunately, the positive control in Figure 23B and C was overexposed due to overload of plasmid DNA

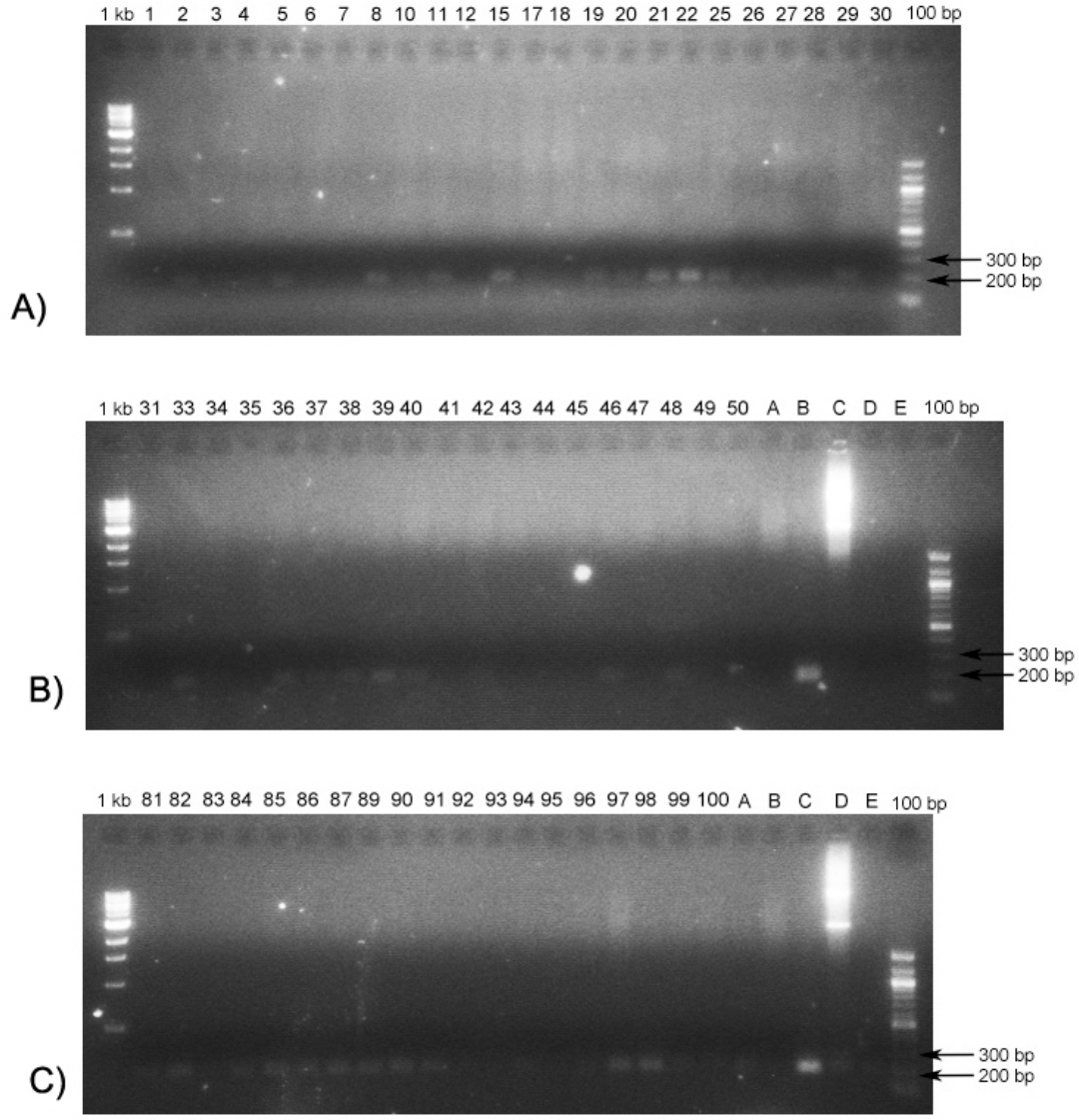

Figure 24: PCR screening of transformants of EDIII3. Genomic DNA was isolated from the plants and screened using the gene specific primers EDIII3 Scr Forward and EDIII3 Scr Reverse. Positive transformants would be identified by a product of 200 bp. Samples 1-50 were run on the same agarose gel, so the controls in gel B are applicable to gel A. The controls in lane A to E contain PCR of genomic DNA extracted from wild type *N. tabacum* (A), pSRN-EDIII3 (B), PCR of pUC57-EDIII3 (C), pSRN-TPSS-T7 (D) and H_2O (E).

Figure 24 shows the transgenic plants screened for the EDIII3 gene with PCR. As seen in the gels, some of the transgenic plants (marked by number) have PCR products of approximately 200 bp, indicating that they are positive. The samples 1-50 (Figure 24A and B) were analyzed together with a single PCR reaction, and run on the same gel, but on different rows. The controls shown in Figure 24B are also applicable as a control to samples in 24A.

Unfortunately, the positive control in Figure 24B and C was overexposed due to overload of plasmid DNA.

The plants that were grown on soil for ethanol induction analysis were number 70, 78 and 89 for pSRN-EDIII1 and 11, 25 and 96 for pSRN-EDIII3. All these plants were confirmed to be transformants by PCR (Figure 23 and 24).

4.2.2 Calculating transformation efficiency

The transformation efficiency was calculated be dividing the number of positive transformants to the total plants screened.

$$\frac{Positive\ transformants}{Plants\ screened}\ x\ 100$$

Due to that many plants died or were subject to infection, no clear statistically data can be made from this, but the calculations seen in Table 12 give an indication. As seen in the table, explants transformed by *Agrobacterium* containing the pSRN-EDIII1 vector were more successful than those transformed with pSRN-EDIII 3.

Table 12: Overview of transformed, screened and positive plants. The table also contains calculated transformation efficiency.

	pSRN-EDIII 1	pSRN-EDIII 3
Plants transformed	100	100
Plants screened	74	86
Positive transformants	70	48
Transformation efficiency	94.6 %	55.8 %

4.2.3 SDS page and coomassie staining of ethanol induced positive pSRN-EDIII 1 and pSRN-EDIII 3 transformants.

When the plants had grown roots and were of appropriate size, about 5-10 cm in height, they were transferred to soil and kept in a greenhouse. 10 plants of each gene that were positive were transferred to soil. Unfortunately, many plants died while on soil, and in the end only 3

plants of each construct were used further in ethanol induction. In addition to these plants, a plant transformed by pSRN-TPSS-T7 and a wild type *N. tabacum* were used as controls.

The plants were sprayed with 5% ethanol and leaves harvested at 0, 1, 4 and 7 days after the induction. Leaves collected before ethanol induction represented day 0. Soluble protein was extracted from the leaves and the samples were run on a SDS gel before being stained by coomassie.

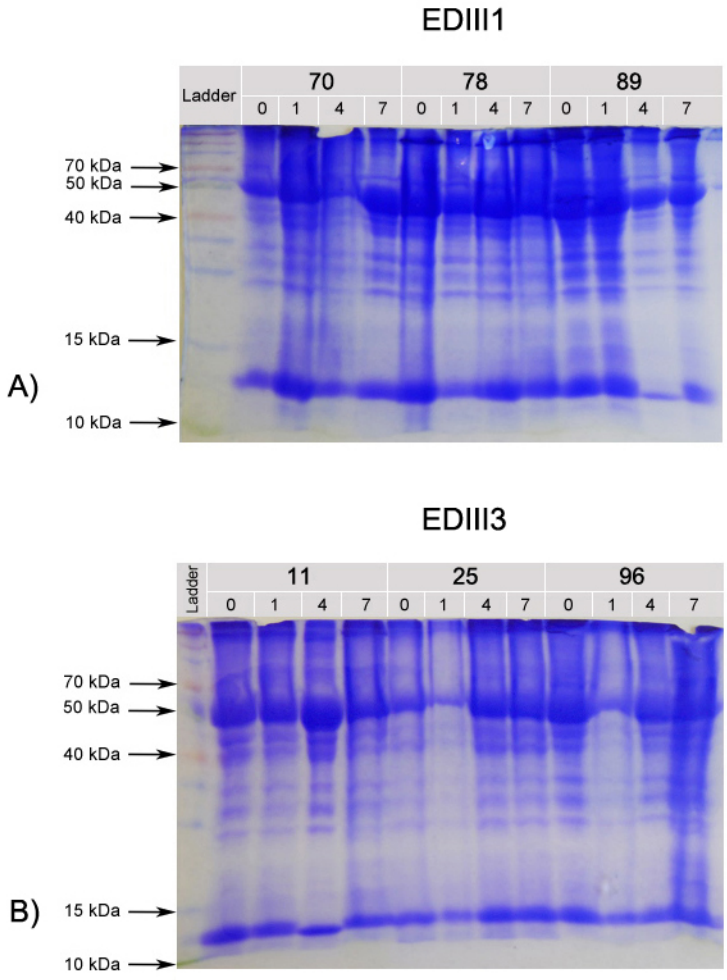

Figure 25: Coomassie stained SDS-Page gels of protein extracted from ethanol induced pSRN-EDIII1 and EDIII3 transformants. The transformants induced by ethanol were number 70, 78 and 89 (EDIII1) and 11, 25, 96 (EDIII3). Leaves were harvested 0, 1, 4 and 7 days post ethanol induction. Leaves harvested at day 0, were collected before ethanol induction. The gel was run with the Spectra™ Multicolor Broad Range Protein Ladder.

Figure 25 shows the coomassie stained SDS gel with soluble protein extracted from plants transformed by pSRN-EDIII1 (A) and pSRN-EDIII3 (B). The estimated size for EDIII1 and EDIII3 is 13 kDa. Due to the poor staining and quality of the gels, and the amount of bands, it is hard to observe any bands of that size that are present at day 1, 4 and 7 and not in day 0 or in the control (Figure 26).

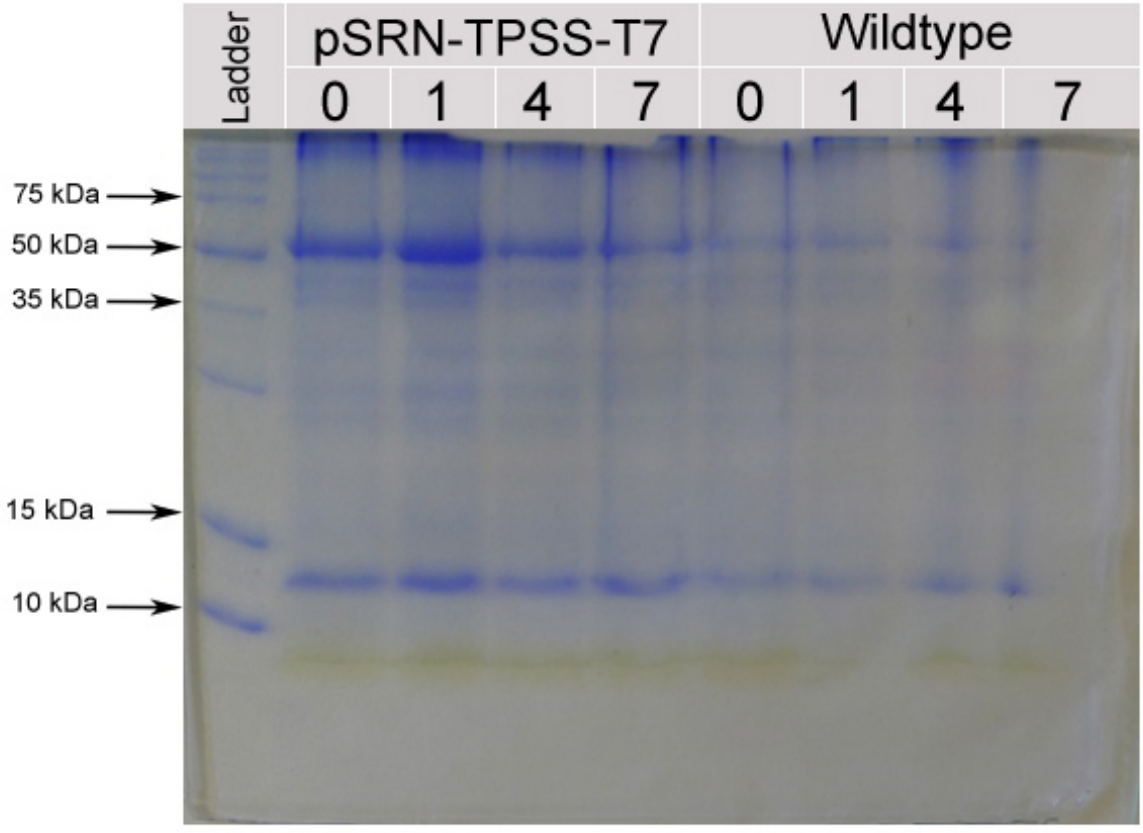

Figure 26: Coomassie stained SDS-Page gel of protein extracted from ethanol induced pSRN-TPSS-T7 transformants and wild type *N. tabacum*. Leaves were harvested 0, 1, 4 and 7 days post ethanol induction. Leaves harvested at day 0, were collected before ethanol induction. The gel was run with the Broad Range Protein Molecular Weight Markers (Promega) as a ladder.

The soluble protein extracted from wild type *N. tabacum* and *N. tabacum* transformed with pSRN-TPSS-T7 run on a gel and coomassie stained is shown in Figure 26.

Comparisons of the SDS gels in Figure 28 and 30 show no discernable bands that would indicate expression of the EDIII1 or the EDIII3 genes.

Chapter 5 Discussion

5.1 The challenge in dengue vaccine development and current status

As mentioned in the introduction, dengue is and increasing global threat that is spreading rapidly and reemerging in several areas. Since there is currently no direct treatment for dengue, an outbreak can put substantial socioeconomic strain on the countries affected and there is great need for a functional dengue vaccine.

Since the 1920s there have been several attempts to develop a dengue vaccine, but so far none have been successful, though a few have reached clinical trials. The possibility to develop a dengue vaccine is not an impossible task as dengue only causes acute infection and persons infected develop a natural, permanent immunity against the specific serotype (Chaturvedi et al. 2005). There are however several factors that hinder the dengue vaccine development such as the lack of an animal model that express dengue disease and the fact that immunity is serotype specific and is only temporarily cross protective. But one of the largest hurdles is the fact that a vaccine needs to be tetravalent as not to cause ADE (Chaturvedi et al. 2005; Guzmán et al. 2004). Another problem is that tetravalent vaccines can experience interference between the serotypes resulting in immunodominance so that immunity is achieved against all serotypes. This can be solved by administering several doses of the vaccine (Lang 2012).

There are currently several projects working towards developing a dengue vaccine. These vaccines can be live attenuated virus, inactivated virus, subunit or DNA vaccines. In 2010 the quest for a dengue vaccine took an important step as a vaccine candidate reached phase III clinical trials, the first dengue vaccine to ever do this. The Sanoti Pasteur CYD tetravalent dengue vaccine is a recombinant live attenuated virus vaccine and is based on a yellow fever vaccine 17D backbone. The vaccine expresses the pre-membrane and envelope genes of each dengue serotype. So far in the clinical trials the vaccine has shown to develop neutralizing antibodies against all serotypes and with the 23,000 individuals vaccinated so far, there have been no safety concerns as of yet. The results from the first phase III efficiency trials are to be known at the end of 2012 and so far the prospects are promising (Lang 2012).

5.2 The start of the art of plant derived vaccines: successes and hurdles

In recent years the interest for creating plant-derived pharmaceuticals have increased drastically. There is a strong demand for low cost vaccines, especially in developing countries where the availability is limited. Producing therapeutic proteins in plants can be used to achieve low cost pharmaceuticals, and plant-derived recombinant proteins now also meet the criteria for current god manufacturing practices (cGMP) for parenteral or mucosal delivery (Pelosi et al. 2012).

The first therapeutic protein to be produced in plants was human growth hormone expressed in transformed tobacco and sunflower callus cultures in 1986. It was not until 1997, when avidin was expressed in transgenic maize, that it was recognized that plants could be used to produce recombinant proteins for commercial use. Since then, many have attempted to produce recombinant protein for therapeutic use in plants with varying degrees of success. The main hurdles for producing recombinant protein in plants have been the limitation of protein yield and non-mammalian glycosylation (Obembe et al. 2011).

There are a number of studies done to express antigens and recombinant proteins in plants. In relation to development of dengue vaccines, consensus EDIII (cEDIII) has been expressed in transgenic callus of rice, and DENV3 polyprotein consisting of capsid, premembrane and envelope protein has been expressed in *Lactuca sativa* cv. Simpson elite lettuce chloroplasts (Kanagaraj et al. 2011; Kim et al. 2012). Another examples is hemagglutinin antigen from low pathogenic avian influenza A that has been transiently expressed in *N. benthamiana* (Kanagarajan et al. 2012).

The examples mentioned above are still at an early stage of development, but in the last decade several important achievements have occurred in the field of plant made pharmaceuticals. The first plant made vaccine to be regulatory approved was the Newcastle disease virus vaccine for chickens produced in tobacco, and it is currently the only licensed plant made vaccine (Pelosi et al. 2012). Other plant made recombinant proteins are undergoing clinical trials or are pending approval. CaroRX is an antibody produced in tobacco preventing tooth decay caused by *Streptococcus mutans.* It has completed phase II clinical trials and it has been approved for use in the EU, though it is not marketed.

Glucocerebrosidase produced by Protalix is a plant produced therapeutic protein to treat Gaucher disease and designed to be administered orally. It is currently pending approval from the Food and Drug administration (FDA) and is expected to be available commercially soon (Xu et al. 2012).

5.3 EDIII antigen as a vaccine candidate and an overview on vaccines developed based on EDIII

EDIII is a potential dengue vaccine candidate as it is both dengue specific and serotype specific. Antibodies bind to all three envelope protein domains, but research with monoclonal antibodies has shown that binding to the EDIII has the highest efficiency in neutralizing virus and block virus absorption (Crill & Roehrig 2001; Crill et al. 2009). EDIII also has low risk in inducing cross-reactive antibodies, thus reducing risk of severe illness during inoculation (Khanam et al. 2006; Simmons et al. 2001). Studies with human immune sera have shown that EDIII binding antibodies are only a small part of the total amount of antibodies present after primary and secondary infection, the main part of antibodies present being whole virus binding, though these are highly cross-reactive (Wahala et al. 2009).

There have been several studies conducted with EDIII as a possible vaccine candidate. A study was conducted on CD46-IFNAR mice that are susceptible to measles virus infection. A vector based on the pediatric measles vaccine, containing the four dengue antigens fused to ectodomain of the membrane M protein, was injected into the mice. After two injections, neutralizing antibodies against all four serotypes had been induced (Mota et al. 2005). In another study BALB/c mice were injected with lapidated consensus dengue virus envelope domain III (LcEDIII), a dengue subunit vaccine, and showed after one dose to have induced neutralizing antibodies for all serotypes. In addition there was a strong memory response after only one injection (Chiang et al. 2011).

More studies have been conducted on monovalent EDIII for specific serotypes to test the functionality of fusions. A study conducted by the Pedro Kourí Institute in Havana, Cuba tested DENV1 and DENV2 EDIII fused to P64k protein from *Neisseria meningitidis* in *Macaca fascicularis*. Both these tests showed good antibody responses when the antigens were paired with Freund adjuvant (Bernard et al. 2008; Hermida et al. 2006). In another study EDIII domain of DENV2 was fused to maltose-binding protein (MBP) from *E. coli* and

administered to BALB/c mice. The mice were later injected with a lethal dose of DENV2 and showed a partial protection against the virus by neutralizing antibody response (Simmons et al. 1998). A recombinant protein containing all four serotypes fused to the MBP was also administered to BALB/c mice as a tetravalent vaccine. The mice developed neutralizing antibodies for all serotypes, but the levels for serotype 4 was significantly lower than with the monovalent antigen (Simmons et al. 2001).

These studies mentioned show that EDIII has potential as a dengue vaccine candidate, and though no EDIII based vaccines have reached clinical trials, some of the newer studies show promise for a tetravalent EDIII vaccine.

5.4 Tobacco plant derived dengue antigens: what have we learnt?

Tobacco is a non-feed/non-food plant that is genetically well studied, easily manipulated and has a high biomass. This makes tobacco an ideal candidate to express and produce recombinant proteins. Tobacco does however contain toxic substances, which are necessary to remove in downstream processing. There are also varieties that can be breed with lower toxicity, but these substances will still be needed to be removed (Xu et al. 2012).

Tobacco can be used to express therapeutic proteins, industrial enzymes and biopolymers, and there is continual development for creating commercially available products (Xu et al. 2012). There are several methods to express recombinant proteins, but in this study the methods used were transient expression and stable nuclear transformation. There are however limited results available due to the time requirements needed for plant growth and necessary analysis.

Transient expression is used for rapid expression of high levels of recombinant proteins. The gene is only temporarily expressed in the plant cell genome, and there are only few instances of nuclear genome integration. Nuclear transformation on the other hand creates transgenic plants with the genes present in all plant tissue, in contrast to transient expression where the recombinant protein is expressed only in areas. This creates a problem due to public concern, as the transgenes are present in pollen and there is a possibility of spreading transgenes to the environment (Xu et al. 2012). A solution is producing transgenic plants that have male sterility (Cho et al. 2001).

This is not a problem with transient expression, but when producing recombinant protein transiently the plants do need to be contained, for example in a greenhouse (Xu et al. 2012). This limits scalability of production and in this instance; nuclear-transformed plants have fewer limits as they can be grown in field crops. Since *Agrobacterium* is often used in transient expression there is a necessity for purification adding to the costs. In most cases, transient expression is used as a method to rapidly study protein expression and folding in plants as well as the effects of fusion proteins. This can save time and costs by doing so before advancing to larger scale expression by nuclear transformation. The main advantage of the transient expression method is the rapid and high yield of recombinant protein and even with its limits in scale, some companies do use this method to produce pharmaceuticals (Xu et al. 2012).

Though both transient expression and nuclear transformation are used today to express recombinant protein, research point to that plastid transformation seems to be the most safe and economical method of production in the long run. Transgenic plants via chloroplasts have the ability for higher yield of product since there are several genomes within one chloroplast in comparison to one nuclear genome per cell. In addition, chloroplasts are maternally inherited and there is no risk of spread in the environment via pollen (Meyers et al. 2010).

5.4.1 Protein analysis

Transient expression

Soluble protein was extracted from leaves infiltrated with constructs harboring denv2 HFBI, SP denv2 HFBI, GFP denv2 and SP GFP denv2, shown in Figure 18, 19, 21 and 22 respectively. Soluble protein extracted from leaves infiltrated with pEAQ-HT-GFP (Figure 21A) and agroinfiltration buffer (Figure 21B), were used as positive and negative controls.

Figure 21A of the Western blot for pEAQ-HT-GFP shows bands corresponding to the estimated mass of GFP of 27 kDa, indicating that extraction and infiltration has most likely been successful as all constructs were infiltrated at the same time and extracted using the same method. This protein seems to be only present in samples from 6, 9 and 12 dpi, further indicating that this is GFP.

In the Western blot of denv2 HFBI (Figure 21C), only a single band is visible in all samples, and in all other Western blots (Figure 21A-D). This is most likely an unspecific band that might have similar epitopes as the target antigens and therefore bind to the antibody. No bands indicating expression of denv2 HFBI can be detected on the blot.

HFBI is as mentioned a class II hydrophobin. Fusions to HFBI have previously been used with insects and fungi to simplify protein extraction by using an aqueous two-phase system (ATPS) (Lahtinen et al. 2008; Linder et al. 2004). HFBI has also been used as a fusion partner to GFP for transient expression in *N. benthamiana*. Data showed that yield of total soluble protein was higher with the GFP-HFBI fusion than with GFP alone and that protein extraction was simplified using a modified version of the ATPS (Joensuu et al. 2010). A different protocol for extraction of proteins was used in this thesis, and not the phosphate buffered saline (PBS) based buffer as used by Joensuu et al. (2010). By using the PBS based buffer it is possible that the extraction of the recombinant proteins might have been more successful and should be attempted at a later occasion.

The Western blot for SP denv2 HFBI (Figure 21D) shows a weak band with an approximate mass of 16-18 kDa. This band is only present in samples extracted from 0, 3, 6, 9 and 12 dpi, and the sample extracted from uninfiltrated leaves does not contain this band. This opens for the possibility that the visible bands are the expressed SP denv2 HFBI fusion genes. However, the estimated mass for this protein is 23 kDa. The relation between protein mass and mobility is not absolute and in some cases proteins can have higher or lower mobility than estimated (Dunker & Rueckert 1969). The assumption is that the proteins in the gel all have equivalent shapes, but that is not always the case. Proteins for SDS gel electrophoresis are prepared by addition of SDS and heating to denature the proteins. SDS is a detergent and acts as denaturing agent, but the binding of SDS to proteins are not constant. Insufficient denaturing of proteins can lead to higher mobility in gels due to a more compact size (Rath et al. 2009).

Other explanations to the lower than estimated molecular mass, could be protease digestion or degradation due to freeze/thaw cycles. However, the samples were only freeze/thawed once, so this was not a reoccurring process, and it is unlikely that it would produce a distinct band (Moore 2009). The sample from 0 dpi also contains the same band. This should not be the case as samples were collected straight after infiltration and no protein would be expressed at

that time. It is possible that this is a contamination from the neighboring well from gel application.

The SP denv2 HFBI construct contains a signal peptide (SP) for direction through the secretory pathway to the endoplasmic reticulum (ER), in addition to a KDEL peptide for ER retention. Fusing proteins to an SP and KDEL has shown to increase protein expression levels noticeably (Fiedler et al. 1997). Research showed that SP and KDEL fusions to dengue envelope glycoprotein gene increased the expression levels in tobacco with 122 % (Martinez et al. 2011). The same increased expression levels was seen with the transient expression of the anti-HIV antibody when fused with SP and KDEL (Sainsbury et al. 2010).

In Figure 21D there are also several large bands visible in the sample extracted from 0 dpi. This is possibly an indication that the concentration of the primary or secondary antibody was too high (Moore 2009).

The samples extracted from leaves infiltrated with GFP denv2 and SP GFP denv2 can be seen in Figure 22. The estimated mass of SP GFP denv2 is 43 kDa, and 40 kDa for GFP denv2. On either of the gels it is not possible to discern any bands of these sizes that are not present in the negative control. To do this one would need a large concentration of protein, as coomassie is a less sensitive method to analyze proteins than Western. An observation is that both these gels contain bands of approximately 13 kDa, visible as a double band and only in lanes containing samples extracted from 6, 9 and 12 dpi in Figure 22A and from 3, 6, 9 and 12 dpi in Figure 22B. It is also possible that the same double band is visible in samples extracted from 0 dpi in Figure 22B, though the clarity of the gel is not optimal. The estimated protein mass for denv2 is 13 kDa, but it's highly unlikely that this band is denv2 expressed without fusions. Due to the large difference between the molecular weights on the gels and the estimated weights it is also highly unlikely that this is the expressed gene retaining its structure. An explanation could be that the band is a part of product degradation, as seen in a study conducted on monoclonal antibodies (Sharp & Doran 2001).

GFP is usually used as a reporter gene, but it is highly stable and can be used as a fusion protein to increase stability. In a study the HIV-1 fusion inhibitor cyanovirin-N (CV-N) was fused to GFP and result showed that the protein yield was increased with this fusion, much to do with GFPs protective effect (Elghabi et al. 2011).

Proteins were not quantified before application and it is therefore not possible to observe any changes or increased protein expression in the blots or the gels. They can however give an indication if the recombinant protein is expressed and Figure 19 shows that further analysis should be done with the SP denv2 HFBI construct, as it seems to possibly express the gene with fusions. The samples with GFP denv2 and SP GFP denv2 constructs should also be analyzed with Western blot to better determine expression and to determine if the ER retention together with the GFP protein has any positive effect.

Nuclear transformation

Soluble protein was extracted from transgenic plants containing EDIII1 and EDIII3 genes shown in Figure 25. The gene expression was induced by ethanol and leaves collected 0, 1, 4 and 7 days post induction. The estimated protein mass is 13 kDa for both EDIII1 and EDIII3, however, no band of this mass can be observed on the gels that are not present in the control, Figure 26, or in day 0 which was collected before induction.

The poor quality of the stained gel might explain some of the reason why it is difficult to discern any specific bands with approximately the correct mass. *Agrobacterium* also inserts the gene randomly into the nuclear genome and it is possible that the gene has been subject to positioning effects. The gene might also be silenced in the plants. In retrospect in is clear that more plants should have been tested with ethanol induction, and an original 10 plants were planed for each construct. The plants proved to grow poor on soil however, and many died, leaving 3 plants for each construct to be analyzed. It was also discovered that the protocol used was incorrect and instead of spraying the plants with ethanol solution once, they should have been sprayed every day for the 7 day period. This probably had an impact on protein expression.

The plants for the control containing only the pSRN-TPSS-T7 vector were induced and analyzed by a fellow master student. The results from this analysis, shown in Figure 26, also shows no sign of expression as it had done previously (Lossl et al. 2005).

Also here the extracted proteins were not quantified before application to the gel. Due to this, it is not possible to say anything conclusive of increased protein expression after ethanol

induction. Also, due to inadequate protocol and amount of tests it is not possible to say that the genes will not be expressed at all. The fellow master student conducted the same test with EDIII4 and Tetra genes and did also not get any conclusive results.

5.5 Future oral vaccines produced in edible crops. Where are we now?

The main goal of this project is to develop a tetravalent vaccine that can be orally administered. There are several advantages to have orally delivered vaccines such as simple and safe administration and reduction of cost. With administering vaccines orally directly with plant tissue there is no need for lengthy processing or purification. Vaccines grown in plants open for the possibility for local production, thus reducing cost and increasing availability.

A problem for recombinant proteins intended to be delivered orally is the gastrointestinal environment. Encapsulating proteins in plant cells offers protection against the gastric fluid and therefore enabling correct uptake by the mucosal surface in the gastrointestinal tract.

In a study cholera toxin B-proinsulin (CTB-Pins) fusion protein was expressed in both lettuce and tobacco chloroplasts. Powdered tobacco leaf material was administered orally to non-obese diabetic (NOD) mice. These mice showed lowered glucose levels in both blood and urine and this was the first report of a therapeutic protein expressed in chloroplasts of edible plants. The total soluble protein expressed in lettuce was however about 6 times lower than in tobacco (Ruhlman et al. 2007). In another study C3H/HeJ hemophilia B mice were orally administered coagulation factor IX fused cholera toxin B, which proved to be effective. This was done to prevent anaphylactic reactions in patients with hemophilia during protein replacement therapy (Verma et al. 2010).

Delivery of vaccines at mucosal surfaces is far more effective than standard needle-based delivery. Most infection agents enter through mucosal surfaces and this is therefore the first line of defense and mucosal immunization is more effectively induced by oral delivery (Neutra & Kozlowski 2006).

In this project both tobacco and lettuce chloroplasts have been transformed with dengue vaccine candidates. The process of plastid transformation is lengthy and demanding and so far little results have been obtained of the antigens.

5.6 Conclusions and future work

EDIII for serotype 1 and 3 were inserted into tobacco nuclear genomes for stable nuclear transformation and leaves were infiltrated with EDIII for serotype 2 to several fusion proteins to improve yield and stability. Fusions to SP and KDEL were also made to target the protein to the ER and for ER retention. From the protein analysis conducted, one of the SP fusions with HFBI is possibly positive, but further analysis would need to be conducted to say anything conclusive. No expressions of the other fusions could be observed, but there is a possibility that the GFP denv2 and SP GFP denv2 constructs were expressed, but have been degraded. This could possibly explain the double band visible in all but the negative control sample.

In this project there has been a lack of proper expression of the antigens so far and the goal with the nuclear stable transformation was to be able to express the antigens so that knowledge of folding and the antigens generally, could be acquired. Transient expression is usually the platform for this, but until now has not had any proper results.

Once the antigens are properly obtained, they can be further studied immunologically to confirm their antigenic properties or if they are cross-reactive. Clinical trials on animals and humans must be conducted to ensure effectiveness and safety and to evaluate the most efficient vaccine conformations. The vaccine must be evaluated if it should be administered as a mixture of the four monovalents, or if it should contain a single peptide made up of the four antigens. As mentioned before, the main goal is to develop an edible vaccine. Tobacco contains toxic substances, but varieties with lower toxicity can be breed. However, the most cost-efficient would be to produce edible crops of lettuce or other edible plants. This reduces the amount of processing needed, which in turn reduces cost. There is no doubt that an orally administered dengue vaccine would benefit those who need it most.

Chapter 6 Literature

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