Expression of candidate dengue antigens in tobacco plants for future production of low-cost dengue vaccine

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Abstract

Dengue virus is a human pathogen transmitted by the mosquito *Aedes Aegypti*, causing a disease ranging from mild flu to potentially lethal hemorrhagic manifestations, with dengue fever and dengue hemorrhagic fever respectively. The virus has four serotypes DEN1-4 with secondary infection of heterologous serotypes that can cause severe disease symptoms due to antibody dependent enhancement (ADE). The virus has spread substantially the last decades, and today the disease is endemic in over 100 countries in tropical and sub-tropical regions, with almost half the human world population living in areas where dengue is at risk. There is no licensed vaccine against dengue commercially available today, due to a challenging immunologic effect that has not been compensated for yet. WHO has emerged that development of a vaccine against dengue is top priority, and the vaccine must be safe, efficient and affordable for those who need it. In order to overcome the threat of ADE, the vaccine must provide immunity against all four serotypes. Vaccine candidates have been designed with four monovalent serotype-specific antigens that will be combined into one "tetravalent" vaccine or one chimeric tetravalent peptide containing all the four monovalent antigens combined together. A sub-unit vaccine of envelope domain III (EDIII) has been demonstrated to be ideal antigens, as they have distinct epitopes in each serotype that has successfully provided immunity against all four serotypes.

Molecular farming by using plants, in particular tobacco plants as green factories have many advantageous, such as (1) being a safe production platform for human vaccines; (2) it has the ability to process peptides similar to the humans processing; and (3) cost-effectiveness which is the most important factor to make vaccines available to poor people who need them. In this study, monovalent antigen EDIII4 and tetravalent antigen "Tetra" by combining all the four monovalent antigens together were introduced into nuclear and chloroplast genomes of tobacco. An ethanol inducible promoter T7 RNAP was utilized to control the expression of EDIII4 and Tetra in tobacco nuclear genome, whereas *Prrn promoter* which drives transcription of the plastid ribosomal RNA (rrn) operon was used to control the expression of EDIII4 and Tetra in tobacco chloroplast genome. For nuclear transformation, *Agrobacterium*-mediated transformation method was used; while biolistic particle gun bombardment was utilized in chloroplast transformation of tobacco. Regenerated putative transformants from both nuclear and chloroplast transformation experiments were produced and molecular methods including DNA and protein analyses as well as morphological characterization were carried out on the nuclear transformants. Verification of transcriptomic chloroplast transformants are in progress. The results obtained so far are reported in this thesis.

Sammendrag

Dengue virus er patogen for mennesker og overføres via myggen *Aedes aegypti* som forårsaker en sykdom som kan variere fra mild influensa til potensielt dødelige hemoragiske manifestasjoner, med henholdsvis dengue feber og dengue hemoragisk feber. Viruset har fire serotyper DEN1-4 med sekundær infeksjon av heterologe serotyper som kan forårsake alvorlig sykdomssymptomer på grunn av antistoff avhengig forsterkning (ADE). Viruset og vektoren har spredd seg betydelig de siste tiårene, og i dag er sykdommen endemisk i over 100 land i tropiske og sub-tropiske regioner, med nesten halvparten av verdensbefolkningen i dengue-utsatte områder. Det er ingen lisensiert vaksine mot dengue tilgjengelig i dag, grunnet den utfordrende immunologiske effekten (ADE) som ikke har blitt kompensert for ennå. WHO har utlyst at utvikling av en vaksine mot dengue er topp prioritet, og vaksinen må være trygg, effektiv og rimelig for de som trenger det. For å overvinne trusselen om ADE, må vaksinen gi immunitet mot alle fire serotyper.

Vaksinekandidater har blitt designet med fire monovalente serotype-spesifikke antigener som skal kombineres til en "fireverdig" vaksine eller et kimerisk fireverdig peptid som inneholder alle de fire monovalente antigenene kombinert sammen. En sub-enhet vaksine av envelope domain III (EDIII) har vist seg å være ideelle antigener for en vaksine, siden de har forskjellige epitoper i hver serotype som har gitt immunitet mot alle fire serotyper.

Molecular farming ved hjelp av planter, særlig tobakksplanter som grønne fabrikker, har mange fordeler. Blant annet er det en trygg produksjonsplattform for vaksiner til mennesker, det har evnen til å prosessere peptider på samme måte som mennesker og det har svært høy kostnadseffektivitet. Det er essensielt å gjøre vaksinen tilgjengelig for fattige mennesker som trenger dem og da må den være rimelig. I denne studien ble det monovalente antigenet EDIII4 og det kombinerte fireverdige antigenet "Tetra" introdusert i cellekjernegenomet og kloroplastgenomene til tobakk. En etanol-induserbar promoter T7 RNAP ble benyttet for å kontrollere genuttrykket av EDIII4 og Tetra i kjernetransformerte tobakker, mens Prrn promoter som driver transkripsjon av plastid ribosomalt RNA (prrn) operon ble brukt til å kontrollere uttrykket av EDIII4 og Tetra i kloroplastgenomene til tobakk. For kjernetransformasjon ble *Agrobacterium*-mediert transformasjon benyttet, mens en genkanon ble benyttet for å transformere kloroplastene. Molekylære DNA metoder ble anvendt for å bekrefte at kjernetransformerte tobakksplanter hadde integrert EDIII4 og Tetra genene. I tillegg ble protein analyser utført, samt morfologisk karakterisering. Det gjenstår å verifisere at de kloroplasttransformerte plantene har innsatt antigenene. Resultatene oppnådd så langt er rapportert i denne avhandlingen.

Table of Content

1. Introduction

During the past thirty years, plant biotechnology has advanced significantly and made considerable contributions to food production, social economic development and establishment of biotechnology-based industries, such as pharmaceutical industries. Plant biotechnology, a useful biotechnological tool, has demonstrated its potential for generation of a number of genetically modified (GM) crops in the last twenty years. Since the first genetically modified (GM) crop was commercialized in 1996, global hectarage of biotech crops has continued to grow, reaching 134 million hectares in 2009 (James 2009). This translates to an increase of 9 million hectares over 2008, demonstrating the significance, economic benefits and great potential of GM crops. In addition to these successful stories, plant biotechnology has also made an unforgettable contribution to the advancement of biotech industry.

Plant has not only been the food sources and natural herbal medicines for mankind in history, but also the feed for animals and raw materials for industries, for instance cooking oils, color agents, perfumes etc. In recent years, plants have been utilized as a green factory for production of high value proteins including biopharmaceuticals, vaccines, expensive enzymes, chemicals etc. This master thesis is a part of a research project aiming to express candidate dengue antigens in tobacco plants for production of a dengue vaccine at low cost.

1.1. DENGUE

Dengue presents a significant financial burden to the public health care systems of developing nations in the Americas and Southeast and South Asia (Beatty, Beutels et al. 2011). Dengue fever (DF) and dengue hemorrhagic fever (DHF) represent a spectrum of febrile syndromes ranging from a mild illness to a severe and life threatening constellation of signs and symptoms highlighted by plasma leakage. Infection occurs when an infected female *Aedes* mosquito passes one of four antigenically related but distinct dengue virus types to the human host.

Dengue virus belongs to the Flaviviridae family. There are four strains of the virus that are named DEN1-4. These are referred to as serotypes which have distinct variations with specific epitopes for each serotype. All serotypes are pathogenic, but virulence of the serotypes can differ. Dengue virus is the most common arthropod-borne virus of human pathogens, transmitted with female mosquitos of *Aedes Spp,* but primarily *Ae. Aegypti* (Gibbons 2010).

Figure 1.1: Dengue vector *Aedes Aegypti*

The viral infection has 2-14 days of incubation time before symptoms occur which results in dengue fever or dengue hemorrhagic fever. Children and young adults are particularly affected by the disease. Dengue fever is defined by flu-like symptoms such as fever, headache, muscle and joint pains, nausea, vomiting and rash. Dengue hemorrhagic fever has additional hemorrhagic manifestations and plasma leakage that can be fatal. World Health Organization (WHO) estimates that 50-100 million people are infected annually with 500 000 cases of dengue hemorrhagic fever, resulting in 20-25 000 deaths. The real numbers are probably higher due to incidents around the world that are not diagnosed and reported (WHO 2012).

1.1.1. Dengue distribution and vaccine development

The spread of dengue virus has increased massively during the last decades. It is estimated that during the three last decades the disease burden has increased at least fourfold (Guzman and Isturiz 2010). Dengue was only reported in a few countries in Southeast Asia until the 1960s but by the 1970s the virus became hyperendemic in most major cities of tropical regions. Increasing population and urban development have allowed the mosquitos to evolve and the extent of the disease has emerged. In addition, the virus has spread to new areas due to increased global transportations and international travelers.

Dengue is found in tropical and sub-tropical regions of the world putting over 2,5 billion people (about 40 % of the world population) at risk. About 70 % of the disease burden is located in South-east Asia and the Western Pacific regions but the virus is now endemic in more than 100 countries in America, Africa, Asia and Australia (WHO 2012).

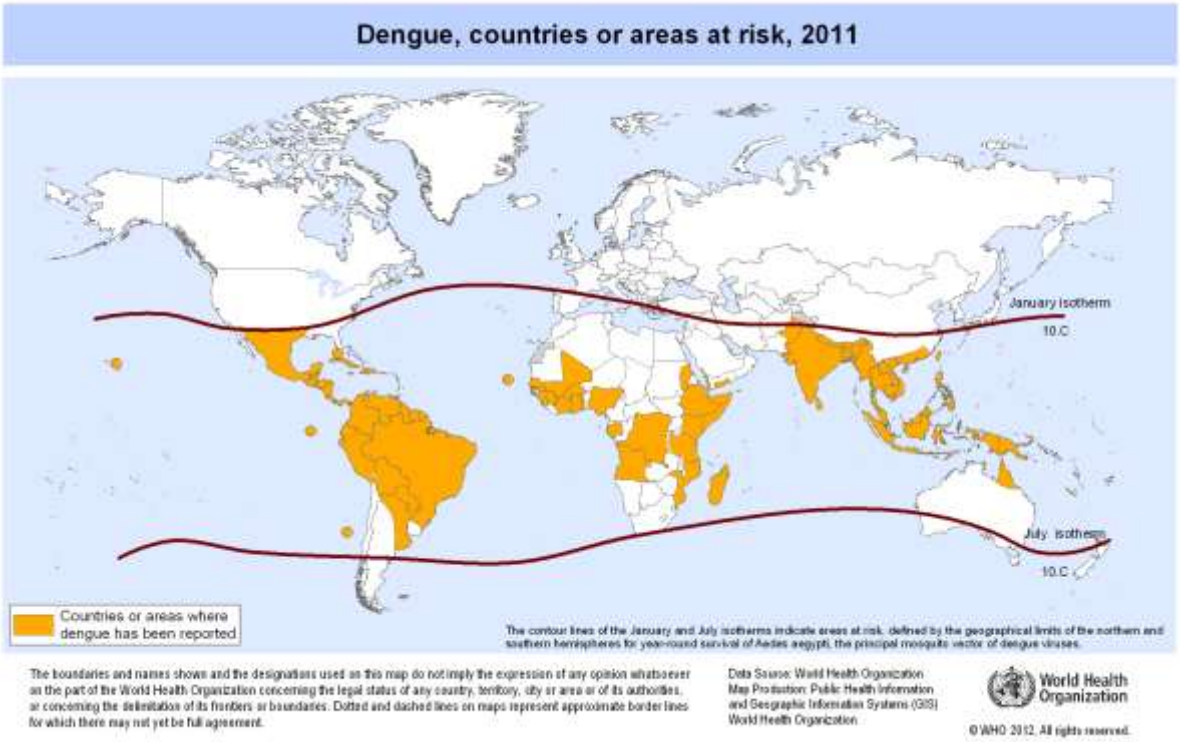


Figure 1.2: Dengue distribution in 2011. The yellow marks show areas where Dengue was reported in 2011 and the red lines indicate where Dengue can be a risk, defined by year-around survival of Aedes Aegypti. (WHO 2012)

A growing number of dengue infections have been reported in Europe from patients that have been infected while traveling abroad in areas with dengue. It is not unlikely that the disease

might spread to Europe as well (Jelinek, Mühlberger et al. 2002). A recent epidemiological alert made by the Pan American Health Organization/WHO in March 2012 reported risk of dengue outbreaks during the second part of 2012, and they strongly suggested the severity of dengue threat and the importance of dengue vaccine development (PAHO 2012).

Up to date, there is no licensed dengue-vaccine available to treat or prevent dengue. Preventative measures are the only approaches which help to reduce the spread of the vector, in addition to necessary education and knowledge about how people that are at risk of transmission can protect themselves. The mosquitos breed in water and vector control programs have helped in preventing uncontrolled spread of the virus vector.

Dengue causes low mortality rates compared to the total number of infected patients and has therefore not been considered an important public health problem. The spread of dengue has changed the picture. Dengue is today a bigger problem than Malaria in terms of morbidity and economic impact and it has become the most severe vector-borne viral disease of humans (Gubler 2012). The WHO has announced dengue as a world health priority and appointed the need for a vaccine.

Dengue virus infection of a single-serotype results in lifelong immunity against the infecting serotype, whereas cross-protection against the other heterotypic serotypes lasts for 2-6 months (Guzman and Vazquez 2010). A secondary infection from another serotype later in life will actually trigger the immune system's mechanism to enhance viral pathogenesis. This is called Antibody Dependent Enhancement (ADE). Primary infections of dengue virus rarely cause severe dengue disease. Studies have showed that secondary infection with heterotypic serotype was observed in up to 99 % of studied cases of severe dengue disease, indicating that severe dengue disease is caused by ADE (Halstead 1982). All four dengue virus serotype are now co-circulating in Asia, Africa and the Americas (Guzman, Halstead et al. 2010; Guzman and Vazquez 2010). In order to make a safe vaccine it must provide immunity against all of the four serotypes. Otherwise, the vaccine may cause ADE in a later infection and result in severe dengue disease.

1.1.2. Envelope Domain III as antigen candidate

The Dengue genome encodes three structural proteins; capsid (C), membrane (M) and envelope (E) and additional seven non-structural proteins (NS) as illustrated below in figure 1.3: the Dengue Genome. The envelope protein is a glycoprotein that has important functional roles in virus attachment to cells and fusion with membranes. Antigen domain from this protein is ideal because antibody neutralization of this protein will affect the virulence of the virus as well as host range and cellular tropism (Crill and Roehrig 2001). Envelope protein contains three domains whereas domain three (EDIII) is chosen for vaccine antigen because it has serotype-specificity, has functions in receptor binding and it is the target of specific neutralizing antibodies (Guzman and Vazquez 2010). The risk of ADE is minimal and the protein is highly stable and soluble. It is confirmed that this peptide as an antigen elicits long-lasting antibody response (Churdboonchart V 1991). The peptide also has independent folding domain and is amendable to tetravalent design.

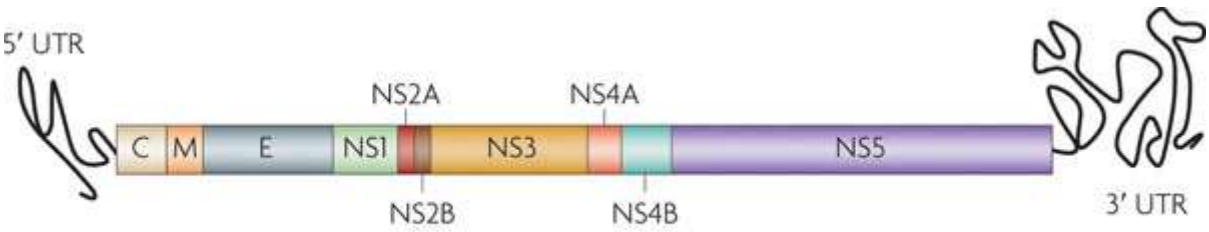


Figure 1.3: Dengue Genome. The E domain encodes envelope glycoproteins where the antigens for the vaccine are derived from. C encodes the capsid and M the Membrane. The seven remaining genes encode non-structural proteins. (WHO/TDR 2010)

1.1.3. Immunity against all four serotypes

Because of the antibody dependent enhancement with secondary infections it is important that the vaccine will induce immunity against all of the four serotypes, if not the vaccine might be harmful. The vaccine must either be constructed as a chimeric tetravalent vaccine or the four monovalent EDIII antigens must be mixed together in one vaccine.

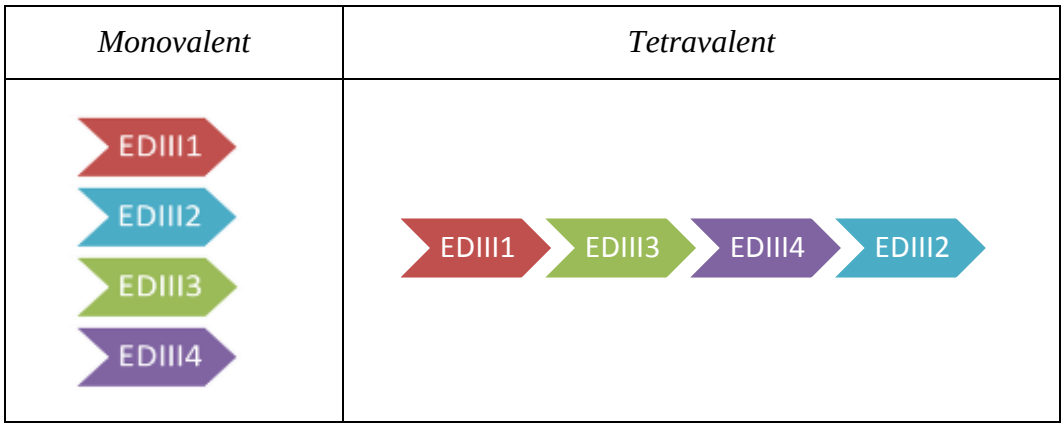


Figure 1.4: Vaccine candidates made of four single monovalent antigens or one chimeric tetravalent antigen.

1.2. TOBACCO, NICOTIANA TABACUM

Nicotiana tobacum is a plant of Solanaceae family. The plant is a model organism commonly used in experimental studies. It is well characterized and very feasible to genetic manipulation. Furthermore, the plant has robust growth and simple seed production as it is self-pollinating. Due to high biomass and rapid growth, production of recombinant proteins in tobacco can be very efficient. It is possible to grow 100 tons tobacco per hectare (Sheen 1983). Tobacco is a non-food and non-feed economic crop with a bad reputation due to the tobacco industry, but now it has a promising future as an ideal candidate green factory for molecular farming.

Figure 1.5: Tobacco, NicotianaTabacum from the studies. From upper left: Mature plants, seed buds, flowering plants, tobacco flower and shoots growing on medium in box.

In addition to the nuclear genetic material, plants also have genetic material in mitochondria and chloroplasts, two important organelles. It is possible to manipulate both nuclear and plastid DNA, but experiments with manipulation of mitochondria genome has so far not been achieved.

The entire genetic material in a nucleus is called the genome whereas in the plastids it is called the plastome. The genome of tobacco is arranged in 48 chromosomes and it comprises about 4500 Mbp with 36 000 genes. The plastome is very small compared to the genome, with only 144 genes and about 156 kbp.

Tobacco has totipotent cells which mean that the cells can divide and produce all the differentiated cells in an organism. By this, a new plant can be regenerated from explant tissue. At first, tissues from tobacco dedifferentiate and form a mass of unorganized cells, called callus. Then the cells in the callus can differentiate to regenerate a new plant. These characteristics are ideal for manipulation of tobacco for production of high value proteins by molecular farming.

1.3. MOLECULAR FARMING IN PLANTS

The last 15 years have had a significant development in plant science and research has shown promising results in molecular farming with economic productions and potential for large-scale synthesis of valuable candidate recombinant proteins and enzymes. Like humans, plants can assemble, modify and secrete complex proteins. Diverse pharmaceutical proteins such as antibodies, vaccine candidates, enzymes, hormones, cytokines, growth regulators and etc. have been successfully produced in plants (Obembe, Popoola et al. 2011; Yusibov, Streatfield et al. 2011)

Traditionally, vaccines are produced by using inactivated or attenuated pathogens which are efficient in immunologic response but there is a risk of potential infections, allergies and autoimmune response. Plant-derived vaccine systems can offer safer and more cost-efficient vaccines compared to traditional vaccine productions. Plant-derived vaccines are made of subunits from an infectious agent, like peptide domains on a protein, eliminating any pathogenic reaction. Since plants are not host of human infectious agents, the risk of contaminations of other human pathogens is also minimal. Traditional vaccine production has

mainly been produced in mammalian or bacterial cell cultures with fermenter-based technology. These systems are very expensive in production and they are dependent on high-cost equipment. Plant-derived vaccine systems can be very cost-efficient compared to traditional vaccines because of low expenses in both establishment and downstream processing. Once a transgenic plant is maintained, the plant can be grown in large-scale where soil, water and sunlight are the only demands. The expression level should be quite high for cost-efficient productions (>1 % of total soluble protein), but in theory the costs can be reduced up to 80 %. (Maliga & Bock 2011; Yusibov et al. 2011).

Plant-derived vaccines can be made as capsules to eliminate the need for syringes, trained medical personal to inject the vaccines and cooled storing, which will make the vaccine even more cost-effective. Edible vaccines can reduce the cost even more as they eliminate or further reduce any downstream processing and they can be cultivated on-site for easier distribution and less transportation.

Molecular farming in plants can be achieved with three different expression systems. For stable expression, the transgenes can be inserted in either the nuclear or the chloroplast genome. A third option is transient expression where the transgene is not integrated in the plant's genome, but is rather infiltrated into the cells as plasmids where it is expressed for a limited time (Yusibov, Streatfield et al. 2011).

1.3.1. Chloroplast engineering for molecular farming

Plastids are intracellular organelles where biosynthesis takes place, such as photosynthesis in chloroplasts. The chloroplast is descended from free-living cyanobacteria that have been evolutionary preserved in plants. Since the chloroplasts originated from bacteria, the preserved genetic material (plastome) is circular with systems for gene regulation, replication, transcription and translation that reflect a prokaryotic system.

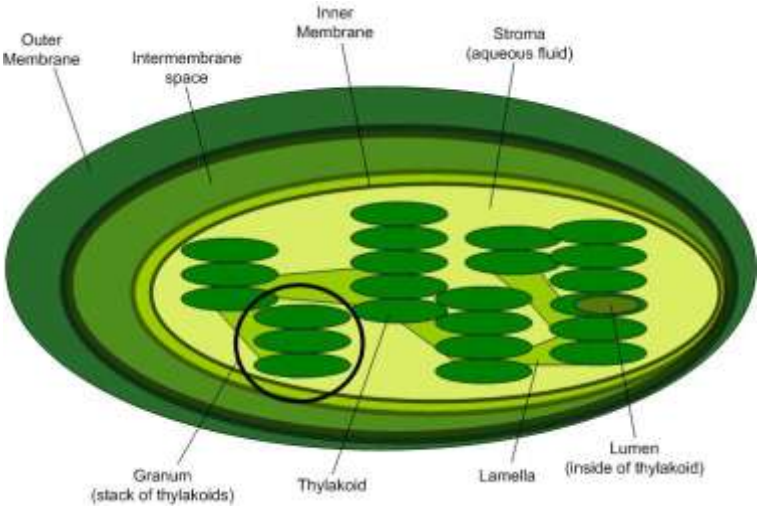


Figure 1.6: Chloroplast illustration. Chloroplasts have double membrane surrounding the photosynthesis machinery. Stacked thylakoid plates make granum and all the grana are connected by lamella. The chloroplast DNA (plastome) is found in the fluid stroma. A plant cell can contain 100 chloroplasts. (Illustration from Jihong Liu Clarke)

More than 100 transgenes have been stably integrated and expressed via the chloroplast genome to confer important agronomic traits, as well as to express industrially valuable biomaterials and therapeutic proteins (Bock 2007; Daniell, Ruiz et al. 2009; Bock and Warzecha 2010). Chloroplast transformation has been accomplished in a number of crops including lettuce, cabbage, oilseed, rape cauliflower, poplar, sugarbeet, tomato, potato and eggplant.

Chloroplast engineered plants are considered safe to both the environment and the plant. Chloroplasts are maternally inherited and transplastomic chloroplasts will thus not spread via pollen and will not be a threat to the environment.(Daniell 2007; Ruf, Karcher et al. 2007; Svab and Maliga 2007). Chloroplast transformation is causing minimal threat to the plant host as well, because of controlled insertion of the transgene with the mechanisms of site-specific homologous recombination. The chloroplast genome is illustrated in figure 1.7, containing two copies of a large inverted repeat (IR) with a small and large single copy region (SSC and LSC, respectively) that separates the IRs. The transgene to be inserted is flanked, for examples with *trnf* gene sequences (as shown in the figure) that bind to homologous *trnf* in the plastome and replace the SSC with the transgene. This controlled insertion mechanisms eliminates any position-effect that can be caused by random insertion and also, by using the chloroplasts sub-cellular compartmentalization for metabolic engineering, minimal of pleiotropic effects in the plant has been reported (Daniell, Kumar et al. 2005; Verma, Kanagaraj et al. 2010). In total, chloroplast engineering is a highly controlled and safe way of genetic manipulation.

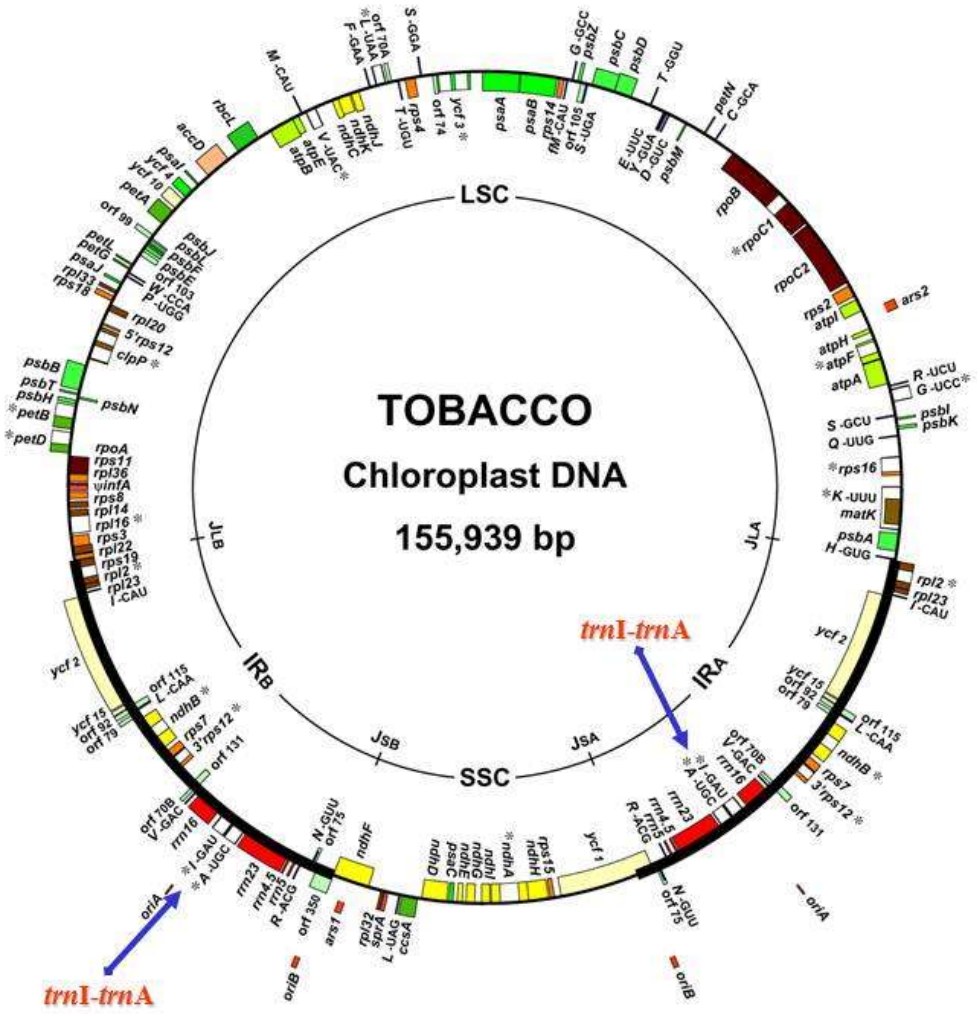


Figure 1.7: Chloroplast genome. Inner circle show large single copy region (LSC), Inverted repeat (IR) A and B, and single copy region (SSC). A chloroplast can contain 100 of these plasmids. The marked trnI (tRNA) genes are used in site specific recombination to replace the sequence in between the sites with the transgene. (Jihong Liu Clarke)

Furthermore, chloroplasts can contain up to 100 chloroplast genomes (plastome) and a single cell can contains about 100 chloroplasts, comprising up to 10 000 chloroplast genomes (100x100) in one cell (Bendich 1987). This high transgene copy number can result in very high expressions. Transgenes can also be arranged in operon to make polycistronic mRNA so that multiple transgenes can be introduced and expressed in a single transformation event (Ruiz, Hussein et al. 2003; Quesada-Vargas, Ruiz et al. 2005). In total, this can result in abundant transgene transcripts and accumulation of the expressed proteins as high as 70 % of total soluble protein (Oey, Lohse et al. 2009; Ruhlman, Verma et al. 2010). Once a plant of

stably integrated transgenes is achieved, it can be grown in large scale and very high yields of the expressed transgene can be harvested cost-efficiently.

1.3.1.1. Chloroplast transformation with biolistic gene gun

Chloroplast transformation of tobacco can be carried out by biolistic particle bombardment using PDS/1000-He System Hepta Adapter DNA delivery system. Inside the bombardment chamber there will be vacuum that reduces friction drag of the DNA-coated particles. Pressurized Helium will build up until a rupture disk break and the pressurized gas enters the bombardment chamber as a shock wave that causes the biolistic bombardment of the DNA towards target explant cells. DNA-molecules coated with gold particles will thus be introduced into targeted cells as shown in the figures 1.8 and 1.9 below.

Figure 1.8: Biolistic PDS-1000/He Particle Delivery System. Helium enters from the top and vacuum is controlled to the left. DNA and explant is prepared and installed inside the bombardment chamber where Helium pressure in vacuum environment shoots the DNA-coated particles towards the explants.

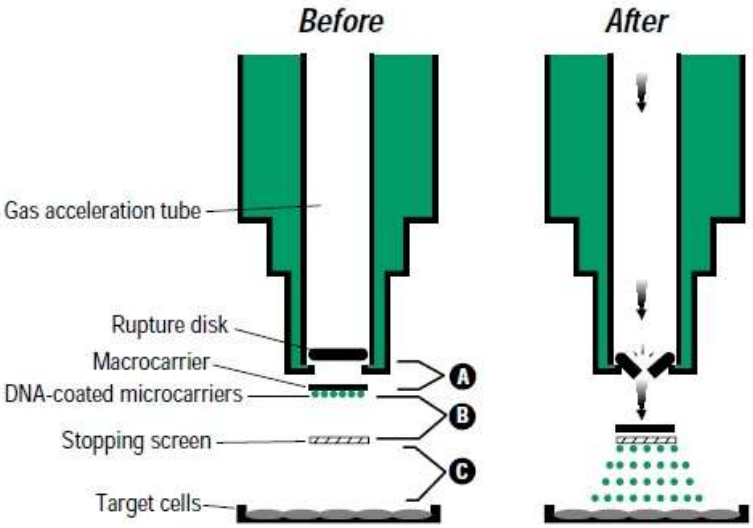


Figure 1.9: Biolistic bombardment before and after shooting. Before shooting, the instrument is prepared with A) Rupture disk that withstands a given pressure. B) Macrocarrier holding seven microcarriers carrying DNA-coated gold particles. C) Stopping screen and target cells. The distances indicated with A, B and C are adjusted for optimal transformation with different target cells. The right illustration shows how the shooting works. High Helium pressure builds up before the rupture disk break and Helium shoots out towards the DNA-coated microcarriers as the arrows indicate. Helium pressure shoots the DNA-coated gold particles trough the stopping screen and bombard the explant target cells (Biorad 2006)

The transformed leafy explants are subsequently selected on callus-inducing and regenerated media supplemented with adequate hormones and antibiotic as described in this study under section 3 (materials and methods). Transformed explants develop callus prior shoot formation. In order to achieve transplastomic transformants, 2-3 rounds of selection are necessary before transferring the bombarded explants into regeneration medium for regeneration of transplastomic plants.

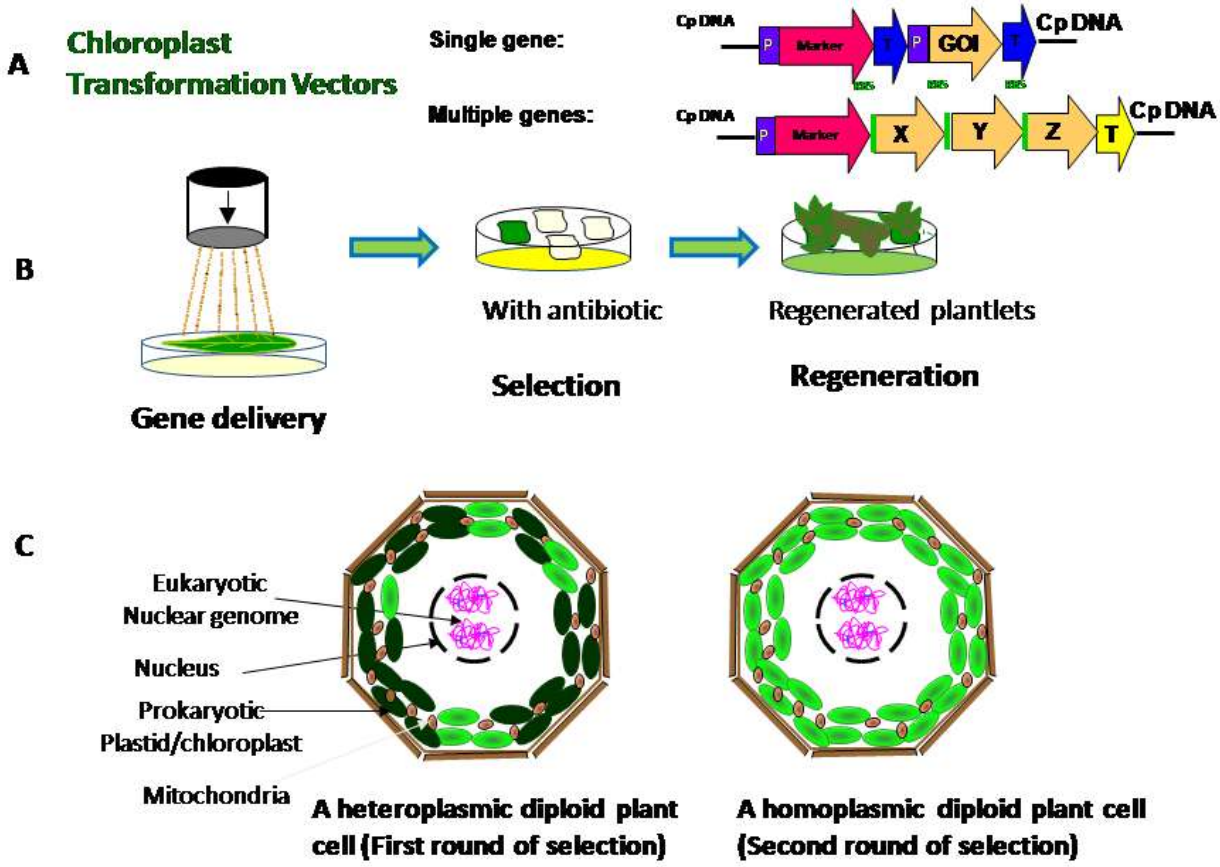


Figure 1.10: Chloroplast engineering. A) Vectors are designed with transgene flanked by chloroplast (Cp) DNA. B) Explant is transformed with the vector and transplastomic tissue is selected and regenerated with antibiotic. C) Several regenerations are necessary to develop homoplasmic cells (Clarke and Daniell 2011).

1.3.2. Engineering of nuclear genome

Plant nuclear transformation can be achieved by three main approaches: *agrobacterium* mediated transformation; biolistic transformation as described for chloroplast transformation and *Agrobacterium*-based floral dip which is used routinely in Arabidopsis transformation. Among those three, Agrobacterium mediated transformation is the most widely used method in many plant species. *Agrobacterium tumefaciens* and related *Agrobacterium* species have been known as plant pathogens since the beginning of the 20th century. However, only in the past two decades has the ability of *Agrobacterium* to transfer DNA to plant cells been harnessed for the purposes of plant genetic engineering. Since the initial reports in the early 1980s using *Agrobacterium* to generate transgenic plants, scientists have attempted to improve this "natural genetic engineer" for biotechnology purposes. Some of these modifications have resulted in extending the host range of the bacterium to economically important crop species. However, in most instances, major improvements involved alterations in plant tissue culture transformation and regeneration conditions rather than manipulation of bacterial or host genes. *Agrobacterium* mediated plant transformation is a highly complex and evolved process involving genetic determinants of both the bacterium and the host plant cell. *Agrobacterium* mediated transformation has high efficiency compared with biolistic transformation method but lower transgene expression with average of 0.01–0.4% of TSP (Beatty, Beutels et al. 2011).

1.3.2.1. Agrobacterium tumefacien

Agrobacterium is a bacterium that can cause crown gall disease in plants. A tumor inducing (Ti) plasmid contains transfer DNA (T-DNA) region that the bacterium can introduce into a plant host's genome. The inserted T-DNA region along with the transgene will be transcribed and further translated by the plant's machinery. The T-DNA region encodes proteins involved in biosynthesis of hormones making the plant tissue proliferate and develop tumors. The T-DNA region will also produce proteins involved in biosynthesis of novel plant metabolites called opines and agropines. These are derivate of amino acids and sugar that provide the *A. tumefacien* with carbon and energy sources that the plant cannot utilize (Slater A., Scott et al. 2008).

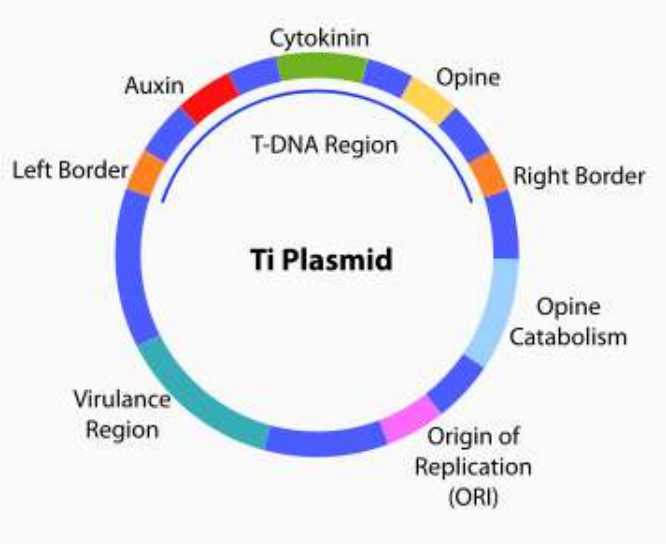


Figure 1.11: Illustration of a Ti plasmid. The T-DNA region between left and right border is inserted in the plants genome. The T-DNA region contains cytokinin that causes tumor in the plant and the auxin and the opins are metabolites that only the agrobacterium can feed on. The virulence region is responsible for transferring the T-DNA region into the plant. (2012)

1.3.2.2. Agrobacterium mediated stable transformation of plants

Strains of *agrobacterium* have been modified to serve as a biological tool for transformation of plants. The pathogenic genes in the T-DNA region of the Ti-plasmid are removed, the genes necessary for insertion are still present and genes for selection and target genes are added. *Agrobacterium* can infect the plant and transfer parts of the T-DNA region containing the target gene into the plant's genome and insert it.

Explants from *in vitro* culture or sterilized explants from in vivo plants are excised and inoculated with *agrobacterium* which will enter the scars of the plant tissue. Co-cultivation will allow the *agrobacterium* to insert the T-DNA region in the tobacco genome. Antibiotics against the *agrobacterium* and for the selective marker are added to kill the *agrobacterium* and to select transgenic tissue. The transgenic tissue will finally grow shoots after about 30 days. From transformation to developing mature plant takes about 3 months for tobacco.

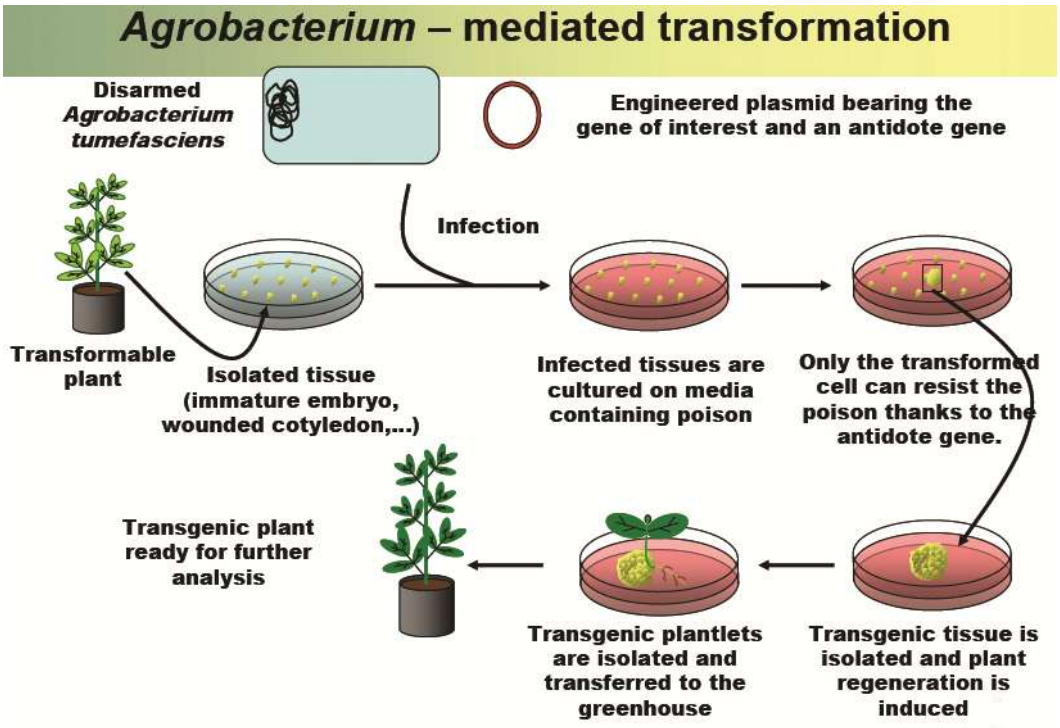


Figure 1.12: Agrobacterium mediated transformation. Explant is inoculated with agrobacterium, and during a period of co-cultivation the agrobacterium enters the plant cells and inserts its T-DNA region. Antibiotics are added to kill agrobacterium, as well as select transformed cells that can regenerate a new plant. (Kan Wang 2006)

Concerning transgene integration, nuclear transformation by *agrobacterium* (also gene gun method) has random transgene integration, whereas chloroplast transformation allows transgene to be introduced into chloroplast genome by site-specific recombination. Random integration has the disadvantages to generate position effects where other genes are affected and can be disabled or silenced. Transgene of nuclear transformed plants can be spread more easily to the environment via pollen which contains the transgene. Nevertheless, nuclear genome transformation has advanced significantly and has successfully managed to express advanced proteins.

1.3.2.3. The Constitutive and ethanol induction transgene expression

Constitutive transgene expression driven by constitutive expressed promoter such as Cali flower mosaic virus (CaMV) 35 S promoter can be harmful to the transformed plants which can then cause deleterious effects of the transgene. If a transgene expression interferes with important metabolic pathways it can cause inhibited growth. High expressions are desired but the plant must sustain healthy as well. The plants are especially vulnerable to metabolism interference during early growth stages. To avoid the deleterious effects caused by

constitutive expression of transgene in chloroplasts, an ethanol inducible T7 RNA polymerase system was developed (Lössl, Bohmert et al. 2005). This inducible system which triggers transgene expression upon ethanol application further enhances the security and control of production in GM plants.

Most inducible systems only work in nuclear compartments. In order to induce expression in chloroplasts, a trans-activating system was invented, engineering plants as both transgenic and transplastomic. The ethanol induces a positively regulated AlcA promoter in the nucleus which regulates the downstream gene encoding a RNA polymerase derived from T7 bacteriophage (T7RNAP). The translated T7RNAP is targeted to the chloroplast with a Rubisco unit bound. Once the protein is transported to the chloroplast, the polymerase will bind to a T7 promoter and translate the transgene in the plastome.

Because of time limitations the ethanol inducible system will be tried on nuclear transformed tobacco only, with no trans-activation of the chloroplast transgene expression. The antigen sequence will be inserted downstream of the ethanol inducible promoter for regulation.

2. Aim of the project

The objective of the thesis was to express candidate dengue vaccine antigens in tobacco, aiming for production of affordable plant-made dengue vaccine at a low cost, in addition to acquire knowledge on tobacco genetic engineering during the master education.

This included constructing plasmid expression vectors containing candidate dengue antigens for both nuclear and chloroplast transformation; introduce the antigens into tobacco chloroplasts by chloroplast genetic engineering, and introduce the antigens under the control of an inducible promoter into the nuclear genome of tobacco plants using nuclear transformation method; and finally molecular analyses of antigens expressed in tobacco nucleus and chloroplasts and immunologic detection of the antigens

3. Materials

3.1. Tobacco

A fast growing tobacco (*N. tobacum*) cultivar Petit Havana was used through all the studies.

3.2. *Esterichia coli*

The JM109 strain of chemically competent *Esterichia coli* cells was used in heat shock transformation. The cells are in a $CaCl_2$ solution and they are freezed in -80 °C with aliquots of 50 µl.

3.3. *Agrobacterium tumefacien*

Agrobacterium tumefaciens LBA4044 strain of electrochemically competent cells were used in electroporation and the transformed cells were used in transforming tobacco.

3.4. Human Dengue Virus Envelope Domain (EDIII) antigens

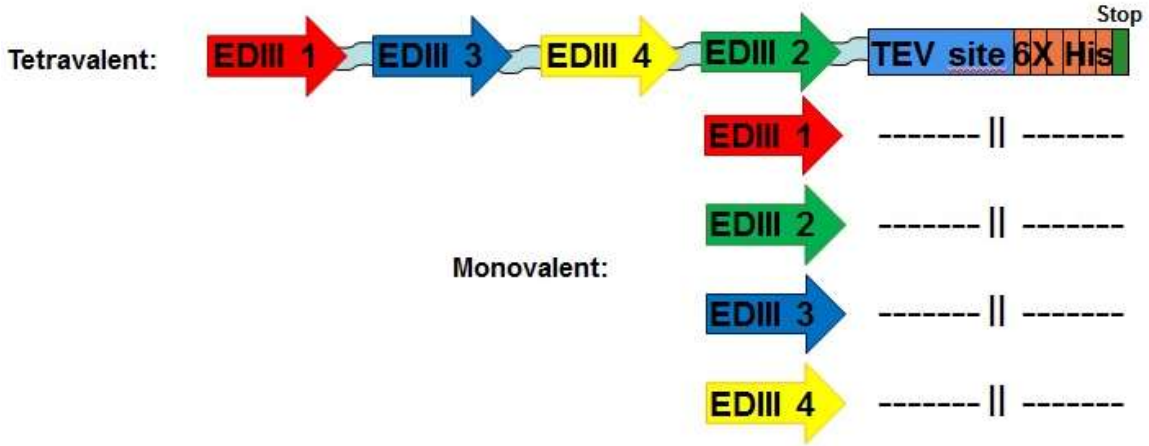


Figure 3.1: Tetravalent and four monovalent antigen constructs of Envelope Domain III. (Even Sannes Riiser)

EDIII4 and Tetravalent EDIII were chosen as main constructs for this thesis. The nucleotide sequence encoding the antigen for EDIII4 is 309 bp and 1290 bp for Tetra. Additional, downstream of the gene is a pentaglycine linker, TEV-protease cleavage site, 6x His-tag and

finally a stop codon. The full construct of EDIII4 is 372 bp and 1356 bp for Tetra. The His-tag is a peptide of six Histamines used for affinity purification of recombinant proteins. TEV-protease cleavage site can be recognized by a site specific protease that will cleave inside the cleavage site and thus remove the His-tag after purification. A pentaglycine linker is a peptide of five glycines which is the smallest of all amino acids with only a hydrogen bound in the side chain. The pentaglycin linker allows a more flexible folding of the peptide chain. In the Tetravalent peptide all the four monovalent peptides are spaced with a pentaglycine linker as well. A tetravalent antigen must fold in a manner where all the monovalent peptides are accessed on the surface of the protein for antibody recognition.

The antigen constructs are codon optimized and the synthesized nucleotide sequences are inserted into pUC57 plasmid for recombinant storing.

3.5. Vectors

EDIII4 and Tetra from pUC57 were to be constructed into pDK60 vector for chloroplast transformation and into pSRN vector for nuclear transformation.

Table 1.1: Antigen of EDIII4 and Tetra used in vector constructs and in transformation

	Constructed vectors	*Transformed tobacco*
Nuclear transformation		pSRN-TPSS-T7 (empty)
	pSRN-EDIII4	pSRN-EDIII4
	pSRN-Tetra	pSRN-Tetra
Plastid transformation		pDK60 (empty)
	pDK60-EDIII4	pDK60-EDIII4
	pDK60-Tetra	pDK60-Tetra

3.5.1. pUC57-EDIII 4 and pUC57-Tetra

The supplier of the synthesized DNA sequences of EDIII 4 and Tetra has introduced the genes into a pUC57-plasmid in a non-directional manner. Vectors of the pUC series are common plasmids for cloning in *E. coli*. The pUC57 vector contains an AmpR gene that encodes resistance against the antibiotic ampicillin. The rep (*pMB1*) gene is responsible for replication of the plasmid.

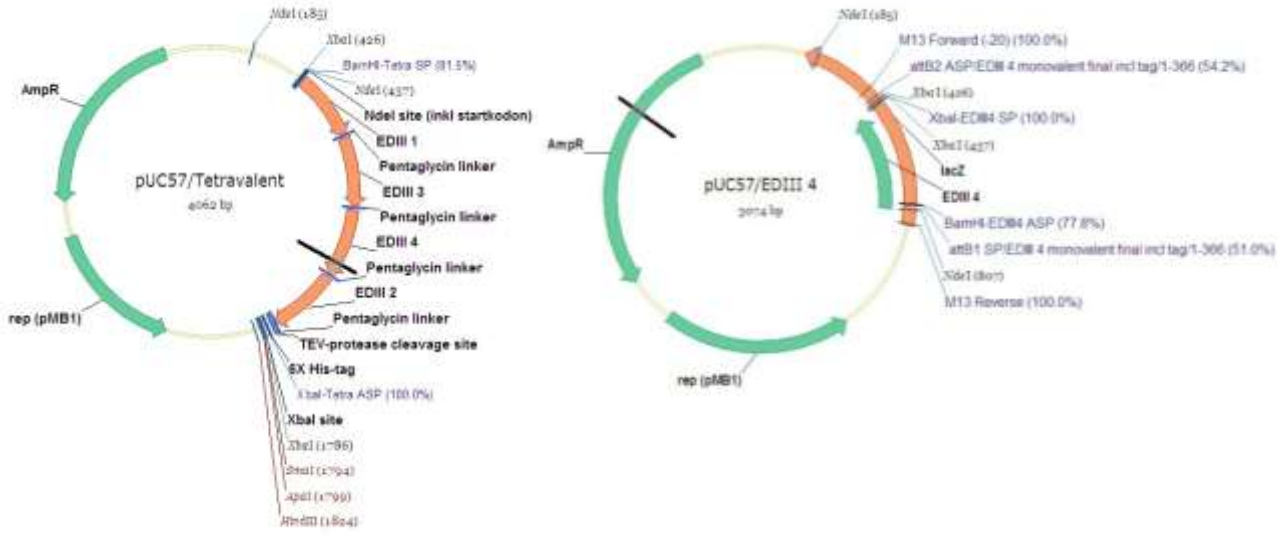


Figure 3.2: EDIII4 and Tetra are stored in pUC57 vector.

3.5.2. pSRN-TPSS-T7 for nuclear transformation

The pSRN-TPSS-T7 vector was received from project partner Dr. Andreas Lössl in Austria, BOKU. The vector is a modified pSRN Ti-plasmid designed for nuclear transformation (Lössl, Bohmert et al. 2005). The pSRN-TPSS-T7 vector is designed to express the *T7 RNAP* gene that encodes a RNA polymerase derived from bacteriophage T7. This gene is not of interest in this experiment and was replaced with EDIII4 and Tetra. Recombined pSRN-EDIII4 and pSRN-Tetra vectors were introduced into *A. tumefacien* in order to transfer the vector into tobacco and insert the T-DNA region in the nuclear genome of tobacco. The *alcA* promoter is a positively regulated promoter that can be activated with ethanol. By inserting EDIII4 and Tetra downstream of the *alcA* promoter the expression of the antigens in tobacco can be manually induced by addition of ethanol.

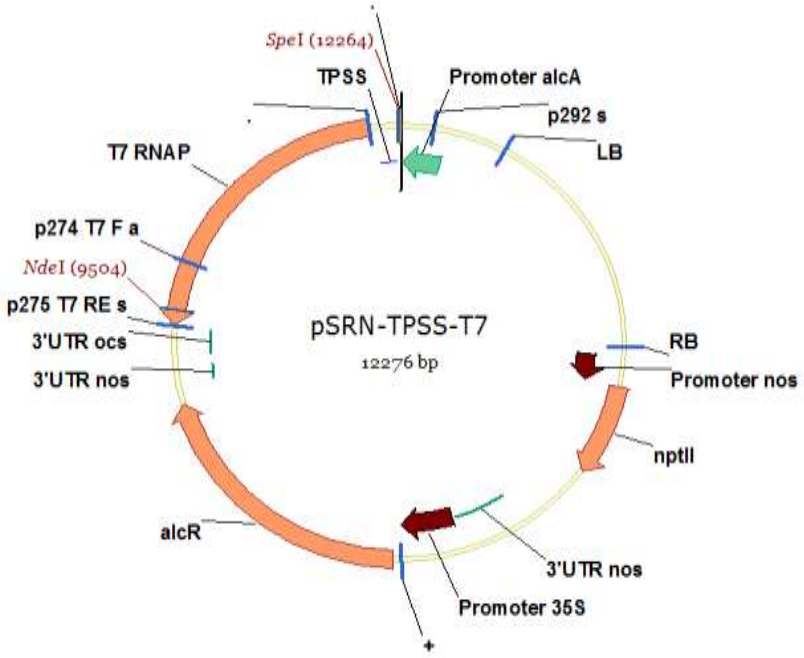


Figure 3.3: pSRN-TPSS-T7 vector for cloning with EDIII4 and Tetra.. The *nptII* is a selective marker encoding Neomycin phospho-transferase II that phosphorylates kanamycin. The *alcR* is constitutively transcribed by the 35S promoter and it encodes a transcription factor necessary for induction of the *alcA* promoter. *T7RNAP* gene for the T7 RNA polymerase is regulated by the *alcA* promoter that can be induced by ethanol. Primer p274 T7 F and p275 T7 Re inside T7RNAP were used for PCR screening of the vector.

3.5.3. pSRN-EDIII4 and pSRN-Tetra for nuclear transformation

The vector was modified by removing the T7 RNAP and replacing it with EDIII 4 or Tetra which then would be under regulation of the ethanol inducible *alcA* promoter.

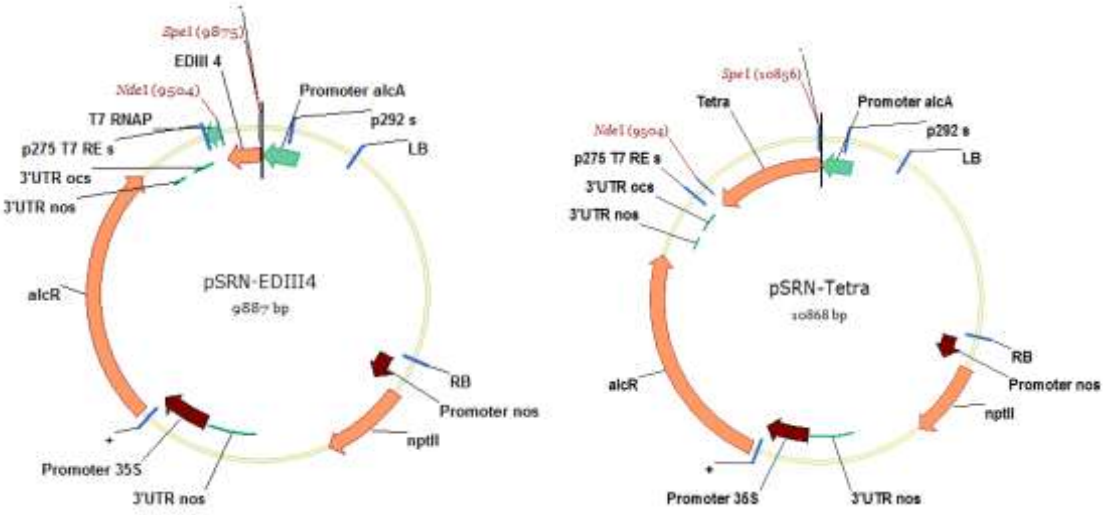


Figure 3.4: Recombined pSRN-EDIII4 and pSRN-Tetra. The EDIII4 and Tetra sequences were inserted into the pSRN between the SpeI and NdeI sites in a directional order. PCR screening to indicate right insertion was done with primers p275 Forward and p292 Reverse.

3.5.4. pDK60 for chloroplast transformation

The pDK60 vector is an expression vector used in plastid transformation. The targeting sequences are homologous to sequences in the chloroplast genome of tobacco and mechanisms of site specific homologous recombination will be able to insert the region between the targeting sequences into the chloroplast genome. *AmpR* gene is selective marker for Ampicillin resistance for cloning. The *AmpR* gene will not be inserted into the chloroplast genome of tobacco. The *aadA* gene is selective marker for Streptomycin resistance that will be selective to transplastomic tobacco tissue. EGFP encodes enhanced green fluorescent protein and it will be replaced with EDIII4 and Tetra. The vector was aimed to make constructs containing EDIII4 and Tetra, and the EGFP was not of interest in this study. *Prrn* is a constitutive promoter that will regulate expression of EDIII4 and Tetra.

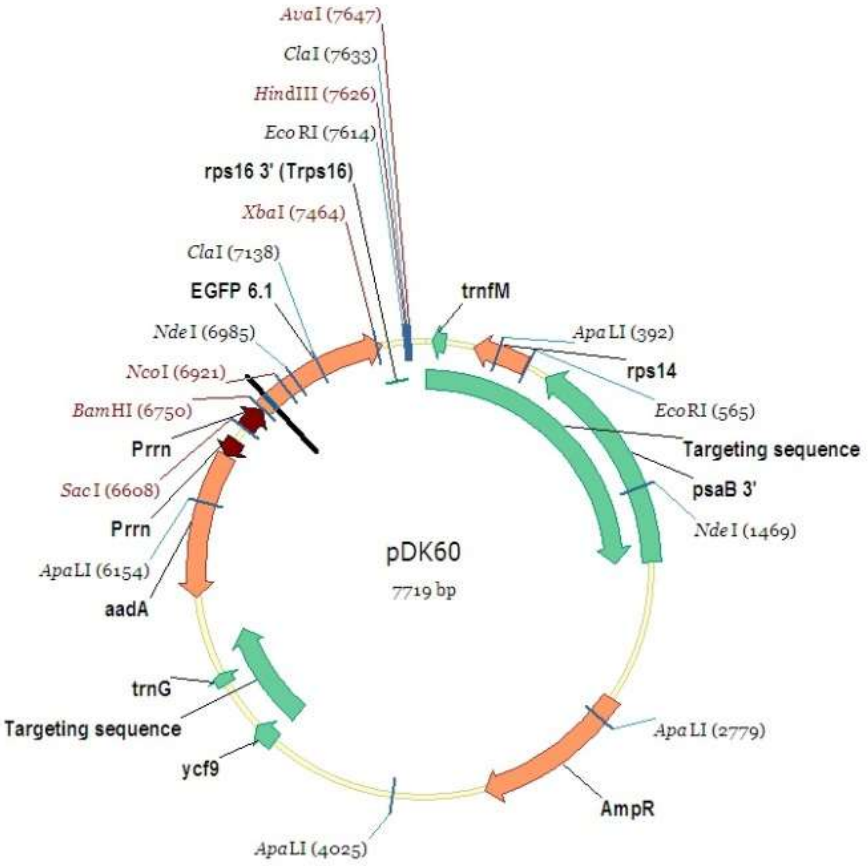


Figure 3.5: Vector pDK60 for cloning with EDIII4 and Tetra. *AmpR* encodes Ampicillin resistance for bacteria cloning and *aadA* are selective marker for Tobaccowith Spectinomycin resistance. *trnfM* and *trnG* are transcription factors. EGFP encodes enhanced green fluorescent protein that will be replaced with EDIII4 and Tetra. *Prrn* are constitutive promoters.

3.5.5. pDK60-EDIII4 and pDK60-Tetra for chloroplast transformation

EDIII4 and Tetra will be inserted between BamHI and XbaI downstream of constitutive promoter *Prrn*. PCR Screening analysis with primers binding in *aadA* and *trnfM* were used to indicate if EDIII4 and Tetra are correctly inserted. The same primers were be used for sequencing.

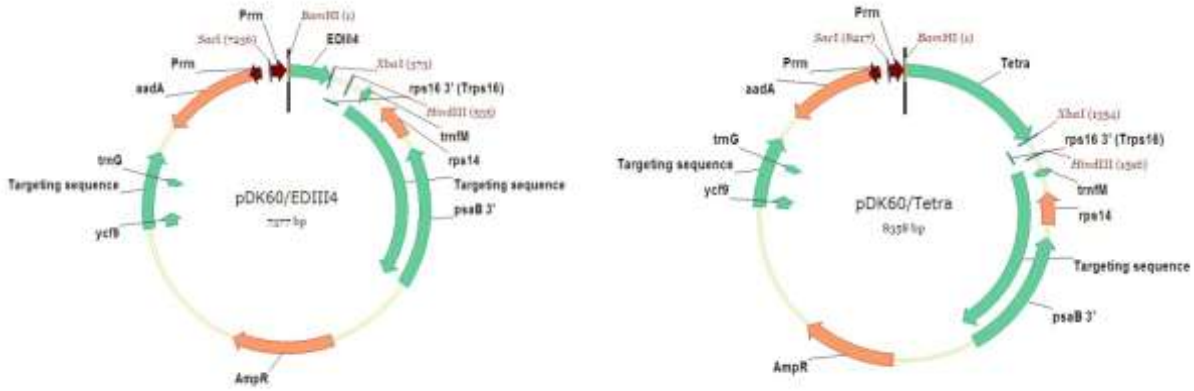


Figure 3.6 pDK60EDIII4 and pDK60Tetra. Recombined pDK60 EDIII4 and pDK60 Tetra were to be constructed with insertion between BamHI and XbaI.

3.5.6. pPCR-Script for subcloning

The pPCR-Script is a cloning vector. *CamR* encodes Chloramphenicol resistance and additional blue-white selection was used in colony selection. EDIII4 and Tetra were inserted between the SmaI sites with the purpose to enhance stability and efficiency of the endonucleases during digestion for further cloning. PCR screening for recombined vectors were done with M13 forward and reverse primers.

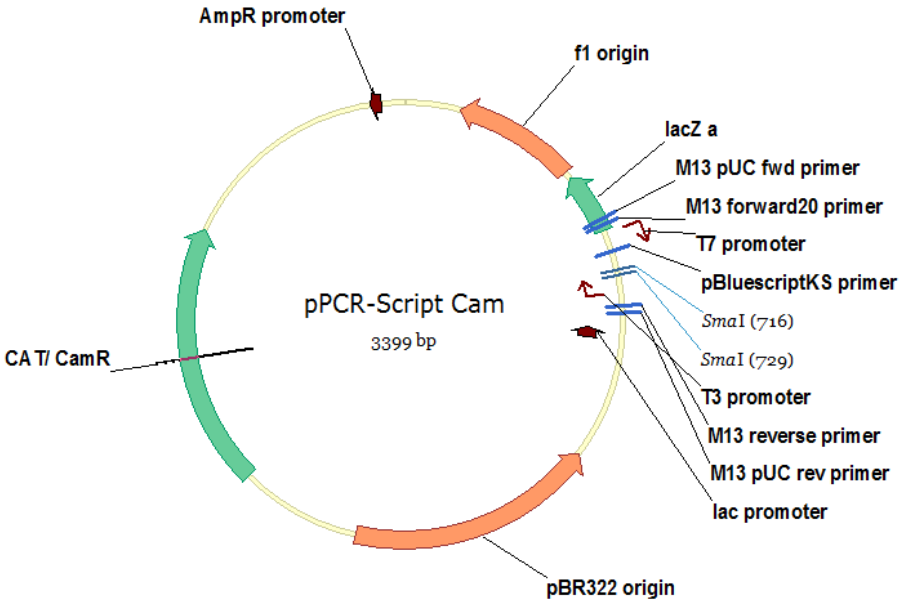


Figure 3.7 pPCR-Script for sub-cloning.

3.6. Kit

Used for	*Name*	*Supplier*	*Catalogue Number*
Isolation of DNA	Wizard SV Gel and PCR Clean-up system	Promega	A9281
	Quantum Prep® Plasmid Miniprep Kit	BioRad Laboratories	732-6100
	Quantum Prep™ Plasmid Midiprep Kit	BioRad Laboratories	732-6120
	Dneasy Plant Mini Kit	Qiagen	69106
PCR	AmpliTaq® DNA polymerase	Applied Biosystems	N808-0152
	Platinum® Pfx DNA polymerase	Invitrogen	11708013
Western Blotting	iBlot Gel Transfer Stacks Nitrocellulose, Regular	Invitrogen	IB3010-01
	AP Conjugate Substrate Kit	BioRad Laboratories	170-6432

3.7. Primers

Template	*Used for*	*Primer name*	*Sequence*
pUC57 EDIII 4 pUC57 Tetra	Adding BamHI site and XbaI site	BamHI EDIII4 ASP	GGATCCATGTCTTATACTATGTGTTCT
		XbaI EDIII 4 SP	TCTAGATTAATGATGATGATG
		BamHI Tetra SP	GGATCCATGTCGTATGTTATGTGCACA
		XbaI Tetra ASP	TCTAGATTAATGATGGTGATG
	Adding SpeI and NdeI site	EDIII 4 SpeI Forward	TGATCTACTAGTATGTCTTATACTATGTGTTCTGGAAAA
		EDIII 4 NdeI Reverse	GATGTACATATGTTAATGATGATGATGATGATGACCTTG
		Tetra SpeI Forward	TCAGTAACTAGTATGTCGTATGTTATGTGCACAG?
		Tetra NdeI Reverse	GTAGTACATATGTTAATGATGGTGATGATGATGTCCTTG
pDK60 EDIII 4 pDK60 Tetra	Screening	aadA P1	CGGCGAGTTCCATAGCGTTA
		aadA SP	GATGACGCCAACTACCTCTG
	Sequencing	aadA SP	GATGACGCCAACTACCTCTG
		pDK60 sekv (aadA)	ATAGTTGAGTCGATACTTCGGCG
		trnfM Sp	CGGGGTAGAGCAGTTTGGTA
		pDK60 sekv (prrn)	AACAAAAGCTGGAGCTCGGT
		pDK60 Tetra Prrn Sp	TAGGGAGGGATCCATGTCGT
pPCR-Script	Screening	M13 Forward	GTAAAACGACGGCCAGT
		M13 Reverse	CATGGTCATAGCTGTTTCC
pSRN-TPSS-T7	Screening	P274 T7 F ASP	ACCACCGTCACGCTCACAGATCTGTGAGCGTGACGGTGGT
		P275 T7 RE SP	AATGCGCTTGCGCTTCAGG
		P292 Forward AS	TGAATTAAGCTTGGCCGCCG
pSRN-EDIII4 pSRN-Tetra	Screening	EDIII4 Scr Forward	AAATGGCTGAAACCCAACAT
		EDIII4 Scr Reverse	TGCAGAATCTCCAACTCCAA
		Tetra Scr Forward	GGACGTCTTATAACCGCTAA
		Tetra Scr Reverse	ATTGTTCCGTGTTGAGTTTC
	Sequencing	P275 T7 RE SP	AATGCGCTTGCGCTTCAGG
		P292 Forward AS	TGAATTAAGCTTGGCCGCCG
		pAlcA Forward 254	CTAGGATTGGATGCATGCG
		pAlcA Forward 157	TATAGAGCAGAGACGGAGC

3.8. Reagents

Name	*Supplier*	*Catalogue Number*
100 mM dNTP Set	Invitrogen	10297-018
1,0 micron Gold	BioRad Laboratories	1652262
1 kb ladder	New England Biolabs	N3232S
100 bp ladder	New England Biolabs	N3231S
1-Naphtalene acetic acid (NAA)	Sigma Aldrich	N-1641
1-Natriumdodecyl sulfat (SDS)	Duchefa	S1377
2-propanol	Merck	1,09634
6-benzylaminopurine (BAP)	Sigma Aldrich	B-3408
Absolute alcohol prima	Kemetyl	E41332
Acetic acid	Sigma Aldrich	A6283
Acrylamide	Sigma Aldrich	A9926
Agarose	Sigma Aldrich	A9539
Ammonium persulfate (APS)	Sigma Aldrich	A-9164
Ampicillin	Sigma Aldrich	A-9518
Bacto agar	BD	214010
Bacto yeast extract	BD	212750
Boric acid	Duchefa Biochemie	B0503.1000
Broad range protein molecular markers	Promega	V849A
Bromophenol Blue	Merck	1,11746
Calcium chloride ($CaCL_2$)	Sigma Aldrich	05292-1ML-F
Chloramphenicol	Sigma Aldrich	C-0378
Dithiothreitol (DTT)	Sigma Aldrich	D-0632
EDTA	Duchefa Biochemie	E0511.0500
Ethidiumbromide 0.07 %	VWR	429932N
Gelzan CM	Sigma Aldrich	G1910
Glycerol	Duchefa Biochemie	G1345.1000
Glycin	Merck	4201
Kanamycin sulfate	Sigma Aldrich	K-1377
Magnesium chloride ($MgCl_2$)	Applied Biosystems	58002032
Mes hydrate	Sigma Aldrich	M2933-25
Methanol	Merck	1,06009
Murashige & Skoog medium	Duchefa Biochemie	M0221.0001
Rectified ethanol	Kemetyl	E41331
Skim milk powder	Fluka	70166
Sodium chloride (NaCl)	Merck	1.06404.1000
Sodium dodecyl sulfate (SDS)	Sigma Aldrich	L4390
Spectinomycin	Sigma Aldrich	S-9007
Streptomycin sulfate	Sigma Aldrich	S-6501
Sucrose crystallized	Duchefa Biochemie	S0809.1000
Tetramethylethylenediamine (TMED)	Sigma Aldrich	T-9281
Thimerosal	Sigma Aldrich	T-5125
Tris base	Duchefa Biochemie	T1501.1000
Tryptone	Duchefa Biochemie	T1332.0500
Tween 20	Sigma Aldrich	P1379
X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside)	Sigma Aldrich	B-4252-250

3.9. Enzymes and Antibodies

Name	*Used on*	*Supplier*	*Catalogue Number*
*BamH*I	pDK60	New England Biolabs	R0136
*Xba*I	pDK60	New England Biolabs	R0145
*Sma*I	pPCR-Script	New England Biolabs	R0141
*Spe*I	pSRN	New England Biolabs	R0133
*Nde*I	pSRN	New England Biolabs	R0111
T4 DNA ligase	All cloning	New England Biolabs	M0202
Extra Avidin -Alkaline phosphatase	Western Blot	Sigma Aldrich	E2636
Affinity Purified Rabbit anti-6His	Western Blot	Immunology Consultants Laboratory , Inc	RHIS-45A-Z
Anti-Rabbit	Western Blot	Made in the lab	

3.10. Antibiotics, Hormones and Vitamins

Name	*Used on*	*Stock concentration*	*Working Concentration*
Ampicillin	E. coli	100 mg/ml	100 µg/ml
Chloramphenicol	E. coli	34 mg/ml	
Kanamycin	E. coli, A. Tumefacien and TobaccoN.	50 mg/ml	50 µg/ml
Cefotaxim	A. Tumefacien	500 mg/ml	500 µg/ml
Streptomycin	TobaccoN.	100 mg/ml	500 µg/ml
Spectinomycin	TobaccoN.	100 mg/ml	500 µg/ml
1-Naphthaleneacetic acid (NAA)	TobaccoN.	1 mg/ml	0,1 µg/ml
6-Benzylaminopurine (BAP)	TobaccoN.	1 mg/ml	1,0 µg/ml
Thiamin-HCl	TobaccoN.	1 mg/ml	0,9 µg/ml

Antibiotics and hormones were dissolved in mQH_2O and sterilized with a 0,2 µm filter.

3.11. Growth medium

Name	*Recipe*
LB (lysogeny broth) solution	10 mg Tryptone, 5 g Yeast Extract and 10 g Sodium Chloride was dissolved in water in a total volume of 1 liter. The pH was adjusted to 7,0 before being autoclaved.
LB (lysogeny broth) agar medium	1 litre LB- solution pH 7,0 was added 15 g Bacto Agar. The medium was autoclaved and then cooled down to about 55 °C before antibiotics was added and the medium was poured in 9 cm petridishes.
MS-II solution	4,71 g MS salt, 20,0 g Sucrose and 0,5 g MES Hydrate was dissolved in water of a total volume of 1 liter with pH adjusted to 5,7-5,8. The solution was autoclaved.
MS-II medium	1 liter MS-II solution pH 5,7-5,8 was added 3,3 g Gelzan before being autoclaved.
Co-cultivation medium	1 liter MS-II medium was cooled to about 55 °C before 0,1 µg/ml NAA and 1,0 µg/ml BAP was added and the medium was poured in 9 cm petridishes.
MS-II Selection medium	1 liter co-cultivation medium with 100 µg/ml Kanamycin and 500 µg/ml Cefotaxim added to the medium along with the hormones.
Hormone free MS-medium for root induction	MS-II medium was prepared with only half MS, 2,36 g MS salt in 1 liter. The medium was autoclaved, cooled and poured into magenta boxes.
RMOP medium	4,71 g MS salt, 30 g sucrose, and 3,3 g Gelzan were dissolved in water with a total volume of 1 liter with pH adjusted to 5,7-5,8. The medium was autoclaved and cooled down.
RMOP medium with selection	RMOP medium was prepared and autoclaved. When the temperature had cooled to about 55 °C, additional 900 µl Thiamine-HCl (1 mg/ml), 100 µl NAA (1 mg/ml), 1000 µl BAP (1 mg/ml) and 5 ml Spectinomycin (100 mg/ml) were added and mixed well. The medium was poured into petridishes.
RMOP medium with double selection	RMOP medium with selection was added additional 5 ml Streptomycin (100 mg/ml) for double selection.

All medium plates and boxes dried to become solid for about 15 minutes and were stored at 4 °C.

3.12. Buffers

Name	*Recipe*
10x TBE pH 8,0	90 mM Tris Base, 90 mM Boric Acid and 2 mM EDTA in water of a total volume of 1 liter.
6x Loading Buffer	25 % Bromophenol blue and 40 % Sucrose in water. Use 1:5 ratio 6x lading buffer:sample
Protein Extraction Buffer	100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl pH 8,0, 0,05% Tween 20, 0,1 %SDS, 14 mM DTT, 200 mM Sucrose and 2 mM PMSF were mixed with water.
5x Running Buffer	Add 15,1 g Tris Base, 94 g Glycin pH 8,3 and 25 ml SDS 20 % in water with a total volume of 1 liter.
4x Loading Buffer	0,7 ml dH2O, 4,8 ml Tris-HCl 0,5 M pH 6,8, 4,0 ml Glycerol 100 %, 0,8 g SDS 20%, 0,4 ml B-Mercaptoethanol and 0,004 g Bromphenol Blue 1 %
Staining Buffer	0,1 g Comassie brilliant blu, 30 ml water, 50 ml Methanol and 20 ml Acetic Acid
Destaining Buffer	65 ml water, 25 ml Methanol and 10 ml Acetic Acid
10x Tris-Buffered Saline (TBS Buffer) pH 7,5	20 mM Tris and 5 M Sodium Chloride in water
1x TTBS buffer	1x TBS with 200x Tween 20.

3.13. Instruments and Special Equipments

Used for	*Name*	*Supplier*	*Catalogue Number*
PCR	T100 Thermal Cycler	BioRad Laboratories	186-1096
	C1000 Thermal Cycler	BioRad Laboratories	185-1148
	PCR Strip Tubes	VWR	53509-309
Centrifuge	Centrifuge	Eppendorf	5810 R
	Haraeus Biofuge Pico	Kendro Laboratory Products	75003280
	Heraeus Fresco 21 Centrifuge	Thermo Fisher Scientific	75002425
Electrophoresis	PowerPac Basic Power Supply	BioRad Laboratories	164-5050
	Mini-Sub Cell GT Base	BioRad Laboratories	170-4360
	Wide Mini-Sub Cell GT Base	BioRad Laboratories	170-4370
	Sub-Cell GT Base	BioRad Laboratories	170-4390
	SDS-PAGE Electrophoresis	BioRad Laboratories	166-5060EDU
	Molecular Imager Gel Doc XR System	BioRad Laboratories	
Plastid Transformation	PDS-1000 He Hepta System	BioRad Laboratories	165-2258
	1.5 ml high-quality tubes	VWR high G-force	211-0015
	BD Falcon Disposable Petri dishes	Becton Dickinson	351005
Electroporator	ECM 630 Electroporator	BTX Harvard Apparatus	45-0001
	Electroporation cuvettes 1mm gap	VWR Scientific Products	47727-640
Spectrophotometer	UV-1601 CE	Shimadzu Corporation	206-67001-34
	Nano Drop 2000	Thermo Scientific	2000
Laf-bench	Hera Safe KS 12	Thermo Electron	51022515

		Corporation	
Blotting Machine	iBlot Gel Transfer Device	Invitrogen	IB1001

3.14. Softwares and Bioinformatic tools

Program	*Supplier*
Vector NTI Advance 11.0	Invitrogen
CLC Main Workbench 6.6.2	CLC Bio
PrimerSelect 5.05	Lasergene
Quantity One v 4.5.0	BioRad Laboratories
Standard Nucleotide BLAST	The National Center for Biotechnology Information (available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi)
Sequence Editor	Nikolaus Pawlowski, Fr33 (available at: http://www.fr33.net/seqedit.php)
Primer 3 Plus	Steve Rozen and Helen J. Skaletsky (2000) (available at: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)
Expasy bioinformatics resource portal	Swiss Institute of Bioinformatics, SIB Web Team (available at : http://expasy.org/)
LIGation CALCulator	Insilico Online Bioinformatics Resources (available at:http://www.insilico.uni-duesseldorf.de/Lig_Input.html)
Gibthon Ligation Calculator	Gibthon (available at: http://django.gibthon.org/tools/ligcalc/)

4. Methods

4.1. MOLECULAR ANALYSIS

Molecular methods were performed to analyze DNA and proteins. The antigen nucleotide sequences were cloned into vectors for amplification in bacteria and for transformation of tobacco. The plants were analyzed to see if they were true transgenic and transplastomic plants. Further analysis would confirm expression of the EDIII genes and quantification of the protein. The proteins would then be analyzed for immunologic recognition by binding to antibodies.

4.1.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a molecular method that can amplify a specific short segment of genomic DNA or cDNA. The technique requires a thermal cycler instrument and sample containing template DNA, buffer, single nucleotides, polymerase for synthesis and primers to define the segment to be amplified. The technique is commonly used to detect a specific gene or DNA segment using desirable primers. DNA is denatured to be single stranded (ssDNA) which allows short sequences of complementary DNA called primers to bind to the ssDNA. Enzymes called DNA polymerase will then bind to the primers and synthesize a complementary sequence from the 3’ end of the primer.

The thermal cycler

The thermal cycler will adjust the temperature in different steps and cycles to amplify the segment. The first step in the thermal cycler is heating the sample to 95 °C in order to denature the double stranded DNA (dsDNA) to ssDNA. Lowering the temperature to the primers annealing temperature will allow the primers to bind to complementary sequences. The annealing temperature is usually 55 °C. One primer is complementary to a segment on the 3’ end of the sense strand (coding strand) and the other primer is complementary to a segment on the 3’ end on the other strand called antisense strand (non-coding strand). The primers bind to the complementary sequence and form a short sequence of dsDNA. The

temperature is increased to 72 °C where DNA polymerase will bind to the dsDNA and synthesize complementary nucleotides to the template strand from the 3' end of the primer. The denaturation, primer annealing and synthase elongation steps will be repeated in 25-35 cycles. In the second step the newly synthesized DNA strands will also be used as template and the syntheses will be terminated as the polymerase reaches the primer sequence in the 5' end. The amplification will grow exponentially as the number of template doubles for each round. One DNA template can be copied to 1 million after 20 cycles and 1 billion after 30 cycles (Acquaah 2004)

Polymerase

Platinum Pfx polymerase is a high quality proof reading polymerase from *Thermococcus* species KOD. The Pfx polymerase has 26 times higher fidelity than Taq DNA polymerase (Invitrogen)

Taq DNA polymerase is derived from *Thermus aquaticus* and commonly used in standard PCR reactions. Using Taq DNA polymerase in PCR detection is very efficient. When amplifying a nucleotide sequence that should be used further in cloning, Pfx polymerase offers higher proof reading.

Template and primers

PCR is a very sensitive technique that can utilize very small amounts of DNA, but the high sensitivity is also a drawback to contaminations. In order to detect a sequence in PCR, some information of the template sequence must be known to choose specific primers. When designing primers the usual requirements is that the GC content should be 40-60 % and the melting temperature, Tm, should be 50-60 °C. Cytosine and guanine are binding together with three hydrogen bonds, whereas adenine and thymine only have two hydrogen bonds between them. The guanine and cytosine needs higher temperature to be denatured and parted than adenine and thymine. The annealing temperature in a PCR is usually 55 °C.

By lowering the annealing temperature in a PCR program the primers bind more easily but the sensitivity decreases. Increased temperatures can increase sensitivity but annealing might be less efficient.

4.1.1.1. Protocol for PCR

PCR with Pfx polymerase for high quality proof reading

PCR-products were amplified with a high quality proof reading Pfx polymerase to maintain the right sequence of the antigens for further cloning work.

Table 4.1: PCR reaction volume with Pfx polymerase

	Reagents	*Stock concentration*	*Final Concentration*
2,5 µl	Pfx Amplification Buffer	10x	1x
3,0 µl	dNTPs	2,5 mM	0,3 mM
0,5 µl	MgSo4	50 mM	1,0 mM
0,5 µl	Primer Sense	20 µM	0,1 – 1,0 µM
0,5 µl	Primer Antisense	20 µM	0,1 – 1,0 µM
0,2 µl	Platinum Pfx DNA polymerase		
1,0- 2,5 µl	Template		
	mQH_2O		
25,0 µl	Total Volume		

Table 4.2: PCR-program with Pfx polymerase

Step	Temperature °C		Time
Initial Denaturation	94		5:00
Denaturation	94		0:15
Annealing	55	X35	0:30
Extension	68		1:00 pr kb
Final extension	68		5:00
Cooling	4		∞

PCR with Taq polymerase for standard screening analysis

Taq polymerase has low fidelity compared to Pfx polymerase but it is common in screening analysis for detection. PCR-screening was performed on colonies to confirm that the gene of interest was cloned into the vector. By amplifying regions outside the insertion site on the vector the sizes of the PCR-products would indicate if the cloning was done successfully.

PCR-screening was also performed on DNA extractions from Tobaccoto confirm that plants were transgenic.

Table 4.3: PCR reaction volumes with Taq polymerase

	Reagents	Stock consentration	Final concentration
2,5 µl	Buffer	10x	1x
1,5 µl	$MgCl_2$	1,5 mM	3 µM
2,0 µl	dNTP	2,5 mM	0,2 mM
0,5 µl	Primer forward Sense	20 µM	0,1 – 1,0 µM
0,5 µl	Primer reverse Antisense	20 µM	0,1 – 1,0 µM
0,125 µl	Taq polymerase		25 units/ml
1,0–2,0 µl	DNA Template		0,01 – 1,0µg
	mQH_20		
25,0 µl	Total Volume		

Table 4.4: PCR-program with Taq polymerase

Step	Temperature °C		Time
Initial Denaturation	95		5-8:00
Denaturation	95		0:30
Annealing	52-55	X30-	0:30
Extension	72	35	1:00 per kb
Final extension	72		4-7:00
Cooling	4		∞

4.1.2. Agarose Gel Electrophoresis

Agarose Gel electrophoresis is a method used to separate and visualize DNA fragments or enzyme digested genomic DNAs. The separation depends on size, structure and charge of the DNA. DNA is negatively charged and can thus be separated in an agarose gel in an electrical field in which the DNA will migrate towards the positive electrode.

Agarose gel

Agarose makes pores in the gel that allow DNA fragments to migrate in the gel. Depending on the sizes of the DNA fragments to be separated, the agarose resolution can be adjusted. A typical gel containing 1% agarose have an optimum resolution of 500 bp to 10 kbp.

Sample preparation

A loading buffer containing dye and sucrose are added to the DNA samples. The sucrose binds to the DNA fragments and makes it denser than the running buffer, allowing the DNA to sink to the bottom of the wells in the gel and prevent diffusion. The dye will visualize the migration efficiency. The samples are loaded onto wells in the agarose gel. A ladder with different DNA markers of certain lengths is also added on the gel to identify the sizes of the separated linear DNA fragments. Ladders can only tell the length of linear DNA fragments where the migration and the sizes correlate, whereas plasmids will coil and then the migration will be unpredictable.

Buffers

Tris-borate-EDTA (TBE) buffer maintains a suitable environment for the DNA. The buffer contains Tris base, boric acid and EDTA. The Tris base keeps the DNA deprotonated and The EDTA prevents the DNA from degradation. Agarose gels are prepared by dissolving agarose in 1x TBE and upon electrophoresis the gel is surrounded by 1x TBE buffer

A loading buffer is added to the samples prior to electrophoresis. Sucrose in the buffer binds to the DNA and ensures that the DNA drops evenly into the wells. Bromophenol blue colours the samples which make it easier to load them onto the gel. The migration in the gel can be traced due to the bromophenol blue dye that has a migration rate that roughly equals the same as for dsDNA fragments of 300 bp.

Electrophoresis and imaging

Current are applied to the gel chamber. The DNA fragments in the samples will be separated according to their size and structure. The smaller fragments, the less resistance in the gel and they will migrate further down the gel compared to bigger fragments. Coiled circular DNA fragments will meet less resistance than linear DNA fragments.

The gel contains Ethidium Bromide for staining. The Ethidium Bromide binds to the hydrogen bonds in the double stranded DNA. UV-lights will make the Ethidium Bromide fluorescence and the DNA fragments will be visualized.

Interpretation of the gel results

DNA ladders on the gel show several bands of known sizes. The length of the fragments in the DNA samples can be identified by comparison with a DNA ladder.

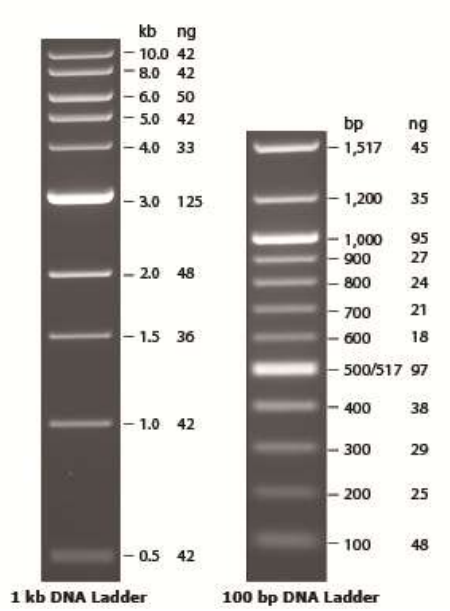


Figure 4.1 DNA Ladders for agarose gel electrophoresis. The ladders are applied on an agarose gel and separated along with DNA samples. After electrophoresis the different fragments in the ladders will be separated as illustrated, and the fragments in the DNA samples are compared to the known sizes in the DNA ladders. (New England Biolabs)

A successful PCR will amplify DNA fragments of a certain size based on the primer pair. A gel electrophoresis can confirm that the right fragment have been amplified and thus is present in the sample. No band on the gel indicates that the DNA sequence to be amplified is not present in the sample or that the PCR were unsuccessful. All controls (both positive and negative) are necessary to be included in the PCR reactions to confirm the results.

4.1.2.1. Protocol for agarose gel electrophoresis

Table 4.5: Agarose Gel

Agarose gel	
1x	TBE buffer
1 %	Agarose
0,6 µg/ml	Ethidiumbromide

Usually a 50 or 100 ml gel was prepared with 1 % agarose.

Agarose was added to 1xTBE buffer and the agarose was dissolved by heating in microwave oven. The gel was cooled down to 60 °C before1 droplet Ethidiumbromide was added for each 50 ml gel volume. The gel was poured into a casting tray and sample comb was inserted. The gel cooled for 20-30 minutes to become solid. The electrophoresis chamber was filled with 1xTBE buffer and the gel was placed in the chamber.

Samples were added 6x loading buffer before being applied on the gel. Voltage of 70-110 was added for 40-90 minutes depending on the gel size and the sizes of the fragments in the samples.

The gel was photographed in UV light to visualize the DNA.

4.1.3. Isolation of DNA

DNA from bacteria or plants has to be extracted and isolated in order to perform any analysis. DNA techniques are depending on pure DNA samples for good results. Isolation of DNA is done with commercialized kits that contain a variety of solutions and columns that can bind the DNA of interest. The kits have different aplications based on DNA sample, size, structure and DNA extracting host cells.

Plasmids from bacteria (Biorad, Quantum Prep® Plasmid Mini/ Midi prep Kit) were extracted with a kit that uses alkaline lysis method for purifying plasmid DNA. The column is made of silicon dioxide exoskeleton of diatoms that bind plasmid DNA. The columns binding capacity for a mini prep is 25 µg DNA from 1,5 ml culture and for midi prep 100-300 µg DNA from 40 ml culture.

Extraction of genomic DNA from Plants (Qiagen, Dneasy Plant Mini kit) was done with columns that have silica-based membrane which bind DNA in the presence of high concentrations of chaotropic salt while carbohydrates, polyphenolics and other plant metabolites are removed. The membrane can bind DNA molecules up to 40 kb in size but the dominating fragments will range between 20-25 kb. The mini kit can process tissue up to 100 mg and the maxi kit can process 1 g tissue. The binding capacities of the columns are 50 µg

for a mini column and 500 µg for a maxi column but the yields are typically 3–30 µg with mini kit and 30–260 µg with maxi kit due to the recommended amounts of starting material.

DNA from PCR-products or gels (Promega, Wizard SV Gel and PCR Clean-up system) were also collected with silica membranes with the principle of DNA binding upon presence of chaotrophic salts. DNA in gel are cut out and melted in a buffer prior to extraction. The column membrane can bind up to 40 µg DNA. Fragments form 100 bp to 10 kb can be recovered and depending on the fragment sizes a recovery up to 95 % of total DNA can be achieved.

4.1.3.1. Isolation of Plasmids from Bacterial culture

Plasmids were isolated by Quantum Prep Plasmid Miniprep Kit or Quantum Prep Plasmid Midiprep Kit from Biorad. 1-5 ml culture was used in a miniprep and 50-100 µl water or TE-buffer was used for elution. 40-50 ml culture was used in a midiprep and the DNA was eluted in 300-600 µl water or TE-buffer.

4.1.3.2. Isolation of Genomic DNA from Plant Tissue

Genomic DNA from the plants was isolated using Dneasy Plant Mini Kit from Qiagen. 20 mg dry weight of homogenized tissue was used for DNA isolation and the DNA was eluted in 50-400 µl elution buffer.

4.1.3.3. Isolation of DNA from agarose gel or PCR-products

DNA from PCR products or from agarose gels were isolated and purified using Wizard SV Gel and PCR Clean-up system from Promega. DNA was eluted in 50 µl nuclease free water.

4.1.4. Quantification of DNA

Nanodrop is a spectrophotometer that offers both retention technology and standard cuvettes for sample measurements. The retention technology can measure samples of high concentrations within the full spectrum of 190-840 nm wavelengths with very small volumes. The technology offers high accuracy and reproducibility. Sample of 0,5-2,0 µl are loaded onto the instrument and the surface tension keeps the droplet in between two optical fibers. The retention technology can measure sample concentrations 200 times higher compared to a

standard cuvette measurement. It is not necessary to dilute samples for measurements with the retention technology and it makes it ideal for measuring small sample volumes.

Nucleic acids have an absorbance maximum at 260 nm wavelength. Concentration of DNA in the samples is determined from the measured absorbance at 260 nm. A blank reference sample will be measured at first to calibrate the readings and eliminate noise. Quality controls measures the purity of the sample. A ratio is calculated from the measurements at 260 and 280 nm wavelength where the desired ratio should be ~1,8-1,9. Lower ratios may indicate presence of contaminations such as proteins. A secondary purity control measures the absorbance at 260 and 230 nm where pure samples have a ratio of 1,8-2,2. Lower ratios may indicate the presence of co-purified contaminations. A pure sample will give higher accuracy. The NanoDrop can measure DNA up to 15 000 ng/µl with high accuracy.

(Thermo Scientific, NanoDrop 2000/2000c Spectrophotometer V1.0 User Manual, 2009)

4.1.4.1. Protocol for NanoDrop

DNA was quantified using NanoDrop Spectrophotometer. The spectrophotometer was set to measure nucleotide which has a maximum wavelength of 260 nm. A blank sample was first measured as a reference. 2 µl sample was loaded onto the spectrophotometer and the absorbance was measured at 260 wavelength. As a quality control for the purity of the sample the A260/280 ratio should have a value of 1,8-1,9. The concentration of the nucleic acids is calculated based on the measurements.

For some samples the NanoDrop measurements seemed unreliable and they were tested on agarose gel by comparing their intensities with mass markers. 6 µl of 1kb ladder was applied on the gel where the band of 3 kb would contain about 500 µg DNA. The band intensity of the 3 kb lane was used as an indication of the amount of DNA in the bands of the samples.

4.1.5. DNA Sequencing

DNA Sequencing is a method to reveal the nucleotide sequence of a gene or a nucleotide. As in PCR, primers will bind to ssDNA template and a polymerase will synthesize a dsDNA molecule. Sequences of Adenine, Thymine, Guanine and Cytosine called deoxyribonucleic triphosphates (dNTPs) make genes and genetic material as dsDNA. DNA sequencing uses a mix of these dNTPs, as well as fluorescently labeled di-deoxyribonucleic triphosphates (ddNTPs) to synthesize nucleotide sequences in order to determine the sequence. The ddNTPs lack 3'–OH and cannot bind a nucleotide. When a ddNTP is incorporated to a sequence during synthesis it will prevent further binding and synthesis, which will terminate the sequence. After multiple cycles of synthesis, products of all lengths will be provided with a fluorescently labelled ddNTP terminating the sequences. Each of the ddNTPs; ddATP, ddGTP, ddCTP and ddTTP have different fluorescent markers.

The amplified DNA fragments are separated according to size with electrophoresis (chromatography) and they will eventually pass a laser detector that will measure a signal from the fluorescent tag. The signals will be converted to digital data and a sequence will be presented. (Klug, 2010)

4.1.5.1. Protocol for sequencing

Sequencing was performed by GATC Biotech. DNA samples and primers were prepared according to GATC's preferences and recommendations. The results were assembled in CLC Workbench to verify the sequences. pDK60-Tetra was sequenced with reverse primer TrnfM, forward primer aadA, Prrn and gene specific Tetra screening primer to cover the entire sequence of Tetra, whereas EDIII4 in pDK60 was sequenced with forward aadA and reverse trnfM primers.

pSRN-Tetra was sequenced with reverse primer p275 reverse and forward primers p292, pAlcA and gene specific Tetra screening to identify Tetra. pSRN-EDIII4 was sequenced with forward pAlcA primer and p275 reverse primer.

4.1.6. SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) is a method for identification and screening of proteins. Proteins are made up of peptide chains of amino

acids. The different amino acids have different charges and structural properties. Polypeptides will make internal bindings between amino acid residues that will cause the peptide to fold according to its nature and form a tertiary structure or alternatively quaternary structure of multi-subunit proteins. In order to distinguish and identify proteins by analysis their structure must be reversed to primary peptide where their properties can be utilized.

Sodium dodecyl sulphate (SDS) is a strong ionic detergent that along with a reducing agent such as mercaptoethanol can unfold proteins. Mercaptoethanol break disulfide bonds within and between peptides and the protein loses its tertiary structure. SDS coats the peptide to keep it an unstructured polymer with uniform negative charge. Proteins with presence of SDS can then be separated according to their length in a polyacrylamide gel with electrophoresis (Watson, Baker et al. 2004). Protein Ladders will indicate the sizes of the proteins in the samples. Protein patterns on the gel of transgenic plants will be compared to wild-type plant proteins to show if there are any differences in the pattern.

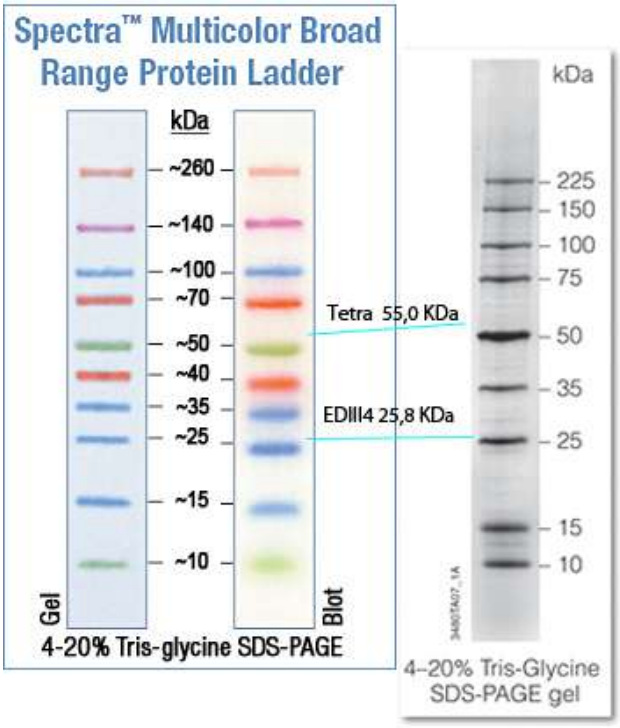


Figure 4.2: Protein ladders for SDS-PAGE. The protein ladder to the left is used for ordinary SDS-PAGE analysis, whereas the protein ladder to the right is used on SDS-PAGE for Western analysis. The expected sizes of EDIII4 peptides is 25,8 KDa and 55,0 KDa for Tetra peptides.

4.1.6.1 Protocol for SDS-PAGE

Isolation of soluble proteins from tobacco

Proteins had to be extracted from the plants in order to analyze the protein expressions. Plant material was homogenized in an extraction buffer that breaks the cell membranes and free

soluble proteins. It is important to work fast and keep the protein samples cold to prevent the proteins to be degraded.

Table 4.6: Protein Extraction buffer

	Reagents	Final Concentration
1 ml	NaCl 1M	100 mM
200 µl	EDTA 0,5M	10 mM
2 ml	Tris-HCl pH 8,0 1M	200 mM
5 µl	Tween 20	0,05 %
50 µl	SDS 20 %	0,1 %
140 µ	DTT 1M	14 mM
2 ml	Sucrose 1M	200 mM
200 µl	PMSF 100 mM	2 mM
Up to 10 ml	Water	

200 µg crushed tobacco tissue was homogenized in 200 µl Extraction Buffer by using a pestel and by vortexing. The tubes were kept on ice during and after homogenization to prevent protein degradation. Centrifugation with 21 000 x g at 4 °C for 3 minutes separated the soluble and insoluble proteins. The tissue was homogenized again to increase the amount of free soluble proteins. Another centrifugation for 10 minutes pelleted the insoluble proteins and left the soluble proteins in the supernatant which was transferred to a new tube. No residue of insoluble protein and tissue should be left in the sample and additional centrifugation was done to remove them if necessary. The samples were boiled for 5 minutes at 95 °C causing some of the samples to precipitate. By adding SDS up to 2 % the samples got more fluid again. The samples were stored at -80 °C.

Polyacrylamide gel

Table 4.7: 12 % Resolving gel

1,5 mm gel	Reagents	Stock
3,296 ml	Water	
1,875 ml	Tris	1,5 M pH 8,8
37,5 µl	SDS	20 %
2,25 ml	Acrylamide	40 %
37,5 µl	APS	10 %
3,75 µl	TMEd	

Table 4.8: 4 % Stacking gel

1,5 mm gel	Reagents	Stock
3,225 ml	Water	
1,25 ml	Tris	0,5 M pH 6,8
25 µl	SDS	20 %
0,5 ml	Acrylamide	40 %
25 µl	APS	10 %
5 µl	TMEd	
7,5 ml		

The 12 % gel was prepared and 7,5 ml gel was immediately poured into the 1,5 mm gel holder. 1 ml 2-propanol was added on the top of the gel. The gel was solid after 20-30 minutes. The 2-propanol was washed off with sterile water and the excess water was dried off. The stacking gel was prepared and immediately poured into the gel holder on top of the resolving gel. The gel comb was installed and the gel was left to dry for 30-40 minutes. The gel was then ready to be used or it could be stored for up to 2 weeks by wrapping the gel (holder) in moist paper in a sealed bag at 4 °C.

Electrophoresis

The gel was installed in the electrophoresis chamber and 1x running buffer was added. 30 µl protein sample was added 10 µl 4x Loading buffer and the samples was loaded onto the gel along with 5 µl ladder. 66 Volts were added for 20-30 minutes to allow the proteins to migrate through the stacking gel. When the proteins had reached the resolving gel the Voltage was increased to 120 for another 40-50 minutes until the proteins had migrated through at least ¾ of the resolving gel.

Staining

The gels were stained with staining buffer for 1-2 hours with shaking. The buffer was discarded and replaced with destaining buffer overnight. The gels were dried and photographed.

4.1.7. Western Blot

Western Blot is an analytical technique used to detect specific proteins with antibody specific binding. Western Blot Analysis was done to detect the antigens of EDIII4 and Tetra with Anti-His immunoblotting and secondary antibody Anti-rabbit.

A SDS-PAGE with separated proteins is put on top of a nitrocellulose membrane. The gel is covered with moist filter paper and a sponge on the top and the total assembly is applied to a closed electric field where current force the proteins towards the membrane due to hydrophobic interactions and charged interactions between the membrane and protein.

After blotting the proteins onto the membrane, a solution containing skim milk powder are added to block the unoccupied sites on the membrane. A solution with primary antibody is then added that will bind specifically to the protein of interest. Excess antibodies are discarded and a secondary antibody is added that will bind specifically to the bound primary antibody. The secondary antibody is biotinylated and has a high affinity for Alkaline Phosphatase. Alkaline phosphatase is added and it will bind in multiple to the biotinylated secondary antibody which in turn will bind more color development reagents and enhance the color visualization.

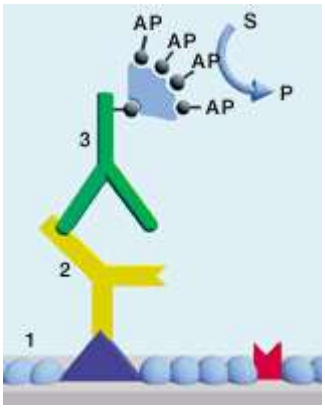


Figure 4.3: Illustration of Western Blot Principle. 1) Milk has blocked the unbound membrane. 2) Primary Antibody bound specifically to the protein (blue triangle). 3) Biotinylated Secondary Antibody bound to the Primary Antibody. Streptavidin-biotinylated Alkaline Phosphatase (AP) complex binds as a precipitate to the Secondary Antibody that will amplify color signal when color development reagent is added (Biorad 2012).

4.1.7.1. Protocol for Western Blot

Blotting

The blotting was done by iBlot Device (Invitrogen) with iBlot Gel Transfer Stacks Nitrocellulose Kit containing nitrocellulose membrane, filter paper, sponge, cathode stack and anode stack. The gel was put on nitrocellulose filter and the gel was covered with a moist filter paper and a sponge. The cathode stack at the bottom and the anode stack on the top made a closed electrical field in the iBlot Device instrument that made the proteins migrate towards the nitrocellulose membrane during six minutes with program P3.

Blocking

The membrane was put in a petridish and TTBS buffer with 2,5-7 % skim milk was added to block the proteins to the membrane. The dishes incubated with modest circulation at room temperature for 30 minutes or overnight at 4 °C.

Primary Antibody binding

Primary Antibody solution was prepared with 1000x Anti-His in 1x TTBS. The blocking solution was discarded and Primary Antibodies of Anti-His was added to the membranes for 2 hours incubation at room temperature with shaking. The membranes were then washed with 1x TTBS to remove excess antibodies with washing every 5 minutes for 5 times with shaking in the meantime.

Secondary Antibody binding

Secondary Antibody solution was prepared with 30 000x Anti-Rabbit in 1 x TTBS. The membranes were added Secondary Antibody Solution and was left for incubation at room temperature for 1 hour with shaking. The solution was then discarded and the antibodies were washed off with additional 5 steps of washing every fifth minute.

Detection

Streptavidin-conjugated Alkaline Phosphatase solution was prepared with 40 000x Alkaline Phosphatase in 1x TTBS. The solution was added to the washed membranes and incubated with shaking for 2 hour at room temperature or overnight at 4 °C. The solution was then washed off with additional 5 steps of washing every 5 minutes.

Colour Development Buffer was made by mixing 1x AP Colour Development Buffer and 100x of each AP colour reagent A and B. 3-4 ml Colour Development Buffer was added to each membrane until the membranes were sufficiently developed. The Buffer was discarded and the membranes were washed in double distilled water for 10 minutes. The membranes were air drying before they were photographed.

4.2. CONSTRUCTION OF RECOMBINED PLASMID VECTORS

The antigen sequences must be integrated in a plasmid vector in order to be introduced into the plant cells. The vector carrying the target gene also contains selectable marker gene and genes necessary for introducing the target gene into the host's genetic material. The target genes are recombined into vector plasmids by digesting the vector and the target gene at specific sites and followed by ligation of the target gene to the vector. The recombined plasmid is transferred into bacteria cells. Transgenic colonies growing on selection medium are cultivated for cloning of the recombined plasmids.

The nucleotide sequences of EDIII4 and Tetra was cloned into pDK60 vector for plastid transformation and in pSRN vector for nuclear transformation.

4.2.1. Primerdesign

When constructing a recombined expression vector, the target gene must be placed downstream of a promoter in the vector. The target gene must be inserted with the same direction as the promoter and establishing the direction is done by using two different restriction enzymes. Restriction enzymes in the vector are chosen for the purpose and they must be added to the target gene if not present. Adding restriction sites to the target gene is accomplished with primers. Primers are designed with a complementary sequence to the start or the end of the gene and the restriction site sequence is added to the 5' end of the primer. Some enzymes cannot cut a site at the start of a sequence and need additional base pairs for stabilization.

Primers for PCR screening analysis was designed in Vector NTI or Primer 3 Plus. Primers were set to have melting temperature of 55-60 °C with GC content of 40-60 % and length of about 20 bp. Some of the screening primers failed in sequencing and new primers were specifically designed for the purpose of sequencing. The requirements for the sequencing primers were length of 17-19 bp, with ideally G/C content of 10 bases for a 17mer, 8-9 for 18mer and 7-9 for a 19mer. Repeats of identical nucleotides should not exceed 4 bases and the primers should have a good mix of all four nucleotides.

4.2.1.1. Primerdesign for addition of restriction site

EDIII4 and Tetra were cloned into pDK60 with BamHI and XbaI site, whereas NdeI and SpeI site was going to be used for cloning in pSRN vector. EDIII4 and Tetra in pUC57 did not match the restriction sites at right place, except from XbaI. Primers were designed to bind the restriction sites to the gene sequences in order to get the right restriction digests for cloning in pDK60 and pSRN. The primers had a complementary sequence of 15-20 bp where the forward primer started at the start codon and the reverse primers started at the stop-codon. At the 5' end of the primers the restriction site sequences was added. The enzyme supplier recommended additional six bp upstream of the restriction site in order for the enzyme to bind and stabilize properly. This was only done for the primers used for pSRN cloning.

The primers designed for cloning in pSRN was named EDIII4 SpeI Forward, EDIII4 NdeI Reverse, Tetra SpeI Forward and Tetra NdeI Reverse. The primer pair for EDIII4 would amplify fragments of 390 bp and the primer pairs for Tetra would amplify fragments of 1371 bp.

The primers for pDK60 cloning were named BamHI EDIII4 Forward, XbaI EDIII4 Reverse, BamHI Tetra Forward and XbaI Tetra Reverse. The primers gave a PCR-product that contained BamHI site upstream of the gene of interest and XbaI site downstream in a total product of 378 bp for EDIII4 and 1358 bp for Tetra.

The primers were designed in Vector NTI. The complementary sequences were adjusted to reach a melting temperature, Tm, of 55-60 °C, before adding the tail for enzyme binding. The primers were also checked for primer hairpin, primer self-dimer and primer pair dimer in DNA Star's Primer Select.

4.2.2. Sub-cloning with pPCR-Script

The restriction enzyme failed to cut the PCR-products of EDIII4 and Tetra, even the PCR-products that had additional basepairs upstream of the restriction site as recommended from the enzyme supplier. In order to digest the nucleotide sequences of EDIII4 and Tetra properly they had to be sub-cloned into pPCR-Script.

Table 4.9: pPCR-Script Reaction

µl	Reagents
2,0	pPCR-Script vector
2,0	T4 ligase buffer
1,0	rATP
0,4	*Sma*I
2,0	T4 DNA ligase
-	Insert product
-	Water
20	Total

*Sma*I digested the vector and left blunt ends where the PCR-products of EDIII4 or Tetra (recombined with BamHI/XbaI for pDK60 cloning and SpeI/NdeI for pSRN cloning) should be inserted and ligated in a non-directional manner. 1-3 µg of the PCR-products were added to the reaction mixture. After 4 hours of incubation at room temperature *E. coli* was transformed with 2 µl of the product in 50 µl cells. Transfomed cells were selected with blue-white screening and Chloramphenicol selection.

6 white colonies from pPCR-Script Tetra were screened with gene specific primers and with M13 primers. Positive colonies were cultured and plasmids were isolated for restriction digest.

4.2.3. Restriction digest of DNA with Endonucleases

Restriction enzymes are endonucleases derived from bacteria. In bacteria the restriction enzymes serve as a defense mechanism that digests unfamiliar DNA to disarm it. The enzymes recognize specific sequences and digest it, leaving either a blunt or a sticky end with a single stranded overhang. These enzymes are adopted as a biotechnological tool to digest specific DNA. When constructing recombined vectors restriction enzymes digest the vector plasmid and the insert gene in order to join them together.

Digestion protocol and incubation parameters for the enzymes used were adjusted according to the supplier's recommendations for each enzyme.

Table 4.10: Restriction Digest

µl	Reagents
	Water
5,0	10 x NEB buffer
0,5	100x BSA
	DNA 5-10 µg
	Enzyme 20 000 U/ml
50,0	Total

Digestion reaction with *SpeI* and *NdeI* for pSRN vector constructs contained 10x NEB 4 buffer, 100 x BSA, *SpeI* and *NdeI* of 20 000 U/ml each in 50 µl reaction. NEB 4 had 100 % efficiency with both enzymes. The enzymes digested for 2,5 hours at 37 °C before heat inactivating the enzymes for 20 minutes at 80 °C.

Digestion with *Xba*I and *BamH*I for pDK60 vector constructs contained 10x NEB3 buffer and 100 x BSA in 50 µl reaction volume. The supplier recommended NEB 3 buffer in a double digest of *Xba*I and *BamH*I which give *BamH*I 100 % efficiency and *Xba*I 75 % efficiency. The different efficiency was leveled out by adding 1,8 µl *BamH*I and 2,4 µl *Xba*I and abundant incubation time. The reactions incubated in room temperature overnight and were heat inactivated for 20 minutes at 65 °C.

4.2.4. Joining fragments with ligation

T4 DNA ligase is an enzyme derived from bacteriophage T4 that joins nucleotide backbone together. It facilitates the binding between a 5' phosphodiester binding and an adjacent 3' hydroxyl terminus. Ligase is used for joining of digested vector backbone and insert DNA to make a recombinant vector.

Table 4.11: Ligation Reaction

µl	Reagents
2 µl	10X T4 DNA Ligase Buffer
1 µl	T4 DNA Ligase
	Nuclease-free water
	Insert DNA
	Vector DNA 50-100 ng
20	Total

50-100 ng vector backbone DNA was used in ligation reactions of 20 µl reaction volume. The amount of insert to be added was calculated based on the molar ratios with the following formula:

$$ng\ Insert = \frac{ng\ Vector\ x\ kb\ Insert}{kb\ Vector} * molar\ ratio\ of\ \frac{\text{Insert}}{Vector}$$

Ligation calculator from Insilico and Gibthon was also used to calculate the amount of insert DNA and vector backbone DNA.

Table 4.12: Ligation Reaction for pDK60 Cloning

pDK60-EDIII4		pDK60-Tetra	
µl	Reagents	µl	Reagents
2 µl	10X T4 DNA Ligase Buffer	2 µl	10X T4 DNA Ligase Buffer
1 µl	T4 DNA Ligase	1 µl	T4 DNA Ligase
	Nuclease-free water		Nuclease-free water
64/200 ng	EDIII4	230/ 370 ng	Tetra
400/1200 ng	pDK60 Backbone	400/ 650 ng	pDK60 Backbone
20	Total	20	Total

Table 4.13: Ligation Reaction for pSRN Cloning

pSRN-EDIII4		pSRN-Tetra	
µl	Reagents	µl	Reagents
2 µl	10X T4 DNA Ligase Buffer	2 µl	10X T4 DNA Ligase Buffer
1 µl	T4 DNA Ligase	1 µl	T4 DNA Ligase
	Nuclease-free water		Nuclease-free water
50 ng	EDIII4	50 ng	Tetra
100 ng	pSRN Backbone	100 ng	pSRN Backbone
20	Total	20	Total

The ligation reactions incubated for 1 hour at room temperature and the ligase was heat inactivated for 10 minutes at 65 °C.

4.2.5. Cloning recombined vectors

The recombined vector is introduced in bacteria and selected transgenic bacteria are grown in cultures. High amounts of the recombined plasmids can then be extracted from the culture for further transformation in plant.

4.2.6. Bacterial techniques

Free DNA can be introduced into bacteria host cells through different processes of transformation, depending on the competency of the bacteria host cells. Some cells are not naturally able to take up DNA but they can be manipulated to be competent.

Plasmids were transformed into *E. coli* cells to amplify the plasmid for isolation and to store recombined plasmids. *A. tumefacien* was transformed to transfer the plasmid further into tobacco.

4.2.6.1. Heat shock transformation of Escherichia coli

Heat Shock transformation is used on chemically competent cells treated with calcium chloride. The exact mechanisms of this transformation are not known. Ice cold cell suspensions are exposed to high temperature of 42 °C that are assumed to cause the cell walls to break down, allowing free DNA to enter the cells interior. This transformation method has a transformation frequency of about 1 transformed cell per 1000 cells. (Acquaah 2004)

1-5 µl ligation reaction or plasmid was added to 50 µl *E. coli* JM109 cells. The cells were incubating on ice for 30 minutes, then 45 seconds at 42 °C and 2 minutes back on ice. The heat shocked cells were added 400 µl LB-medium and incubated for 1 hour at 37 °C with shaking of 180 RPM. 100-200 µl of the cell suspension was plated on LB-agar plates containing selection and the plates were incubating for 16-20 hours in 37 °C to grow transformed cells.

Colonies of transformed cells was cultured for cloning and plasmids could be isolated. Control cells were transformed with water, empty vector and digested vector if available in order to tell if the transformation was successful.

4.2.6.2. Making competent E. coli cells

Competent *A. tumefacien* cells were already available whereas competent *E. coli* cells had to be prepared. JM109 *E. coli* competent cells were first cultured in 5 ml LB solution overnight and 1 ml of the culture was then transferred to 100 ml LB solution for further cultivation. When OD_{600} reached about 0,35 the culture was aliquoted into two 50 ml tubes and centrifuged at 4000 RPM with 4 °C for 10 minutes. The supernatant was discarded and the cell pellet was dried off for 1 minute before being carefully re-suspended in 30 ml $MgCl_2$-

$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$). The cell suspension was centrifuged at 4000 RPM with 4°C for 10 minutes. The supernatant was discarded and the pellet was dried off for 1 minute. The cells were re-suspended in 2 ml 0,1 M $CaCl_2$ and 355 µl 100 % Glycerol and 82 µl DMSO was added and carefully mixed by swirling. The cells were put on ice for 15 minutes before another 82 µl DMSO was added and mixed by swirling. The cells were put on ice-bath while the cell suspension was aliquoted in cooled 1,5 ml tubes of 50 or 100 µl. The aliquots were put in liquid Nitrogen and they were stored in -80 °C.

4.2.6.3. Electroporation of A. tumefacien

Transformation with electroporation is performed on electro competent cells. The cells are put in an electroporator instrument that applies a pulse of strong electric field to the cells. The mechanisms are not exactly known for this process either. It is believed that transient pores are formed in the cell wall after the electric shock and free DNA can enter the cell. The transformation efficiency varies between 10-6 to 10-9 depending on the plasmid size (Acquaah 2004)

The electroporator was set to 2000 V, 200 Ω and 25 µF. Electro competent LBA4044 *A. tumefacien* cells were used for transformation of pSRN-TPSS-T7, pSRN-EDIII4 and pSRN-Tetra. Cells mixed with plasmids were added in a cuvette. The cuvette was placed in the electroporator where current was applied to electroporate the cells. Two samples of isolated pSRN-TPSS-T7 were used. 35 µl cells was added to 1 µl of 17 ng/µl pSRN-TPSS-T7 and another 35 µl cells was added to 2 µl of 11 ng/µl. 2 µl pSRN-Tetra of 24 ng/µl was added to parallels of 21 µl and 40 µl cells and both were successfully electroporated. 1 µl pSRN-EDIII4 of 29 ng/µl was added to 21 µl cells and was successfully transformed. Additional empty vector and water was transformed into *A. tumefacien* to control that the transformation was successful.

1 ml LB solution was added to the cuvette after successful transformation and the cells were mixed carefully with the LB solution by aspirating. The cell suspension was then transferred to a 15 ml tube and incubated for 2 hours at 28 °C with 180 RPM shaking. 100 – 200 µl of the cell suspension was plated on LB-medium containing selection. Transformed cells were grown in 28 °C for 2-3 days to select colonies.

4.2.6.4. Selection of bacteria with antibiotics

Selection system identifies transformants or transgenic cells by establishing their preferential growth over non-transgenic cells. Cloning vectors contain selectable markers such as antibiotic resistance and the marker agent is added to the growth medium. Only circular plasmids will be functional and be expressed by the bacterium. Cells expressing the marker gene are able to grow on the antibiotic medium, while non-transgenic cells will die. A small amount of non-transgenic plants might survive on the selection medium due to mutations. The selected cells need to be analyzed for detection of the transgene.

Different types of antibiotic were used to grow recombined bacterial cells.

Table 4.14: Antibiotic concentrations in selection medium

Antibiotics	Vector	Concentration
Ampicillin	pDK60	100 µg/ml
Kanamycin	pSRN	50 µg/ml
Chloramphenicol	pPCR-Script	34 µg/ml

4.2.6.5. Blue white selection

Empty vectors also contain the selectable marker gene and will grow on selection medium. Blue-white screening can distinguish these false positive colonies from the true transformants by screening the color of bacterial colonies. Vectors used in this system are designed to insert the target gene inside the gene LacZ that encodes the enzyme β-galactosidase. Bacterium transformed with empty vectors containing the LacZ gene will grow blue colonies with presence of X-gal (5-bromo-4-chloro-3-indolo-β-D-galactosidase) because the β-galactosidase hydrolyzes the X-gal and a oxidized blue insoluble pigment (5,5'-dibromo-4,4'-dichloro-indigo) appears. Recombined plasmids do not have functional LacZ and X-gal will not be hydrolyzed by β-galactosidase. Bacteria containing the recombined plasmids will grow white colonies. Antibiotic in the medium will kill cells that are not transformed.(Sambrook and Russell 2001)

4.2.6.6. Growth and storage of bacteria

Cells were stored at – 80 °C in stocks of 1 ml cell with about 15 % glycerol. *E. coli* Cells were usually cultured for 12-16 hours. During this period, called log phase, the cells grow exponentially and can be harvested for extraction or to prepare stocks. When growing the cells from the stock again they were streaked on selection medium and incubated at 37 °C for 12-24 hours to grow colonies. Colonies were then cultured in 3-10 ml LB solution with selection. Bigger cultures where grown from a mini-culture by using 250 µl of the culture in 100 ml LB solution with selection. *A. tumefacien* grow slower and plates needed 2-3 days at 28 °C to grow colonies, 2 days to grow a mini-culture and 3 days to grow a maxi-culture from a mini culture.

4.3. TRANSFORMATION OF TOBACCO

Tobacco was transformed with biolistic particle gun for chloroplast transformation and with *Agrobacterium* mediated stable transformation for nuclear transformation.

4.3.1. Growing tobacco

Seeds of *Nicotiana tabacum* was soaked in 25 % chlorine for 10 minutes and then in 70 % Ethanol for 2 minutes. The chlorine and ethanol was washed of in sterile water before soaking the seeds in sterile water for one hour. The seeds were then dried of and 10-20 seeds were sowed on medium with half MS-2 in magenta box.

The seeds were allowed to grow in dark until shoots appeared. When shoots had grown roots single shoots were transferred to new magenta box with half MS-2 and light intensity of x lux at x °C. When plants had grown about 2/3 in the box they were optimal for transformation or ready to be put on soil and placed in the greenhouse.

4.3.2. Selection and growth of tobacco

Plant hormones control the growth of the plants. 1-Naphthaleneacetic acid (NAA) is a hormone used extensively to promote root formation on stem and leaf cuttings while 6-Benzylaminopurine (BAP) induces shoot formation. Combining BAP and NAA in a 10:1 ratio will induce growth and root formation.

4.3.3. Chloroplast transformation with biolistic particle gun

DNA plasmid

High concentration plasmids was isolated with midi prep kit and eluted in milliQ water. The concentration should be 1 500 – 2 500 ng/µl and minimum 20 µg was needed for one biolistic shooting.

Gold wash

For 1 heptashot:

A eppendorf tube was put on ice. 1.5 mg gold was weighed in a 1.5 ml tube and 140 µl EtOH 100% was added. The tube was vortexed with maximum power for 1 minute before being centrifuged at 4000 RPM in a microcentrifuge for 1 second. The ethanol was carefully removed and the gold was resuspended in 1 ml sterile water while keeping it cold. The gold solution was centrifuged again at 4000 rpm for 1 second. The water was removed and the gold was resuspended in 175 µl sterile water and 172 µl was transferred into the cold eppendorf tube on ice.

DNA coating

20 µg plasmid DNA was added to the gold solution and vortexed. 175 µl 2.5M $CaCl_2$ was added to the sample and it was mixed well by vortexing. 35 µl 100 mM Spermidine was then added and vortexing was mixing the reagents well. The tube was incubating on ice for 10 min with brief vortex once every minute. The sample was centrifuged at 3500 RPM for 1 second and the supernatant was completely removed. 600 µl EtOH 100% was added to the coated gold and the gold was resuspended by pipetting and vortexing vigorously. Centrifugation at 5000 RPM for 1 second separated the coated gold from the ethanol The supernatant was removed completely and the ethanol washing was repeated three times. DNA-gold particles was finally resuspended in 50 µl EtOH 100% and mixed well by pipetting. The DNA coated gold was used directly in shooting transformation or stored at -20°C for maximum two weeks.

Preparing the gene gun

Equipment and instruments were sterilized and prepared for transformation. 6-7 µl DNA-coated gold solution was applied onto seven macrocarrier disks in the hepta holder. A rupture

disk that should withstand 11 000 psi was inserted into the hepta adapter which was installed in the bombardment chamber below the Helium supply. The hepta holder was installed below the hepta adapter.

Preparing the plant tissue

Healthy leafs from plants of 1/2 to 2/3 heights in magenta boxes were used for transformation. Explants were to be placed with the abaxial side facing up on RMOP plate without selection and explants were cut to fit the plate with minimal gaps and overlaps. The plate was placed in position of 6 or 9 cm below the macrocarrier.

Bombardment

Vacuum was added to the bombardment chamber and it was fixed (?) at above 25 inches Hg. Helium pressure of 1360 psi was applied to the chamber and the rupture disk broke at about 1100 psi causing the DNA-coated gold particles to be bombarded onto the explant. The vacuum pressure was immediately turned off to allow the pressure to drop.

Regenaration

Filter paper was put on RMOP plates without selection to keep moist. Explant was transferred to the filter paper for cutting into pieces of 5x5 mm which was placed on RMOP plates containing spectinomycin with 12 explants on each plate. The abaxial side should still face up and the complete explant should be in contact with the selection medium. The plates were sealed and stored in 23 °C.

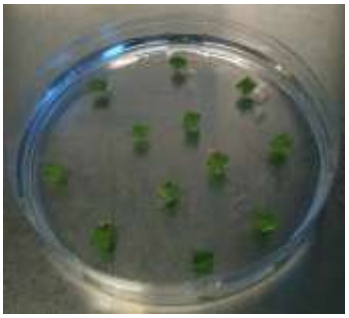


Figure 4.4: Transformed explant placed on RMOP for growth and selection of transformed cells.

Growing transplastomic plants

Green callus tissue would appear 4-8 weeks after transformation. Shoots were cut into smaller pieces of 1,5x1,5 mm to 2,0x2,0 mm and put on new medium for a new regeneration round. The callus was transferred to new medium but it was still the same regeneration round.

Transformants were tested on double selection of both Spectinomycin and Streptomycin to eliminate spontaneous mutants. Explant on double selection was cut into 2x2 mm to 2x3 mm.

4.3.4. *Agrobacterium* mediated stable transformation

pSRN-EDIII4 and pSRN-Tetra were transformed into the nuclear genome of tobacco to express the antigens upon ethanol induction. *Agrobacterium* was used to mediate the insertion of the transgenes into the nuclear genome of tobacco.

Agrobacterium suspension

Agrobacterium cells were grown in miniculture of 5-10 ml LB-medium containing 50 µg/ml Kanamycin for 16-20 hours at 28 °C and 180 RPM. 250 µl of the culture was transferred to 100 ml LB-medium containing selection. The culture grew one day with 150 RPM shaking in 25 °C and then the temperature and shaking was increased to 28 °C and 180 RPM for another 16-20 hours.

The cultures were grown to reach OD_{600} (optical density at 600 nm wavelength) of about 1,0 in absorbance. The cells were then pelleted by centrifugation at 2000 RPM for 15 minutes. The supernatant was discarded and the pellet was first washed and then resupended in MS-II solution. The OD_{600} were measured again and the cell suspension was diluted to obtain absorbance of 0,5-1,0.

Explant preparation

Simultaneously, the tobacco to be transformed was prepared. Both the cell suspension and the tobacco explant should be fresh upon transformation. Wild type tobacco plants of about 2/3 height in magenta boxes were used to stamp out circular tissues from the leaves of about 10x10 mm. The tobacco explant was put on moist filter paper for storing up to the transformation.

Inoculation and co-cultivation

Tobacco explant inoculated with agrobacterium suspension for 60 seconds to allow agrobacterium to enter the wounded tissue. Then the tobacco tissues were rinsed briefly in MS-II solution and dried of on a sterile filter paper before plated on MS-II medium with NAA and BAP

with 10 explants on each plate. The plates were sealed and put in dark at 23 °C for 3 days of co-cultivation to allow the agrobacterium to transfer the T-DNA containing the gene of interest into the plant genome.

Growing transgenic tissue

After 3 days the tobacco tissues were transferred to MS-II medium containing 500 µg/ml cefotaxim and 50 µg/ml Kanamycin in addition to the hormones. The plates were put back in 23 °C with light intensity of 42 µM/m^2/sec (2-3000 lux). Every 9-12 days the medium was changed. As the transgenic tissue grew they had to be cut into smaller pieces when being transferred to fresh medium. When clones of the same tissue were cut and put on new plates they were given the same number. After about 30 days the tissue grew shoots that were isolated and put on new medium. When they grew roots the plants were transferred to magenta boxes containing half MS-II without hormones and selection. When the plants grew about 2/3 of the box they were transferred to soil. At least two clones of each line were kept to make sure there was enough tissue for analysis as well as growing seeds.

Ethanol induction

10 selected transgenic tobacco plants of EDIII4 and Tetra, as well as a pSRN-TPSS-T7 transgenic tobacco and a wild type tobacco were sprayed with 5 % Ethanol. Leaf tissues were harvested right before Ethanol induction, at day 0, and then at 1, 4 and 7 days after spraying. After harvesting the tissues were put straight in liquid nitrogen.

Figure 4.5: Ethanol-induction of tobacco. 5 % ethanol was sprayed on the tobacco plants

5. Results

5.1. Selection of candidate dengue antigens for tobacco transformation

The entire NFR-funded project aims to produce tetravalent dengue vaccine by expressing the four monovalent antigens either alone and combined proportionally once they are produced in tobacco chloroplasts or nucleus to generate tetravalent dengue vaccine, or together in one construction called Tetra. This master study was assigned (1) to express EDIII-4 monovalent dengue antigen alone in tobacco chloroplasts and nucleus and (2) to express all four together as a tetravalent antigen candidate, whereas the project team and another master thesis were report the other dengue antigens. Vectors containing candidate EDIII-4 and EDIII-Tetra were constructed for chloroplast and nuclear transformations.

As described in the introduction, chloroplast transformation has a unique advantage to achieve a high-level expression and maximal recombinant protein accumulation in chloroplasts. Thus, tobacco chloroplast transformation was carried out in current study to express the EDIII antigens in monovalent and tetravalent forms. However, it is reported previously that some foreign proteins can be toxic to chloroplasts causing problem in the recombinant protein expression and accumulation in tobacco chloroplasts. Moreover, unstable protein accumulation and protein degradation were also known in chloroplast transformation system. Therefore, nuclear transformation of tobacco to express those candidate dengue antigens under the control of T7 ethanol inducible promoter was designed and carried out parallel with the tobacco chloroplast transformation.

5.2. NUCLEAR TRANSFORMATION OF TOBACCO

5.2.1. Constructing pSRN-EDIII4 and pSRN-Tetra vectors for Agrobacterium mediated nuclear transformation of tobacco

Expression vectors containing EDIII4 and Tetra antigen-encoding genes were constructed for tobacco nuclear transformation using *Agrobaterium*-mediated transformation method. EDIII-4

monovalent antigen and the tetravalent antigen (Tetra) containing all the four monovalent antigens linked by pentaglycin linker in an order (EDIII-1, 3, 4 and 2 as shown in the figure 3.1 in Materials) and 6x His-tagged were codon optimized for tobacco plant expression and synthesized by Bio Basic (www.biobasic.com). The products in pUC57-EDIII4 and pUC57-Tetra were used as template for amplification of EDIII4 and Tetra.

Primer pairs containing SpeI and NdeI sequences were used on pUC57-EDIII4 and pUC57-Tetra in PCR with high quality proof reading Pfx polymerase. The primers for Tetra were tested with a temperature gradient for the annealing temperature in the PCR with 52,0 - 52,5 - 53,4 - 54,8 - 56,5 - 57,8 - 58,6 – 59,0 °C. The eight parallels of Tetra were applied on a gel and figure 5.1 A show that the primers were annealing at all temperatures. The primers for EDIII4 were not tested with temperature gradient because they were designed with the same parameters as for the Tetra primers. Since the Tetra primers annealed well at temperatures ranging from 52 to 59 °C, EDIII4 were amplified with annealing temperature of 55 °C and with four parallels to obtain sufficient amounts of the PCR-products (figure 5.1 B).

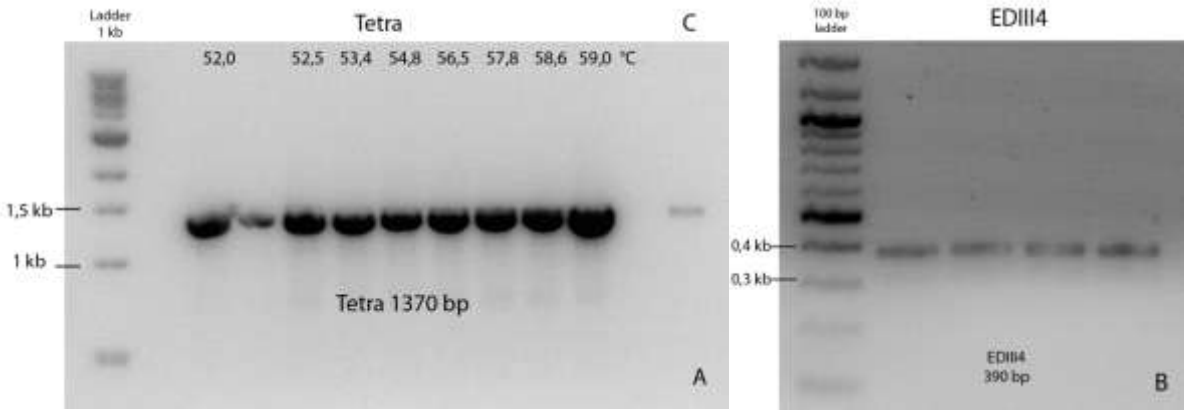


Figure 5.1: PCR products of Tetra and EDIII4 on agarose gel A) PCR-Product of Tetra have the right size of about 1400 bp. The C sample to the right is an old PCR-product of Tetra added on the gel to indicate the right length. The thermal cycler used a temperature gradient of 52,0 – 52,5 – 53,4 – 54,8 – 56,5 -57,8- 58,6 – 59,0 °C to test the best annealing temperature of the primers. B) PCR-product of EDIII4 have the right size of about 400 bp according to the 100 bp ladder.

EDIII4 was expected to be 390 bp and Tetra was expected to be 1370 bp. The markers in the gel indicated the correct sizes of the fragments. PCR products of EDIII4 and Tetra were recollected from the gel for subsequent cloning. The purified PCR-products were digested with restriction enzymes. EDIII4 and Tetra were subcloned into pPCR-Script vector and *E. coli* was transformed with the recombined pPCR-Script vectors. Seven white colonies from

pPCR-Script-EDIII4 and 6 from pPCR-Script-Tetra were screened. Both pPCR-Script-EDIII4 and pPCR-Script-Tetra were screened with gene specific primers and Tetra was in addition also screened with pPCR-Script specific primer M13 to confirm the correct insertion. The screening results are shown in the figure 5.2 below.

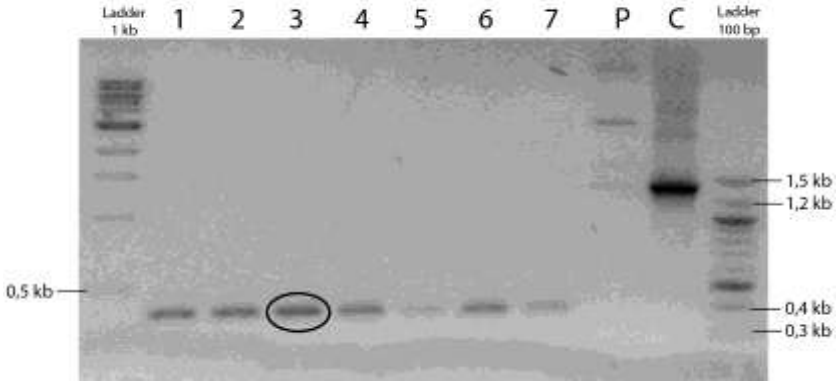


Figure 5.2: PCR Screening for EDIII4 in pPCR-Script. Seven colonies of pPCR-Script-EDIII4 were screened with EDIII4 specific primers. The bands have the correct sizes of about 400 bp and P is pPCR-Script DNA and C is pPCR-Script colony. Additional amplification with vector specific primers was carried out to confirm if EDIII4 was correctly inserted into the vector. Colony number 3 was cultivated for isolation of the plasmid.

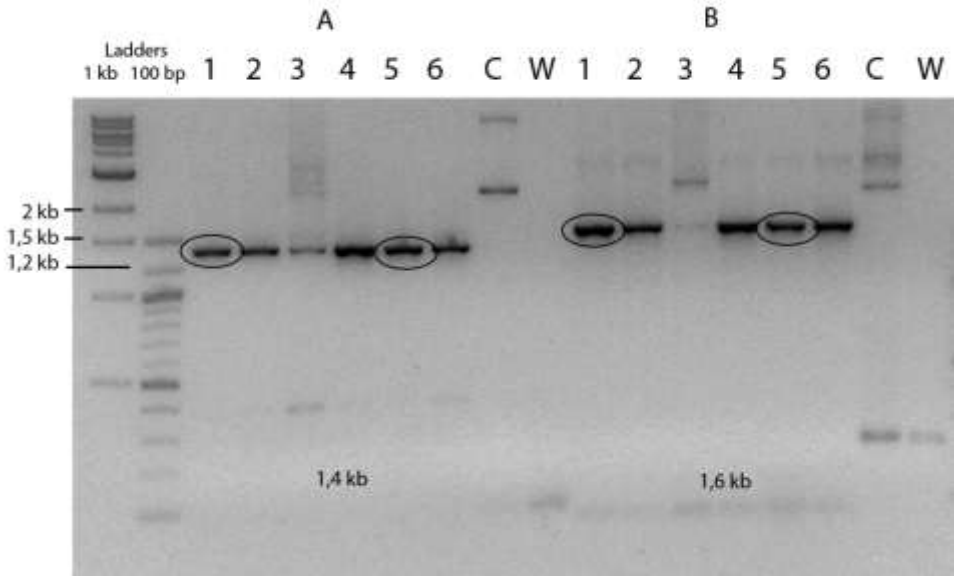


Figure 5.3: PCR Screening for Tetra in pPCR-Script. Six colonies of pPCR-Script-Tetra were screened with Tetra specific primers and with M13 pPCR-Script specific primers to confirm that Tetra was inserted into pPCR-Script. The PCR-products had the correct sizes in all colonies but colony number 3 had additional bands as well. C is PCR with pPCR-Script as positive control and W is PCR with water as no template control.

One positive colony of pPCR-Script-EDIII4 and two positive colonies of pPCR-Script-Tetra were cultivated and the plasmid DNAs were isolated from the bacterial culture. The plasmids

pPCR-Script-EDIII4 and pPCR-Script-Tetra were digested with *Spe*I and *NdeI* to release EDIII4 and Tetra, while pSRN-TPSS-T7 was also digested with *Spe*I and *NdeI* . The digested plasmid samples were applied on agarose gels to separate and recollect the EDIII4, Tetra and pSRN-TPSS-T7 backbone for ligation.

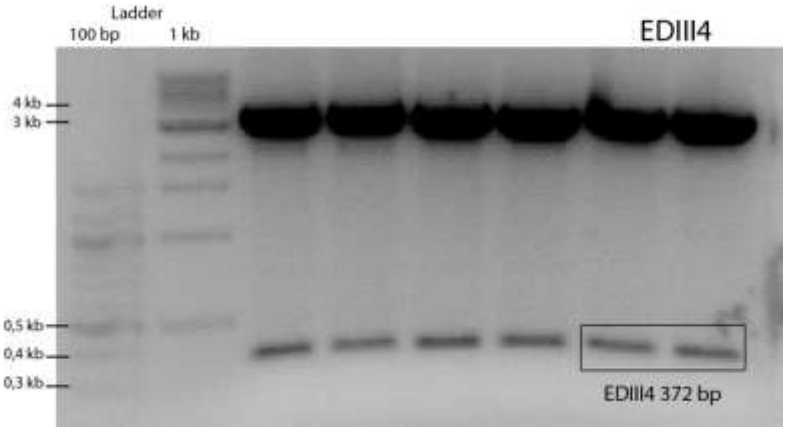


Figure 5.4: Digested pPCR-Script-EDIII4. The two lanes to the far right are EDIII4 and the rest are from other monovalent digestion. The 400 bp of EDIII4 were cut out from the gel. The upper fragments are pPCR-Script backbone with a size of 3,4 kb.

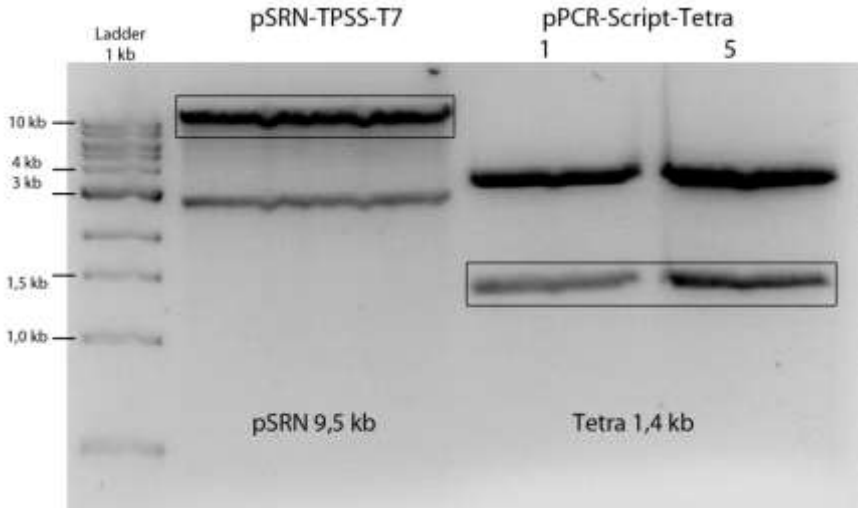


Figure 5.5: Digested pSRN-TPSS-T7 and pPCR-Script Tetra. Marked pSRN-backbone of 9,5 kb and Tetra of 1,4 kb were cut out from1 the gel and purified before the ligation experiments.

The digested fragments of EDIII4, Tetra and pSRN backbone were isolated and purified before being ligated together to form pSRN-EDIII4 and pSRN-Tetra. *E. coli* was transformed with the ligated plasmids and recombined cells were selected with kanamycin (50 mg/L). Colonies from pSRN- EDIII4 transformations were picked for PCR-screening.

PCR verification of pSRN-EDIII4 and pSRN-Tetra vectors was performed with gene specific primers as shown in the figure 5.6 A and B. The blank PCR sample (B) confirms no contaminations in the PCR reaction and positive controls confirmed the correct insertions of

EDIII4 and Tetra. The plasmids were sent to sequencing and the sequencing results identified the correct insert of EDIII4 and Tetra in pSRN-EDIII4 and pSRN-Tetra respectively.

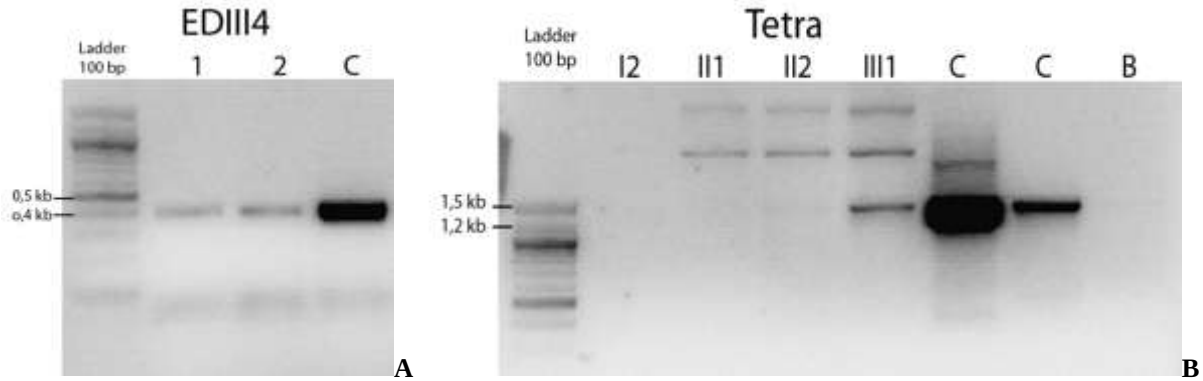


Figure 5.6: Screening for EDIII4 and Tetra in pSRN. A) EDIII4 detected in pSRN. Colony 1 and 2 are positive with the right size of ~400 bp as indicated by the right size with the marker. C is pUC57 EDIII4 which also confirms the right size of EDIII4. B) Screening for Tetra with four colonies picked from three different transformations; I/II/III. C is pUC57-Tetra. B is Blank

Electroporation was conducted to introduce both pSRN-EDIII4 and pSRN-Tetra expression vectors under the control of T7 promoter into the disarmed *A. tumefacien* strain LBA4404 for a*grobacterium* mediated stable transformation. Kanamycine (50 mg/L) selection was supplemented in the LB culture medium**.**

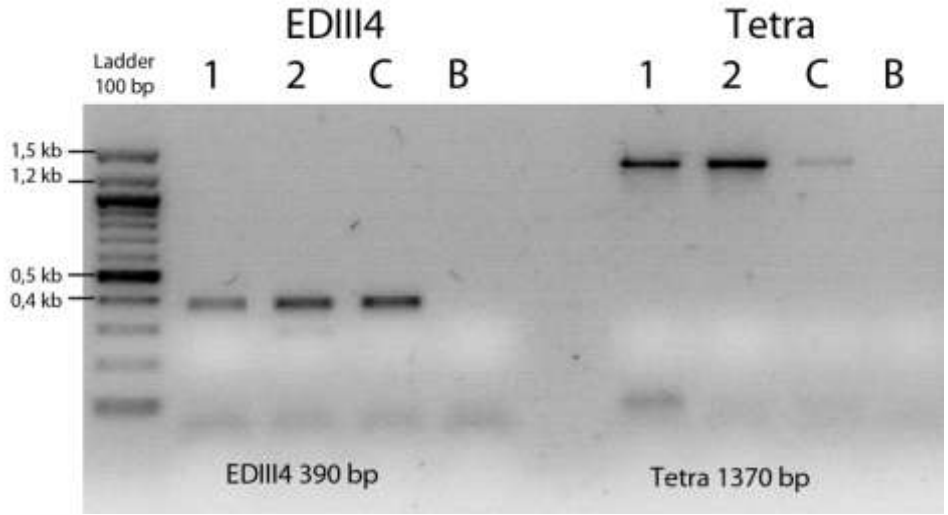


Figure 5.7: PCR-screening of pSRN-EDIII4 and pSRN-Tetra in Agrobacterium. pSRN-EDIII4 1, pSRN-EDIII4 2, pUC57-EDIII4 positive control (C) and blank PCR (B). pSRN-Tetra 1, pSRN-Tetra 2, pUC57-Tetra positive control (C) and blank PCR (B). The right sizes of the PCR-products confirmed that EDIII4 and Tetra was present in the plasmids.

5.2.2. *Agrobacterium* mediated stable transformation

A. tumefaciens strain LBA4404, harbouring the plasmids pSRN-EDIII4 and pSRN-Tetra, was grown overnight in 15 ml liquid LB medium supplemented with 50 mg l^{-1} kanamycin at 28°C with shaking at 200 rpm until an OD_{600} of 0.6-0.8 was reached. The bacterium suspension was collected at 2700 rpm for 10 min, washed twice with MS basal medium supplemented with 2% sucrose (MS-2), and re-suspended in 10 ml MS-2. The pSRN-TPSS-T7 vector was also introduced into *A. tumefaciens* by electroporation and transformed as a control reference plasmid parallel with pSRN-EDIII4 and pSRN-Tetra to monitor the transformation methodology and efficiency in case the candidate dengue antigens have severe impact on plant growth, protein accumulation or unpredicted protein degradation. Transgenic pSRN-TPSS-T7 plants were named as T7-plants.

Tobacco seeds were sterilized and sowed on half-strength MS (Murashige & Skoog, 1962) basal medium. Leaves of 4-week-old tobacco grown *in vitro* were excised and used as explants for transformation. *Agrobacterium*-mediated transformation of tobacco was carried out basically as described in the Materials and Methods. After a 3-day antibiotic-free co-cultivation period, the explants were rinsed and placed on selection medium consisting of MS basal medium supplemented with 2 % sucrose, 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and antibiotics: 500 mg l^{-1} claforan (Aventis Pharma Ltd, Norway) and 100 mg l^{-1} kanamycin (Sigma-Aldrich, St Louis, USA). Shoots and plantlets regenerated on selection medium were subsequently cultured on hormone free ½ strength MS medium for root induction and were subsequently transferred to soil and grown in the greenhouse.

For controls: wild-type tobacco leaf explants were put directly on selection medium to control the selection and to compare growth with transformed plants. Wild-type tobacco did not grow on the selection medium. Figure 5.8 illustrates the growth of wild-type and transformed explants. Transformed tobacco explants developed fast and had to be subcultured every 10 days. After 20-30 days callus were developed from the explant tissues , and shoots were differensieted from the callus tissue consequentely. After 30-40 days shoots were removed and put on new medium. For rooting, the shoots were transferred to hormone free 1/2 strength MS medium for further root formation before it was transferred to the greenhouse pots.

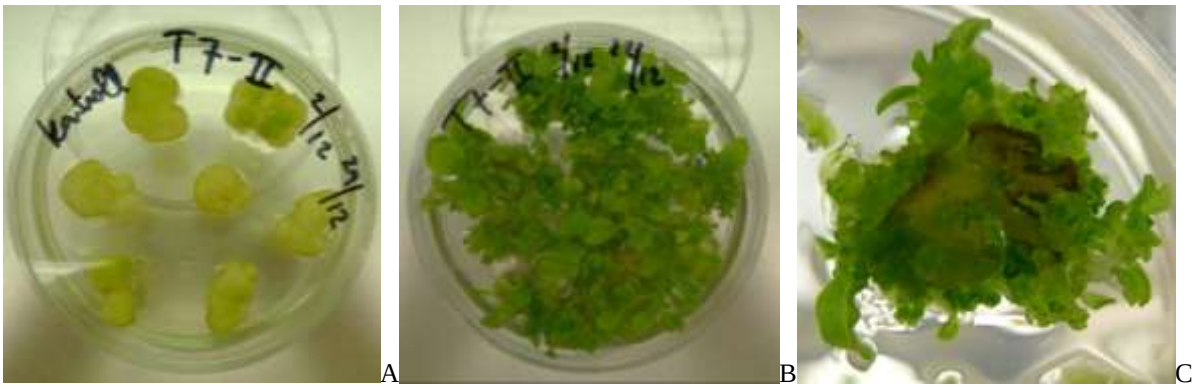


Figure 5.8: Selection of transgenic tobacco. Illustrations from pSRN-TPSS-T7 transfomed tobacco. A) Wild-type tobacco explant did not survive on the selection medium. B) Transformation efficiency were very high with most of the transfomed tissue growing on the selection medium. C) Transgenic tissue developed shoots from the callus. Single shoots were removed and transferred onto new medium for root formation.

5.2.2.1. Screening of tobacco nuclear transformants

The T7-expressing plants were screened with p275 Reverse and p274 forward primer and p275 reverse primer. The region to be amplified is localized in the T7 RNA polymerase gene. PCR of a true transformant will amplify a region band of 601 bp.

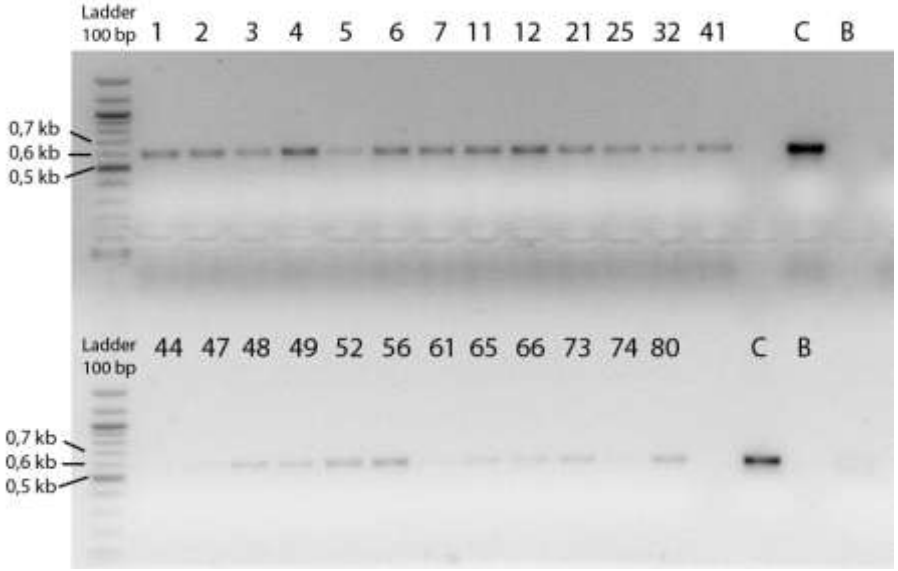


Figure 5.9: Screening for pSRN-TPSS-T7 transformants. C is pSRN-TPSS-T7 plasmid positive control and B is blank negative control PCR. All bands are of correct size of ~600 bp. Some bands are weak but only number 44 were empty confirming that 24 of the total 25 are transformants.

Figure 5.9 show that 24 out of 25 screened plants were confirmed T7-transformants. The size markers and the size of the amplified fragment from pSRN-TPSS-T7 plasmid (C) indicated the correct size of the fragments.

The primers used for PCR screening of pSRN-EDIII4 and pSRN-Tetra transformants gave an expected product of 224 bp of EDIII4 and 893 bp of Tetra as shown in figure 5.10 with EDIII4 and figure 5.11 with Tetra below.

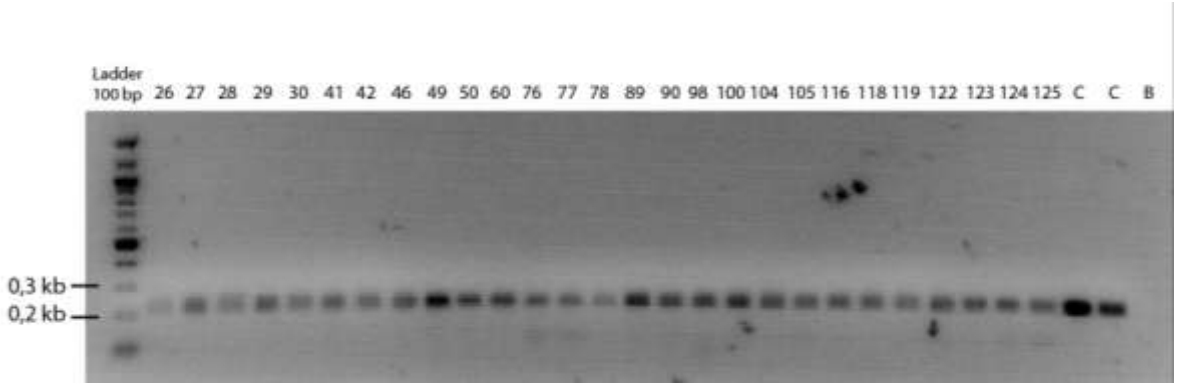


Figure 5.10: Screening for pSRN-EDIII4transgenic tobacco. The PCR product on the gel showed the right sizes. C is control EDIII4 in pUC57 and in pSRN as positive references. B is blank PCR. All plants screened are positive.

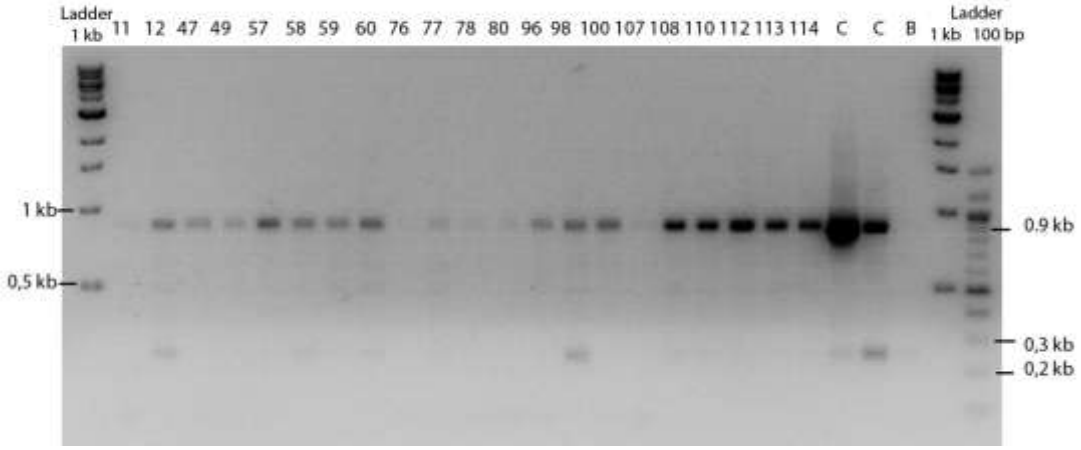


Figure 5.11: Screening for Tetra transgenic tobacco. Expected sizes of Tetra positive plants are 893 bp. C is control of Tetra in pUC57 and in pSRN as positive references. B is blank PCR.. B: Blank PCR was negative as expected.

Tobacco nuclear transformants containing EDIII4 and Tetra were PCR confirmed with the correct sizes of the amplified fragments. Good transformation protocol followed by effective antibiotic selection and regeneration allowed to generate a large number of tobacco nuclear transformants expressing EDIII4 and Tetra antigen-encoding genes.

5.2.2.2. Transformation efficiency

Statistics cannot be made out of the transformation efficiency because not all of the plants were screened. However, the transformation was quite efficient. Out of 150 pSRN-EDIII4-expressing putative transformnats, 26 were screened first. The result showed that 26 out of 26

putative transformants PCR screened were positive and showed the correct PCR product, suggesting the high transformation efficiency For generation of pSRN-Tetra-expressing tobacco transformants, 120 tobacco leaf explants were transformed with pSRN-Tetra construct. PCR screening showed that 17 out of 21 screened plants were positive. The original pSRN-TPSS-T7 was used to transform 120 tobacco leaf explants. The PCR screening revealed that 24 transformants were verified out of 25 screened regenerated tobacco plants. 80-100 % of the screened plants were positive but only 15-20 % of the total regenerated putative transformants were screened because there was no need to screen more plants after enough transformants were verified.

Table 5.1: Transformation efficiency

	T7	*EDIII4*	*Tetra*
Transformed explants	120	150	120
Screened plants	25	26	21
Positive transformants	24	26	17
Transformation Efficiency of screened plants	96%	100 %	81 %

5.2.2.3. Growing transgenic plants

When shoots started to develop roots on the confirmed transgenic plants, they were transferred into Mega boxes with hormone free root induction medium. The plants were transferred to soil when the roots had established and eventually they had to be transferred to bigger pots (figure x A,B and C).

A B C

Figure 5.12: Growing transgenic plants. Shoots from the transgenic plants were grown to fully mature plants. A) When shoots were starting to generate roots they were transferred to boxes. B) Shoots reaching the end of the box were ready to be put on soil. C) Finally, the plants were transferred to pots to grow mature. Analysis was conducted on the mature plants and they were grown further for seed harvests.

In order to harvest seeds from transgenic tobacco plants, the plants developed buds that were covered with a bag and when the bud was flowering it pollinated the ovary inside the bud. The fertilized seeds grew in the pod and dried up before they were harvested (figure 5.13).

Figure 5.13: Growing transgenic seeds. A) tobacco bud B) Flowering tobacco with pollen. C) Seed bud almost ready for seed harvest. Before the buds were flowering they were covered with a thin bag for self-pollinating. The grewing seed pods contained fertilized seeds that were harvested when the seeds dried.

5.2.3. Morphologic evaluation of transgenic tobacco

It was observed morphologic changes of some of the plants. Wild-type (WT) tobacco has one main branch covering with leaves and it flowers on the apical top. Some of the transgenic plants produced additional branches from the main-branch that developed buds and flowers as compared with control wild type tobaccos. These plants had thinner branches and smaller leaves compared to WT tobacco plants. These morphologic changes are probably caused by position-effect of the inserted transgene, but they did not seem to cause any harm to the plants.

Figure 5.14: Morphology of tobacco transformants compared with the wild type. A) Illustrations of tobacco with normal morphology with bud growing on top of the main-branch and no branching. B) Some transgenic tobacco plants developed abnormal branching and possibly earlier and more flowering

5.2.4. Ethanol induction of dengue –antigens EDIII4 and Tetra expression

One wild-type (WT) tobacco plant, one T7-transformant, 10 EDIII4 and 10 Tetra transformants were sprayed with 5 % ethanol to induce expression of EDIII4, Tetra and T7. Leaf materials of every plant were harvested just before they were sprayed with ethanol, as a reference that should not express the transgenes. Plant materials were harvested after 1, 4 and 7 days after the ethanol spray to evaluate the expression of the EDIII4 and Tetra. Comparison at day 0 and day 7 are illustrated for EDIII4 in figure 5.15 and for Tetra in figure 5.16. All plants grow very well during that week and no significant changes are observed.

Figure 5.15: EDIII4 tobacco day 0 (left) and day 7 (right) after ethanol induction.

Figure 5.16: Tetra tobacco day 0 (left) and day 7 (right) after ethanol induction.

5.2.4.1. SDS-PAGE with transgenic plants of EDIII4 and Tetra

Proteins were extracted from the tobacco transformants harvested at day 0, 1, 4 and 7. Soluble proteins were analyzed with SDS-PAGE. Expected size of the EDIII4 peptide is 25,8 KDa and 55,0 KDa for Tetra. The SDS-PAGE results are presented in figure 5.17 for EDIII4 and figure 5.18 for Tetra. The expected sizes are indicated with an arrow.

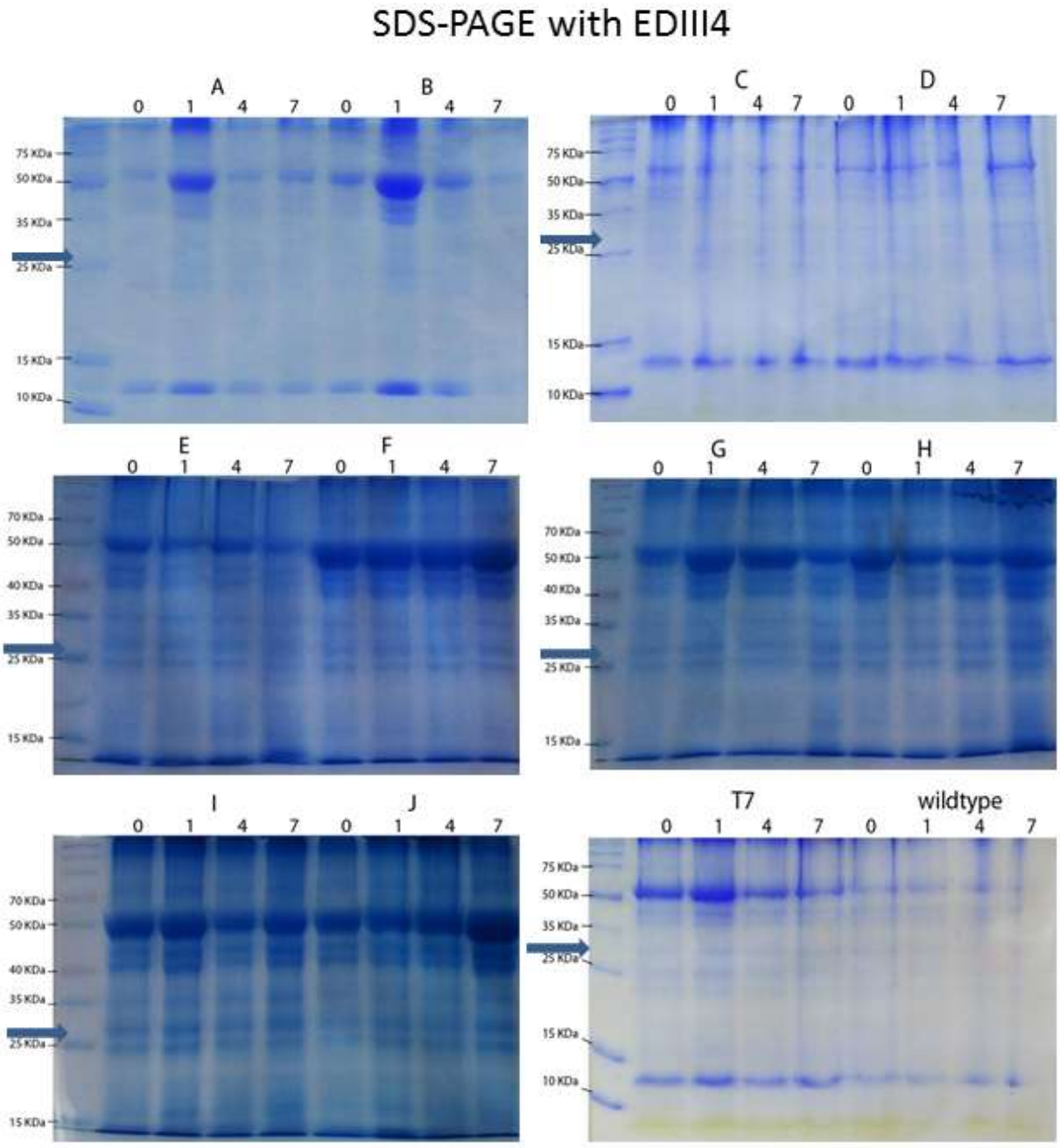


Figure 5.17: SDS-PAGE results with ten EDIII4 transgenic plants (A-H) from day 0, 1, 4 and 7 after ethanol induction. Expected size for EDIII4 were 25,8 KDa as indicated with the arrows.

Figure 5.17 show that in all the gels there are two bands around 25 KDa and the upper band could have matched the expected size, but the control gel with T7 plants and wild-type plants does also have the same bands. No unique bands are observed in the EDIII4 samples compared to the control gel and no single peptides are observed with increased expression.

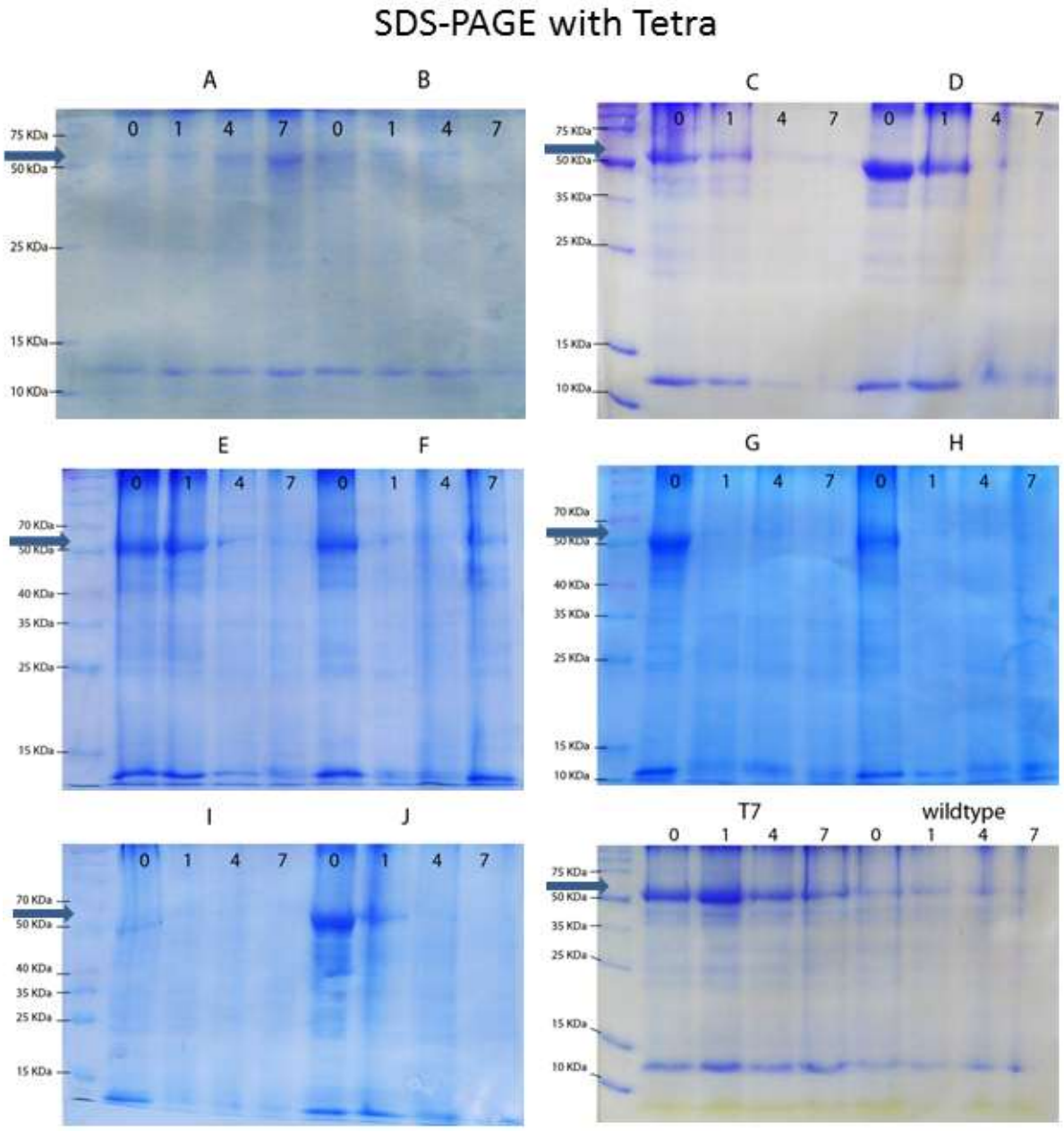


Figure 5.18: SDS-PAGE results with ten Tetra transgenic plants (A-H) from day 0, 1, 4 and 7 after ethanol induction. Expected size for Tetra were 55 KDa as indicated with the arrows.

Figure 5.18 show the same dominating peptide in all samples of about 50 KDa. It could have matched Tetra, but it is also present and dominating in the control gel samples. It is neither

observed any increased expression of any single peptide or any unique bands compared to the control gel.

In addition, by comparing EDIII4 and Tetra (Figure 5.17 and 5.18) they both have all the same peptides.

5.2.4.2. Western Blot with EDIII4 and Tetra transgenic plants

Expression in plant A, B, C and D of both EDIII4 and Tetra were analyzed with western blot, and additional control blot with wild-type and T7-plant. Figure 5.19 present EDIII4 results with peptides of about the expected size present and dominating. Tetra results are presented in Figure 5.20 with no bands that seem to match the right size as indicated with the arrows. The promising EDIII4 bands are also present and dominating in Tetra peptides. It seems that all the bands are unspecific bindings with the antibodies. The control gel with T7 and wild-type plant was not successfully blotted, with blank membrane and proteins still present in the gel (figure 5.21).

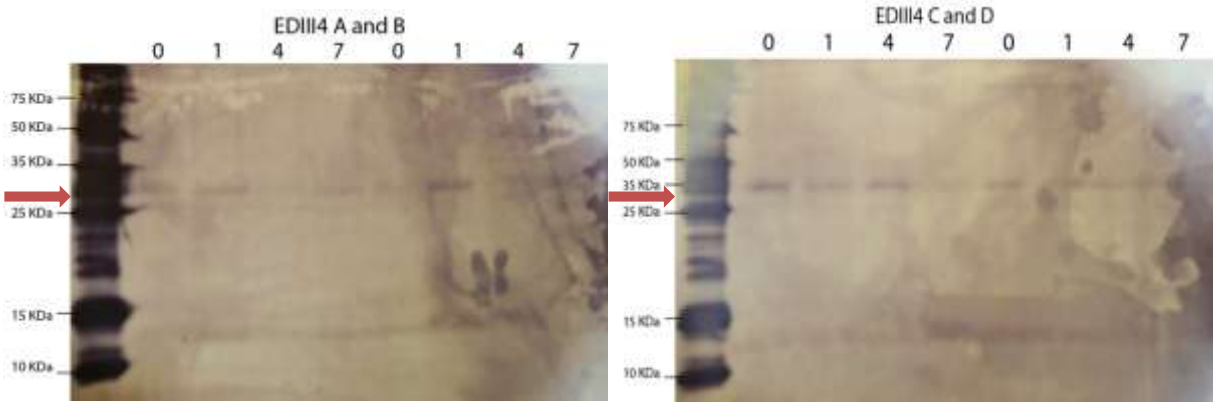


Figure 5.19: Western Blot with Anti-His on EDIII4 plant A, B, C and D from day 0, 1, 4 and 7 after ethanol induction.

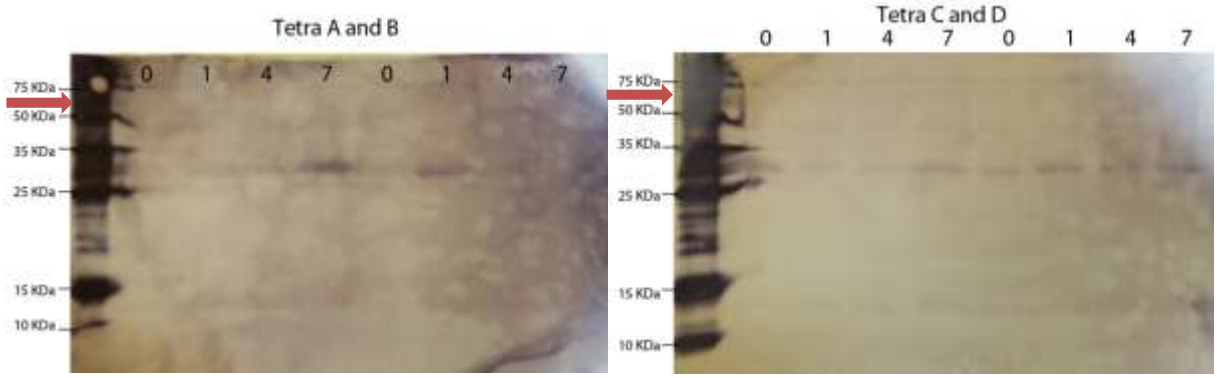


Figure 5.20: Western Blot with Anti-His on Tetra plant A, B, C and D from day 0, 1, 4 and 7 after ethanol induction. The expected sizes of Tetra should be above the red line but is not observed.

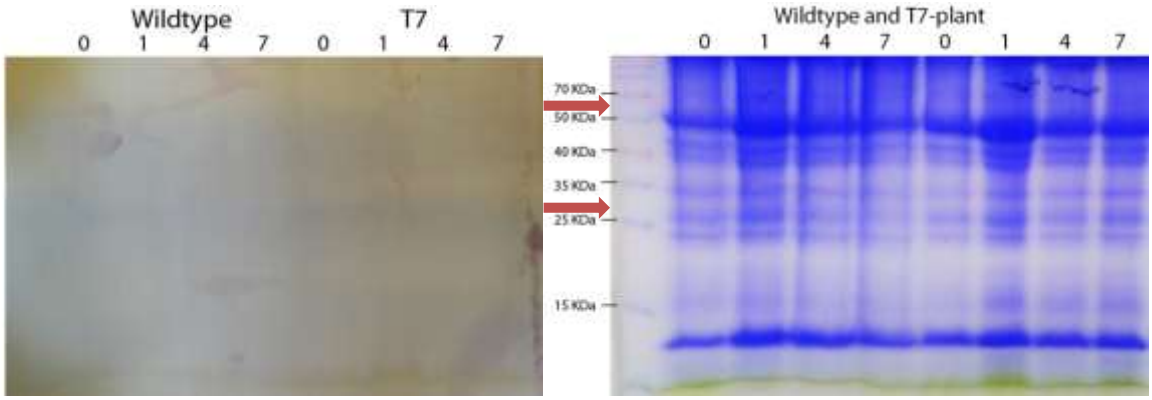


Figure 5.21: Western Blot (left) and stained gel after blotting (right) with wild-type tobacco and T7-plant from day 0,1,4 and 7 after ethanol induction. The blotting did not work as the proteins were not transferred to the membrane. The red arrow on the stained gel show that the band sizes that could have matched EDIII4 (bottom) and Tetra (top) are also present in the wild-type and the T7 plants.

Based on figures above, any visible induction of EDIII4 and Tetra expressions in the transgenic tobacco containing EDIII4 and Tetra transgenes could not be detected, from this very first ethanol spray experiment. The reason could be several due to the unsuccessfully spray; the low induction level which was not detectable; or the time interval shall be prolong to 10 days or 14 days. This experiment will be repeated in the future and the reason(s) shall be revealed.

5.3. TRANSFORMATION OF TOBACCOCHLOROPLASTS

Plasmid pDK60-EDIII4 harboring monovalent antigen EDIII4 and pDK60-Tetra containing the tetravalent dengue antigen were introduced into tobacco chloroplasts by chloroplast transformation. The results are presented below.

5.3.1. Constructing pDK60-EDIII4 and pDK60-Tetra expression vectors for tobacco chloroplast transformation

EDIII-4 monovalent antigen and the tetravalent antigen (Tetra) containing all the four monovalent antigens linked by pentaglycin linker in an order (EDIII-1, 3, 4 and 2 as shown in the figure 3.1 in Materials) and 6x His-tagged were codon optimized for tobacco chloroplast expression and synthesized by Bio Basic (www.biobasic.com). The products pUC57-EDIII4 and pUC57-Tetra were used as template for amplification of EDIII4 and Tetra with the primers containing *BamH*I and *Xba*I sites. Platinum Pfx polymerase was used in PCR

reactions to achieve high nucleotide sequence accuracy.

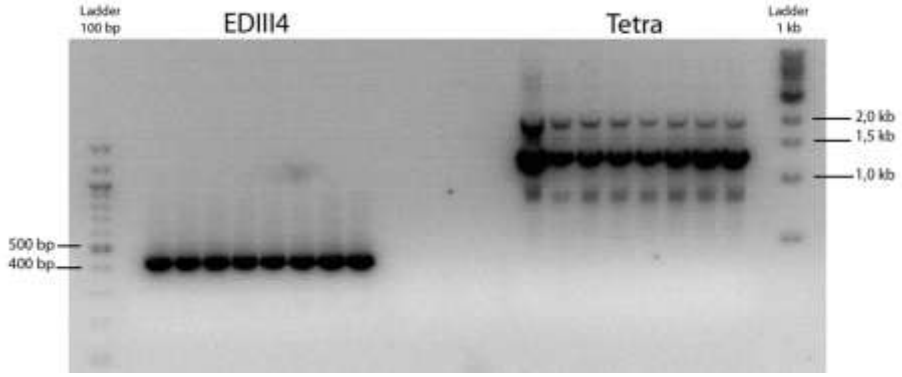
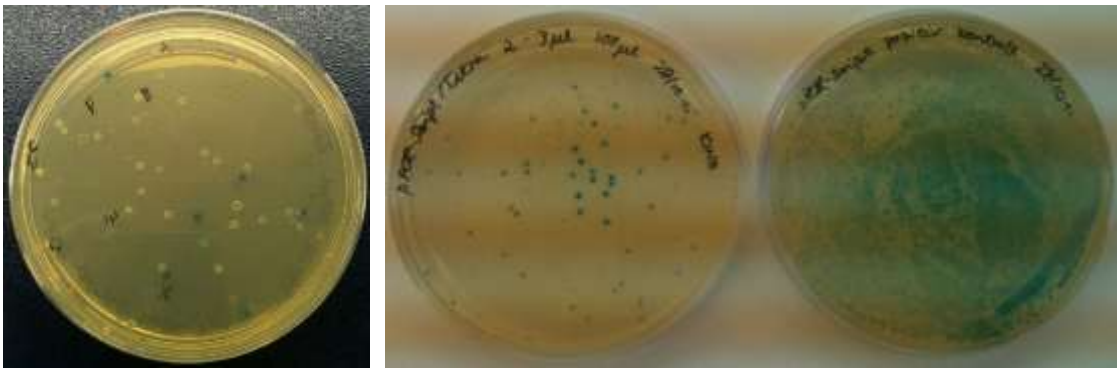


Figure 5.22: Gel image of EDIII4 and Tetra PCR-products. Eight parallels of both EDIII4 and Tetra are applied on the gel. EDIII4 was expected to be 390 bp. The primers for Tetra have generated the expected 1370 bp product as shown in the figure here. A couple of unspecific amplified fragments were also detected in the PCR but was eliminated. . The EDIII4 and Tetra bands were cut out of the gel and the DNA was isolated for subsequent cloning and vector construction.

The EDIII4 and Tetra were cloned into pDK60 tobacco chloroplast expression vector by restriction digest and ligation. PCR-products of EDIII4 and Tetra were first sub-cloned into pPCR-Script prior their digestion and ligation to pDK60 with the pPCR Script plasmid containing either EDIII-4 or Tetra was transformed into *E. coli* cells and they were plated in LB-plates with 33 µg/ml chloramphenicol with X-gal on the surface.



A B C

Figure 5.23: Selection of subcloned EDIII4 and Tetra in pPCR-Script. A) *E. coli* transformed with pPCR-Script EDIII4 shown as white colonies. Totally 19 colonies were selected. B) Cells transformed with pPCR-Script Tetra had high density growth of recombined cells. In total 16 white colonies were selected. C) *E. coli* transformed with undigested pPCR-Script showing only blue colonies which confirm the transformation efficiency and that no recombined plasmids or false positives are present.

Transformation was successful and blue-white screening indicated that the pPCR-Scripts had recombined with EDIII4 and Tetra due to the white colonies (figure 5.23). Totally, 19 colonies of pPCR-Script-EDIII4 and 16 colonies of pPCR-Script-Tetra were selected for vector construction. Even though the white colonies should confirm recombination, additional PCR analysis was performed with vector specific M13 primers to confirm the correct inserts. The amplified products of vector specific primers were expected to be 227 bp of empty vector,

598 bp of EDIII4 and 1582 bp of Tetra respectively.

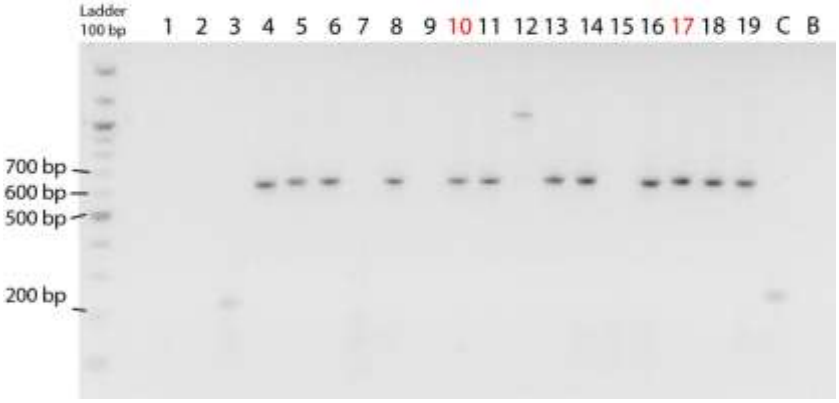


Figure 5.24: PCR screening of EDIII4 in pPCR-Script with M13 primers. 12 out of 19 colonies had the right size on the gel with 598 bp. There was no control sample to match the expected product size. The C sample to the right is empty pPCR-Script that would amplify 227 bp with the M13 primers. Sample 3 seems to be empty vector. Sample 1,2,7,9 and 15 are not detected at all. Sample 12 is too big. Sample B to the right is no amplification control with $H_2O$and no product was obtained as expected. Colony 10 and 17 containing EDIII4 were selected for further analysis.

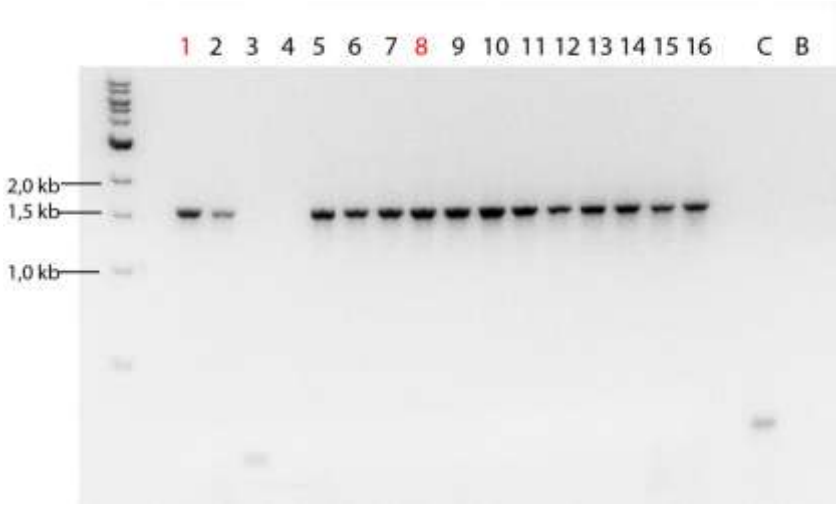


Figure 5.25: PCR product of pPCR-Script-Tetra with M13 primers. 14 out of 16 screened colonies had the right size of the insert as the 1 kb ladders indicate the sizes to be ~1,6 kb. C is control with empty pPCR-Script that was expected to be 227 bp. Sample 3 seems also to be empty vector. 4 is not detected at all. B is no amplification control PCR. Colony 1 and 8 containing Tetra were chosen for further cultivation.

Two positive colonies of pPCR-Script EDIII4 and pPCR-Script Tetra were cultured, and amplified plasmids were isolated from the cells. High concentrations of 2-5 µg of pDK60, pPCR-Script EDIII4 and pPCR-Script Tetra were digested with *BamH*I and *Xba*I. Digested samples were separated on an agarose gel and the bands of the correct sizes were excised from the gel.

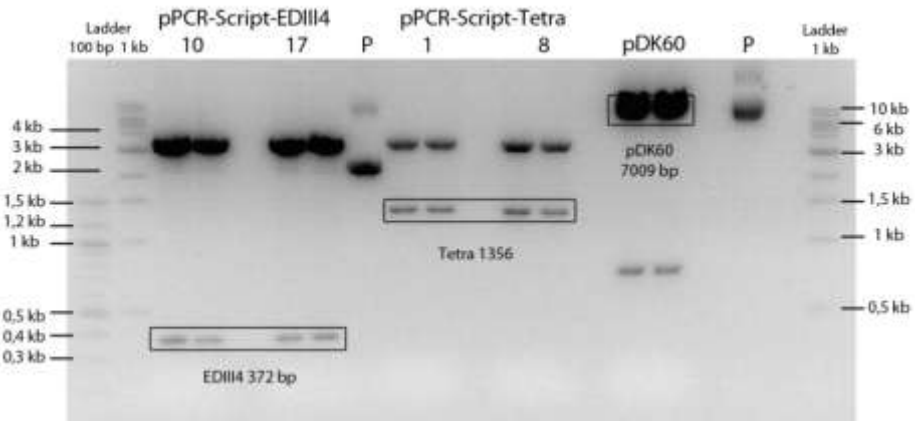
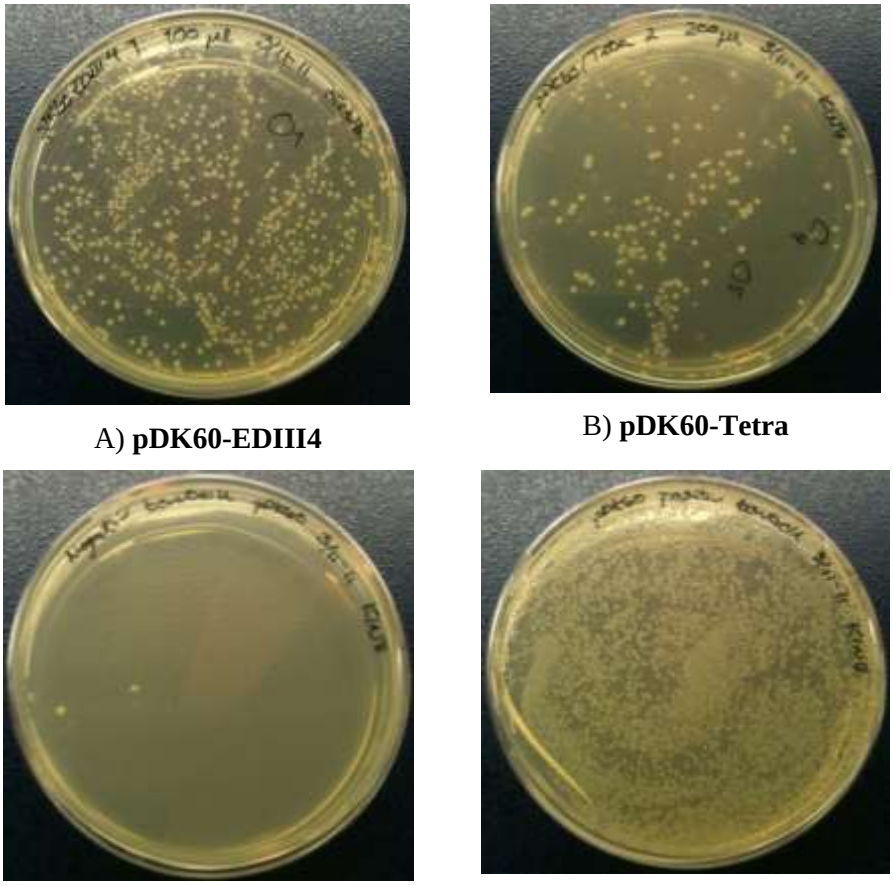


Figure 5.26: pPCR-Script EDIII4, pPCR-Script-Tetra and pDK60 digested with *BamH*I and *Xba*I. P is a control sample of uncut plasmid following its respective vector. EDIII4 of 372 bp, Tetra of 1356 bp and pDK60 backbone of 7009 bp were cut out of the gel

Ligation of EDIII4 and Tetra with pDK60 plasmid vector: EDIII4 and Tetra excised from the gel were ligated with pDK60 using T4 ligase and plasmid pDK60 containing EDIII4 and Tetra were transformed into *E. coli*. The transformed cells were plated on LB-agar plates containing 100 µg/ml ampicillin to select transformants.



A) **pDK60-EDIII4**

B) **pDK60-Tetra**

C) **Blank control**

D) **Positive control**

Figure 5.27: Selection of *E. coli* transformed with pDK60-EDIII4 and pDK60-Tetra. Both A) pDK60-EDIII4 and B) pDK60-Tetra had high growth. C) Negative control grew one colony D) Transformation of pDK60 confirmed that the transformation was successful.

The transformation were succesfully transformed, with growth of pDK60-EDIII4 and pDK60-Tetra on antibiotic selection medium whereas the negative control was total blank, confirming the ligation was working as well. Negative control had one colony, mostly likely due to the contamination during the practical work . Positive control showed very dense growth confirming that the transformation was efficient. For further analysis there were 5 colonies selected from pDK60-EDIII4 and 8 colonies of pDK60-Tetra. After being confirmed by antibiotic selection of colonies, they were further PCR screened to verify the inserts. Colony 3 and 7 from pDK60-Tetra and colony 1 and 5 from pDK60-EDIII4 were cultivated and plasmid DNAs were isolated for sequencing of the expression vectors. The plasmid sequences verified the correct insertion of both pDK60-EDIII4 and pDK60-Tetra.

5.3.2. Tobacco chloroplast transformation with pDK60-EDIII4 and pDK60-Tetra

Two separate independent transformation experiments were carried outs with pDK60-EDIII4. They were successfully selected and tobacco shoots were regenerated for two rounds. Additional regeneration on double selection was also conducted to discriminate mutant resistance from true transplastomic cells. Explants were successfully growing on the medium, indicating successful transformation of EDIII4.

In the primary selection there has been very little growth, as observed in figure 5.27 A. Once the primary transformants have been cloned for regeneration, the growth has been uniform in almost all clones (figure 5.27 B and C, figure 5.28 A, B and C). Picture D, E and F in figure 5.27 and 5.28 illustrates growth on double selection. The explants dedifferentiate to callus which develops green tissue and eventually shoots.

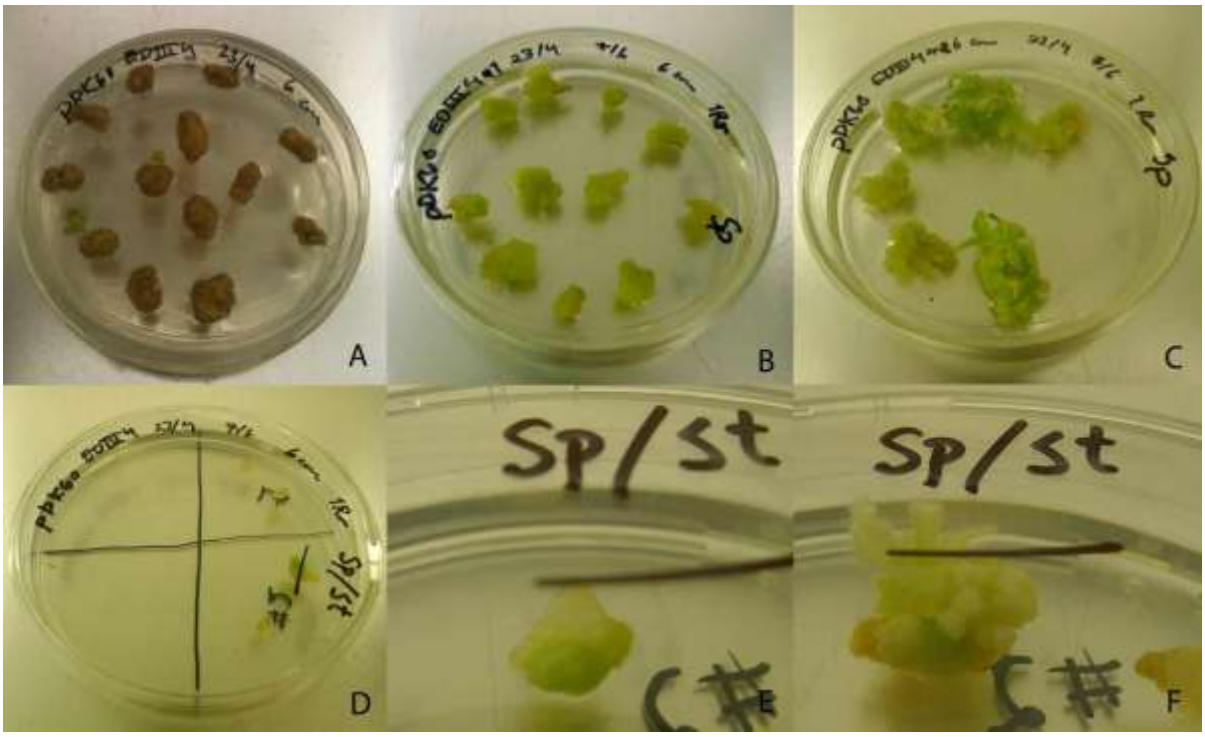


Figure 5.27: Selection and regeneration of EDIII4 transformed tobacco experiment #1. A) Two putative transformants grown on selection medium containing 500 mg/L spectinomycin. B and C) The two shoots were cut into small pieces and put on the second round selection with the same section pressure. Tissue has developed into callus shown green D) Double selection with 500 mg/L spectinomycin and 500 mg/L streptomycin. E) True transformed callus tuned into green on double selection. F) The same callus four weeks later has developed a shoot.

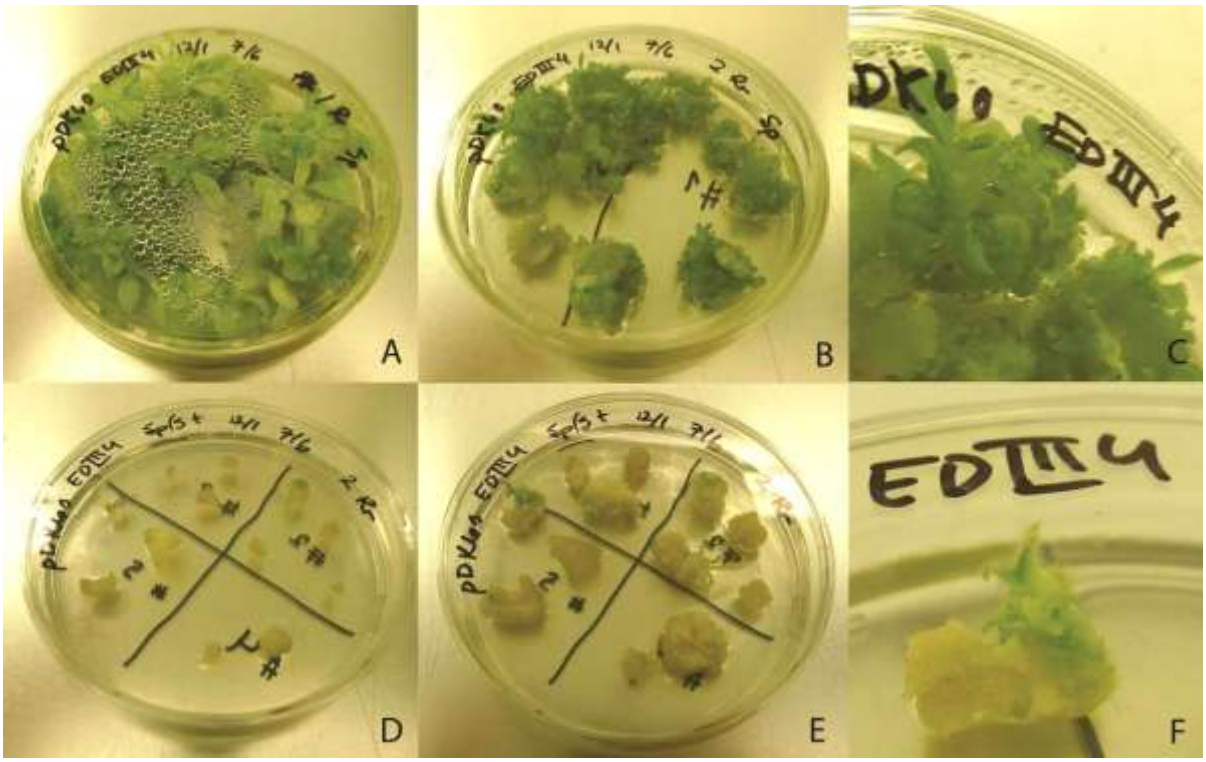


Figure 5.28: Selection and regeneration of EDIII4 transformed tobacco, experiment #2. A) Primary selection has been regenerated and growths of first regenerations were very good. B) The shoots have been regenerated in a second round and the growth were still good. C) Shoots from second regeneration round D) Growth of first regenerations on double selection after 3 weeks. E) The same plate after additional 4 weeks F) Shoots are growing from callus on double selection.

Tobacco chloroplasts were also transformed with pDK60-Tetra as shown in the summarized table.

Table 5.2: Statistics from chloroplast transformed tobacco

Vector	*Distance*	*Plates*	*Explants*	*Regenerations*	*Lines*
pDK60 EDIII4	6 cm	28	336	2	15
pDK60 EDIII4	9 cm	27	324	2	11
pDK60 Tetra	6 cm	16	192	2	0
pDK60 Tetra	9 cm	18	216	2	0

EDIII4 with 6 cm shooting distance had 15 regenerated explants from the second regeneration and EDIII4 with 9 cm shooting distance regenerated 11 tissues from the second regeneration.

Due to the lack of time and the growth of plants take time, the tobacco chloroplast transformation experiment with pDK60-Tetra is still ongoing.

6. Discussion

This thesis was aimed to express dengue virus EDIII-based antigens in tobacco chloroplasts and in the nucleus of tobacco with an inducible promoter. In this study, monovalent antigen number four (EDIII4) and the chimeric tetravalent antigen (Tetra) has been used, whereas other dengue antigen candidates were investigated by the project team and another master student.

The two antigens (EDIII4 and Tetra) have been introduced into nuclear and chloroplast expression vectors, i.e pSRN for nuclear transformation and pDK60 for chloroplast expression system.

6.1. Vaccine candidate: Envelope Domain III

Researchers have found that EDIII domain is best suited as antigen candidate. Monoclonal antibodies (mAbs) have been tested on the E protein where EDIII was demonstrated to be the most efficient blocker of virus infectivity (Crill and Roehrig 2001). EDIII is localized on the surface of the virus and it has epitopes that elicits binding with host cell. Studies have confirmed that antibodies can neutralize these epitopes and cause immunity against the virus. The four serotypes have minor variations in these epitopes that make EDIII a suitable vaccine candidate for immunity against all four serotypes (Etemad, Batra et al. 2008). In addition, expressing EDIII in a plant will be feasible with minor demands for peptide processing, as the structural integrity of EDIII depends on one single cysteine binding (Roehrig, Volpe et al. 2004). Also, EDIII has an independent folding domain, and it exhibits a very high degree of stability (Bhardwaj, Holbrook et al. 2001) which make it advantageous for recombinant protein production.

Up to date, a number of studies have described the expression of EDIII –based tetravalent dengue vaccine using yeast expression system and its immunogenicity. Unfortunately, a number of problems have been encountered, despite of the promising progress in recent years. Batra et al (2011) showed that the incorporation of the EDIII 1-4 serotypes into a single chimeric tetravalent antigen did not show any better effect than a physical mixture of the four

single antigens. Therefore, the current study has chosen the same strategy, to try a single chimeric tetravalent antigen and the physical mixture of the four single antigens.

6.2. Challenges in dengue vaccine production and potential solutions

Despite of that the attempt of dengue vaccine development initiated for almost a half century ago, no licensed effective dengue vaccine has been made available in the market. As a consequence of globalization and climate change, dengue has rapidly spread to more than 100 countries in the past 30 years. *A. aegypti*, the dengue-causing mosquito, was previously found normally at a height of 500 meters above sea level, but has recently been identified at an altitude of 2,200 meters in Darjeeling and 4,000 meters in Nepal, according to the WHO. This demonstrates the challenges and necessity of dengue vaccine development. As a result of global warming, vector borne diseases will have new destinations where populations were earlier unexposed to such diseases. Therefore, combating re-emerging infectious diseases like dengue is a very challenging task, and developing an affordable and effective dengue vaccine is highly prioritized.

Vaccination is considered to be the most efficient and cost-effective solution to combat infectious diseases for human health. Vaccination is only possible, however, under two essential conditions: The functional vaccine must (1) be available and (2) be affordable to all those people who need it. Unfortunately, vaccines are not affordable to all, especially as the price of vaccines has been on the increase over the past decade. If the vaccines can be made in a cheaper way, more children would benefit and more lives could be saved. Looking into plants (in particular tobacco plants) for solutions to these two points addressed above, especially aiming to bring the price down and make the vaccine accessible and affordable to poor people in developing countries has been the solely future goal of this project.

As described in the introduction, a functional dengue vaccine will have to be tetravalent in order to provide a stable and satisfactory immunologic protection against the four infecting dengue serotypes (DEN-1 to DEN-4). One encouraging example is DENVax, a dengue vaccine made by Inviragen that has reached clinical trial (Brewoo, Kinney et al. 2011). Hopefully, a dengue vaccine will reach the market in the future. Nevertheless, the dengue

vaccine has to be produced in a cost-effective manner so that it can reach the poor people who need them the most.

6.3. Tobacco plant as a green factory for production of low cost and affordable dengue vaccine

The major advantage of plant expression systems over other vaccine production systems is the reduced manufacturing cost. Fermenters and bioreactors can be replaced with contained plant growth rooms, greenhouses or plants can be grown in the field in a well-controlled environment. Tobacco is a non-food and non-feed crop with excellent biomass, which makes it an ideal choice for the production of vaccine antigens. To common knowledge, tobacco can cause damage to human health (such as lung cancer), so it is considered of great importance for tobacco farmers to be aware that tobacco can also be utilized as a plant factory for production of vaccines and biopharmaceuticals. With the consideration of biosafety, tobacco is a self-pollinating economic crop and transgenes that are introduced into the chloroplast genome cannot spread through pollen because chloroplasts and their genetic information are maternally inherited and pollen doesn't contain chloroplasts. Therefore, tobacco is no doubt a green vaccine factory.

This current study by manipulation of both tobacco nuclear and chloroplast genomes has demonstrated the feasibility and possibility for the future production of dengue vaccine in tobacco plants. Although only limited results are made available due to the time requirement for growing tobacco plants and the necessary analyses, both nuclear and chloroplast transformants are successfully produced and they are under investigation. The two candidate dengue antigens (EDIII4 and Tetra) were introduced into the tobacco nucleus and chloroplasts demonstrating the methods and tobacco plants we have used are highly feasible. To date, many laboratories worldwide have utilized tobacco plants to produce high value proteins for medical purpose, biofuel and cosmetic industries etc. (Clarke and Daniell 2011; Yusibov, Streatfield et al. 2011).

Developing a vaccine against dengue virus is challenging, because of the advanced immunologic response with ADE, along with the demands of cost-efficiency, safety and immunologic efficiency.

6.4. Development of oral vaccines against dengue fever in edible crops

Edible crops are ideal green factories for the production of therapeutic proteins and vaccines for oral delivery, eliminating the need for expensive fermentation, purification, cold storage, transportation, and sterile delivery. Oral delivery of recombinant plant-produced vaccines is a desirable way of vaccination due to the simplicity and safety of administration. The cost of downstream processing is usually about 80% of the production cost for plant-made pharmaceuticals and vaccines (Clarke and Daniell 2011; Yusibov, Streatfield et al. 2011). Thus, edible crops offer unique advantages in significantly reducing the production costs, facilitating the low cost of local production and minimum plant processing which are the most desirable and essential issues for affordable vaccines to the poor people in low-income countries. Stability of antigens at room temperature is a desired property of vaccines. The technology of freeze-drying plant biomass can be used, if necessary, to both concentrate the antigen and stabilize it in a dried form that can be stored at room temperature. To date, a number of vaccines have been produced in edible plants such as rice, maize, potato, carrot, lettuce, spinach, *Brassica*, goosefoots (*Chenopodium*), green pepper etc. and have shown immunological responses in mice (Ruhlman, Ahangari et al. 2007). Furthermore, oral delivery of tobacco-derived vaccines has also shown promising immunogenicity in mice and a number of them have entered clinical trial Phases I, II or III (Yusibov, Streatfield et al. 2011). This is the next step of the current project to produce edible dengue vaccine in lettuce.

Vaccine production in plants are still a young production system for proteins, but there are currently several vaccines and antibodies produced in plants that have reached clinical trials. Among these are vaccines against Norwalk virus, Newcastle disease virus, Rabies virus, H5N1and H1N1 influenza virus and *E. coli* LT-B. In addition, plants have also produced antibodies for HIV and Hepatitis B, as well as therapeutic proteins such as; insulin, glucocerebrosidase, gastric lipase, lactoferrin and intrinsic factor (Yusibov, Streatfield et al. 2011). A new era of the molecular farming in plants is approaching, as several plant-produced vaccines will be available within the next few years, paving way for acceptance and approval.

6.5. Evaluation of chloroplast vs nuclear engineering for a dengue vaccine

Chloroplast engineering is very promising in producing a safe, efficient and affordable vaccine against dengue. The biggest disadvantages and limitations to chloroplast engineering are low transformation efficiency and time-demanding development of transformants. Stable nuclear or transient transformation can be a necessary supplement to express the transgenes while waiting for transplastomic plants to be selected. The antigens must be studied for acceptance of the plant, stability, expression rate, correct folding and immunologic affinity. Based on these outcomes, the antigen might need to be modified in order to produce a stable, safe, efficient and functioning vaccine.

Using chloroplasts as a bioreactor for production of pharmaceutical proteins has been demonstrated to be very cost-efficient, and therefore it is an ideal production platform for highly cost-efficient vaccines, within reach for those who currently need it the most. As mentioned, chloroplasts can have up to 10 000 copies of the transgene in one cell, and transgenes can be arranged in operon to make polycistronic mRNA so that multiple transgenes can be translated simultaneously. This can result in abundant transgene transcripts and accumulation of the expressed proteins as high as 70 % of total soluble protein (Oey, Lohse et al. 2009). In contrast, nuclear transformed cells have only few copies in a cell, depending on chromosome number, and the transgenes are transcribed as monocistronic mRNA. Studies with expression of human serum albumin (HSA) in plant found that chloroplast engineering increased the expression 500-fold compared to previous attempt with nuclear genome engineering (Daniell, Khan et al. 2002).

Another advantage that has been demonstrated is the chloroplasts' ability to process peptides. Even though chloroplasts are prokaryotic, they have the ability to form disulfide bonds and correctly fold human proteins (which make them ideal bioreactors for vaccines and pharmaceuticals). Transgenes expressed from the nuclear genome must be transported to endoplasmic reticulum to form disulfide bonds. Bacteria cells used for expression of recombinant pharmaceuticals or vaccines does not have the ability to make proper bonds and fold the peptides correctly in order to make functional proteins, and additional processing of the peptides *in vitro* may be necessary. Such processing is very expensive and can make up over half the total production cost (Petrides, Sapidou et al. 1995).

Due to the potential for transgenes outcrossing with wild relatives or other crops via pollen, field production of many GM crops engineered via nuclear transformation has encountered strong opposition in several countries.

Using chloroplasts for genetic manipulation is more environmentally friendly than nuclear transformed plants, because chloroplasts are maternally inherited, which eliminates the risk of transgene spread with pollen (Hagemann 2010). This provides a better solution for engineering of agronomical important crops (Daniell 2007). Transgene containment via maternal inheritance would, however, will not be applicable to a few crops that show biparental inheritance of chloroplast genomes (e.g. *Pelargonium*).

Also, transformed chloroplasts are usually safer to the plant as well. Insertion of a transgene into the plastome is site-specific, which eliminates any position effects as often seen in nuclear transformation (which randomly inserts the transgene and can result in variable transgene expression levels). Position effects can cause gene silencing, either transcriptional or post-transcriptional, but this has not been observed in chloroplasts.

Nuclear transformed plants are generally more sensitive to foreign protein expressions in the cytosol because of interference with metabolism, and pleiotropic effects due to toxicity to the cell can be a problem. Foreign protein expressions in chloroplasts are generally more feasible because of simpler metabolism in chloroplasts. Also, this makes it possible to produce extreme amounts of foreign proteins in chloroplasts, as reported. Still, expression can reach high levels that can be toxic to the chloroplasts and cause male sterility, retarded growth and chlorosis (insufficient chlorophyll production that reduces photosynthesis) (Mohammad T. Waheed and Elke Lössl 2011). This has led to development of the trans-activation system (since chloroplasts are not feasible for chemical induction of inducible promoters), aimed as a solution to eliminate harmful expressions during critical growth stages.

According to the result from transplastomic chloroplast transformants produced in this study, the EDIII4 plants which grew on double selection, as demonstrated in figure 5.27 and 5.28, showed promising results, but since the plants did not reach adequate developmental stage in time, analysis could not be performed. The chloroplast transformed plants need to be screened with PCR to examine if EDIII4 and Tetra are present. A southern blot analysis of the DNA is planned to reveal the homoplasmy. Heteroplastic plants must be regenerated additional rounds

in order to prevent that the transplastomic chloroplasts are eliminated by segregation. Chloroplasts transformed with pDK60 will contain GFP (green fluorescent protein) that can be examined in microscopy for fluorescence, to confirm that the chloroplasts are transplastomic. When protein expression has been detected, the antigens need to be examined further. Our preliminary results from chloroplast-expressed dengue antigens have revealed that the antigen-proteins seem to be toxic to chloroplasts and the transplastomic tobacco plants expressing monovalent EDIII 1-4 and tetravalent grow poorly and show severe negative impacts on the plant development (data not shown). In this study, we observed that regeneration of tissue was generally very slow and selected tissue was often pale, which can be explained by the toxicity to the chloroplasts.

Further research is planned to confirm if the antigens are actually toxic to the chloroplast, and new solutions must be prepared. The ongoing research with trans-inducible expression of the antigens in tobacco by Andres Lössl will tell if the toxicity to the chloroplasts can be eliminated by delaying the antigen expression until plants are mature. So far, preliminary results are promising with normal growth and healthier regenerated plants, but expression level is lower than expected (unpublished).

6.6. Nuclear transformed plants with inducible expression

6.6.1. Protein expression analysis

Protein analysis with SDS-PAGE of the nuclear transformed plants did not detect the accumulation of antigen peptides of EDIII4 and Tetra. Expected size of EDIII4 were 25,8 KDa and all the gels had two bands around 25 KDa. Expected size of Tetra were 55,0 KDa which also was present in all gels of Tetra. Further study is going to be carried out soon to clarify this preliminary result. It did not show any pattern of increased expression of these peptides after ethanol induction. The wild-type and T7-plant have highest expression on day 1, also with the stained gel from the western blot. Still, it is not valid to estimate any increased expression because all the band intensity in a sample varies equally. These variable intensities on the gels are indication of the concentration of each extraction. The amount of protein used for extraction was not measured correctly and during the extraction procedure and application

on the gel some protein could have been lost, resulting in different concentrations of proteins in the samples. The sample preparation for the gel analysis was incomplete in most samples. After loading buffer was added to the samples, they should be heated (75°C for 3 minutes) to solubilize precipitates. When this was left out before application on the gel, the precipitated proteins accumulated on the top of the wells and it reduced the amount of soluble proteins for separation in the gel. Only the samples of EDIII4 E, F, G, H, I and J (shown in the results session) was prepared correctly and the gel results clearly differs from the other gels, as the protein amount is more even in all the samples. It cannot be observed any single bands with changed amount of expression compared to the other bands in the sample in these gels either.

Because of inadequate performance, the gels are poor and any comparing of the gels does not give any trustworthy observations. No increased expression levels are seen in any of the gels and all gels basically contain the same bands. Further study will take into consideration of these problems.

Another possible explanation to the undetectable EDIII4 and Tetra expression after ethanol spray (induction) is due to insufficient induction. They were mistakenly only sprayed once, at day 0, to observe how the expression rates increase during the 7 days, but according to the protocol, ethanol spray shall be carried out every day and then the expression will increase throughout the period. The T7 plants should at least have showed expression, as it has previously been demonstrated by co-workers (Lössl, Bohmert et al. 2005).

6.6.2. Western blot analysis

The sample preparations prior to gel application were performed correctly and the protein amount in the gels was more evenly present. The blotting procedure of EDIII4 (A, B, C and D) and Tetra (A, B, C and D) was successfully performed, but the control gel with T7 and wild-type plant was all blank. The quality of the finished membranes was poor because it was overexposed, with ladders that were difficult to predict the sizes, a lot of noise in the background/film and in addition the bands were weak and not of expected size. The EDIII4 and Tetra gels were not stained after blotting, but it would have given an improvement of the blotted results.

With the unsuccessful blotting of wild-type and T7 plants it is difficult to predict anything from the gels. Since the results from the protein gels did not give any indication of expressed

antigens either, it was decided not to go further with western blot on the remaining plants of EDIII4 and Tetra (E, F, G, H, I and J) and the control gel was not redone.

It might be that the anti-His was not specifically binding to the antigens, but since the gels did not indicate that they were expressed either they were probably not present. If there had been more time, all plants would have been analyzed with western blot and the gels used for blotting should have been stained. Other antibodies could have been tested as well. Antibodies against dengue virus, anti-DENV 1-4, are available in the lab, but previous studies have not shown immunologic affinity for the EDIIIs with these antibodies. Since folding of the EDIIIs and Tetra is difficult to predict, specific antibodies for the EDIIIs or Tetra antigens cannot be produced before the antigens are expressed, isolated and purified.

In conclusion, the induction was probably not sufficient, which resulted in undetectable expression of the antigens. It could also be owned to that the transgene could have been silenced and prevented expression, or expressed peptides could have been degraded.

6.7. Concluding remarks and outlook

In summary, EDIII4 and Tetra have been inserted in the nuclear genome of tobacco and confirmed that it is feasible to use tobacco for

Because the chloroplast engineering is demanding, restricted knowledge and results of the antigens has been gained so far (in the project). Transformation of the nuclear genome of tobacco with inducible expression was aimed to express the antigens more easily and rapidly to gain more knowledge of the antigens.

Little is known of how the antigens are expressed and folded, and how the plant will respond to the foreign protein. If the antigens are toxic to the chloroplast, it might necessary to modify the antigens, or combine it with trans-activating inducible expression system or plant species can be changed.

Once the antigens are being expressed, they need to be analyzed for immunologic response, to confirm that they can be neutralized by antibodies. A clinical trial with testing on animals and finally humans must be performed to confirm efficiency and safety of the vaccine candidate. A clinical trial will tell which of the chimeric tetravalent antigen or mixed vaccine of the four antigens have best efficiency and safety.

Then, modifications can be performed to optimize the transgene expression. A cost-effective vaccine is depending on high yields and minimal processing. Vaccines can be made as injections, capsules for oral digestion or edible vaccines. Tobacco cannot be consumed due to the nicotine and the vaccine proteins must be extracted into injections. Processing and purification can be costly. Vaccine produced in tobacco species of low nicotine containment (or other crops) can be produced more cost-efficient by introducing the vaccine in capsules which reduces processing and purification. For even more cost-efficient vaccines, edible crops, such as lettuce or tomato can further reduce processing or eliminate it.

7.Referances

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