

Transient expression of poliovirus-like particles in plants.

Developing a synthetic polio vaccine

by

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This thesis is submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy at the University of East Anglia.

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DECLARATION

I hereby certify that the work contained within this thesis is my own original work, except where due reference is made to other contributing authors. This thesis is submitted for the degree of Doctor of Philosophy at the University of East Anglia and has not been submitted to this or any other university for any other qualification.

Johanna D. Marsian

ABSTRACT

Plants, or cell suspension cultures derived from them, are a promising platform for the production of biologics and pharmaceuticals. In this work transient expression utilising the pEAQ vector system was deployed for the expression of virus-like particles (VLPs) in *Nicotiana benthamiana* or *N. tabacum* BY-2 cell suspension cultures. The results presented in this thesis demonstrate the potential of plant systems for the production of VLP-based vaccines.

VLPs of the fish virus, Nervous necrosis virus (NNV), were successfully produced in plants by transient expression of the coat protein. The protein self-assembled into T = 3 particles, which appeared to be morphologically identical to the wild-type NNV when analysed by high resolution microscopy but were devoid of nucleic acid. In addition, transgenic BY-2 cell suspension lines were generated expressing correctly assembled NNV VLPs.

Poliovirus (PV)-like particles from all three PV serotypes, containing either the wt coat protein or coat proteins with stabilising mutations, were successfully expressed in plants. These were generated by co-expression of the structural polyprotein P1 and the proteinase 3CD. Sufficient quantities of purified particles could be obtained for structural and immunological analysis. Mice carrying the gene for the human PV receptor were protected from wild-type PV when immunised with the plant-made stabilised PV VLPs. Structural analysis of the stabilised mutant of PV3 at 3.6 Å resolution revealed a structure almost indistinguishable from wild-type PV3, with the stabilising mutations having no effective on the antigenic surface of the particle.

To make the product more attractive to the vaccine industry, tobacco BY-2 cells have been successfully tested for the transient expression of the above-mentioned PV mutant VLPs using the cell-pack method.

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LIST OF ABBREVIATIONS

AFP	Acute flaccid paralysis
BTV	Bluetongue virus
BY-2	Bright Yellow-2
CaMV	Cauliflower mosaic virus
CNS	Central nervous system
CPMV	Cowpea mosaic virus
Cryo-EM	Cryo-electron microscopy
cVDPV	Circulating vaccine-derived polioviruses
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpi	Days post infiltration
D_{AgU}	D antigen units
ELISA	Enzyme-linked immunosorbent assay
EV	pEAQ empty vector control
eVLP	Empty virus-like particle
FMDV	Foot and mouth disease virus
GFP	Green fluorescent protein
GMP	Good manufacturing practise
GPEI	Global Polio Eradication Initiative
HBV	Hepatitis B virus
hGL	Human gastric lipase
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HT	Hyper-Trans
IRES	Internal ribosome entry site
KBP	Kentucky BioProcessing LLC
LB	Left border
kDa	Kilodalton
LB media	Laura-Bertani media
MCS	Multiple cloning site
mRNA	Messenger RNA
MS	Murashige and Skoog
MWCO	Molecular weight cut-off
NDV	Newcastle disease virus
NIBSC	The National Institute for Biological Standards and Control
NNV	Nervous necrosis virus
nos	Nopaline synthase
OD₆₀₀	Optical density at 600 nm
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
pEAQ	Easy and quick plasmid
PRRSV	Porcine respiratory and reproductive syndrome virus
PV	Poliovirus

PVX	Potato virus X
RB	Right border
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssRNA	Single-stranded RNA
TBE	Tris-borate-EDTA
T-DNA	Transfer DNA
TEM	Transmission electron microscopy
TBSV	Tomato bushy stunt virus
UNICEF	United Nations Children's Fund
UTR	Untranslated region
VAPP	Vaccine-associated paralytic poliomyelitis
VDPV	Vaccine-derived poliovirus
VHH	Variable region of the heavy chain of a heavy-chain antibody
VLP	Virus-like particle
VNN	Viral nervous necrosis
WHO	World Health Organization
Wt	Wild-type

Chapter 1: GENERAL INTRODUCTION

1.1 MOLECULAR PHARMING

Molecular pharming is a modern buzzword that refers to the recombinant expression of pharmaceutically useful proteins in plants. It started with the production of chimaeric human growth hormone in 1986 via transgenic tobacco and sunflower (Barta et al., 1986), followed by the synthesis of monoclonal antibodies in transgenic tobacco in 1989 (Hiatt et al., 1989) and human serum albumin in transgenic tobacco and cell cultures (Sijmons et al., 1990). Protein-based pharmaceuticals are big business with a potential market of \$US 278.2 billion by 2020 (PMR, 2015). In recent years plants have become an attractive alternative to more conventional cell-culture based approaches (see below section 1.1.1) for the production of a wide array of pharmaceutical components as many researchers have discovered the advantages of plants as bioreactors. One of the reasons for the renewed interest in plants as bioreactors is the development of methods for the rapid production of candidate proteins via transient expression, obviating the requirement for lengthy transformation and regeneration procedures.

1.1.1 PLANTS AS EXPRESSION SYSTEM

In the last 20 years plants have become serious competitors to other production systems for pharmaceuticals, such as bacteria, yeast or mammalian cells. They offer a safe, inexpensive and potentially limitless way to produce therapeutics in a quick and flexible manner. Plants require only simple inorganic nutrients, water, carbon-dioxide and sunlight for efficient growth. Furthermore, they bring a low risk of contamination with endotoxins or mammalian pathogens (Lico et al., 2008; Ma et al., 2003; Twyman et al., 2003) which can be an issue with mammalian cell cultures. Unlike prokaryotic expression systems, plants are able to introduce post-translational modifications such as glycosylation (Ma et al., 2005). In insect and yeast cells glycosylation is limited to very simple and inconsistent high mannose glycoforms (Chen and Lai, 2013). It is of advantage if a production system for pharmaceutical components, especially vaccines, is quick in the response to a sudden increase in demand. Transient expression in plants can be adjusted rapidly with low manufacturing costs (Giddings, 2001; Twyman et al., 2003).

Nicotiana benthamiana (*N. benthamiana*) has long been the favourite host when it comes to the production of recombinant proteins since it is well studied and agro-infiltration is easy without causing great damage to the tissue (Goodin et al., 2008). Additionally, it allows a wide number of plant viruses to replicate which makes it a versatile host for virus-derived expression vectors. However, protein production is not limited to *N. benthamiana*. Several types of plants and plant tissues have been used for the production of protein such as potatoes, alfalfa and *Arabidopsis thaliana* but lettuce stands out as a VLP production host that combines all the benefits from *N. benthamiana* but furthermore contains fewer phenolics and alkaloids which can be difficult to remove (Lai and Chen, 2012; Platis and Labrou, 2008; Rybicki, 2009). A decade or two ago the idea of edible vaccines, in which plant tissue expressing the recombinant protein would be directly consumed, sparked a lot of interest, but has now lost its popularity due to problems of dose and quality control which is difficult to guarantee without purification (Rybicki, 2014; Sala et al., 2003).

Despite the major risks and flaws, mammalian cells dominate the biopharmaceutical industry because they can produce high titres (1-5g/L) of complex proteins with mammalian glycan structures (Chu and Robinson, 2001); with Chinese hamster ovary cells culture being the most successful manufacturing platform for biologics. In direct comparison, plants produce at least 10-fold less recombinant protein and it can take up to 18 months to create a stable transgenic plant line. This means so far plants produce less of the desired product than the other platforms and it takes longer to do so (Thomas et al., 2011). However, this can be overcome with the development of transient expression system in plants. Large amounts (50 mg/kg fresh weight) protective antigens of influenza were transiently produced in plants within less than three weeks from the release of the viral sequence to the end product (D'Aoust et al., 2010; D'Aoust et al., 2008).

In 1982 the first commercially produced therapeutic recombinant protein was insulin made in *Escherichia coli* (*E. coli*). Its success is down to the simplicity and low costs of the product (Santos et al., 2016). The first plant-made therapeutic (PMT) protein approved by the US Food and Drug Administration (FDA) for human use was Protalix's Eleylso (taliglucerase alfa) in 2012 and the first plant-made vaccine got approval in 2006 by the US Department of Agriculture for the use in chicken against Newcastle disease virus

(NDV) (FDA, 2012; Sparrow et al., 2007). However, both pharmaceuticals were not produced in whole plants but in plant cell suspension cultures.

Plant suspension cultures are a potentially great alternative to whole plants for recombinant production of pharmaceuticals. I will introduce their characteristics, benefits and flaws in the next section.

1.1.2 PLANT SUSPENSION CULTURES

Despite the breakthrough of Protalix's Eleyso- the first licensed PMT, plant suspension cultures are still very much neglected and their capability not fully exhausted. Even though plant suspension cultures are around for over four decades, with the first reported recombinant protein made in 1990 by Sijmons and colleagues (Sijmons et al., 1990), efforts have lagged to exploit their potential. Companies and investors are wary of this unfamiliar production method (Maxmen, 2012).

Plant suspension cultures combine the advantages of whole plants with those of traditional fermenter systems. They grow in contained, controlled and sterile environment with almost no limitation for mass culture and are capable of high quality protein production with complex glycosylation. The growth media is inexpensive, lacking animal components but unlike whole plants, suspension cultures are not phototrophic and therefore need a carbon source. The cultivation strategies are versatile and range from shake flasks, stirred tank reactors (Doran, 1999), wave reactors (Eibl et al., 2010) to bubble columns (Terrier et al., 2007). Most commonly used is the batch fermentation where the medium is inoculated and after inoculation the reactor is a closed system except for added oxygen (Santos et al., 2016). All this makes production of therapeutics in plant suspension cultures compatible with pharmaceutical good manufacturing practice (GMP).

Plant suspension cultures are not limited to one plant species; the most commonly used ones derived from tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*) and carrot (*Daucus carota*). Bright Yellow-2 (BY-2) is a widely used tobacco cultivar which can multiply up to 100-fold within 7 days with a doubling time of 16-24 h under ideal conditions (Santos et al., 2016) (Figure 1.1-1). Developed in 1968 in the Japan Tobacco Company (Kato, 1972) transformation with *Agrobacterium tumefaciens* (*A. tumefaciens*) has proven to be highly efficient (Nagata et al., 1992) in this cultivar; allowing the synthesis of several

biopharmaceuticals like Hepatitis B surface antigen (Smith et al., 2002) or the Norwalk virus capsid protein (Zhang and Mason, 2006).



Figure 1.1-1: BY-2 cells under the light microscope.

1.1.3 STABLE- VS. TRANSIENT-EXPRESSION

There are two different ways to express pharmaceuticals, including VLPs, in plants and suspension cultures: (i) stable transformation of either the nuclear or plasmid genome or (ii) transient expression. Though used for seminal studies in the early days of molecular pharming, the use of stable transformation has declined as the process is time-consuming and therefore not suitable for rapid screening of a wide variety of different constructs or a quick response to emerging epidemics (Kusnadi et al., 1997). Transient expression uses the soil bacterium *A. tumefaciens* which causes crown gall disease, to deliver foreign sequences to plants. Since the 1970's it has been known that this gram-negative bacterium can transfer a part of its special Ti (tumor-inducing) plasmid into the plant cell where it then integrates into the host genome (Chilton et al., 1977). The gene of interest is inserted into the T-DNA region of a binary plasmid which will then be expressed in the plant cell. This allows screening and production in a matter of days. However, expression is limited to the infiltrated tissue but large-scale production and processing has been developed (D'Aoust et al., 2010). This is in contrast to stable transformation where the foreign gene becomes part of the genome and making the transgene heritable through succeeding generations.

All three platforms have advantages and disadvantages—despite their great scalability, transgenic plants have a slow development cycle due to the need to regenerate plants and produce homozygous true-breeding lines. Also regulations of transgenic plants handicap the possibilities of this system. However, a great example for a successful

therapeutic made in transgenic plants is the HIV-neutralizing human monoclonal antibody 2G12 which is currently undergoing phase I clinical trials (Ma et al., 2015). Transient expression is not as tightly regulated as transgenic plants; mainly because the gene of interest is not introduced into the germline and hence not heritable and also transmission through pollen is impossible. Transient expression is more established and accepted with Medicago's influenza vaccine currently undergoing phase 3 clinical trials (D'Aoust et al., 2010). It allows the rapid production of high protein yields ideal for emergencies such as vaccines and prophylactic antibodies, as seen in the recent outbreak of Ebola virus disease in West Africa (Arntzen, 2015). Although scalability has shown to be possible it is still a very niche production system. In contrast plant cell suspension cultures allow contained production very similar to the commonly used prokaryotic or mammalian cell based expression systems. Plant cells have been overshadowed by whole-plant platforms but the approval of taliglucerase alfa for use in human adults in 2012 and then for paediatric use in 2014 (Tekoah et al., 2015) has opened the way to the full acceptance of this technology and all plant-based pharmaceuticals in general.

1.2 VIRAL VECTORS USED FOR VLP PRODUCTION

A number of plant virus-derived systems have been employed for transient expression of VLPs. These range from vectors based on the complete viral genome to which a foreign gene has been added, through deconstructed vectors in which non-essential viral functions have been deleted, to non-replicating virus-based systems. Full viral vectors behave like wild type virus by moving within their host and producing infectious virus particles but are also able to express heterologous proteins. Their ability to replicate and move from cell to cell and systemically results in extremely high levels of protein expression. However, during replication the gene insert can undergo mutation or deletion and it is difficult to express several genes in the same cell. Two full virus vectors, the potato virus X (PVX) and the cowpea mosaic virus (CPMV) have mostly been used for epitope display (Canizares et al., 2006; Marusic et al., 2001) but also for the plant-based expression of hepatitis core-like particles (Mechtcheriakova et al., 2006). The use of full virus vectors has been reviewed by Porta and Lomonossoff (Porta and Lomonossoff, 1996, 2002).

The creation of deconstructed viral vectors involves the removal of viral genes which are not essential for the production of the target protein, for example the viral coat protein, and may be replication-competent or replication-deficient. They are designed for rapid, high level production of recombinant proteins in plants, without the problems of biocontainment. The history and use of deconstructed viral vectors has recently been reviewed (Peyret and Lomonossoff, 2015) and the pEAQ and magnICON®- systems have, in particular, been used to produce VLPs in plants (Matic et al., 2012; Thuenemann et al., 2013b).

The pEAQ vectors are based on CMPV; a bipartite virus whose genome consists of two separately-encapsidated RNA molecules: RNA-1 (6.0 kb) and RNA-2 (3.5 kb). Each RNA molecule has a single open reading frame encoding one polyprotein. RNA-1 carries the sequence for the viral replication machinery and the 24K protease, which is responsible for processing of the polyproteins. RNA-2 encodes the coat proteins plus the movement protein but depends entirely on RNA-1 for its replication. The first major step towards the development of the pEAQ vectors was the finding that most of the RNA-2 polyprotein can be deleted and replaced with a sequence of choice without impairing its replication by RNA-1 (Canizares et al., 2006; Rohll et al., 1993). This so called delRNA-2 construct had to be introduced into leaves by agro-infiltration (Liu and Lomonossoff, 2002) together with RNA-1 (Canizares et al., 2006). It was found that keeping the first 512 nucleotides at the 5`end of RNA-2, including two in-frame AUGs at positions 161 and 512 (the main translation initiation site), as well as the entire RNA2 3`UTR is necessary to sustain the ability of the delRNA-2 construct to be replicated by RNA-1 (Rohll et al., 1993). However, this required the precise in-frame positioning of the foreign sequence between AUG 512 and the 3`UTR which made insertion of heterologous sequences into the delRNA-2 construct a troublesome two-step process (Sainsbury et al., 2009). Another important finding was that co-infiltration of the delRNA-2 construct with P19, the suppressor of gene silencing from Tomato bushy stunt virus, stabilised the mRNA transcribed from the T-DNA. The effect is so powerful that replication by RNA-1 could be suspended. Studies originally intended to simplify cloning, revealed that removal of AUG 161 caused a 10-fold increase in yield of an inserted GFP gene through increased translational efficiency of the mRNA (Peyret and Lomonossoff, 2013; Sainsbury and Lomonossoff, 2008). Further improvement was gained when another out-of-frame upstream AUG at 115 was removed, even though removing only AUG 115 weakened translation compared to the wildtype. The explanation behind these

results could be that AUG 161 inhibits translation initiation at AUG 512. This also supports the finding that removing AUG 115 initiates an open reading frame that extends past AUG 161: AUG 115 might allow ribosomes to bypass AUG 161 and re-initiate at AUG 512 (Sainsbury and Lomonossoff, 2008). This new and improved expression system showed to enhance expression of GFP, DsRed, the Hepatitis B virus core antigen (HBcAg), and human anti-HIV antibody 2G12 (Peyret and Lomonossoff, 2013; Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2010b). For the reason that the high yields were due to increased translation the system was named *CPMV-hypertrans* (*CPMV-HT*). This early HT expression system, based on a shortened version of CPMV RNA-2 with a mutated 5'UTR, is the direct precursor to the pEAQ vector series.

In order to make cloning and expression with *CPMV-HT* easier the plasmid backbone, originally derived from pBINPLUS, had been reduced to less than half its original size without compromising transient expression levels; resulting in a 5.2 kilobase backbone. The reduction in size resulted in greatly improved yields during cloning procedures. To ease addition of further expression cassettes into the T-DNA, three restriction sites (AsiSI, MluI and FseI) adjacent to the original Ascl site of pBINPLUS were added. The resulting vector pEAQbeta was the starting point in the Lomonossoff lab for the design of a series of vectors, including pEAQ-*HT* (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2009).

The pEAQ-*HT* expression vector (published on GenBank under accession number GQ497234.1) was used for the work described in this thesis. It carries the P19 suppressor of gene silencing, NPTII eukaryotic kanamycin resistance gene and a multiple cloning site within the *CPMV-HT* cassette; which allows replication of the plasmid in *E. coli* and *A. tumefaciens* along with high levels of expression in plants (see Figure 1.2-1). The pEAQ vectors have been deployed in the production of a number of different proteins through several different approaches. A variety of enveloped and non-enveloped VLPs have been expressed in plants using the pEAQ vectors. These include hepatitis B virus (HBV), papillomaviruses and porcine respiratory and reproductive syndrome virus (PRRSV) (Thuenemann et al., 2013a). Furthermore, active enzymes have been synthesised through agroinfiltration of pEAQ, like human gastric lipase (hGL) (Vardakou et al., 2012). Even entire synthetic pathways have been manipulated using pEAQ through co-expression of multiple enzymes which showed full functionality *in planta* (Kanagarajan et al., 2012). This allows the analysis of their modes of action in the

biosynthetic pathway, as well as potentially allowing metabolic engineering. The pEAQ vectors have proven to not only work in whole plants but also in a variety of transgenic cell suspension cultures (Larsen and Curtis, 2012; Sun et al., 2011).

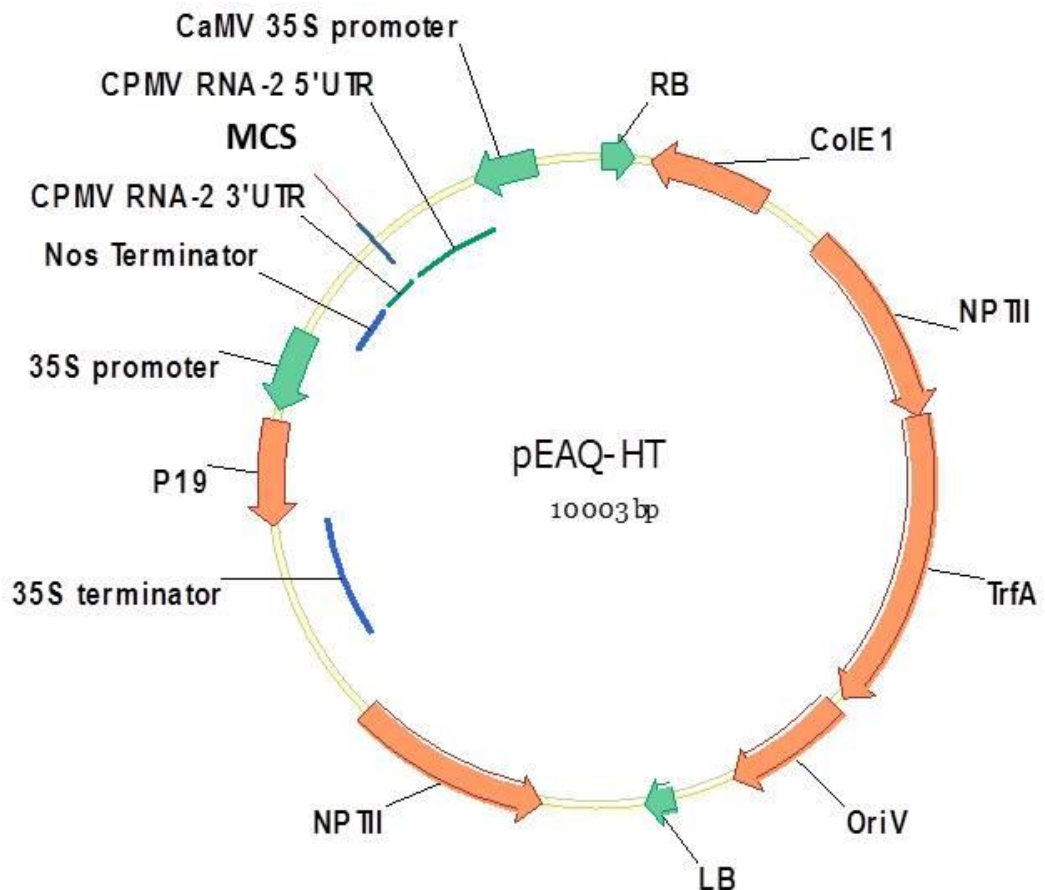


Figure 1.2-1: Diagram of the pEAQ-HT expression vector. The vector backbone harbours essential elements for plasmid replication in *E. coli* and *A. tumefaciens* and the T-DNA carries the NPTII kanamycin resistance gene and the P19 suppressor of gene silencing. The gene of interest is inserted in the multiple cloning site (MCS) between the modified CPMV RNA-2 5'UTR containing the *HT* mutations, and the native CPMV RNA-2 3'UTR. These UTRs are themselves flanked by the CaMV 35S promoter and the nos terminator.

Behind the high expression potential of the pEAQ vectors lays a modification of the 5'UTR in the CPMV cassette. It was found that removal of AUG 161 caused a 10-fold increase in yield of an inserted GFP gene through increased translational efficiency of the mRNA transcribed from the T-DNA. A further enhancement was obtained when an additional out-of-frame upstream AUG at 115 was also removed (Sainsbury and Lomonosoff, 2008).

1.3 VIRUS-LIKE PARTICLES AS VACCINES

VLPs are convincing look-alikes of viruses but are lacking infectious genomic material. However, VLPs retain the native antigenic conformation of the immunogenic proteins and the repetitive structure of the original virus particle. They thus combine both safety and the potency to elicit a strong immune response (Grgacic and Anderson, 2006; Kushnir et al., 2012) due to their size, shape and surface where they display epitopes in a dense repetitive array (Bachmann and Jennings, 2010; Chen and Lai, 2013). Following the recombinant expression of the viral proteins many self-assemble spontaneously into VLPs, though some require a maturation step. Currently used non-recombinant vaccines against viral diseases are made from lab-cultured pathogens which have to be attenuated or inactivated. This comes with bio-containment concerns because of the risk of residual pathogenic activity due to incomplete attenuation or inactivation. It is of fundamental importance for a vaccine to be unable to cause disease without compromising immunogenicity. A potentially safer approach is therefore the synthesis of vaccines based on the expression of specific components of pathogens which can mimic the immunological properties of the original virus without its pathogenic properties and VLPs are excellent candidates for such synthetic vaccines. In addition, certain VLPs have also been investigated as potential drug-delivery vehicles and as imaging agents for biomedical purposes.

The concept of using plants for the synthesis of pharmaceuticals is more than two decades old and several different VLP based vaccine candidates have been produced:

Bluetongue virus (BTV) is a non-enveloped, insect-transmitted virus causing the Bluetongue disease in ruminants. Its complex, multi-layered capsid is built out of four structural proteins arranged in three concentric shells; each protein is present in a different stoichiometry. Despite this complex setup, Thuenemann et al. successfully produced BTV-8 VLPs in *N. benthamiana* which effectively protected sheep from challenge. The approach to their production involved the co-expression of the four different viral structural proteins using pEAQ-*HT* with carefully adjusted ratios in order to obtain large quantities of correctly-assembled VLPs (Thuenemann et al., 2013b).

Human Papillomavirus (HPVs) is a non-enveloped tumour-inducing DNA virus that accounts for approximately 5% of all human cancers (Munger et al., 2004; Parkin and

Bray, 2006). The capsid is about 55 nm in diameter formed by the major (L1) and minor (L2) capsid protein. The L1 protein self-assembles into highly immunogenic VLPs or into capsomers made of five L1 monomers (Kirnbauer et al., 1992). Three VLP based vaccines against HPV are already on the market (Gardasil, Gardasil-9 (Merck) and Cervarix (GSK)) based on the expression of L1 in insect or yeast cells (Harper et al., 2004; Villa et al., 2006). However, high production costs hinder their use in developing countries as most people at risk cannot afford them. Matic et al. aimed to develop an alternative, less expensive vaccine made in plants using the pEAQ system (Matic et al., 2012). They expressed full-length HPV-8 L1 and a truncated version, produced by deleting the C-terminal nuclear localization signal. Electron microscopy proved that assembly of the expressed HPV-8 L1 into VLPs of T = 1 or T = 7 symmetry had occurred (Matic et al., 2012) and thus represents another candidate vaccine.

Norwalk virus (NV) causes acute gastroenteritis in humans. The major problem for developing a viable vaccine against NV is that there is currently no method of propagating the virus in cell culture and hence traditional killed or live-attenuated vaccines have not been developed. Most recently, Mathew et al. confirmed the production of Narita 104 virus capsid protein VLPs in *N. benthamiana* which generate a significant mucosal and serum antibody response when inoculated into mice demonstrating that the authors had made a potential plant-derived vaccine candidate against NV (Mathew et al., 2014).

Probably the most successful plant-produced vaccine candidate, currently undergoing phase III clinical trials, is against **influenza virus**. D'Aoust et al. showed that plants are capable of producing enveloped influenza VLPs which elicit a protective immune response in mice (D'Aoust et al., 2008; Landry et al., 2010). Recently Medicago USA Inc. adapted the plant-based influenza VLP expression to large-scale production allowing the purified end product to be obtained only 3 weeks after receiving the sequence (D'Aoust et al., 2010).

The fact that complex particles such as multishelled orbivirus capsids, as well as enveloped influenza VLPs can be synthesized in plants shows that plant-based expression of such therapeutics can deliver to at least the same level as conventional systems.

Table 1.3.1: Transiently expressed VLP-based vaccine candidates made in plants.

Virus	Summary	Reference
Influenza	Influenza virus-like particles induce a protective immune response against a lethal viral challenge in mice, produced for H7N9 outbreak virus.	D`Aoust et al., 2010
Papillomavirus	HPV-16 L1 VLPs via agroinfiltration-mediated transient expression or via transplastomic expression.	Maclea et al., 2007 Fernandez-San Millan et al., 2008
Papillomavirus	Expression of HPV-8 L1 VLPs.	Matić et al., 2012
Papillomavirus	Transient expression of chimaeric L1::L2 VLPs and proof of increased breadth of immune response.	Pineo et al., 2013
Bovine papillomavirus	Transient expression of BPV L1 VLPs.	Love et al., 2012
Human norovirus	NaVCP VLPs which generate a mucosal and serum antibody response.	Mathew et al., 2014
Bluetongue virus	Protective bluetongue virus-like particles.	Thuenemann et al., 2013b

Table 1.3.1 summarises the potential lying within the transient expression of VLPs in plants. In this work two very different viruses were chosen in order to explore the possibilities of plant-based vaccine production. The rather simple nervous necrosis virus and the more complex poliovirus will be further described in the following chapters of this thesis.

1.4 NERVOUS NECROSIS VIRUS

Nervous necrosis virus (NNV) infection is a serious disease known to affect over 40 cultured marine fish species worldwide (Tan et al., 2001; Walker and Winton, 2010), causing extensive economic losses to the aquaculture industry each year (Lin, 2007). The disease caused by NNV is commonly known as viral nervous necrosis (VNN). Infected fish display extensive cellular vacuolation and neuronal degeneration in the central nervous

system and the retina, due to NNV induced apoptosis (Figure 1.4-1) (Chen et al., 2006; Wang et al., 2010). Symptoms of VNN are abnormal swimming behaviour and muscle tremors (Roy, 2010).

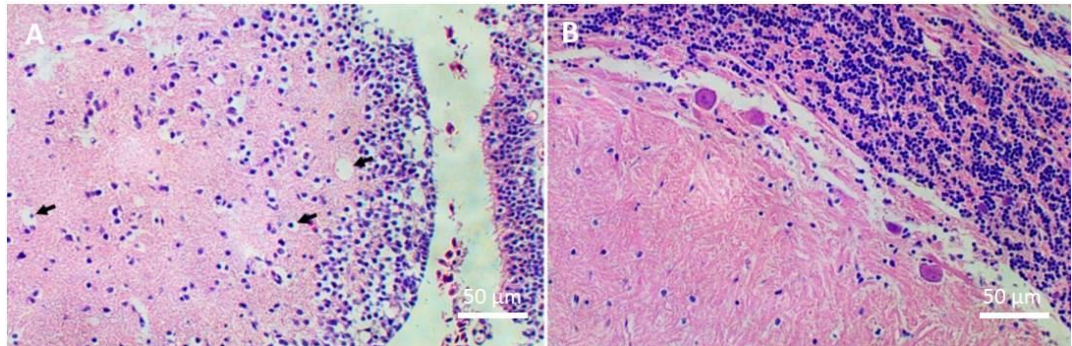


Figure 1.4-1: Histopathology of Mandarin fish infected with NNV. A) Brain tissue of NNV-infected Mandarin fish. Black arrows point to numerous vacuoles caused by NNV infection. **B)** Brain tissue of mock-infected Mandarin fish. (Tu et al., 2016).

NNV is member of the genus Betanodavirus and can be classified into five genotypes: striped jack NNV (SJNNV), tiger puffer NNV (TPNNV), barfin flounder NNV (BFNNV), red-spotted grouper NNV (RGNNV) (Nishizawa et al., 1997) and turbot NNV (TNNV) (Johansen et al., 2004). Each of these five genotypes infects a number of different fish but all of them share the same features (Roy, 2010). They are small (25-30 nm in diameter), non-enveloped, spherical viruses with two single-strand positive-sense RNA molecules (Lin et al., 2001); a RNA-dependent RNA polymerase is encoded by RNA1 and RNA2 contains the information for the coat protein (Mori et al., 1992). 180 copies of the coat protein self-assemble into a T = 3 capsid (Cheng et al., 1994).

NNV infects larvae and juvenile stages with lethality up to 99% but full-grown fish are susceptible as well (Lin, 2007). Though, the infection rate of NNV is temperature dependent. Chi and colleagues found that between 24-32 °C virus titre increased in fish with an increase of the temperature. However, the virus replication rate was down regulated at 20 °C although infections still took place (Chi et al., 1999). The outbreak of the disease depends on the temperature which again is depending upon the virus strain and the fish species (Chi et al., 1999). Studies showed that NNV is spread horizontally and also vertically (Nishizawa et al., 2012).

Several kinds of NNV vaccines candidates have been developed including recombinant capsid protein (Husgag et al., 2001; Lin, 2007; Sommerset et al., 2005), synthetic peptides (Coourdacier et al., 2003), virus-like particle (Liu et al., 2006; Thiery et al., 2006)

and inactivated virions (Kai and Chi, 2008; Yamashita et al., 2005). However, the biggest problem so far is that no effective vaccine has been developed that does not need to be injected into each fish separately. The final aim of this work is to design a vaccine which protects all developmental stages of the fish against NNV and that can be administered orally.

1.5 POLIOVIRUS

Poliovirus (PV) is the causative agent of poliomyelitis, a crippling disease known to humankind since antiquity (Landsteiner and Popper, 1909). Children with a naïve immune system are more susceptible to the disease but polio can strike at any age. It infects primarily via the faecal-oral route and poor hygienic conditions facilitate the spread. Once PV is ingested it multiplies in the oropharyngeal and intestinal mucosa (Sabin, 1956), from there it enters the lymph nodes and then causes blood viremia (Racaniello, 2006). Most PV infections cause asymptomatic viral replication that is limited to the gut and only about 24% of those infected develop minor symptoms such as fever, headache and sore throat. In even rarer instances, (only 1-2%) the virus enters the central nervous system (CNS) where it destroys motor neurons causing paralysis of extremities or even death (Racaniello, 2006). The spinal cord is a thick bundle of nerve fibres that emerges from the brain and runs downwards with the function to connect the body with the brain. Grey matter occupies the centre of the spinal cord and has the shape of a butterfly with outstretched wings. The anterior horns represent the forewings of the butterfly and their role is the coordination of limb movement. If PV enters the CNS it replicates in the anterior horns of the grey matter causing lesions and therefore the affected muscles lose their function due to a lack of nervous activation, resulting in irreversible paralysis. The condition is known as acute flaccid paralysis (AFP). In the most severe cases PV attacks the motor neurons of the brain stem, reducing breathing capacity and causing difficulty in swallowing and speaking. Without respiratory support, bulbar polio can result in death (WHO, 2015). PVs invasion of the CNS is accidental as it does not present a benefit for its normal life cycle. Polio arrives at the CNS via retrograde axonal transport with a pace of up to 12 cm/day (Ohka et al., 1998); PV containing vesicles are brought to the spinal cord by CD155 dependent axonal transport (Mueller et al., 2002; Ohka et al., 2004). It is known that physical trauma like

hard exercise stimulates retrograde axonal transport of PV and therefore facilitates viral invasion of the CNS (Gromeier and Wimmer, 1998).

Poliovirus occurs in three serotypes (PV1, PV2 and PV3) and is classified as a member of the *Picornaviridae*, a large family of small RNA viruses which includes foot and mouth disease virus, hepatitis A virus, rhinovirus and coxsackie virus. Its single stranded (+)-sense RNA genome (~7500 nucleotides) contains a single large open reading frame encoding one 220 kDa polyprotein which is subsequently processed by a series of proteolytic cleavages to generate the viral proteins (Kitamura et al., 1981). One of the processing intermediates, P1, is a ~100 kDa protein containing the sequences of all capsid proteins. P1 is subsequently processed by the viral proteinase 3C^{pro}/3CD to produce the mature capsid proteins (Ypma-Wong et al., 1988). Poliovirus is composed of 60 copies each of four proteins: VP1, VP3 and an immature precursor, VP0 which gets further processed into VP2 and VP4 during RNA uptake (Basavappa et al., 1998; Holland and Kiehn, 1968; Jacobson et al., 1970). Finally, all 4 proteins form an icosahedral capsid 27-30 nm in diameter (see Figure 1.5-1) (Hogle et al., 1985).

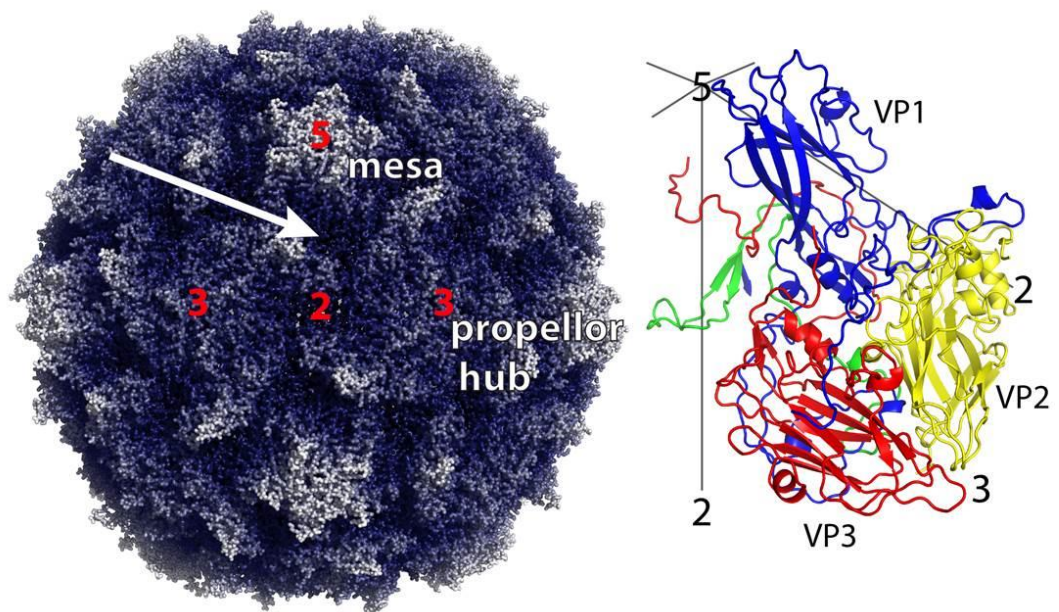


Figure 1.5-1: Poliovirus capsid structure. The figure in the left panel is a full-atomic model of the structure of the Mahoney strain of type 1 poliovirus. The figure has been radially-depth cued with atoms colour coded according to their radius in the virus particle. Features that are closer to the centre appear dark; features furthest from the centre appear white. In the right panel, VP1 is blue, VP2 is yellow, VP3 is red, and VP4 is green. Note the star-shaped mesas at the fivefold axes, the three-bladed propellers at the threefold axes, and the deep canyon that separates the star-shaped mesas from the nearest blades of the propellers. Figure courtesy of Jim Hogle and Mike Strauss, Harvard Medical School.

Two distinct antigenic forms of poliovirus exist, named D and C antigen (Mayer et al., 1957) or also referred to as N (native) and H (heated) (Hummeler and Hamparian, 1958) because D/N can be converted to C/H by heating (Le Bouvier, 1955). The D antigen is found on infectious virus whereas the C antigen is expressed on non-infectious empty particles (Le Bouvier, 1955; Schwerdt and Fogh, 1957). C particles do not display the antigens responsible for eliciting protective immune responses and are ineffective as vaccines.

Empty particles, such as VLPs, do not undergo the final maturation cleavage of VP0 into VP2 and VP4, which reorders the proteins on the inside of the virus capsid with a concomitant increase in particle stability (Basavappa et al., 1994). The cleavage occurs simultaneously with RNA insertion which, of course, does not occur in VLPs. Consequently, although empty capsids are initially in the desirable D conformation they can be easily converted to the C form during extraction and purification (Marongiu et al., 1981). Therefore, to produce an effective vaccine from recombinantly expressed empty particles, it will be necessary to stabilise the particles to reduce/eliminate conversion from D to C form while maintaining the desired antigenic phenotype.

1.5.1 200 YEARS OF POLIO HISTORY

The crippling disease of poliomyelitis is known to humanity since ancient times. An Egyptian carving dated back to 1403-1365 BC is displayed in the Ny Carlsberg Glyptoteket Copenhagen depicting Ruma, guardian priest of the Temple of Astarte in Memphis (Figure 1.5-2). His weak and deformed leg is believed to be the result of polio (Williams, 2013). However, the roots of the disease stayed a mystery for a long time, even though severe outbreaks in the 19th century in Europe and America sparked increased scientific interest. Polio first appeared in the literature in 1789 (Underwood, 1789) describing the disease to be only affecting children, which led German doctors and researchers to name it “Kinderlähmung” which translates to infantile paralysis. Kinderlähmung is a term which is still used in Germany even though since 1858 it is known that polio also attacks adults when the first recorded case of polio in an adult man was published in Switzerland (Vogt, 1859). Poliomyelitis had many titles one of which was Heine-Medin disease, named after two influential European researchers. Jacob von Heine (1800-1879) wrote the first coherent description of the disease in 1840, however mistaking teething to be the reason for the paralysis that he observed in his young

patients. Karl Oscar Medin (1847-1927) was a Professor of paediatrics in Stockholm and the first who called polio a systemic illness that did not paralyse every sick person. His assistant Ivar Wickmann (1872-1914) also played an important role in the understanding of the polio spread. His thorough investigations of polio cases in Sweden in the early 1900 lead him to the conclusion that polio was contagious and can spread from person to person and he was the first to have the idea of asymptomatic carriers; an idea that has been highly controversial so far (Wickmann, 1911; Williams, 2013).



Figure 1.5-2: Carving dated 1403-1365 BC showing an Egyptian man with a withered leg. (Reproduced with kind permission of Glyptoteket. Man with a Crippled Leg. Digital image. Glyptoteket, n.d. Web. 10.09.2016 <<http://www.glyptoteket.com/>>).

Researchers devoted their lives to discover the reason behind the paralysis but the advances in science did not allow the researchers to come up with an explanation to the emerging disease. Until in 1870 Jean-Martin Charcot and Alfred Vulpian analysed samples of polio victims discovering lesions in the grey matter of the anterior horns of the spinal cord (Charcot, 1870; Vulpian, 1870). A few years later the German physician Adolph Kussmaul (1822–1902) first used the term poliomyelitis which translates to inflammation of the spinal cord (Compston, 2016). A name that stuck with the disease ever since.

Discovering the lesions in the anterior horns was a great leap forwards in the understanding of the disease but the cause of poliomyelitis was still a big point of

discussion and disagreement between scientists. Until 1908 at the meeting of the Royal and Imperial Society of Physicians in Vienna Karl Landsteiner (1868-1943) reported the successful experimental transmission of poliomyelitis from a 9-year old boy who had died of polio to monkeys. Until then most people believed that a bacterium is the causative agent of the disease and the idea of an even smaller organism, like a virus, was still very new. Landsteiner had filtered the dead boy's homogenised spinal cord through a Berkefeld candle which is a hollow cylinder filled with silica clay, but the holes are too small to let bacteria through. He injected the filtrate into two Old world monkeys which developed paralytic symptoms a couple of days later and under the microscope the spinal cord of the monkeys showed the typical lesions of poliomyelitis (Eggers, 1999). Therewith he proved that the disease causing agent cannot be a bacterium but it was not until the 1952 when the microscopy caught up and for the first time PV particles could be seen under the electron microscope that the voices of sceptics were silenced (Goldsmith and Miller, 2009; Reagan and Brueckner, 1952).

Meanwhile poliomyelitis went on a rampage through the world with a peak in 1952 with 58,000 cases of paralytic poliomyelitis and 9,000 deaths in America (Williams, 2013), leaving the hospitals unable to cope without a cure or treatment. Severe cases of poliomyelitis, where the brain stem got infected and breathing became immobilised, had to stay in respirators that mimicked the breathing movement for the paralysed patient. The first iron lung was developed 1929 by Phillip Drinker, proving to be a life saver for thousands of polio patients (Drinker and Shaw, 1929). Some patients never recovered and their life relied on the iron lung, like June Middleton who died at the age of 83 having spent 60 years connected to the respiratory device (DailyMail, 2009).

The next big milestone for poliovirus research was the design by Enders and Weller 1949 of a method to cultivate poliovirus in vitro (Enders et al., 1949). Based on this progress the first vaccine was developed by Jonas Salk and introduced in 1955 (Salk et al., 1954). The vaccine consists of all three serotypes which are inactivated (killed) with formaldehyde. IPV is given by intramuscular injection and needs to be administered by a trained health worker. In 1954 the Cutter incident shattered the trust of the population in vaccination when 200 000 children in the USA received incompletely inactivated polio vaccine and a high frequency of initial paralysis was observed in the inoculated limb (Nathanson and Langmuir, 1963). However, improvements of the production reinstalled IPV and in the second year of routine IPV vaccination the numbers of paralytic cases had

fallen to 5, 500 and only 220 deaths in America (Williams, 2013). In parallel Salk's biggest rival Albert Sabin was working on a less expensive, orally administered vaccine (OPV) which got released 1961 and became the most successful tool in the fight against poliovirus (Sabin, 1957). OPV is a live attenuated virus which carries point mutations causing lower neurovirulence and reduced transmissibility but the ability to replicate allows high immune responses.

The fast improvement of molecular biology enabled deeper insights into the composition and life cycle of viruses. The big interest in poliovirus made it the first animal virus to have its genome sequenced in 1981 (Kitamura et al., 1981), revealing a RNA genome with only 7440 nucleotides encoding 11 genes. Only a couple of years later the crystal structure was solved by Hogle and his colleagues in 1985 (Hogle et al., 1985) depicting a detailed image of the capsid. In 1989 the human poliovirus receptor (CD155) that interacts with poliovirus during infection was discovered by Mendelsohn and further characterized by Koike (Koike et al., 1990; Mendelsohn et al., 1989). Discovering the PV receptor was an integral part of poliovirus research because this knowledge allowed the engineering of transgenic mice carrying the receptor: an inexpensive model to study poliovirus infection (Ren et al., 1990).

Despite over 200 years of research the story of polio has not come to an end. Work on poliovirus is ongoing (Chan et al., 2016) as the virus is still a thread and there is plenty more to reveal and discover.

1.5.2 NEED FOR NEW VACCINES

The development of the first polio vaccine was down to the support by the March of Dimes, a non-profit organisation funded 1938 by President Franklin D. Roosevelt. Roosevelt himself was a polio victim; he contracted the disease as an adult and was never able to use his legs again. His personal commitment for this fundraising campaign with the aim to fight the disease and to support its victims made it a massive success. In the first year \$1.8 million was raised most of which was dimes directly sent to the White House in envelopes (Williams, 2013). However, the biggest success of the campaign was the development and release of IPV. From this point, polio declined rapidly from tens of thousands of new cases per year to a mere handful in America (March of Dimes, 2007).

However, failure to implement strategic approaches led to ongoing spread of polio paralysing more than 350 000 people in 125 endemic countries (WHO, 2015). To set an end to the dilemma the Global Polio Eradication Initiative (GPEI) was created. Their aim is to completely eradicate and contain all wild, vaccine-related and Sabin polioviruses worldwide, such that no child ever again suffers paralytic poliomyelitis (GPEI, 2010). GPEI is a public-private partnership led by national governments and spearheaded by the World Health Organization (WHO), Rotary International, the US Centers for Disease Control and Prevention (CDC), and the United Nations Children's Fund (UNICEF). Their efforts payed off, decreasing the cases by over 99% since the initiative's foundation in 1988 and eradicating it from 123 countries down to only two remaining countries with endemic polio: Afghanistan and Pakistan. The GPEI estimates having saved about 10 million people from paralysis (GPEI, 2010).

OPV has been the instrument of choice in the Global Polio Eradication Initiative, especially in developing countries because it is relatively inexpensive and easy to administer, and it induces gastrointestinal mucosal immunity which prevents virus transmission as well as protecting recipients from disease. However, OPV has flaws: one of them is its genetic instability through which it can regain neurovirulence by reversion mutations. This can cause vaccine-associated paralytic poliomyelitis (VAPP) in the vaccine recipient, albeit at very low frequency (Burns et al., 2013; Henderson et al., 1964; Kew et al., 2002; Kew et al., 1998). Clinical signs and severity of paralysis associated with VDPV and wild-type (wt) PV are indistinguishable and they pose the same risk on the population: VDPV associated outbreaks happen all over the world (Burns et al., 2013; Gumedde et al., 2013; Kew et al., 2002; Kew et al., 2005). In addition, these attenuated strains may undergo genetic recombination during replication with other polio or non-polio human enteroviruses, resulting in circulating vaccine-derived polioviruses (cVDPVs) that display neurovirulent phenotypes similar to wild-type polioviruses (Minor, 2009). Usually people stop shedding vaccine virus 2-4 weeks after vaccination, yet vaccinees with the immunodeficiency disorder are known to shed the virus for much longer without getting sick from it which makes them very hard to find (Alexander et al., 2009; Bellmunt et al., 1999; Kew et al., 1998); there is currently a man living in the UK who has shed type 2 vaccine derived PV for 28 years (Dunn et al., 2015). He is excreting highly virulent and antigenically drifted virus and there is no strategy to deal with this problem which raises questions about the safety of the population especially after the recent changes in vaccine production.

Furthermore, OPV also lacks thermostability and requires the utilisation and maintenance of a cold chain. The Salk inactivated vaccine (IPV) induces effective humoral immunity which protects against disease in the vaccinee but does not prevent replication of the wt virus encountered subsequently, thus allowing continued transmission within a population. Importantly, the IPV Salk vaccine is significantly more expensive than the Sabin vaccine. Production of both vaccines requires propagation of large amounts of infectious poliovirus which carries the risk of an accidental reintroduction. Failure to implement strategic approaches has led to ongoing transmission of the virus.

Global eradication of PV is close, with no recorded wt cases of type 2 since 1999 and no case of type 3 since November 2012 but several cases of type 1 in this year (2016) already (GPEI, 2016) (Table 1.5.1). After more than two years without wild poliovirus in Nigeria, the Government reported in August 2016 two children paralysed by the disease. This leaves Pakistan, Afghanistan and Nigeria as the last reservoirs of wt PV. Until poliovirus transmission is interrupted in these countries, all countries remain at risk of importation of polio, especially vulnerable countries with weak public health and immunisation services. Re-introduction of these wild polio strains continues to occur in countries that have previously eliminated wild virus circulation via world travellers, and VDPVs may continue to evolve from the cryptic replication of attenuated OPV strains. The WHO is therefore seeking novel polio vaccines with the overall objective of developing genetically stabilised poliovirus virus-like particles as a cheap and viable source of vaccine to replace current IPVs. Such virus-free vaccines will become increasingly relevant as wt polio is eradicated also in order to eliminate the risk of misusing polio as a bioweapon.

Table 1.5.1: Wild type 1 and cVDPV cases, September 2016 (GPEI, 2016).

Total cases	Year-to-date 2015		Year-to-date 2016		Total 2015	
	Wt PV	cVDPV	Wt PV	cVDPV	Wt PV	cVDPV
Globally	26	3	41	14	74	32
In endemic countries	26	0	41	3	74	3
In non-endemic countries	3	3	0	9	0	29

1.6 AIMS OF THIS THESIS

The overall aim of this project was to explore the capabilities of plants to express VLPs as vaccines and to investigate the efficacy and properties of the plant-made product with the focus on potential industrial application. In particular, I started off with the expression of a rather simple virus: Nervous necrosis virus (NNV) which builds itself out of 180 copies of one single protein. NNV infects over 40 marine fish species world-wide and is a major problem for the fish farm industry. The objective was to develop an inexpensive vaccine against NNV using a plant expression platform, which would possibly allow oral vaccination of the fish. Once the general techniques were established and the production of pure and structurally sound VLPs in plants were proven I moved onto a more complex task: the development of a synthetic plant-produced vaccine against poliovirus. This requires the expression of not only the structural proteins but also the protease needed for their correct processing. The goal was the generation of non-infectious poliovirus-like particles (PV VLPs) that have the capability of eliciting a protective immune response against polio. Together with the WHO-funded consortium we developed stabilised mutant versions of the poliovirus gene P1, which keep the correct antigenicity during expression and purification.

In order to make the product more attractive to the vaccine industry, I adopted tobacco BY-2 cells for the transient expression of the above-mentioned PV mutant VLPs using the cell-pack method. BY-2 cells grow in suspension culture and have no limit for mass production which is a potential advantage over whole plants.

Chapter 2: MATERIALS AND METHODS

2.1 MEDIA, BUFFERS AND SOLUTIONS

The recipes for bacterial growth media, buffers and solutions are summarised in Table 2.1.1.

Table 2.1.1: Recipes of media, buffers and solutions.

Name	Recipe
Antibiotics	100 µg/ml kanamycin (in water) 50 µg /ml rifampicin (in methanol) 500 µg/ml carbenicillin (in water)
5 x Formaldehyde gel running buffer (FGRB)	100 mM MOPS, 40 mM sodium acetate, 5 mM EDTA, adjust to pH 7
1 x HEPES buffer	238.3 g/l HEPES, add solid NaOH until the pH is ~6.8, adjust pH to 7.0 with 5N NaOH, sterile filter and store at 4 °C
Luria Bertani (LB) media	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, adjust to pH 7.0
LB agar	as LB, plus 10 g/l agar
MMA	10 mM MES buffer, pH 5.6; 10 mM MgCl ₂ , 100 µM acetosyringone, pH 7.5
Murashige and Skoog (MS)- media	4.6 g/l Murashige and Skoog Plant Salt Mixture, 30 g/l sucrose, 100 mg/l Inositol, 1 mg/l Thiamine, 0.2 mg/l 2.4-dichlorophenoxyacetic acid, 200 mg/l KH ₂ PO ₄ , adjust pH to 5.8 with 1N NaOH, autoclave
Orange-G loading buffer (for agarose gels)	0.025 g orange-G dissolved in 25 ml 60% (v/v) glycerol in 1x TBE
Phosphate buffered saline (PBS)	140 mM NaCl, 15 mM KH ₂ PO ₄ , 80 mM Na ₂ HPO ₄ , 27 mM KCl, pH 7.2
PBS + EDTA buffer (10L)	13.7 mM NaCl, 2.685 mM, KCl 0.885 mM, CaCl ₂ •2H ₂ O, 0.495 mM MgCl ₂ •6H ₂ O, 1.685 mM Na ₂ HPO ₄ •12 H ₂ O, 2.27mM KH ₂ PO, 20 mM EDTA
10 x PIPES buffer	0.58 g/l NaCl, 3 g/l PIPES (piperazine-N,N'bis[2-ethanesulfonic acid]), 0.2 g/l MgCl ₂ •6H ₂ O, adjust pH to 6.5 with 1N NaOH, sterile filter and store at 4 °C

Protein extraction buffer (P-buffer)	305 ml of 0.2 M Na ₂ HPO ₄ , 195 ml of 0.2 M NaH ₂ PO ₄ , 500 ml of Milli-Q water, Complete Inhibitor Cocktail tablet (Roche), pH 7
Protein loading buffer (for SDS gels)	750 µl 4x LDS sample buffer (Invitrogen), 250 µl β-mercaptoethanol
SOC (Life technologies)	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , and 20 mM glucose
TBE buffer	10.8 g/l Tris-HCl, 5.5 g/l Boric, 2 mM EDTA, pH 7.6
10 x TBS buffer	0.5 M Tris, 3 M NaCl, 10 mM EDTA, adjust pH to 8.5
TEN/NET buffer	100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5
Western blot block buffer	5% (w/v) dry milk, 0.1% (v/v) Tween-20, in PBS
Western blot transfer buffer	3.03 g/l Tris-HCl, 14.4 g/l glycine, 20% (v/v) methanol
Western blot wash buffer	0.1% (v/v) Tween-20 in PBS

2.2 MOLECULAR CLONING TECHNIQUES

Standard molecular biology procedures were performed as described by Sambrook et al. (Sambrook, 1989) and the manufacturers instructions were followed when specific reagents were used.

2.2.1 PLASMID CONSTRUCTION

Sequences were obtained from collaborators in the WHO consortium, codon-optimised for *Nicotiana benthamiana* and ordered for synthesis from GeneArt (Life Technologies) with flanking AgeI and XhoI sites. The genes were inserted into pEAQ plasmids for expression.

2.2.2 DNA/PLASMID ISOLATION AND PURIFICATION

Plasmid DNA was isolated from bacteria using the QIAprep Spin Miniprep Kit (Qiagen). DNA fragments from PCR reactions were prepared with the QIAquick PCR Purification Kit

(Qiagen) and from agarose gels using QIAquick Gel Extraction Kit (Qiagen). Purified DNA was stored at -20 °C.

2.2.3 RESTRICTION DIGEST

Digestions of DNA fragments and plasmids was performed using restriction enzymes supplied by NEB (New England Biolabs) and were carried out as per manufacturer`s instructions.

2.2.4 DNA LIGATION

Purified DNA fragments were quantified using a NanoDrop spectrophotometer and an insert: vector molar ratio of 3:1 was calculated. Ligation was carried out using T4 ligase (NEB) either at room temperature for 30 minutes or at 16 °C overnight.

2.2.5 POLYMERASE CHAIN REACTION (PCR)

DNA fragments for subsequent cloning were amplified using high-fidelity polymerase Phusion (NEB) with primers listed in the Appendix 1. The reactions were set up as follows:

Phusion HF buffer (5x)	10 µl
dNTPs (10 nM)	1.0 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
DNA template (20-25 ng)	1.0 µl
Phusion polymerase	0.5 µl
dH ₂ O	32.5 µl
	50.0 µl

The following program was run to amplify DNA using a variable annealing temperature (XY):

1	98 °C	2 mins
2	98 °C	10 sec
3	XY °C	20 sec
4	72 °C	15-30 sec/kb
5	Go to step 2	25 x

6	72 °C	5 min
7	4 °C	∞

DNA fragments for subsequent analysis by agarose gel electrophoresis were amplified using GoTag Green Master Mix (Promega) with primers listed in Appendix 1. When colony PCR was performed a small amount of bacterial culture replaced the template DNA.

GoTag Green MM	10 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
Reverse primer (10 µM)	2.5 µl
DNA template (20-25 ng)	1.0 µl
dH ₂ O	8.0 µl
20.0 µl	

The program was run as follows with a variable annealing temperature (XY):

1	95 °C	5 mins
2	95 °C	1 min
3	XY °C	45 sec
4	72 °C	1 min/kb
5	Go to step 2	31 x
6	72 °C	5 min
7	4 °C	∞

2.2.6 AGAROSE GEL ELECTROPHORESIS

For analysis and purification of PCR products or plasmid digests, agarose gel electrophoresis was conducted. Generally, 0.8% (w/v) agarose was dissolved in TBE buffer and HyperLadder I (Bioline) used as a standard DNA marker. Samples were mixed with 5x loading buffer and migrated on gels for 45 minutes at 100 V.

2.2.7 DNA SEQUENCING

Sequencing reactions were prepared according to Eurofins Genomics (Wolverhampton, UK) requirements. Sequencing was carried out by Eurofins Genomics.

2.2.8 SITE-DIRECTED MUTAGENESIS (SDM)

Point mutations were introduced into plasmids using specifically designed primers in order to change individual nucleotides. The GeneArt Site-directed mutagenesis system (Invitrogen) was used and instructions were followed closely. After verification of the mutation by sequencing the construct was transformed into agrobacteria for expression in plants.

2.2.9 GROWTH, STORAGE AND TRANSFORMATION OF COMPETENT CELLS

One Shot® Top10 chemically competent *E. coli* (Life Technologies) was used for propagation of plasmids. They were grown at 37 °C in LB media with respective antibiotic selection. *E. coli* was transformed using heat-shock. It was allowed to recover in SOC in a shaking incubator at 37 °C before being plated on LB agar plates with appropriate antibiotics.

A. tumefaciens LBA4404 (Hoekema et al., 1983) was propagated at 28 °C in LB media containing 50 µg/ml kanamycin and 50 µg/ml rifampicin. Transformation of *A. tumefaciens* LBA4404 was carried out by electroporation at 2.5 kV; recovered in SOC at 28 °C for an hour before being plated on LB agar plates with the respective antibiotic selection.

After colony PCR and sequencing, Glycerol stocks of positive colonies of *E. coli* and *A. tumefaciens* were prepared by mixing an aliquot of the culture in the stationary phase with glycerol for a final concentration of 25%. They were snap-frozen in liquid nitrogen and stored at -80 °C.

2.2.10 PLASMIDS USED

Genes synthesized by GeneArt (Life Technologies) were sent to us in the small and simple pMA plasmid, carrying ampicillin resistance.

Rapid and high level production of recombinant protein in plants was achieved using the deconstructed viral vector pEAQ-HT which contains a polylinker for simple insertion of the gene of interest, P19 to suppress gene silencing and nptII that enables kanamycin resistance. At least 10-fold reduction in expression of the gene of interest was achieved

by removing the HT-mutation AUG161 using site directed mutagenesis (see 2.2.8). This pEAQ vector was utilized for the expression of toxic genes to keep their negative impact on the plant down (Thuenemann et al., 2013b).

2.3 TRANSIENT EXPRESSION OF PROTEINS IN *NICOTIANA BETHAMIANA*

2.3.1 PLANT GROWTH CONDITIONS

N. benthamiana plants were grown in glasshouses maintained at 25 °C with supplemental lighting to guarantee 16 hours of daylight and watered daily.

2.3.2 AGROINFILTRATION

A. tumefaciens containing the respective constructs were grown to stable phase in LB medium supplemented with the appropriate antibiotics. The culture was pelleted by centrifugation at 2000 x g for 10 minutes and re-suspended in MMA buffer to the desired optical density at 600 nm (OD₆₀₀). Routinely, an OD₆₀₀ of 0.4 was used unless several constructs were co-expressed. For co-expression the constructs were mixed in a 1:1 ratio. The agro-suspension was left at room temperature for 0.5–3 hours prior to pressure infiltration into the extracellular space of 3-4 week old *N. benthamiana* leaves (as described in Thuenemann et al., 2013b).

2.3.3 PROTEIN EXTRACTION

Small-scale extractions were done to test protein expression or for time-course purposes. Four leaf discs (approximately 100 mg) were sampled and placed into a 2 ml screw-cap microcentrifuge tube together with a ¼-inch ceramic bead (MP Biomedicals). The leaf tissue was homogenised in an Omni Bead Ruptor 24 (Camlab) at speed setting 4 for 30 sec with 3 x the volume of protein extraction buffer. A clarification spin (16, 00 x g for 10 minutes) followed to separate the soluble protein from the plant cell debris.

For the purpose of VLP preparation, large-scale extraction was carried out using infiltrated leaf material 4-7 dpi. It was weighed and homogenised in a Waring blender with 3 x the volume of extraction buffer. The crude extract was filtered over 2 layers of

Miracloth (Calbiochem) followed by a clarification spin at 9,500 x g for 15 minutes at 4 °C. Afterwards, the supernatant was further purified with density gradients.

2.4 TRANSIENT EXPRESSION IN TOBACCO BY-2 CELLS

2.4.1 GROWTH CONDITIONS

BY-2 cells were grown in Erlenmeyer flasks at 28 °C in the darkness whilst shaking at 170-190 rpm. In order to maintain a healthy BY-2 suspension culture every 7 days 1 ml of culture was transferred into 50 ml fresh MS media.

2.4.2 CELL-PACK FORMATION AND TRANSIENT EXPRESSION

For the purpose of transient expression of recombinant proteins in BY-2 cells, larger suspension cultures had to be set up. MS media was inoculated with 1/10 of its volume of 4-7 days old BY-2 suspension culture. After 72–144 h the cells were harvested using a Büchner funnel lined with filter paper and a vacuum pump to remove the media and form a tight cell pack which was consequently transferred into a petri dish. *A. tumefaciens* were prepared as described in 2.3.2 and dropped onto the cell-pack making sure to fully saturate it. Together with a water container, for stable humidity levels, the cell-packs were incubated in a lightproof box for 8-10 days at 28 °C.

2.4.3 PROTEIN EXTRACTION

Small-scale extractions were carried out to test expression of recombinant proteins or for time-course experiments as described in Section 1.13 using a cork borer (11 mm in diameter) to sample 4 discs of infiltrated cell-packs. For VLP preparations infiltrated cell-packs were spread out thinly on aluminium foil and dipped in liquid nitrogen until frozen. Extraction buffer was added and the cells ground to a smooth paste. The crude extract was filtered over 2 layers of Miracloth (Calbiochem) followed by a clarification spin at 9,500 x g for 15 minutes at 4 °C. Afterwards, the supernatant was further purified with density gradients.

2.5 PROTEIN ANALYSIS

2.5.1 POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein samples were boiled 5-10 minutes in protein loading buffer before being loaded onto NuPAGE Bis-Tris pre-cast gels, of 4% (w/v) or 4-12% (w/v) acrylamide. As protein standard SeeBlue Plus 2 (Invitrogen) was used. Gels were run in NuPAGE MOPS or MES buffer at 200 V for 47 minutes. After electrophoresis the gels were either used for Western blotting (see below) or stained with InstantBlue (Expedeon) for one hour. The gels were destained in water for at least one hour before imaging. Protein bands of interest were cut out from stained PAGE-gels and identified by Mass Spectrometry by Dr. Gerhard Saalbach (John Innes Centre Proteomics facility).

2.5.2 WESTERN BLOTTING AND IMMUNODETECTION

Western blotting was performed using Western blot transfer buffer to transfer the proteins from the gels to Amersham Hybond-ECL nitrocellulose membranes (GE Healthcare) for one hour at 100 V whilst maintaining a constant cooling by ice bath. Later on the BioRad Trans-Blot® Turbo™ Transfer System was adopted for faster transfer times.

Membranes were blocked in Western blot block buffer for 30-60 minutes at room temperature followed by adding the appropriate primary antibody for one hour. The membranes were washed three times for 5 minutes in wash buffer before being blotted in the appropriate HRP-conjugated secondary antibody for 30-60 minutes, followed by another three washes. Antibodies used in this study are listed below in Table 2.5.1.

Immobilon Chemiluminescent HRP substrate (Millipore) was used to detect bound secondary HRP-conjugated antibody. The emitted luminescence was detected digitally on an ImageQuant LAS 500 (GE Healthcare).

Table 2.5.1: Antibodies used in Western blots.

Name	Target	Species	HRP	Supplier
ab26812	NNV	rabbit	no	Abcam
Sh16	PV VP1	sheep	no	NIBSC
MAB8566	PV VP1	mouse	no	Millipore
Anti-mouse	Mouse IgG	goat	yes	Life Technologies
ab97125	Sheep IgG	donkey	yes	Abcam
Anti-rabbit	Rabbit IgG	goat	yes	Promega

2.6 VLP PURIFICATION

2.6.1 POLYETHYLENE GLYCOL (PEG) PRECIPITATION

After the clarification spin, the crude plant extract was transferred to a glass beaker and particles were precipitated at 4 °C overnight by adding a solution of PEG 6000 (to the desired final concentration) and NaCl (final concentration of 0.2 M). The PEG precipitate was obtained by centrifugation for 20 min at 13,000 x g and resuspended thoroughly in the respective buffer.

2.6.2 DENSITY GRADIENTS

2.6.2.1 *Sucrose cushion*

In Ultra-Clear 25x89 mm ultracentrifuge tubes (Beckman Coulter) the clarified extracts were underlain with sucrose solutions prepared in the appropriate buffer. The bottom fraction was 1 ml of 70% (w/v) sucrose solution and topped with 5 ml of 25% (w/v) or 35% (w/v) sucrose solution depending on the VLP density. Centrifugation was carried out in a SureSpin 630/36 ultracentrifuge swing-out rotor (Sorvall) at 167,000 x g for 3 hours at 4 °C. The fractions were retrieved by piercing the bottom of the tube with a needle.

2.6.2.2 *Sucrose gradient*

The extracts were underlain with sucrose solutions prepared in the corresponding buffer in either Ultra-Clear 25x89 mm or 14x89 mm ultracentrifugation tubes, depending on the sample volume. The gradient was made out of sucrose solutions increasing in concentration (20%-60% w/v) from top to bottom. The volume of each layer was

adjusted according to the tube size. Centrifugation was carried out in a SureSpin 630/36 ultracentrifuge swing-out rotor (Sorvall) at 167,000 x g for 3 hours at 4 °C or for smaller sample volumes in a TH641 ultracentrifuge swing-out rotor (Sorvall) for 2, 5 hours at 4 °C. The bottom of the tube was pierced with a needle to retrieve the fractions and the material was collected as it dripped out.

2.6.3 NYCODENZ GRADIENT

Further purification of VLP samples was achieved by Nycodenz gradient ultracentrifugation. Nycodenz solutions of 20% to 60% (w/v) concentrations were prepared in the appropriate buffer and used to underlay the samples in Ultra-Clear 14x89 mm ultracentrifugation tubes. Centrifugation was carried out in a TH641 ultracentrifuge swing-out rotor (Sorvall) for 22-24 hours at 247,103 x g at 4 °C enabling separation of VLPs from persistent plant contaminants. Desired fractions were collected either by piercing the sides of the tubes and sucking out the material or by piercing a hole in the bottom and collecting the sample as it dripped out.

2.6.4 DIALYSIS

Dialysis was used as a method to remove the density gradient medium from samples and for buffer exchange. Two different methods of dialysis were used depending on the sample. SnakeSkin dialysis tubing (Thermo Scientific) with 10 kDa molecular weight cut-off (MWCO) was used as per manufacturer's instructions. Buffer was exchanged twice within at least 24 hours of dialysis. Also PD10 columns (GE healthcare) were used and the manufacturer's spin protocol was followed.

2.6.5 PROTEIN CONCENTRATION

For small amounts of temperature-insensitive samples the SpeedVac (Savant) was used to reduce the sample volume by vacuum evaporation. Larger volumes of durable material were pelleted using a TH641 ultracentrifuge swing-out rotor (Sorvall) for 1, 5 hours at 197,819 x g at 4 °C, followed by resuspension in a small volume of buffer. Temperature sensitive and fragile samples were concentrated using the Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a 100 kDa MWCO. This method allows buffer and

small solutes to pass through the membrane while gently concentrating the VLPs at a controlled temperature.

2.7 VLP ANALYSIS

2.7.1 TRANSMISSION ELECTRON MICROSCOPY

TEM was routinely used to assess the integrity of VLPs. Samples were adsorbed onto plastic and carbon-coated copper 400 mesh grids, washed with several drops of water and then stained with 2% (w/v) uranyl acetate for 15-30 seconds. Grids were imaged using a FEI Tecnai G2 20 Twin TEM with bottom-mounted digital camera.

2.7.2 NATIVE AGAROSE GEL ELECTROPHORESIS

For the detection of encapsidated nucleic acid, purified VLPs were mixed with Orange-G loading dye and run in duplicate on native agarose gels (1% w/v in TBE) for 2 hours at 60 V. One gel was stained in a 0.5 µg/ml ethidium bromide bath for 20 minutes and to detect nucleic acid bands it was visualised under UV light. The other gel was stained in InstantBlue (Expedeon) to visualize protein.

2.7.3 LOWRY ASSAY

In order to determine protein concentration of purified VLP samples the Modified Lowry Assay Kit (Thermo Fischer Scientific) was applied, closely following the manufacturer's microplate procedures. The OD₇₅₀ of the wells was read on a POLARstar Omega plate reader (BMG Labtech). A standard curve was created in Microsoft Excel by plotting the average blank-corrected 750 nm values for each BSA standard against its concentration in µg/ml. This standard curve was employed to determine the protein concentration of the samples.

2.7.4 FURTHER ANALYSIS

For experiments that could not be carried out at the John Innes Centre samples were sent to collaborators. Immunological tests in animals were conducted at The National

Institute for Biological Standards and Control (NIBSC) and for cryo-electron microscopy (Cryo-EM) particles were sent to the University of Oxford (polio) or the University of Leeds (NNV).

2.8 EXTRACTION AND ANALYSIS OF RNA

2.8.1 RNA EXTRACTION FROM VIRUS PARTICLES

Purified virus particles and virus-like particles were incubated in TEN/NET buffer at 60 °C for 5 minutes together with 2% (w/v) SDS to denature the coat proteins. The same volume of a 1:1 mixture of phenol and chloroform was added and mixed amply by vortex. The mixture was centrifuged at 16,000 x g for 10 minutes and afterwards the aqueous layer was transferred into a fresh tube. To precipitate RNA from the aqueous fraction, 0.2 volumes of 3 M sodium acetate at pH 5.5 and 2.5 volumes of ice-cold 100% ethanol were added and the sample was kept at -20 °C overnight. The sample was centrifuged at 16,000 x g for 15 minutes at 4 °C to recover the RNA pellet. The pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in a small volume (5-10 µl) of DEPC-treated sterile water. The concentration of extracted RNA was measured using a NanoDrop spectrophotometer and the samples were stored at -80 °C.

2.8.2 ELECTROPHORESIS OF ENCAPSIDATED RNA

Extracted RNA was analysed by agarose gel electrophoresis under denaturing conditions. Samples were prepared as follows:

Extracted RNA	4.5 µl
5 x FGRB	2.0 µl
Deionised formamide	10.0 µl
12.3 M Formaldehyde	3.5 µl
	20.0 µl

The samples were heated at 65 °C for 10 minutes to denature RNA and placed on ice immediately. 0.5 µl ethidium bromide (10 mg/ml) and 2 µl Orange-G loading dye were mixed into the samples and loaded on a 1.2% agarose gel prepared in 1x FGRB and 2.2 M formaldehyde. Additionally, 5 µl of Transcript RNA marker from Sigma was

prepared as above and run as a standard. Samples were electrophoresed at 60 V for 1.5–2 hours and RNA bands visualised under UV light (wavelength = 302 nm) and imaged using Gene Snap (Syngene).

2.9 PHOTOGRAPHY

Photography was used to document detrimental effects on leaf tissue of certain constructs, to visualize density gradients and to display proteins bands on stained SDS-PAGE gels. Excitation of fluorescent proteins was achieved using Blak-Ray lamps (Ultra-Violet Product Ltd). Unless otherwise specified, I took the photographs.

2.10 SOFTWARE

Vector NTI Advance 11.5 (Invitrogen) was used for vector design, *in silico* cloning strategies, sequence analyses and alignments, as well as predictions of protein sequences, sizes and attributes.

The BLAST function of the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used both for nucleotide and protein sequence alignments.

The GenBank database of the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/genbank/>) was used to find sequences of interest.

Microsoft Office software suite was used for data analysis and making figures.

Chapter 3: PRODUCTION AND CHARACTERISATION OF NNV VLPs

3.1 INTRODUCTION

Nervous necrosis virus (NNV) is a member of the family Nodaviridae that consists of two genera: *Alphanodavirus*, which usually infects insects, and *Betanodavirus*, also known as piscine nodavirus (Mori et al., 1992). NNV infects over 40 marine fish species and is a massive threat for the fish industry as it causes mass mortality in many economically important fish species worldwide (Tan et al., 2001; Walker and Winton, 2010), such as the Japanese parrotfish (*Oplegnathus fasciatus*) (Yoshikoshi and Inoue, 1990), barramundi (*Lates calcarifer*) (Glazebrook, 1990), European sea bass (*Dicentrarchus labrax*) (Breuil et al., 2001), turbot (*Scophthalmus maximus*) (Bloch, 1991), redspotted grouper, (*Epinephelus akaara*) (Mori et al., 1991) and Atlantic halibut (*Hippoglossus hippoglossus*) (Grotmol et al., 1997). The annual market of for example grouper larvae is worth more than 300 million US dollars (Kai and Chi, 2008).

Wild-type NNV is a small, spherical, nonenveloped virus carrying a bipartite positive-sense RNA genome. RNA1 (3.1 kb) encodes two proteins: the RNA-dependent RNA polymerase (102 kDa), important for RNA transcription and replication and also protein B (10 kDa) which is expressed from a subgenomic RNA3 (0.4 kDa) (Friesen and Rueckert, 1982; Guarino et al., 1984; Saunders and Kaesberg, 1985). Protein B is a non-structural protein involved in inhibition of host RNA interference (Chao et al., 2005; Guo et al., 2003; Mori et al., 1992). RNA2 (1.4 kb) encodes the coat protein precursor alpha (43 kDa) and has additional function in regulating the synthesis of RNA3 (Dasgupta et al., 1984; Friesen and Rueckert, 1984; Mori et al., 1992). The major protein produced during viral infection is coat precursor protein alpha which rapidly self-assembles into provirions (Friesen and Rueckert, 1981; Gallagher and Rueckert, 1988). Provirions mature by spontaneous autocatalytic cleavage of the coat protein alpha post assembly; producing proteins beta (38 kDa) and gamma (5 kDa) which remain part of the mature virion (schematic representation Figure 3.1-1) (Hosur et al., 1987). It is assumed that maturation cleavage plays a role in infection when the RNA gets delivered into the host cell, a model that is similar to that proposed for poliovirus where VPO gets cleaved into VP2 and VP4 during RNA uptake (Cheng et al., 1994; Schneemann et al., 1992).

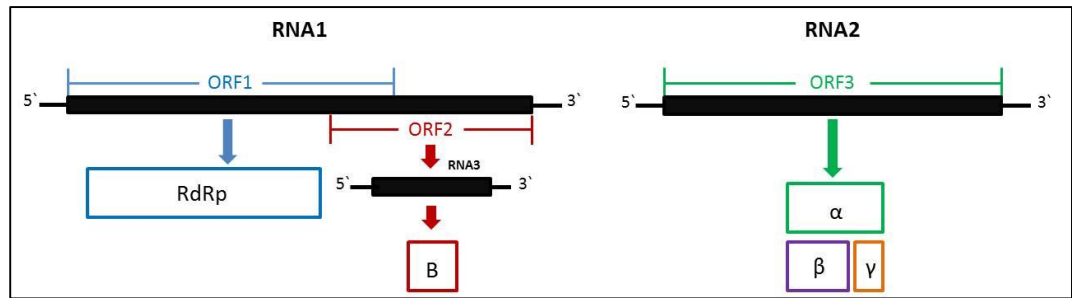


Figure 3.1-1: Genome organisation of NNV. RNA1 encodes two proteins: the RNA-dependent RNA polymerase and also protein B, which is expressed from a subgenomic RNA3. RNA2 encodes the coat protein precursor alpha and has additional function in regulating the synthesis of RNA3. The major protein produced during viral infection is coat precursor protein alpha which rapidly self-assembles into provirions. Provirions mature by spontaneous autocatalytic cleavage of the coat protein alpha post assembly; producing proteins beta and gamma.

NNV infection causes viral nervous necrosis (VNN) due to NNV-induced lesions in the central nervous system and in the retina. The results are abnormal swimming behaviour, dark colouring and massive mortality (Bovo et al., 1999; Grotmol et al., 1997; Thiery et al., 1999). VNN often causes mortality rates higher than 99% in larvae and early juvenile stages and can result in rapid loss of the hatchery; hence the development of an effective and viable vaccine is of great interest of the aquaculture industry. Adult fish are susceptible as well but mortality rates are lower (Lin, 2007).

In general, NNV can be transmitted in two ways: vertically and horizontally. In vertical transmission the broodfish transfers the virus to their offspring by shedding viruses through gonadal fluids whereas the transmission between individual fish of the same generation is called horizontal transmission. Vertical transmission of the virus from the broodfish to the eggs has been reported in several fish species like, striped jack (*Pseudocaranx dentex*), sea bass (*Dicentrarchus labrax*), and barfin flounder (*Verasper moseri*) (Grotmol and Totland, 2000; Mushiake et al., 1994; Peducasse et al., 1999). The only available control strategies are segregation or elimination of NNV carrier-spawners (Mushiake et al., 1994; Watanabe et al., 2000). Furthermore, sterilisation of the eggs has been tested using several techniques, including washing the eggs with ozonated sea-water, which efficiently inactivated NNV. None of the larvae from virus-exposed eggs washed with ozonated sea-water developed VNN (Arimoto et al., 1996; Grotmol and Totland, 2000). However, such harsh treatment may enhance the risk of causing abnormal fry, delay of hatching and reduced hatching rate (Arimoto et al., 1996) and the safe dosage of ozone or chemicals differs between species. Horizontal transmission has been demonstrated experimentally within the same species (Glazebrook, 1990;

Peducasse et al., 1999) but also between species by using filtered homogenates from diseased redspotted grouper juveniles to transfer NNV to striped jack larvae (Arimoto et al., 1996; Mori et al., 1991). These experimental results strongly support the assumption that horizontal transmission of NNV takes place under natural conditions where healthy fish cohabitate with carriers. Their cannibalistic behaviour facilitates the horizontal spread of the virus.

All jawed vertebrates share the same fundamental parts of host defence. There are two main layers of immune responses: innate immune responses and adaptive immune responses. The innate immune system creates a fast, non-specific reaction to the pathogen infecting the host organism. If the pathogen cannot be defeated with innate defences, the adaptive immune system will attack the pathogen with specificity and memory (Rauta et al., 2012). The adaptive (or acquired) immune system employs two types of lymphocytes: T cells are major components of cell-mediated immunity whereas B cells produce immunoglobulins (Ig) that are key elements of humoral immune responses (Mashoof and Criscitiello, 2016). T cell receptors and B cell receptors (plasma membrane anchored Ig, as opposed to secreted Ig that is also known as antibody) are extremely diverse molecules at their antigen binding tips.

In principle the immune system of the teleost fish is very similar to that of higher vertebrates. However, their immune system is conditioned by their aquatic habitat and also by their poikilothermic condition (Tort et al., 2003). Fish are free living organisms from early embryonic stages of live. Thus, compared to terrestrial animals, fish have a greater challenge coping with high microbial loads, which bombard their mucosal barriers. Consequently, innate immunity is their fundamental defence mechanism and the skin, gills and the gut are the first line of defence against pathogens (Hitzfeld, 2016; Uribe et al., 2011). They are covered in mucus, which contains antibodies as well as antimicrobial proteins, like lysozyme, phosphatases and trypsin (Fast et al., 2002). In addition, the epidermis carries defending cells like lymphocytes and macrophages (Uribe et al., 2011). In mammals, nonspecific cellular immune responses are executed by cytotoxic cells, known as natural killer cells. The nonspecific cytotoxic cells of fish are morphologically distinct from the large granular lymphocytes of mammals but they are suggested to be functionally similar (Evans and Jaso-Friedmann, 1992). The complement system in higher vertebrates can be activated in three ways: via the classical, the alternative and the lectin pathway. All three pathways have also been described in

several teleost groups and it appears to be one of the central immune responses in fish (Endo et al., 1998; Nonaka et al., 1984). However, the main difference in fish is that they possess multiple isoforms of the key activation molecule C3, whereas mammals carry only one isoform (Gongora et al., 1998; Kuroda et al., 2000). The biological meaning of the variability may be the specialisation to bind specific surfaces and to increase efficiency in eliminating immunogens (Tort et al., 2003).

Same as other vertebrates, fish have organs whose main function is immune defence. Most of the lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow (Tort et al., 2003). The immune organs in teleost fish comprise the thymus, kidney, spleen, and liver. The thymus is responsible for the production of T lymphocytes which are involved in macrophage function and B-cell stimulation (Hitzfeld, 2016; Uribe et al., 2011). The functional equivalent of the bone marrow in vertebrates is the kidney of teleost fish. It mostly contains antibody-producing B lymphocytes, which are important in the development of humoral immunity and immunological memory. The spleen is the major site of macrophage phagocytosis of blood-borne antigens and the liver of teleost fish is involved in presentation of particulate antigens (Hitzfeld, 2016).

As in mammals, teleost fish possess adaptive immune systems based on antigen recognition by Igs, T cell receptors (TCR), and major histocompatibility complex molecules (MHC) I and MHC II molecules. The basic structure of Ig consists of two identical heavy (H) chains and two identical light (L) chains (exceptions in shark and camelids). Both heavy and light chains contain one N-terminal variable domain and one or more C-terminal constant domains. Variable domains participate in antigen recognition and the domains that comprise the constant regions mediate effector functions of the antibody molecule. Such humoral immunity mediated by Ig includes tagging of pathogens for destruction by phagocytes, neutralization of toxins and viruses, and activation of the complement cascade (Mashoof and Criscitiello, 2016). Three Ig heavy chain isotypes have been described in teleosts: IgM, IgD, and IgT. While IgM constitutes the main systemic immunoglobulin, the role of IgD is unknown. IgT functions analogously to mammalian IgA by protecting mucosal surfaces (Sunyer, 2013). Both IgM and IgD are expressed in B cells found both in systemic and mucosal lymphoid areas, whereas IgT is uniquely expressed by a B cell subset devoid of IgM and IgD (Magadan et al., 2015; Rombout et al., 2014). Ig of teleost fish is found in the skin, gut, gill mucus,

bile, and systemically in the plasma (Lobb and Clem, 1981; Rombout et al., 1986). Most fish respond to primary antigenic challenges by producing specific antibodies, whereas the response to secondary challenge is generally faster and of greater magnitude. This makes vaccination an attractive strategy for control of infectious disease in the aquaculture industry (Mashoof and Criscitiello, 2016; Morrison and Nowak, 2002). Studies indicate that a transfer of maternal antibody takes place in many fish species. The finding raises the possibility that female fish could be vaccinated to provide their offspring with protection against various pathogens. However, it is still questionable whether transfer of maternal antibody confers protection against specific infection (Castillo et al., 1993; Lillehaug et al., 1996; Sin et al., 1994).

Adaptive immunity based on B and T cells is present in both systemic and mucosal immune systems in teleost fish. At the mucosal barriers, e.g. the skin, gills and the gut, the B and T lymphocytes induce and regulate the secretion of antibodies (Kunisawa and Kiyono, 2005). Biochemical analyses showed differences in mucosal and serum Igs of fish, revealing the presence of a specialised mucosal antibody: IgT (Lobb, 1987; Zhang et al., 2011). However, the story of how mucosal immune systems defend teleosts is far from understood, especially the mechanisms by which memory is established and maintained at the mucosa (Salinas, 2015). Peyer's patches, antigen transporting M cells, IgA- and the IgM joining J chain, which are essential components of the mammalian mucosal immune system, are not yet reported in teleost fish (Rombout et al., 2011; Rombout et al., 2014). Novel tools will hopefully lead to conclusions in this area of research, thus benefit the use of oral vaccines in fish (Salinas, 2015).

The most common vaccination methods in fish are injection and immersion. Vaccines that have been administered via injection, elicit mostly an internal immune response and can therefore be considered as systemic vaccines. However, in fish intramuscular injection can also induce a certain degree of mucosal responses (Rombout et al., 2014; Valdenegro-Vega et al., 2013). Immersion vaccination of fish leads to uptake by the skin, the gills and the gut, subsequently inducing local responses (Wendelaar Bonga, 1997). Oral vaccines however, can only be effective if the antigens reach the correct inductive sites. If an adequate amount of antigen reaches the end gut, local as well as systemic antibody responses can be induced in fish (Rombout et al., 1986). This can be achieved with the support of adjuvants.

Adjuvants can be classified into two groups: group 1 adjuvants influence the fate of the vaccine antigen in time, place, and concentration, ultimately improving its immune-availability. Group 2 adjuvants co-stimulate signals during antigen recognition that will provide an environment for the most favourable antigen-specific immune response (Schijns, 2001; Tafalla et al., 2013). Despite severe side effects, traditional adjuvants such as mineral oils are routinely used in different vaccines for fish. Particularly interesting for the work presented in this thesis is the use of adjuvants in orally administered vaccines. Microparticles belong to group 1 adjuvants and offer a promising alternative to oil emulsions (Sinyakov et al., 2006). Biodegradable Poly-(lactide-co-glycolide) (PLGA) polymers have successfully been deployed in oral fish vaccines (Lavelle et al., 1997; Tian and Yu, 2011). PGLA microparticles enclose the antigen and protect it against destruction in the stomach. The antigen is then released by diffusion through pores and by matrix degradation. Adjuvants of the second group, which have successfully been used in oral administration of vaccines are β -Glucans. They are known to stimulate the non-specific immune response of both mammals and fish (Tafalla et al., 2013).

In order to control the spread of NNV and to protect fish farms from the virus a vaccine has to be developed which is inexpensive and potent but, most importantly, easy to administer. Several vaccine candidates have been engineered but they need to be injected into each fish separately. One example is the VLP-based vaccine of Liu and colleagues who expressed NNV VLPs in *E. coli* for intramuscular immunisation of grouper. The results reveal that 1 μg VLPs/g of fish body weight is enough to stimulate a full-scale immune response and the antibody titre was detected at 1 week and increased thereafter (Liu et al., 2006). The VLPs induced a rapid and specific humoral immune response that protected grouper against virus infection. This demonstrated the potency of NNV VLPs as candidate vaccines

Besides the fact that injecting individual fish is usually not feasible, the highest mortality occurs in larvae and the size of most fish species at the larval stage is too small to be immunised by injection (Lin, 2007). One alternative is the bath immunisation with inactivated virus. Kai and colleagues investigated the potential of bath immunisation of grouper larvae against VNN disease with inactivated NNV (Kai and Chi, 2008). After inactivation the virus particles were mixed with melted hydrogenated plant oil to form solid lipid nanoparticles which encapsulates and stabilises the particles; making it more

resistant to stomach acids. The encapsulated vaccine was able to enter the fish through its body surface or via the digestive tract. The results showed effective protection of the fish when bath immunised with nano-encapsulated inactivated betanodavirus (Kai and Chi, 2008). The biggest enemy of oral vaccines is the gastrointestinal tract. The success of an oral vaccine depends on a method that protects the antigen from gastrointestinal digestion and effectively delivers antigen to the hindgut of fish. Lin and colleagues came up with a very clever technique to protect their recombinant subunit vaccine from the stomach acids of the fish. They expressed the capsid protein in *E. coli*, killed the bacteria and then fed it to *Artemia nauplii*; a shrimp which naturally feeds on bacteria and is a common starter feed for fish larvae. The *Artemia* containing the bacteria were used alive for oral vaccination or were frozen at $-20\text{ }^{\circ}\text{C}$ for later use (Lin, 2007). In this case two biolayers, the *E. coli* cell wall and the *Artemia* cuticle, protected the antigen from gastrointestinal degradation and enabled delivery of sufficient antigen to the hindgut resulting in protection of grouper larvae from VNN (Lin, 2007). Another hands-off vaccination technique has been tried where fish were reared at $17\text{ }^{\circ}\text{C}$ and challenged with live NNV. Even though the virus successfully infected the fish, no mortality was observed. This finding is based on the temperature-dependent multiplication rate of NNV, because of the low temperature its virulence was reduced (Nishizawa et al., 2012). However, this depends upon the virus strain and the fish species (Chi et al., 1999) and is therefore not totally reliable.

Overall it becomes clear that several potential vaccines have been developed but none has been marketed yet. The reasons are many-sided but mostly feasibility of administration, costs and effectiveness are the factors that stop those vaccine candidates from making it to industrial production. The aim of this project was to express NNV VLPs in plants which can function as a vaccine protecting all developmental stages of fish against NNV. Recombinant expression of NNV VLPs has already been achieved by Lin et al. in 2001. Using Sf21 insect cells and a recombinant baculovirus vector, they expressed the NNV capsid protein which assembled into VLPs. However, they recognized differences in the density of the VLPs and later found out that their insect cell-produced VLPs encapsidate random RNA of different sizes (Lin et al., 2001). This result is consistent with the results obtained with insect nodavirus VLPs which are known to package random cellular RNAs when recombinantly expressed (Schneemann et al., 1993). Lin and colleagues used the VLPs for cryo-EM and generated a $\sim 23\text{ \AA}$ reconstruction. Though by modern standards this is a rather low resolution structure, it

was sufficient for the authors to state that their VLPs closely resemble the native virus in size and geometry (Lin et al., 2001).

This chapter describes recombinant expression of NNV coat protein in plants with the aim of generating properly assembled NNV VLPs which might subsequently be used as a vaccine against VNN. It also describes the development of optimal extraction and purification methods for the plant-expressed NNV VLPs as well as the characterisation of their properties.

3.2 FIRST EXPRESSION OF NNV COAT PROTEIN IN PLANTS

In order to express NNV VLPs in plants the region of RNA2 encoding the capsid protein (GeneBank accession number EF617326.1) was codon optimised for the expression in *N. benthamiana* and ordered from GeneArt (Life Technologies) with flanking *AgeI* and *XhoI* restriction sites allowing cloning via restriction digest into our expression vector of choice forming pEAQ-HT-NNV. The construct was transformed into *E. coli* for propagation and then into agrobacteria. Before agroinfiltration the sequence was confirmed by sequencing (see section 2.2.7). Infiltrated leaves were harvested 6 dpi when they started to show signs of yellowing (Figure 3.2-1). Out of experience yellowing (not to be mistaken for necrosis) is a positive sign for high expression levels. After homogenisation and crude purification, the plant extract (see section 2.6) was run through a sucrose gradient with fractions ranging from 20% (w/v) to 60% (w/v) as shown in Figure 3.2-1 B.

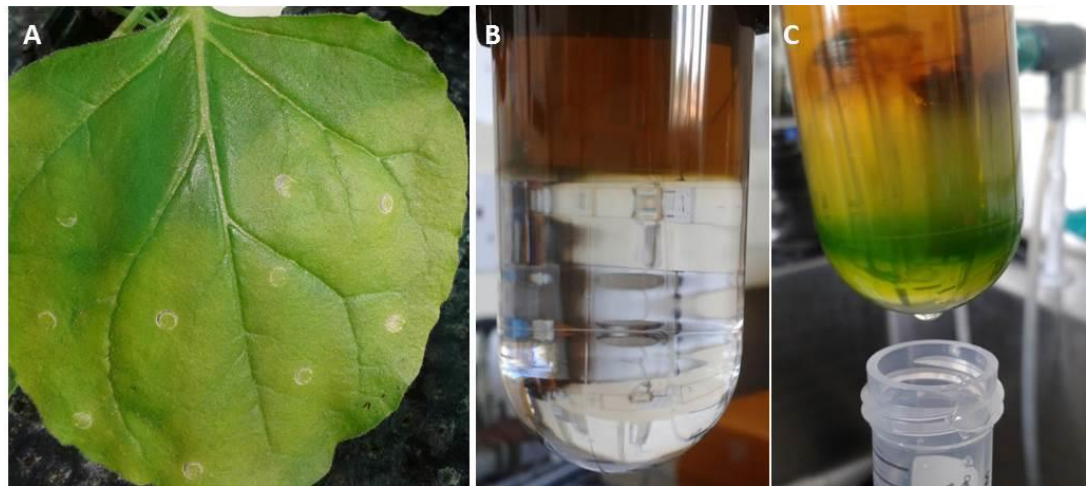


Figure 3.2-1: Overview of the workflow. **A)** Leaf infiltrated with pEAQ-HT-NNV 6 dpi. **B)** Clarified plant extract was underlain with a discontinuous sucrose gradient. **C)** After ultracentrifugation the sucrose gradient was fractionated by piercing the bottom of the tube.

After ultracentrifugation the sucrose gradient was fractionated from the bottom and each fraction was run on a SDS-PAGE gel, which was stained with InstantBlue and also transferred onto a membrane for Western blotting to analyse the protein expression (Figure 3.2-2).

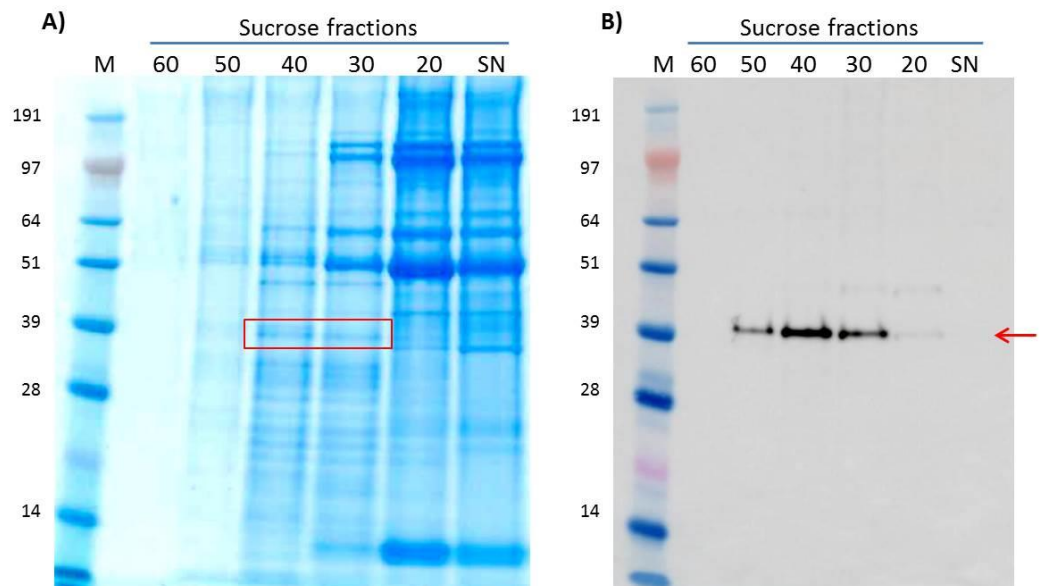


Figure 3.2-2: Analysis of NNV coat protein expression. Clarified leaf extract was separated on a discontinuous 20%-60% (w/v) sucrose gradient and after ultracentrifugation fractionated from the bottom. **A)** Sucrose fractions were run on SDS-PAGE and the protein stained with InstantBlue. **B)** A duplicate of the SDS-PAGE was transferred on a nitrocellulose membrane for immunodetection. The Western blot was probed with anti-NNV (ab26812) antibody. M = SeeBlue Plus 2 with molecular weights indicated. SN = Supernatant. The red box and arrow indicate position of NNV coat protein.

Detecting expression of a particular protein in a crude plant extract on a SDS-PAGE gel can be difficult because of the large background of endogenous plant proteins which are still in the sample. It is particularly difficult when the desired protein is not well expressed. However, faint bands of roughly the right size for the NNV coat protein alpha of 43 kDa can be detected in the 30% and 40% sucrose fraction on the SDS-PAGE gel shown in Figure 3.2-2 A. These bands were excised from the stained gel, sent for mass spectrometry analysis and the protein was identified as nodavirus coat protein. A more precise method is Western blotting, where specific antibodies are used for protein detection. Therefore, a duplicate of the SDS-PAGE gel was transferred onto a membrane and blotted with anti-NNV antibody Figure 3.2-2 B. It shows signals in the 50%, 40% and 30% (w/v) fraction and the strongest band is in the 40% (w/v) fraction, which confirms the results collected so far.

The above results confirm expression of the NNV coat protein in plants. The coat protein was detected in a region of the gradient with higher sucrose concentrations which implies that the protein has been incorporated into structures of significant size; however, it does not automatically confirm VLP assembly. This can only be verified by TEM which is presented later in this chapter.

It is significant that the protein detected after plant-based expression has an apparent size of 43 kDa. During NNV infection the major protein produced is the precursor protein alpha (43 kDa) which self-assembles into provirions (Friesen and Rueckert, 1981; Gallagher and Rueckert, 1988). Provirions mature by spontaneous autocatalytic cleavage of the coat protein alpha; producing proteins beta (38 kDa) and gamma (5 kDa) which remain part of the mature virion (Hosur et al., 1987). VLPs are non-infectious, as they do not contain genomic material, and therefore do not undergo maturation cleavage. This explains the larger molecular mass of the coat protein observed in the plant made VLPs compared to the coat protein isolated from infectious virus where it gets cleaved into proteins beta and gamma.

Summary of section 3.2: NNV coat protein is expressed in plants and when run through a discontinuous sucrose gradient it collects in the 30% and 40% (w/v) fraction. An indication towards assembled particles but this has to be confirmed by TEM.

3.3 OPTIMISATION OF PROTEIN YIELD

3.3.1 TIME COURSE EXPERIMENT

In the previous section expression of the NNV coat protein was confirmed (Figure 3.2-2). In order to determine the day with the highest level of desired protein a time course analysis was conducted. For this purpose, a plant was infiltrated with pEAQ-*HT*-NNV and leaf discs were sampled 1 to 10 dpi. Protein was extracted and expression analysed on a Western blot (Figure 3.3-1).

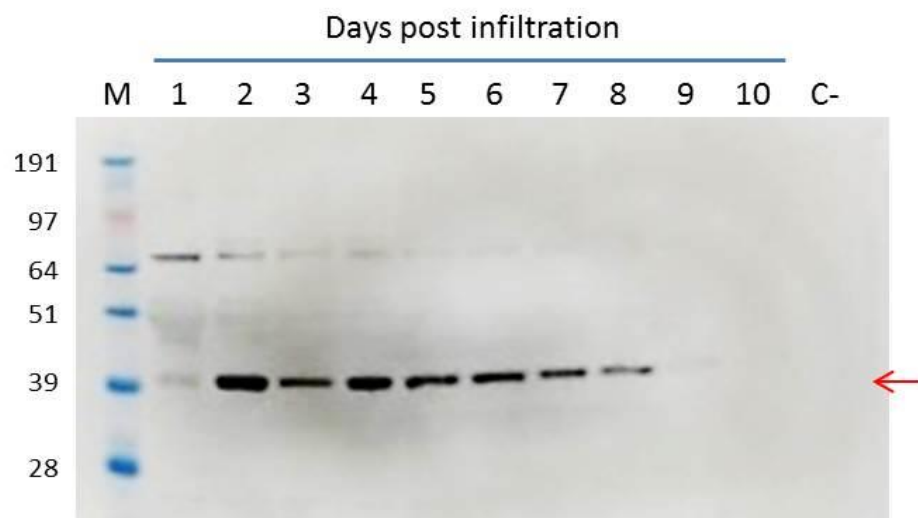


Figure 3.3-1: Expression of NNV coat protein over time. Exactly the same amount of infiltrated leaf material was sampled from a plant expressing NNV coat protein 1 to 10 dpi. The protein was extracted and analysed on a Western blot which was probed with anti-NNV (ab26812) antibody. M = SeeBlue Plus 2 with molecular weights indicated. C- = leaf material infiltrated with pEAQ-*HT*-EV (empty vector control). Red arrow indicates position of coat protein (43 kDa).

The above Western blot demonstrates accumulation of NNV coat protein already 2 dpi with a steady level until 5-6 dpi after which it decreases and completely disappears at 9 dpi. One can also observe bands of 70-75 kDa appearing already 1 dpi and slowly decreasing whilst the amount of coat protein rises. These bands could be dimers of the coat protein but this would need to be confirmed. Similar experiments using the same vector but expressing GFP instead of the NNV coat protein revealed that the highest levels of expression on day 6 (Saxena, 2010). Combining this knowledge with the result of the time course analysis (Figure 3.3-1) results in 4-6 dpi for maximum NNV coat protein yield. It also suggests that accumulation of NNV coat protein occurs more quickly than GFP and shows the value of time courses.

3.3.2 OPTIMISATION OF THE AGROBACTERIUM SOLUTION

Another factor that might influence protein expression is the OD_{600} of agrobacterium suspensions used for infiltration. As default we use an OD_{600} of 0.4 in the lab which has proven to work best when expressing GFP (Saxena, 2010). However, I was curious to know if a lower or higher OD has an impact on the expression level of the NNV coat protein. For that reason, *A. tumefaciens* harbouring pEAQ-HT-NNV were resuspended in MMA buffer to an OD_{600} of 0.2, 0.4, 0.6 or 0.8 and infiltrated into separate plants. Leaf discs were sampled 5, 6 and 7 dpi and protein was extracted.

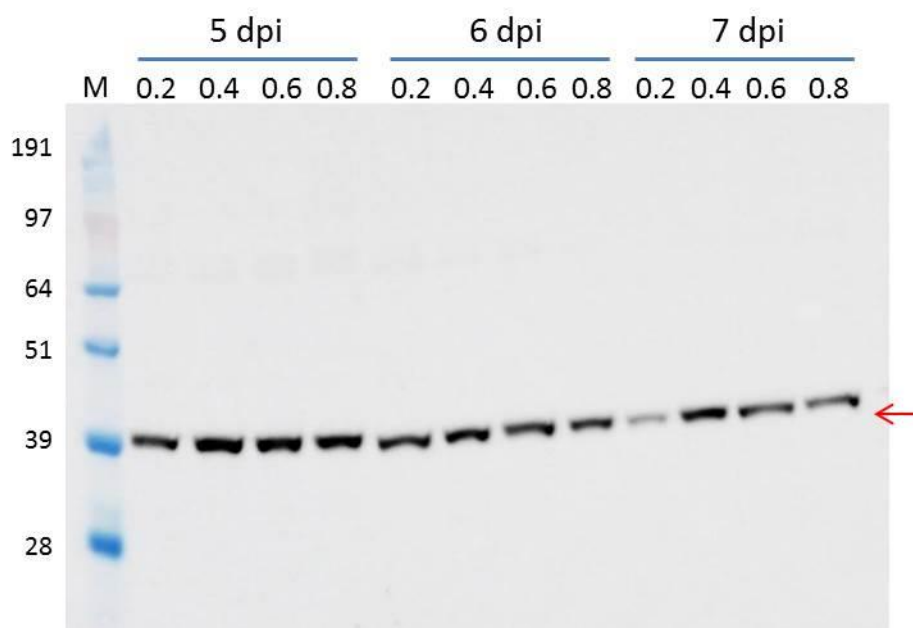


Figure 3.3-2: Finding the most efficient OD_{600} for agro-infiltration and expression of NNV coat protein. *A. tumefaciens* carrying pEAQ-HT-NNV was resuspended in MMA buffer to an OD_{600} of 0.2, 0.4, 0.6 or 0.8 and infiltrated into separate plants. Leaf discs were sampled 5, 6 and 7 dpi and protein was extracted. Western blot was probed with anti-NNV (ab26812) antibody. M = SeeBlue Plus 2 with molecular weights indicated.

The protein samples were run on a SDS-PAGE and transferred on a membrane for immunodetection using anti-NNV antibody. The blot is depicted in Figure 3.3-2 but no striking differences between the samples are apparent. Though especially on 5 dpi and 7 dpi there is a slightly increased amount of protein detected in the sample of OD_{600} 0.4. Overall the lowest (0.2) and highest (0.8) OD_{600} appear least beneficial which confirms that an OD_{600} of 0.4 is the best concentration of agrobacteria for expressing NNV coat protein in plants.

Summary of section 3.3: Using an *A. tumefaciens* solution with an OD₆₀₀ of 0.4 achieves highest yields of NNV coat protein 4-6 days after infiltration.

3.4 OPTIMIZATION OF PARTICLE PURIFICATION

Precipitation with polyethylene glycol is a standard method used for the concentration of virus particles when handling large volumes of liquids (Leberman, 1966). Therefore, I wanted to test if it would also work with the NNV VLPs and tried different PEG concentrations. Crude plant extracts were mixed with 2%, 4% and 6% (w/v) PEG overnight at 4 °C, centrifuged and the pellets resuspended in TBS buffer followed by standard sucrose gradient ultracentrifugation. As a control one sample was not treated with PEG. The sucrose fractions were run on SDS-PAGE gels and shown in Figure 3.4-1.

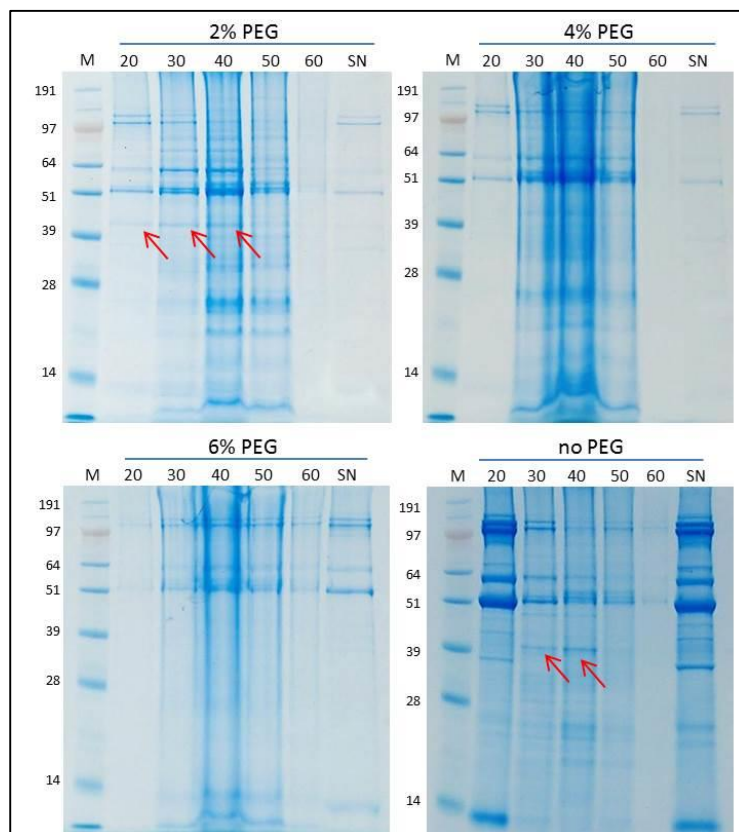


Figure 3.4-1: Trying PEG for NNV VLP concentration. Crude plant extracts containing NNV VLPs were mixed with 2%, 4% and 6% (w/v) PEG following the standard PEG precipitation protocol. Afterwards the resuspended samples were separated on a standard sucrose gradient. As a control one sample was not treated with PEG. After ultracentrifugation each fraction was retrieved by piercing the bottom of the tube and run on SDS-PAGE. Red arrows indicate the NNV coat protein. M = SeeBlue Plus 2 with molecular weights indicated. SN = Supernatant.

Even though precipitation with 4% (w/v) PEG works well with many viruses the result shown in Figure 3.4-1 indicate a problem with NNV VLPs using this method. When applying the lowest concentration of only 2% (w/v) PEG, bands of the expected size are detected in the 30% and 40% (w/v) sucrose fraction; an outcome which is in accord with the untreated control sample. However, there is also a ~43 kDa band in the 20% (w/v) sucrose visible which suggests non assembled coat protein or broken particles (see red arrows in Figure 3.4-1). Samples that were treated with higher PEG concentrations are completely free of coat protein. No bands of the correct size are apparent on the SDS-PAGE gels. One can conclude that PEG precipitation does not benefit purification or concentration of NNV VLP samples; it rather seems to have a destabilising effect on the particles. In the 2% (w/v) PEG treated sample coat protein appears in the 20% (w/v) sucrose fraction which can be a result of broken VLPs. Even more evident becomes the negative effect in the 4% and 6% (w/v) PEG treated samples, where NNV coat protein cannot be seen at all. It is likely that the PEG destroyed the VLPs and the unassembled coat protein remained in the SN but is too dilute to be detectable on the SDS-PAGE gel. Another possible explanation is failure to resuspend the pellets after PEG precipitation.

Because PEG precipitation did not help concentrating the sample another approach was tried. Clarified plant extract was centrifuged through a sucrose gradient and the 30% and 40% (w/v) sucrose fractions containing the NNV VLPs (see SDS-PAGE and Western blot Figure 3.4-2) were combined and dialysed. After dialysis the large sample volume was subject to ultracentrifugation in order to pellet and simultaneously concentrate the VLPs.

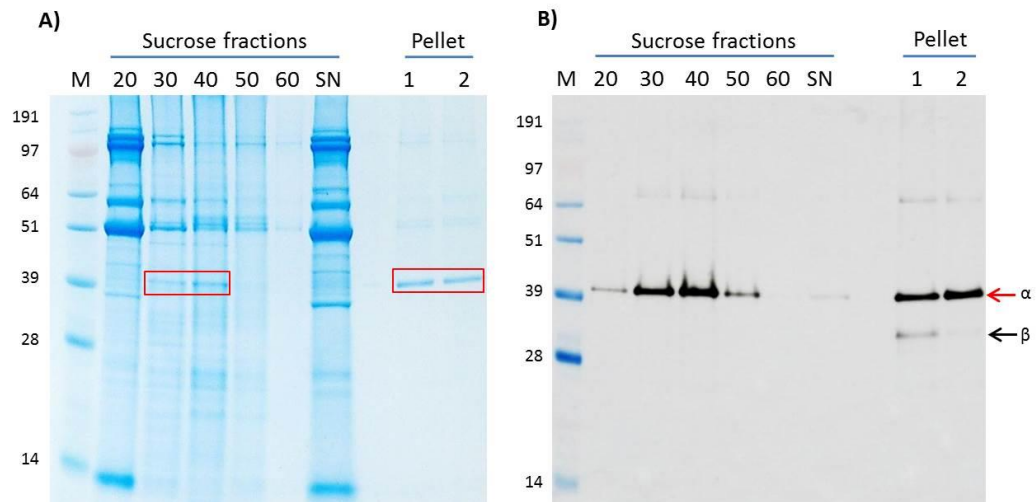


Figure 3.4-2: Pelleting NNV VLPs for concentration and purification. Clarified plant extract containing NNV VLPs was run through a sucrose gradient. The 30% and 40% (w/v) sucrose fraction harbouring the NNV VLPs were combined, dialysed and then pelleted. Two different buffers were used for resuspension. Lane 1 = PBS buffer. Lane 2 = TBS buffer. Western blot was probed with anti-NNV (ab26812) antibody. M = SeeBlue Plus 2 with molecular weights indicated. Red boxes and arrow indicate NNV coat protein alpha. Black arrow indicates mature NNV coat protein beta.

Pelleting is only successful when the particles are very stable, otherwise they can break under the strong forces applied in the ultracentrifuge. Here, after ultracentrifugation the pellet was thoroughly resuspended in either PBS buffer or TBS buffer in order to test which buffer stabilises the VLPs best. The samples were run on a SDS-PAGE and also a Western blot was conducted to analyse the purity of the sample and also the stability of the NNV VLPs. In Figure 3.4-2 A the pelleted samples are very clean and no obvious contamination is detectable. When comparing the pelleted samples to the original 30% and 40% (w/v) fraction it becomes apparent how well the pelleting step improved purity. However, there are no apparent differences between the results obtained with the two buffers as determined by the SDS-PAGE. Looking at the Western blot (Figure 3.4-2 B) in lane 1 the NNV VLP pellet which was resuspended in PBS-buffer not only has a strong band of the correct size for the coat protein but also a slightly less distinct band of lower molecular mass. This band is not present in lane 2 where TBS buffer was used for resuspension. Most likely this lower molecular mass protein which was detected by the anti-NNV antibody is degraded coat protein or even the mature coat protein beta. Nodavirus provirions are known to be less stable until they undergo maturation, where the coat protein gets cleaved into two smaller proteins of 37 kDa and 5 kDa (see 3.1) (Hosur et al., 1987; Schneemann et al., 1992). As there is no such band in lane 2 one can conclude the reason for the degradation is the PBS buffer and not the pelleting. The

biggest difference between PBS and TBS buffer is their pH value. TBS is with a pH of 8.5 closer to the natural pH of ocean water (pH 8.2-8.4) than PBS buffer with pH 7.2. It seems like the TBS buffer stabilises the VLPs or hinders maturation.

Summary of section 3.4: In summary the results of this chapter taught me that the NNV VLPs survive pelleting which is an easy way to concentrate them when dealing with larger sample volumes; whereas PEG precipitation seems to destabilise the VLPs or render them insoluble. Most comfortable are NNV VLPs in a buffer with a rather alkaline pH like TBS. The preference for an alkaline pH may reflect the adaptation of the particles to the pH of ocean water, which is 8.2 to 8.4.

3.5 CHARACTERISATION OF NNV VLPs

3.5.1 TRANSMISSION-ELECTRON MICROSCOPY (TEM)

Until now the focus was on characterising the NNV coat protein and the results presented so far strongly indicate VLP assembly, but correct assembly of the protein and finally formation of NNV VLPs still had to be confirmed via TEM. The following drawing (Figure 3.5-1) explains the process adopted for NNV VLP purification in order to produce a concentrated sample which could be further analysed with the electron microscope.

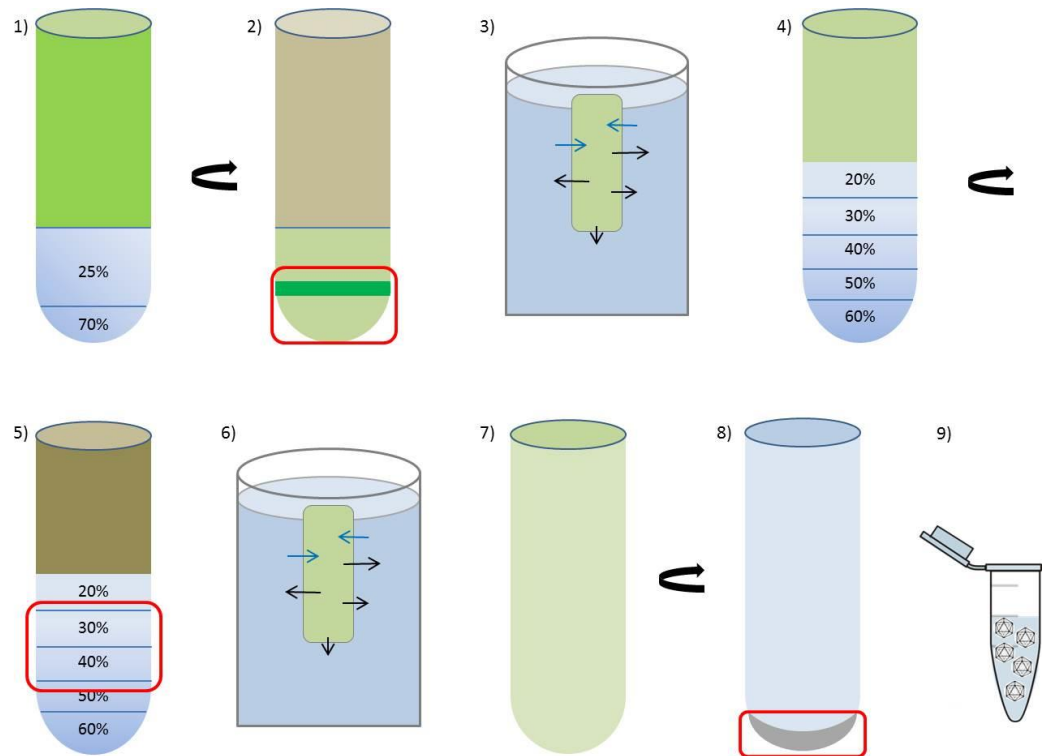


Figure 3.5-1: Sketch of NNV VLP purification. Clarified plant extract containing NNV VLPs was underlain with 5 ml of 25% (w/v) sucrose and 1 ml 70% (w/v) sucrose and ultracentrifuged. The bottom fraction was retrieved and after dialysis run through a discontinuous sucrose gradient of 20%-60%. Routinely NNV VLPs were located by immunodetection and the respective fractions pooled and dialysed. The VLPs were concentrated by pelleting and resuspended in a small volume of TBS to obtain a pure sample of NNV VLPs.

The NNV VLPs shown in Figure 3.5-2 came from clarified plant extract derived from 60 g infiltrated leaf material. It was centrifuged through a sucrose cushion as a first clean up step, followed by a sucrose gradient centrifugation. Localisation of the VLPs in the 30% and 40% (w/v) fraction was confirmed via Western blot (see Figure 3.4-2). After dialysis the sample was pelleted and resuspended in 500 µl of TBS buffer and visualised under the TEM to assess the integrity, appearance and approximate size of the NNV VLPs.

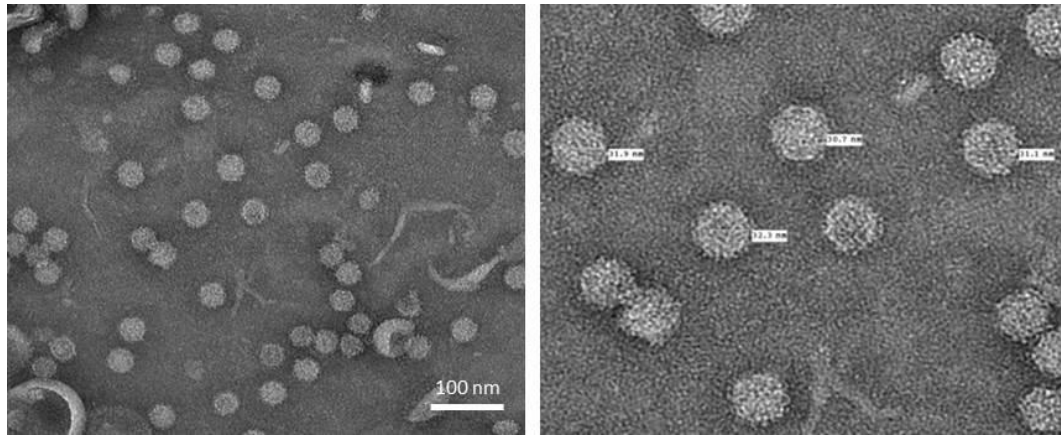


Figure 3.5-2: Electron micrographs of NNV VLPs. Plant extract containing NNV VLPs was purified through two sucrose gradients and concentrated by pelleting. NNV VLPs are visualised by negative staining and transmission electron microscopy.

The TEM images show properly assembled NNV VLPs of correct size (25-30 nm) and form. They are evenly shaped and of great abundance. Wild type NNV forms a T = 3 icosahedral capsid made out of 180 identical subunits (Cheng et al., 1994) and the plant produced NNV VLPs are indistinguishable from the native particles by electron microscopy. Interestingly the NNV VLPs appear full on the electron micrographs; no dark spot can be seen in their centre. Often the stain penetrates and accumulates inside of empty particles, whereas in viruses containing nucleic acid this does not occur. Even though stain penetration can be influenced by the buffer used, it is an indication that the plant-made NNV VLPs encapsidate nucleic acid during particle assembly. To quantitate the amount of VLPs produced and to avoid possible effect of nucleic acid in the sample, modified Lowry protein assay has been carried out using this sample. A yield of 10 mg/kg fresh weight of the highly purified VLPs was calculated.

Summary of section 3.5.1: NNV coat protein expressed in plants spontaneously assembles into evenly shaped and correctly sized VLPs. Plant produced NNV VLPs are indistinguishable from wild type NNV particles by electron microscopy.

3.5.2 NATIVE GEL ELECTROPHORESIS

In 2003 Lu and colleagues expressed NNV VLPs in *E. coli* and found that their VLPs encapsidated various sizes of host RNA (Lu et al., 2003). As discussed above, the TEM images in Figure 3.5-2 indicated that the plant-produced NNV VLPs may also contain nucleic acid. To confirm this, two NNV VLPs samples were run on native agarose gels together with approximately 10 µg each of CPMV and empty VLPs of CPMV (eVLPs) as

controls. One gel was stained with Coomassie blue protein stain, while a duplicate gel was stained with ethidium bromide to visualize nucleic acid (Figure 3.5-3).

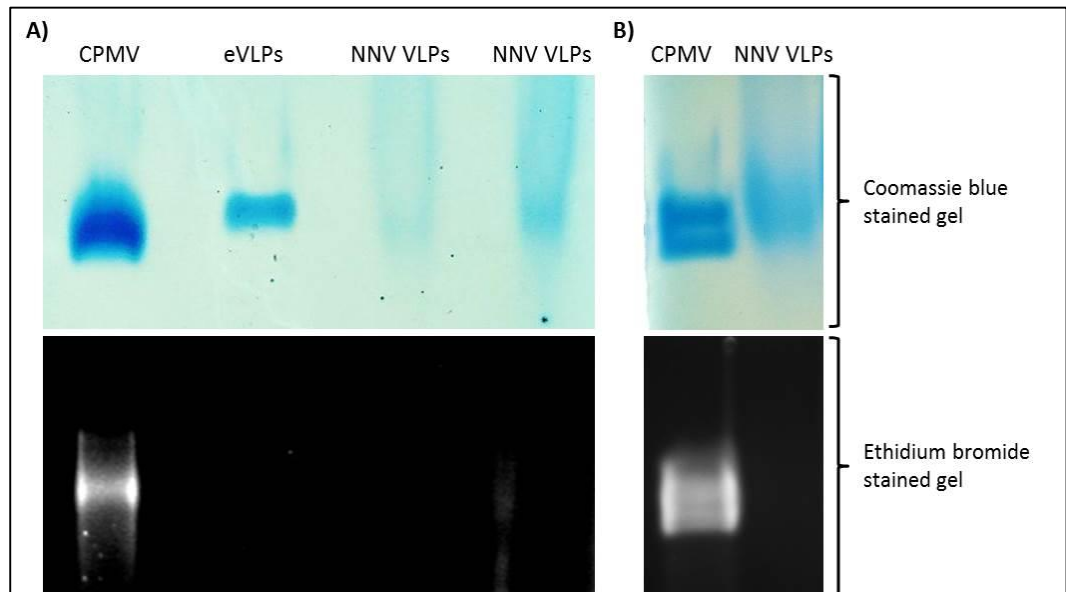


Figure 3.5-3: Native gel electrophoresis of NNV VLPs to analyse encapsidation of nucleic acid. A) Native gel electrophoresis of NNV VLPs, CPMV and eVLPs. Approximately 10 μg of CPMV and eVLPs and 3-5 μg purified NNV VLPs were loaded per lane. **B)** Native gel electrophoresis of NNV VLPs and CPMV. Approximately 10 μg of CPMV 6 μg purified NNV VLPs were loaded per lane. The particles were separated on 1% agarose gels and stained with Coomassie blue or ethidium bromide.

Gel electrophoresis of VLPs on agarose gels is an effective method for analysing the nucleic acid content of particles. RNA-containing CPMV particles can be stained with both ethidium bromide and Coomassie blue, whereas CPMV eVLPs, which do not contain any nucleic acid are only be stained with Coomassie blue. Because the concentration of NNV VLPs was too low in Figure 3.5-3 A to obtain a definitive result, the experiment was repeated, but this time using a more concentrated NNV VLP sample (Figure 3.5-3 B). Even though the concentration of the NNV VLPs is still low compared with CPMV controls, and thus the Coomassie blue stain relatively faint, Figure 3.5-3 shows that the plant-produced NNV VLPs do not appear to encapsidate significant nucleic acid. However, for a more accurate result the nucleic acid extraction could be conducted using purified NNV VLPs and analysed for RNA/DNA content.

Summary of section 3.5.2: Plant produced NNV VLPs are free of nucleic acid.

3.5.3 CRYO-ELECTRON-MICROSCOPY (CRYO-EM)

In the family *Nodaviridae* 180 coat proteins assemble and form a T = 3 capsid with a diameter of ~25-35 nm. For more detailed structural analysis, cryo-EM was performed by Daniel Hurdiss at the University of Leeds, UK on NNV VLPs prepared as described in Figure 3.5-1. This technique allows the determination of 3D structure of a particle to close to atomic resolution through computational averaging of multiple particle images. The results obtained using this technique are summarised in Figure 3.5-4.

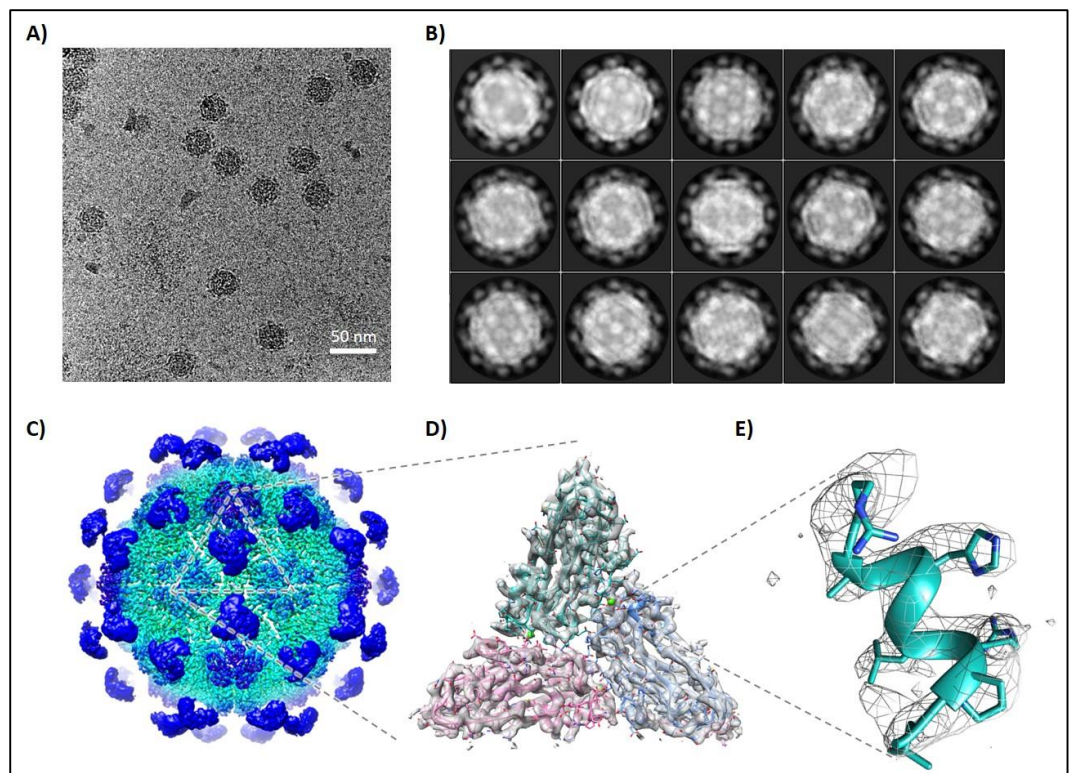


Figure 3.5-4: Cryo-EM analysis of NNV VLPs. NNV VLPs were loaded on copper grids, frozen rapidly by plunging into liquid ethane and imaged. **A)** NNV VLPs unstained in vitreous ice. **B)** Reference free, 2D class averages of NNV VLP generated in Relion. **C)** 3D map of NNV with a global resolution of 3.8 Å. **D)** Density for a single asymmetric unit of the T = 3 NNV-VLP with fitted atomic model (4WIZ). **E)** Representative alpha-helical density with a local resolution of ~3.4 Å showing clear side chain density.

Images of unstained, frozen-hydrated NNV-VLPs were imaged using an electron microscope fitted with a direct electron detecting camera (Figure 3.5-4 A). Single-particle image processing was carried out in Relion (Figure 3.5-4 B), which allowed us to determine the solution structure of the NNV-VLP to a global resolution of 3.8 Å (Figure 3.5-4 C). This revealed that the NNV-VLP is an isometric particle with T = 3 symmetry (Figure 3.5-4 D), consistent with previously reported structures (Chen et al., 2015; Cheng

et al., 1994). Three P-domains of the capsid protein interact and form spikes on the particle surface. They are attached to the rest of the capsid protein but the linkers are unstructured. The shell domain displayed a higher local resolution of 3.4 Å, with well-resolved side-chain density (Figure 3.5-4 E).

Summary of section 3.5.3: The above cryo-EM studies represent the first time plant-produced NNV VLPs have been visualised in such high resolution. It revealed a 3.8 Å structure of authentic looking NNV VLPs.

3.6 TOWARDS TRANSGENIC EXPRESSION OF NNV VLPs

The aim is to generate a transgenic line of *N. benthamiana* for NNV VLPs. Stable transformation can be done using the same pEAQ-*HT*-NNV construct that was used for transient expression, however it carries the suppressor of silencing P19 which derives from the tomato bushy stunt virus (TBSV) (Voinnet et al., 2003). P19 is one of the reasons behind the high expression levels obtainable with the pEAQ-*HT* vectors. It is a 19 kDa protein that specifically sequesters siRNAs and thereby blocks post-transcriptional gene silencing and leads to up-regulation of protein expression (Omarov et al., 2006; Ye et al., 2003). However, when trying to regenerate plants from transformed leaf discs, P19 interferes with miRNA pathways involved in growth and development causing severe developmental defects (Chapman et al., 2004; Dunoyer et al., 2004; Saxena et al., 2011). Thus, wild-type P19 cannot be deployed when generating stably transformed plants.

The mutation R43W in P19 was first identified in an attenuated strain of TBSV in *N. benthamiana* (Chu et al., 2000). A single amino acid substitution of arginine at position 43 with tryptophan still allows P19 to bind siRNA duplexes but the binding is unstable, resulting in reduced suppression of RNA silencing (Omarov et al., 2006). This mutant form of P19 has been shown to be tolerated in homozygous transgenic *N. benthamiana* plants and has been deployed to make the pEAQ vectors suitable for the creation of lines of transgenic plants (Saxena et al., 2011).

In order to create the point mutant R43W/P19, primers P19-R43W-SDM-F and P19-R43W-SDM-R (listed in Appendix 1) were used to change 'CGG' (encoding arginine) to 'TGG' (encoding tryptophan) generating pEAQ-*HT*-NNV-P19m via site-directed

mutagenesis (SDM). After verification of the mutation by sequencing, pEAQ-*HT*-NNV-P19m was transformed into agrobacteria for transient expression in plants.

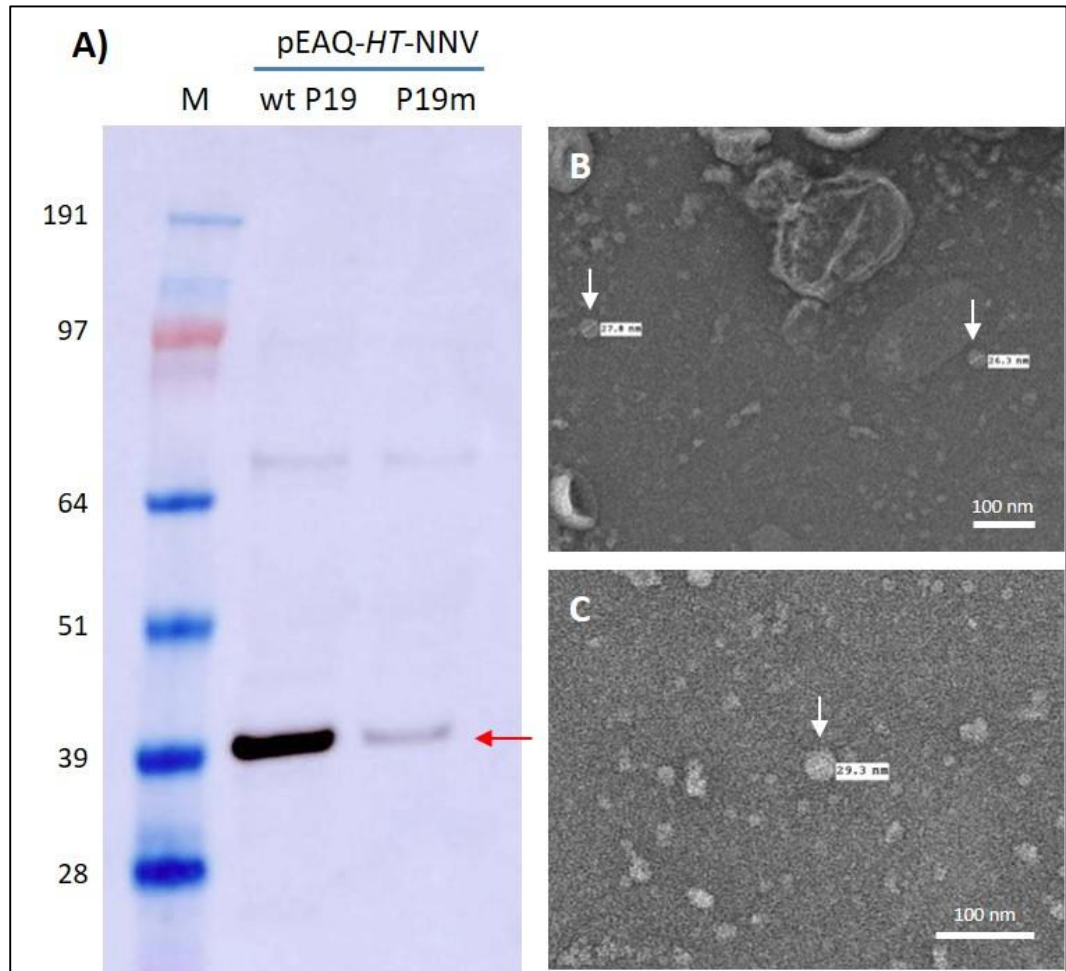


Figure 3.6-1: Transient expression of NNV VLPs in presence of wt P19 and P19m. A) Comparison of protein expression using either pEAQ-*HT*-NNV or pEAQ-*HT*-NNV-P19m on a Western blot. Western blot was probed with anti-NNV antibody. M = SeeBlue Plus 2 with molecular weights indicated. Red arrow indicates position of NNV coat protein. B, C) Verifying correct assembly of NNV VLPs under the TEM when using pEAQ-*HT*-NNV-P19m. NNV VLPs are visualised by negative staining and transmission electron microscopy. White arrows indicate NNV VLPs.

In order to verify if NNV VLPs are still expressed and assemble correctly when using the construct pEAQ-*HT*-NNV-P19m and to compare the protein expression level to the original pEAQ-*HT*-NNV, separate plants were infiltrated with the constructs and protein was extracted. In Figure 3.6-1 A the amount of NNV coat protein that accumulated in the presence of wt P19 and the R43W P19m was compared. The levels of NNV coat protein detected in the presence of R43W P19m were noticeably lower than was found with wt P19, a result consisted with that obtained with transient GFP expression by Saxena et al. (2011). The lower levels of coat protein accumulation is likely to mean that

fewer NNV VLPs will form and this was apparent when visualizing VLPs in samples obtained using the two different version of P19 under the electron microscope (Figure 3.6-1 B, C).

Summary of section 3.6: The suppressor of silencing P19 cannot be deployed when generating stably transformed plants as it interferes with miRNA pathways involved in growth and development causing severe developmental defects. Therefore, a point mutation (R43W) was inserted into wt P19 generating pEAQ-*HT*-NNV-P19m. Expression of the coat protein is reduced but correct assembly of VLPs was confirmed.

3.6.1 FUTURE WORK

Due to time limitations the aim of creating a stable transgenic *N. benthamiana* line expressing NNV VLP has not been reached. However, the first step towards this goal has been made (see section 3.6) by generating pEAQ-*HT*-NNV-P19m which can directly be used for stable integration of the NNV coat protein sequence into the *N. benthamiana* genome. For this purpose, the “leaf disc method” first described by Horsch and Klee in 1986 will be followed.

3.7 DISCUSSION

Several vaccine candidates against NNV have been developed but none has been commercialised yet. However, the expanding world population and over-fishing of the oceans has led to a growing demand for farmed fish, making it a very high value industry. NNV causes mortality rates with up to 99% turning it into an enemy for the fish industry and the interest in a prophylactic treatment such as a vaccine, is high (Lin, 2007). In order to produce a practical fish vaccine, feasible administration and cost efficiency of production, as well as immunological efficacy have to be prioritised. Intramuscular injection of single fish is laborious and costly. Therefore, alternative vaccination techniques, like oral vaccination through feeding the fish with the vaccine or bath immunisation should be explored.

The aim of this project was to investigate the feasibility of producing a plant-produced NNV VLP vaccine. This involved characterising plant-produced NNV VLPs and optimising their expression level, purification and stability. Transiently expressed NNV VLPs

accumulate to relatively high levels in plants within 4-6 dpi. Their relatively simple set-up of only one protein that self-assembles into an icosahedral structure of 180 copies makes them an attractive model for studies of VLP production in plants.

The absence of genomic material in VLPs can make them less stable than their parent virus and therefore delicate to purify (see instability of wt PV VLPs in section 4.6.4). The NNV VLPs, however, proved to be stable and survived pelleting in the ultracentrifuge which simplified their concentration and purification when handling large sample volumes. Though, the adopted purification technique described in Figure 3.5-1 does not succeed in totally removing contamination with plant-derived proteins. Other purification techniques, like Nycodenz gradient ultracentrifugation, were tried but without success. More work has to be put into optimising the purification in order to obtain a cleaner result without compromising on the concentration. The plant-produced NNV VLPs showed no evidence of the encapsidation of nucleic acid, unlike their counterpart produced in *E. coli* (Lu et al., 2003). Absolute purity and no residue of the expression host are features to aim for when developing a vaccine where contaminations could potentially tamper the immune response of the recipient or cause a health risk. Though the recombinant subunit vaccine developed by Lin and colleagues provided complete protection from VNN, when fish were fed the oral vaccine without it ever having been extracted or purified from the expression host (Lin, 2007). An advantage of plants over other conventional expression systems is that they bring a low risk of contamination with endotoxins or mammalian pathogens (Lico et al., 2008; Ma et al., 2003; Twyman et al., 2003), unlike the above-mentioned *E. coli*. Therefore, absolute purity might not be necessary for a plant-produced fish vaccine especially when it keeps the costs low but still elicits a protective immune response.

The high-resolution structural studies (carried out by Daniel Hurdiss at the University of Leeds, UK) revealed that the plant-made NNV-VLPs are of isometric shape with $T = 3$ symmetry, consistent with previously reported structures and authentic looking when compared to the wild-type NNV (Chen et al., 2015; Cheng et al., 1994). To date, animal studies have not been carried out using this plant-produced vaccine candidate but it is of very high priority and the requirements for testing have been discussed with Dr. Richard Paley at CEFAS, Weymouth, UK. It is important to test if the VLPs do elicit an immune answer when administered to fish. Oral vaccination seems to be the easiest when working with a large number of fish. Possibilities like pressing plant material harbouring

NNV VLPs into pellets and feeding it to the animals should be investigated. It would be very simple, inexpensive and quick to do as it does not require any purification of the VLPs. If this is applicable depends on the concentration needed for complete protection and whether the plant-produced amounts are sufficient.

Generating transgenic *N. benthamiana* lines expressing NNV VLPs was an idea that was considered late in the project. The first step of the procedure, preparing and testing an expression construct that can directly be used to transform the plants, has been completed; however, generating stable transgenics is a lengthy process and could not be undertaken during this study. Nonetheless, the creation of stably transformed lines of plants would make the idea of fabricating pellets for oral vaccination against NNV easier because once a stable transgenic plant for NNV VLPs has been developed agro-infiltration is unnecessary. The disadvantage of transgenic plants is the strict regulation that comes with this technique, which modifies the germline of plants, which is likely to hinder the commercial development of such a product. The possibility of generating stable transgenics for NNV VLPs with an inducible promoter has been considered. The heat inducible promoter (EC71016) which can be activated with a single, short heat shock treatment of 37° C, a temperature which is totally acceptable for the Australian plant *N. benthamiana*, appears to be particularly attractive. The advantage of an inducible promoter is that protein expression can be controlled externally by simple methods. One could either place the NNV coat protein under the control of an inducible promoter or the wild-type suppressor of silencing, P19. As discussed above, P19 cannot be deployed when generating stably transformed plants but under the control of an inducible promoter its expression could be switched on after the critical stages of plant development. This would most likely have a positive impact in the expression of the desired protein rather than using the mutated version P19m which leads to a significant decrease in expression level (Figure 3.6-1).

NNV is a relatively simple virus which is composed out of 180 copies of the same protein (Cheng et al., 1994). Its simplicity made it a great tool for me to study general techniques of VLP production in plants which I could apply to my main project: the development of a synthetic, plant produced VLP vaccine against poliovirus which is described in the following chapters.

Chapter 4: EXPRESSION OF POLIO VLPs IN PLANTS

4.1 INTRODUCTION

Poliovirus contains a single stranded (+)-sense RNA molecule of 7410 nucleotides containing a large open reading frame (Kitamura et al., 1981; Racaniello and Baltimore, 1981). Once the RNA enters the host cell it is translated into a single and highly autocatalytic 220 kDa polyprotein. Translation is initiated at the internal ribosome entry site (IRES), a structured RNA *cis*-acting element within the 5' UTR (Wimmer et al., 1993). The polyprotein can be sectioned into two parts: the structural and non-structural region. The structural region includes the proteins that form the capsid, whereas the non-structural region contains proteinases and polymerases important for replication and infection. Processing of the polyprotein takes place in several steps which are sketched in Figure 4.1-1. First the proteinase 2A cuts and therewith separates the structural from the non-structural region, releasing P1 (Kitamura et al., 1981; Toyoda et al., 1986). Then P3 gets released through *cis*-cleavage by the proteinase 3C^{pro}/3CD (Lawson and Semler, 1992) which is also responsible for the processing of P1 into VP0, VP1 and VP3 (Hellen et al., 1989; Ypma-Wong et al., 1988).

Particularly important for the work described in this chapter is the proteinase 3CD which actually is a precursor molecule of the proteinase 3C^{pro} and the polymerase 3D^{pol}. Interestingly 3CD does not possess any polymerase activity, but it cleaves sites in the structural region more efficiently *in vitro* than 3C^{pro} (Jore et al., 1988; Ypma-Wong et al., 1988). Additionally 3C^{pro} is known to be involved in apoptosis of the host cell (Barco et al., 2000), an important process during infection as it facilitates the exit of virus progeny from cells (Tolskaya et al., 1995). It also may protect progeny viruses from host immune defences and suppresses inflammatory responses (Teodoro and Branton, 1997; Tolskaya et al., 1995). However, the activity of 3C^{pro} is not limited to apoptosis; it cleaves a variety of host proteins such as different transcription factors and inhibits host cell RNA and protein synthesis (Baltimore and Franklin, 1962; Clark et al., 1991; Kaariainen and Ranki, 1984; Yalamanchili et al., 1996). In order to reduce the toxic effect of 3C^{pro} its precursor 3CD was deployed in this project for the processing of P1.

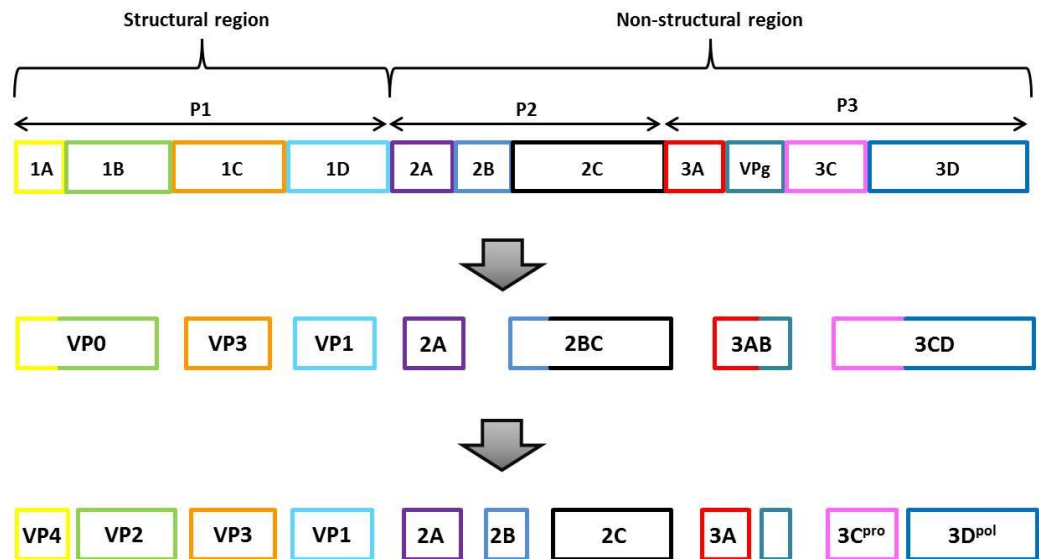


Figure 4.1-1: Schematic representation of the PV polyprotein processing and maturation. First the proteinase 2A cuts and therewith separates the structural from the non-structural region, releasing P1. Then P3 gets released and cleaved by the proteinase 3C^{pro}/3CD which is also responsible for the processing of P1 into VP0, VP1 and VP3. During RNA uptake VP0 undergoes a maturation step to yield VP4 and VP2.

Studies suggest that the formation of PV capsids proceeds through several intermediates: first a 5S promoter subunit with one copy of VP0, VP1 and VP3 assembles and then 5 of those come together and form a 14S pentamer (Phillips and Fennell, 1973). These pentamers are the main building blocks of the empty 75S PV procapsid consisting of 60 copies each of VP0, VP1 and VP3 (Bruneau et al., 1983; Jacobson and Baltimore, 1968; Putnak and Phillips, 1981). The last stage in the formation of infectious particles is the autocatalytic cleavage of the immature precursor VP0 to yield VP4 and VP2, also referred to as the final maturation step during RNA uptake (Basavappa et al., 1998; Basavappa et al., 1994; Hogle et al., 1985; Holland and Kiehn, 1968; Jacobson et al., 1970). This processing step reorders the proteins on the inside of the virus capsid with a concomitant increase in particle stability (Basavappa et al., 1994). Finally, all 4 proteins form an icosahedral capsid of 27-30 nm in diameter (Hogle et al., 1985). However, poliovirus assembly is not that straightforward. A number of intermediates accumulate including the empty capsid, consisting of 60 copies of each of VP0, VP1 and VP3, and the provirion consisting of VP0, VP1, VP3 plus the viral RNA (Fernandez-Tomas and Baltimore, 1973; Guttman and Baltimore, 1977a). Empty particles, such as VLPs, do not undergo the final maturation event and therefore consist only of VP0, VP1 and VP3. Particles with uncleaved V0 are less stable than mature virions (Guttman and Baltimore, 1977a) but the outside architecture of empty particles is virtually indistinguishable from the mature virus (Basavappa et al., 1994; Jiang et al., 2014).

Poliovirus occurs in three serotypes (PV1, PV2 and PV3) which share 71% identical nucleotides in the RNA genome. Most of the nucleotide differences occur in the third position of codons, resulting in low frequency of amino acid differences (Toyoda et al., 1984). The highest amino acid variations can be detected in the P1 region whereas non-structural proteins are more highly conserved (Toyoda et al., 1984). The three main coat proteins (VP1, VP2 and VP3) form structurally similar 8-stranded β -barrels decorated with loops connecting the beta-strands (Hogle, 2002). Structural differences among the serotypes can primarily be found in the loop regions of VP1 that cluster near the fivefold axes of the capsid (Lentz et al., 1997). All three poliovirus serotypes recognise a common cellular receptor: CD155 (also known as poliovirus receptor PVR). The human poliovirus receptor CD155 was first identified by Mendelsohn in 1989 as an integral membrane protein of the immunoglobulin superfamily (Mendelsohn et al., 1989). It is expressed in a wide range of tissues and usually works as a cell-cell adhesion molecule through interaction with nectin-3 (Koike et al., 1990; Mendelsohn et al., 1989; Mueller and Wimmer, 2003).

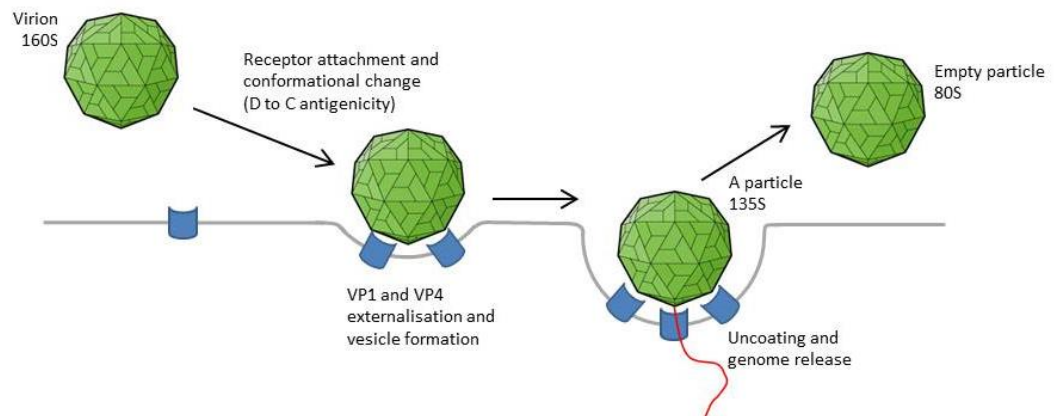


Figure 4.1-2: Schematic representation of the PV cell entry with conformational transition from D to C antigenicity. Infection is initiated by attachment to the PV receptor (CD 155), which induces the conformational transition from D to C antigenicity in the virus. Also vesicle formation is initiated upon receptor binding which internalises the PV particle. The N-terminus of VP1 and VP4 are externalised to anchor the particle in the membrane. The viral RNA gets translocated into the cytoplasm where it is replicated to yield progeny RNAs and translated to yield viral proteins.

When poliovirus binds the receptor it triggers irreversible conformational changes resulting in the formation of the so called A particle or 135S particle (versus 160S for the native virion) (De Sena and Mandel, 1977). In the A particle the capsid protein VP4 and the N-terminus of VP1 are externalised (Chow et al., 1987; De Sena and Mandel, 1977). Both externalised peptides allow the particle to anchor in the membrane independently of the receptor (Danthi et al., 2003; Fricks and Hogle, 1990; Tosteson and Chow, 1997;

Tosteson et al., 2004). After binding to the cell the PV particles are internalised in vesicles and the genome is released leading to the formation of empty particles that sediment at 80S (Belnap et al., 2000a; Brandenburg et al., 2007).

The outer shell of poliovirus is dominated by star-shaped mesas along the fivefold axis and by three-bladed propeller-like structures at the threefold axes (see Figure 1.5-1). Between these features lies a surface depression known as the “canyon” which surrounds each of the twelve fivefold axes of the capsid and is the site of receptor attachment (Bernhardt et al., 1994; Colston and Racaniello, 1994; Colston and Racaniello, 1995). At the base of the canyon is a hydrophobic pocket which is occupied in wt virus by a fatty acid-like ligand or “pocket factor”. It has been suggested that the pocket factor plays a role in the thermal stability of the virion for transport from host to host but as soon as CD155 binds, the pocket factor gets dislodged and uncoating can proceed (Filman et al., 1989; Grant et al., 1994; Rossmann et al., 2002).

PV VLPs have been produced in various recombinant expression systems (e.g. vaccinia virus, baculovirus, *Saccharomyces cerevisiae*) (Ansardi, 1991; Brautigam et al., 1993; Jore et al., 1994), but this is the first time recombinant expression of PV VLPs has been attempted in plants. However, the observation that it is possible to produce VLPs of a plant-infecting member of the order *Picornavirales*, CPMV, by transient expression of the capsid protein precursor (VP60) and the CPMV equivalent of 3C^{pro}, the 24K protease (Saunders et al., 2009), suggested that a similar approach could be viable for poliovirus.

This chapter is about the expression of wt PV 1, 2 and 3 VLPs in *N. benthamiana* aimed at defining the optimal conditions for expression and purification. The focus of this chapter is on balancing the toxic effect of 3C^{pro}/3CD in plants without compromising VLP yield and on increasing the purity of the sample. Furthermore, the plant produced PV VLPs will be described and examined for stability and use as potential vaccines.

4.2 VERIFY EXPRESSION OF POLIO CAPSID PROTEINS

For the recombinant production of poliovirus particles two components are required: the P1 region which encodes the capsid proteins and the poliovirus proteinase that processes P1 into VP0, VP1 and VP3. Without the cleavage of P1 particles cannot form (Jiang et al., 2014). However, as mentioned above, the proteinase 3C^{pro} responsible for

these cleavages has toxic side effects when expressed in a wide variety of different cells; therefore, the more efficient and less harmful precursor 3CD was chosen in this work. The sequence information for wt PV1-P1 and 3CD were provided from our collaborators from the University of Leeds. The wt PV1-P1 is highly similar to the Mahoney strain. The sequences for wt PV2-P1 (MEF-1/Lansing) and wt PV3-P1 (Saukett) were received from our collaborators at the National Institute for Biological Standards and Control (NIBCS) (listed in Appendix 2: Sequences). All sequences were codon-optimised for the expression in *N. benthamiana* and cloned into pEAQ-HT via restriction digest.

In order to test P1 expression, plants were infiltrated with agrobacterium suspensions harbouring pEAQ-HT-wt-PV1-P1. Leaf discs were sampled 3 to 7 dpi and protein extracted. As a positive control leaves were co-infiltrated with pEAQ-HT-wt-PV1-P1 and pEAQ-HT-3CD and sampled 6 dpi. The negative control is plant leaf material infiltrated with pEAQ-HT-EV (empty vector); this was also sampled on day 6 after infiltration. The protein was transferred on a nitrocellulose membrane for immunodetection using the anti-VP1 MAB8566 antibody (Figure 4.2-1).

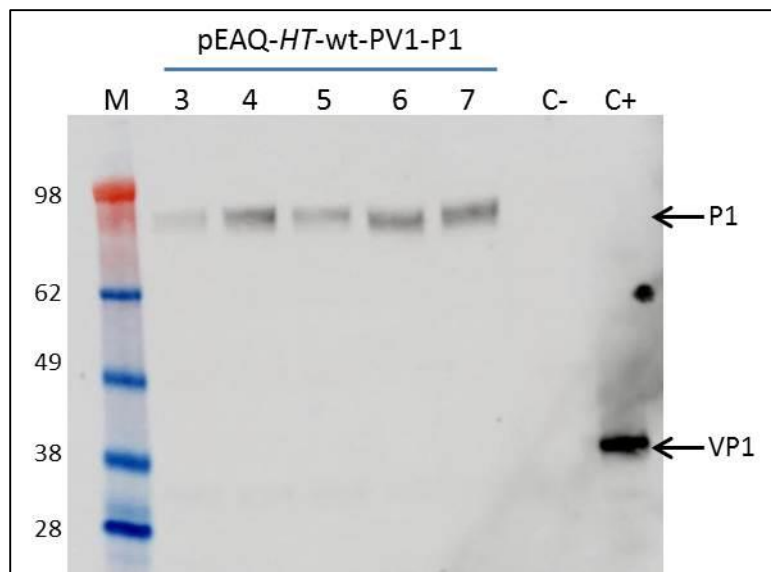


Figure 4.2-1: Verifying expression of P1 and demonstrating the necessity of 3CD for correct processing of the polyprotein. Samples were taken 3-7 dpi from leaves expressing pEAQ-HT-wt-PV1-P1 and the protein extracted. As a positive control (C+) leaves were co-infiltrated with pEAQ-HT-wt-PV1-P1 and pEAQ-HT-3CD and sampled 6 dpi. The negative control (C-) is plant leaf material infiltrated with pEAQ-HT-EV (empty vector); also sampled on day 6 after infiltration. The protein was transferred onto a nitrocellulose membrane for immunodetection using the anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated.

Plants agroinfiltrated with only pEAQ-HT-wt-PV1-P1 produced a high molecular band of approximately 96 kDa when protein extracts were analysed by Western blotting (Figure

4.2-1). This is consistent with the size of unprocessed P1. When pEAQ-*HT*-3CD was co-inoculated with pEAQ-*HT*-wt-PV1-P1, the band corresponding to P1 disappeared and a band corresponding in size to VP1 with a molecular mass of 37 kDa can be detected on the blot (C+). This indicates that that processing of P1 occurs in of co-expressed 3CD.

Summary of section 4.2: PV P1 can be transiently expressed in plants and processing can be achieved by co-expression of the proteinase 3CD.

4.3 OPTIMISING CO-EXPRESSION OF P1 AND 3CD

4.3.1 THE TOXICITY OF 3CD

The toxic effect of the proteinase 3CD became apparent in the infiltrated tissue with severe necrosis occurring; however, the results above demonstrate that the use of 3CD is indispensable (Figure 4.2-1). In order to reduce the negative impact of 3CD on the leaf without losing its proteinase activity for the correct processing of P1, the AUG 161 mutation on the pEAQ-*HT*-3CD plasmid was reversed to decrease 3CD expression 10-fold (Sainsbury and Lomonosoff, 2008). This approach had previously used successfully to downregulate expression of a Bluetongue virus (BTV) protein (Thuenemann et al., 2013b). Using agrobacterium suspensions with a high OD₆₀₀ for P1 and a lower one for 3CD should also help to balance out the impact of 3CD's toxicity. Reverting AUG 161 was achieved with SDM using the primer pair: Rev AUG161_F and Rev AUG161_R (listed in Appendix 1). In Figure 4.3-1 the accumulation of wt PV1 VP1 is shown when using 3CD in either a *HT* or non-*HT* construct. Here, equal amounts of agrobacterium suspensions harbouring pEAQ-*HT*-wt-PV1-P1 (OD₆₀₀ = 0.8) or pEAQ-*HT*-3CD/ pEAQ-3CD (OD₆₀₀ = 0.4) were mixed and infiltrated into leaves. Samples were taken over a period of time of 10 days and the protein analysed on a Western blot using anti-VP1 MAB8566 antibody. Also, a picture was taken 6 dpi to compare the effect of *HT* 3CD with non-*HT* 3CD on the infiltrated leaves.

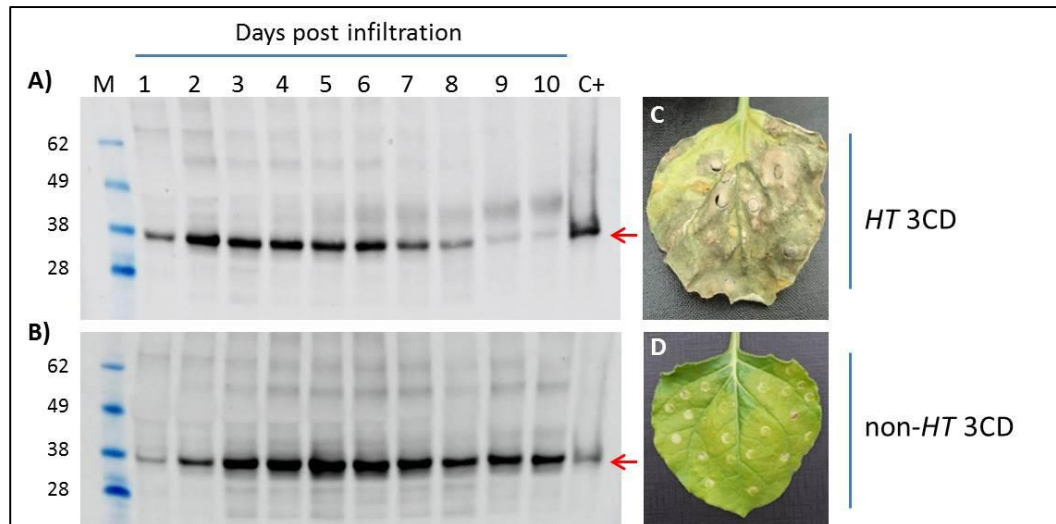


Figure 4.3-1: Comparison of *HT* 3CD with non-*HT* 3CD when co-infiltrated with wt PV1 P1. **A)** Equal amounts of agrobacteria solutions harbouring pEAQ-*HT*-wt-PV1-P1 ($OD_{600} = 0.8$) or pEAQ-*HT*-3CD ($OD_{600} = 0.4$) were mixed and infiltrated into leaves and a picture was taken 6 dpi (**C**). The Western blots were probed with anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV1. Red arrows indicate location of VP1. **B)** Equal amounts of agrobacteria solutions harbouring pEAQ-*HT*-wt-PV1-P1 ($OD_{600} = 0.8$) or pEAQ-3CD ($OD_{600} = 0.4$) were mixed and infiltrated into leaves and a picture was taken 6 dpi (**D**). Samples were taken for 10 days and the protein analysed.

When co-expressing pEAQ-*HT*-wt-PV1-P1 and pEAQ-*HT*-3CD correct processing took place and VP1 accumulated (Figure 4.3-1 A). However, severe necrosis killed the infiltrated tissue within 4-6 days (Figure 4.3-1 C). This limits the expression of the PV capsid protein to the early days post-infiltration, a fact which becomes particularly obvious when comparing the expression time-course to the experiment where non-*HT* 3CD was used (Figure 4.3-1 B, D). Lowering the amount of proteinase available did not appear to affect P1 processing but increased the yield of VP1; extended the duration VP1 is expressed and decreased the necrosis in the infiltrated tissue. The time course experiment in Figure 4.3-1 B demonstrated that expression of PV capsid protein VP1 took place over the entire time of the experiment with highest levels of the protein being observed 5-6 dpi when non-*HT* 3CD was utilised. In all future experiments the non-*HT* 3CD was used and infiltrated plants harvested on 5 or 6 dpi for optimal yield of wt PV1.

Summary of section 4.3.1: The proteinase 3CD causes severe necrosis in the plant tissue when expressed at high levels. Reducing the amount of 3CD by reversing the *HT*-mutation increases the yield of VP1 and extends the time over which it is expressed. Given the fact that both P1 and 3CD are expressed from separate vectors and no

unprocessed P1 is detected, suggests a high degree of specificity between P1 and the proteinase.

4.3.2 PROOF OF VLP ASSEMBLY AND COMPARISON OF YIELD DIFFERENCE

Most obvious becomes the positive impact the non-*HT* 3CD has on expression and yield of VLPs when comparing two wt PV1 VLP samples under the TEM. In the following TEM images the first plant-made PV VLPs are presented and I compare the difference in yield between either wt PV1 VLPs produced with *HT* 3CD (Figure 4.3-2 A, B) or non-*HT* 3CD (Figure 4.3-2 C, D). The same number of plants was agroinfiltrated with either pEAQ-*HT*-wt-PV1-P1 and pEAQ-*HT*-3CD or pEAQ-3CD and crude VLP extraction was conducted 6 dpi. The samples were visualised by TEM.

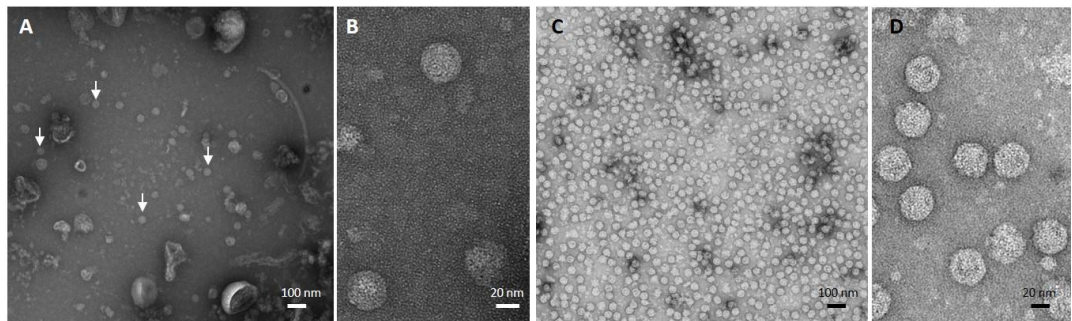


Figure 4.3-2: Comparing VLP yield when either *HT* to non-*HT* 3CD was applied. A, B) The same number of plants was agroinfiltrated with either pEAQ-*HT*-wt-PV1-P1 and pEAQ-*HT*-3CD (white arrows indicate PV VLPs) or pEAQ-3CD (C, D). The samples were visualised by TEM.

The TEM images above prove successful assembly of PV VLPs, the first report of such produced in plants. The wt PV1 VLPs are of authentic icosahedral shape and correct size (27-30 nm). Even though this is not a quantitative comparison, the TEM images show a striking difference in yield when 3CD is less abundant. The proteinase causes severe necrosis in the infiltrated leaf tissue which restricts productivity. When damage caused by 3CD is limited, the productivity of the plant cells is enhanced and therefore more wt PV1 VLPs are produced.

Summary of section 4.3.2: Expression and assembly of PV VLPs is successful in plants, properly shaped VLPs were visualised by TEM. Reducing the expression of 3CD in the experiment increases the yield of PV VLPs.

4.4 OPTIMISATION OF EXTRACTION AND PURIFICATION

4.4.1 SUCROSE CUSHION

Wt PV1 VLPs were purified by centrifuging clarified plant extract through a sucrose cushion constructed of 5 ml 25% (w/v) sucrose and a 1 ml 70% (w/v) sucrose bottom fraction. After ultracentrifugation the bottom of the tube was pierced and the fractions collected. The samples were analysed by SDS-PAGE followed by staining with InstantBlue and Western blotting using anti-VP1 Sh16 antibody.

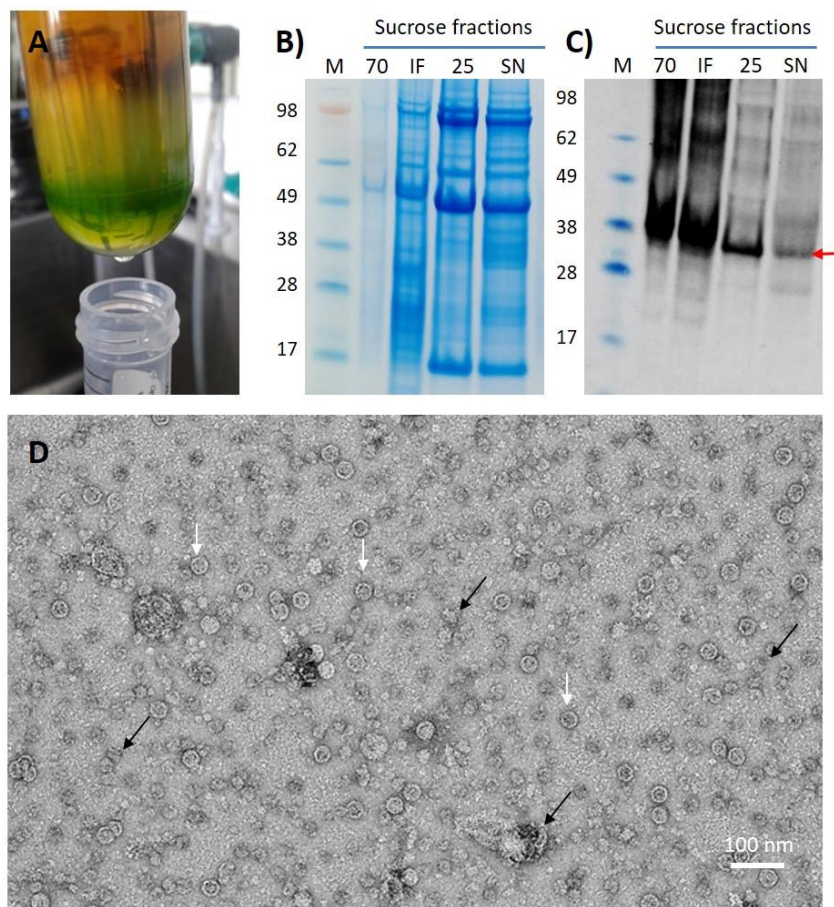


Figure 4.4-1: First step in the purification process of PV VLPs. **A)** Clarified plant extract was run through a sucrose cushion made out of 5 ml 25% (w/v) sucrose and a 1 ml 70% (w/v) sucrose bottom fraction. The 70% (w/v) bottom fraction, the interface (IF), the 25% (w/v) fraction and the supernatant (SN) were retrieved by piercing the bottom of the tube with a needle. **B)** For the detection of the PV capsid protein the fractions were run on a SDS-PAGE. **C)** A duplicate was transferred on nitrocellulose membrane for immunodetection using anti-VP1 Sh16 antibody. M = SeeBlue Plus 2 with molecular weights indicated. Red arrow indicates position of VP1. **D)** TEM images of wt PV1 VLPs after sucrose cushion purification in negative stain. White arrows indicate VLPs and black arrows indicate contamination.

The Western blot shows that the VP1 sediments to a position that is consistent with the coat proteins assembling into higher order structures, such as VLPs. The VLPs sediment mainly in the 70% (w/v) sucrose fraction but also in the interface (IF) between the 70% and 25% (w/v) sucrose fractions which also contains plant cell-derived protein (Figure 4.4-1 B). Even though the IF contains many host proteins, it still carries large amounts of valuable PV VLPs and was therefore combined with the bottom fraction. A small signal of VP1 is detected in the 25% (w/v) sucrose fraction which could represent non-assembled PV capsid protein, incorrectly assembled or broken PV particles. After dialysis the combined sample was visualised by TEM (Figure 4.4-1 D). In the TEM image evenly sized wt PV1 VLPs of 27-30 nm in diameter can be seen with a dark centre where the stain has penetrated the capsid. This is an indicator for truly empty VLPs not carrying plant RNA or DNA. Though, also visible are smaller particle-like structures of approximately 10-20 nm and uneven shape (see black arrows in Figure 4.4-1 D). This is thought to be RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) which can assemble into octameric structures and therefore pass through the 25% (w/v) sucrose fraction.

Summary of section 4.4.1: Though a sucrose step gradient is a valuable first measure for concentrating VLPs, it fails to clearly separate the VLPs from plant contaminants.

4.4.2 NYCODENZ GRADIENT

In order to remove the contamination and further concentrate the sample it was run through a discontinuous gradient of 20% to 60% (w/v) Nycodenz. Three bands formed during ultracentrifugation, whereof two were grey and the top one green. Each band was removed by piercing the side of the tube with a needle (as shown in Figure 4.4-2 A) and analysed via immunodetection on a Western blot using anti-VP1 MAB8566 antibody.

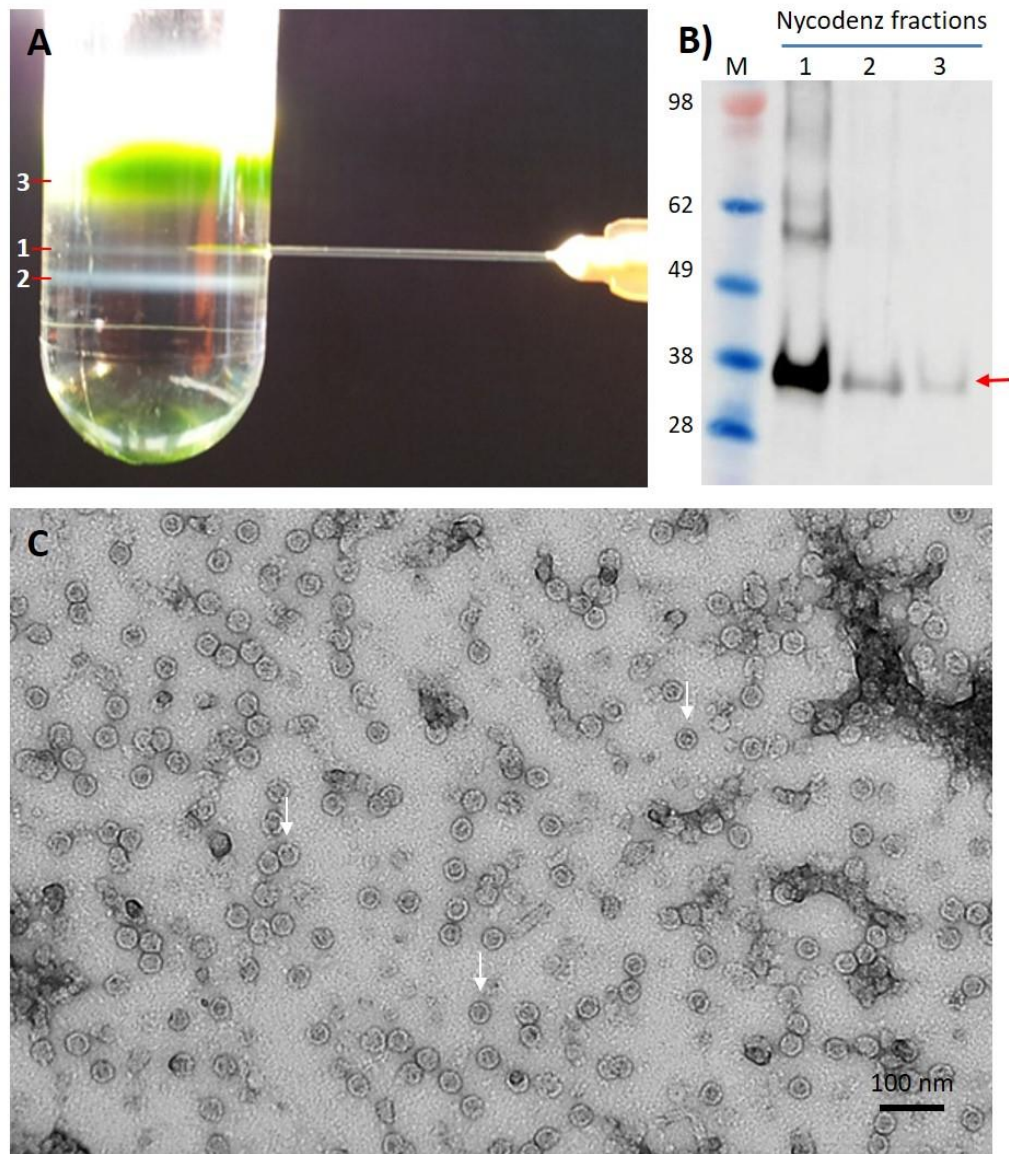


Figure 4.4-2: Second step in the purification process of PV VLPs. A) For further purification the sample was run through a discontinuous Nycodenz gradient and the fractions of interest retrieved by piercing the side of the tube with a needle. **B)** PV VP1 was detected on a Western blot by using anti-VP1 Sh16 antibody. M = SeeBlue Plus 2 with molecular weights indicated. Red arrow indicates position of VP1. **C)** TEM images of wt PV1 VLPs after sucrose cushion and Nycodenz gradient purification. White arrows indicate PV VLPs.

After several attempts the optimal duration of the ultracentrifugation step was found to be 22-24 h at 247,103 x g in a TH641 swing out rotor after which three bands formed within the gradient. Analysis of the content of each band by Western blotting revealed that the middle band contains concentrated VP1 (Figure 4.4-2 B). A small signal of VP1 can also be detected in band 2 and 3; however, this is down to cross-contamination when retrieving the fractions and/or incorrectly assembled particles. Centrifugation of the samples for 22-24 h allows separation of the VLPs from contamination, like RuBisCo.

When visualising band 1, which was tested positive for containing VP1, the TEM image illustrates a very clean sample of plant produced wt PV1 VLPs (Figure 4.4-2 C).

The following illustration (Figure 4.4-3) explains the process adopted to gently purify and concentrate PV VLPs from crude plant extract.

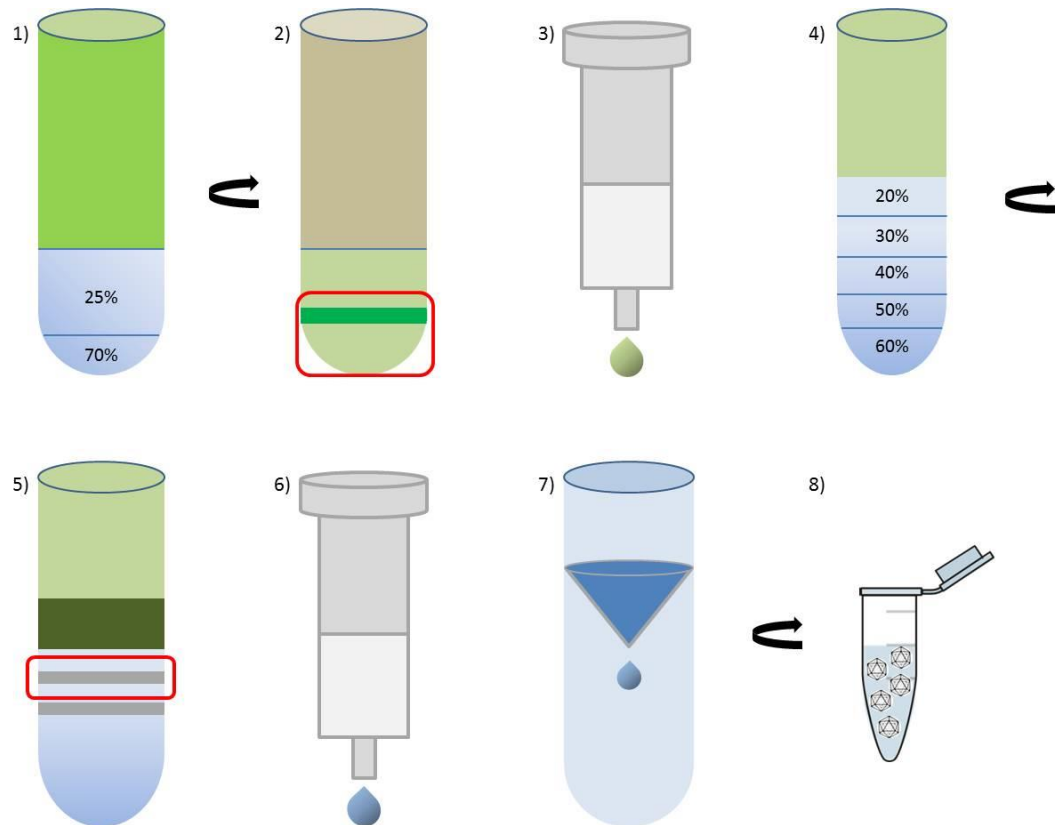


Figure 4.4-3: Diagram of PV VLP purification. Clarified plant extract containing PV VLPs was underlain with 5 ml of 25% (w/v) sucrose and 1 ml 70% (w/v) and spun in the ultracentrifuge. The bottom fraction was retrieved and after dialysis run through a discontinuous Nycodenz gradient of 20%-60% (w/v). Routinely PV VLPs were localised with immunodetection on a Western blot and the respective fractions dialysed. The sample volume was reduced with centrifugal filter units to increase VLP concentration.

Clarified plant extract containing PV VLPs was routinely run through a sucrose cushion as the first step in the purification protocol. The bottom fraction was retrieved and the sucrose removed using PD10 desalting columns (GE Healthcare). For further purification and concentration, the sample was centrifuged through a discontinuous Nycodenz gradient which allowed the separation of VLPs from particulate plant contaminants. Again PD10 desalting columns were utilised for the removal of Nycodenz and the sample volume was reduced to the desired concentration using Amicon centrifugal filter units (Millipore).

Summary of section 4.4.2: Sequential centrifugation through a sucrose cushion and a Nycodenz gradient allows the removal of particulate plant contaminants when the duration of the ultracentrifugation exceeded 22 h at 247,103 x g in a TH641 swing out rotor.

4.4.3 FINDING THE BEST BUFFER

To further optimise sample purity and also to improve long term stability of the PV VLPs, various buffers were tested. Three buffers were chosen on the basis of their pH: HEPES (pH 7.0), PBS (pH 7.5) and PIPES (pH 6.5). They were used throughout for VLP extraction including the final purification steps. In this experiment the same amount of infiltrated leaf material expressing wt PV1 VLPs was separated into three parts and purification and extraction was conducted according to the standard protocol (see Figure 4.4-3); with a sucrose cushion followed by a Nycodenz gradient but using the three different buffers.

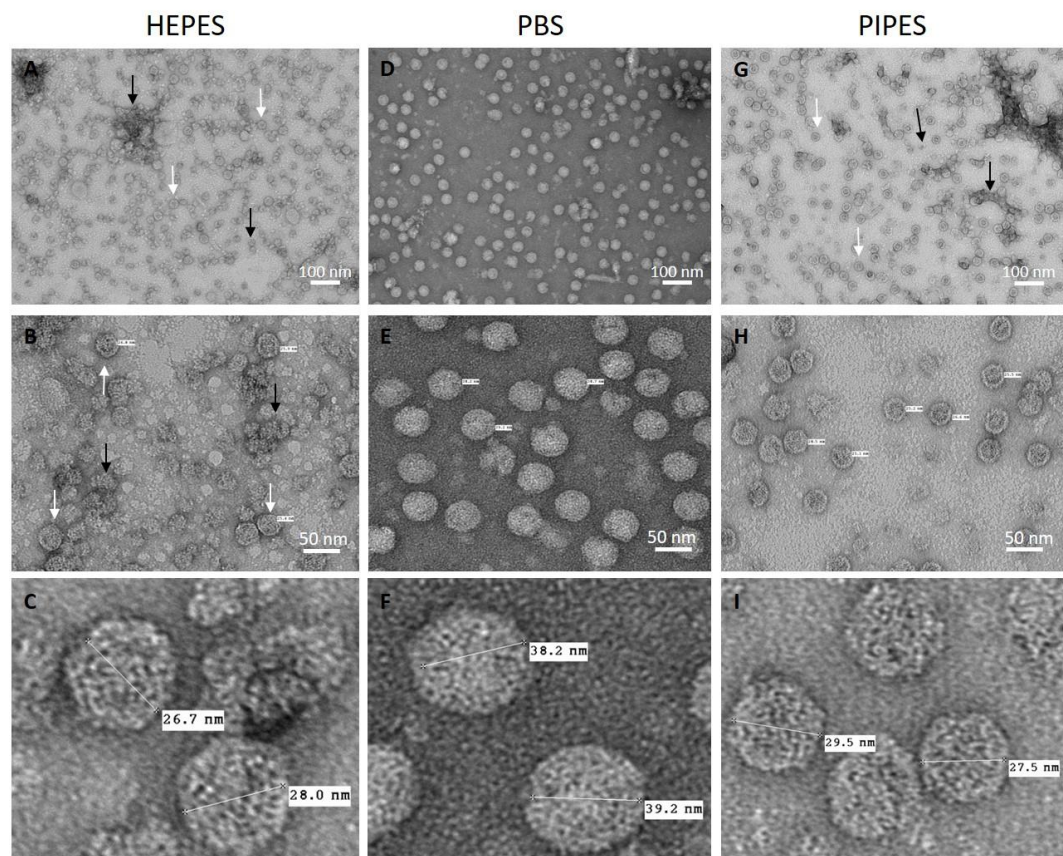


Figure 4.4-4: TEM images of wt PV1 VLPs in different buffers. In order to optimise sample purity and to investigate the influence of buffers on PV VLP stability they were extracted and purified in three different buffers: **A, B, C**) HEPES (pH 7.0), **D, E, F**) PBS (pH 7.5) and **G, H, I**) PIPES (pH 6.5). The VLPs were visualised by TEM using negative staining. White arrows show correctly assembled VLPs and black arrows show contamination.

In the figure above, TEM images are presented of wt PV1 VLPs extracted and purified in three different buffers. There are clear differences in purity and appearance of the VLPs. Figure 4.4-4 A, B and C show obvious contamination of the sample which has been extracted and purified using HEPES buffer (black arrows). One can see particulate structures of uneven size and form between the characteristic-looking VLPs. These unwanted contaminants are thought to be RuBisCo or other plant-derived proteins with higher-order structures. By contrast the wt PV1 VLP sample extracted using PBS buffer looks very clean and pure with almost no visible contamination (Figure 4.4-4 D, E, F). Particles are even in shape and of high abundance; however, for unknown reasons the VLPs appear larger than typical PV VLPs being approximately 30-38 nm in diameter. They appear swollen and very round without the typical icosahedral form (Figure 4.4-4 F). Furthermore, the stain did not penetrate the particles, which is a sign that their centre is occupied or the particles are impermeable to stain. When PIPES buffer was used, a pure sample with correctly sized PV VLPs of characteristic shape and appearance was obtained (white arrows Figure 4.4-4 G, H, I). The variations in purity are most likely down to the pH of the respective buffer. It influences protein extraction and can have a negative impact on the sample's clarity (Vardakou et al., 2012).

Overall the best result was achieved using PIPES buffer which had the lowest pH (6.5) of the buffers tested. With the aim of further increasing the quality of the plant-made PV VLPs, the use of a different buffer for the initial extraction was investigated. Leaves expressing wt PV1 VLPs were homogenized using P-buffer and the VLPs were then transferred into PIPES during the sucrose cushion. The P-buffer has already successfully been utilised for NNV VLP extraction. In order to remove plant-derived contaminations the sample was run through a Nycodenz gradient and then visualised by the TEM (Figure 4.4-5).

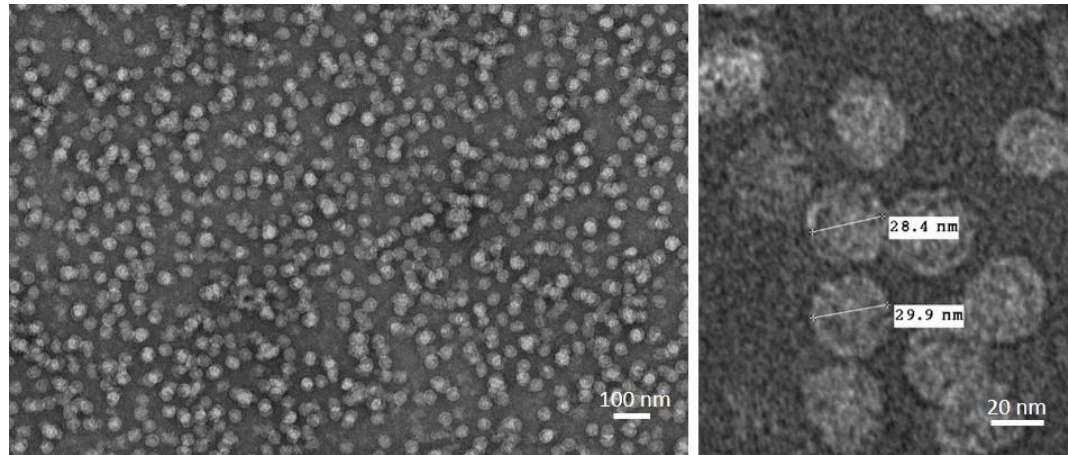


Figure 4.4-5: TEM images of wt PV1 VLPs extracted with P-buffer. VLPs were extracted using P-buffer followed by gentle transfer of the VLPs into PIPES buffer.

The above electron micrograph shows an improvement in purity of the wt PV1 VLP sample when P-buffer was used for the initial protein extraction compared to Figure 4.4-4. Almost no contamination appears in this TEM image and most importantly, the particles seem correctly shaped.

Summary of section 4.4.3: In summary the best results can be achieved when using P-buffer for the initial protein extraction but then for further purification and long term storage PIPES buffer is the best option.

4.5 EMPTY?

Encapsulation of host DNA or RNA is common in recombinantly expressed VLPs. Usually empty capsids allow stain to penetrate which makes them appear with a dark centre in TEM images. So far no consistent pattern could be observed when expressing wt PV1 VLPs. Stain penetration seems to depend on the buffer (Figure 4.4-4) but this may not be the only reason. In order to check if the plant produced PV VLPs encapsulate host nucleic acid, native agarose gel electrophoresis was carried out.

Native gel electrophoresis of virus particles or VLPs in an agarose matrix is a technique routinely used to assess the protein and RNA contents of particles. The method has already been described in section 3.5.2. In this experiment approximately 5 µg and 10 µg of purified wt PV1 particles were analysed.

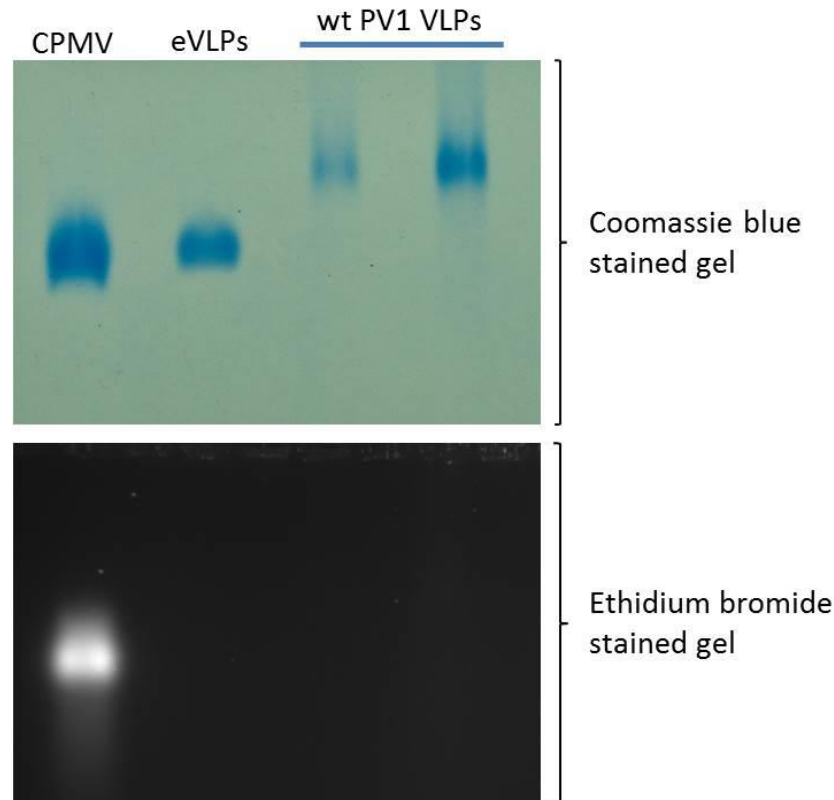


Figure 4.5-1: Native gel electrophoresis of wt PV1 VLPs to analyse encapsidation of nucleic acid. Approximately 10 μg of CPMV and eVLPs and 5-10 μg purified wt PV1 VLPs were loaded per lane. The particles were separated on 1% agarose gels and stained with Coomassie blue or ethidium bromide.

Native gel electrophoresis is an effective method to find out if the VLPs encapsulate nucleic acid during assembly. RNA containing CPMV can be stained with both ethidium bromide and Coomassie blue, whereas eVLPs do not contain any genomic material and can therefore only be stained with Coomassie blue. The same was found to be true for wt PV1 VLPs (Figure 4.5-1); the capsids stained strongly with Coomassie blue but there is no signal in the ethidium bromide stained gel under conditions which clearly revealed the RNA in the CPMV control. This indicates that the PV1 VLPs contain little or no nucleic acids.

Summary of section 4.5: The plant made wt PV1 VLPs appear free of nucleic acid.

4.6 CHARACTERISATION OF PLANT PRODUCED WT PV1, 2 AND 3

In the chapters above optimisation of expression, extraction and purification of plant-made PV VLPs has been described using wt PV1 as an example. However, the aim of this

project is to express all three PV serotypes in plants. The following sections will mainly focus on the expression and characterisation of type 2 and 3 but will also compare the differences between all three serotypes.

4.6.1 ANALYSING THE EFFECT OF WT PV1, 2, 3 EXPRESSION ON PLANTS

Figure 4.6-1 presents the effect of the expression of P1 from the three PV serotypes on plants when co-expressed with the same non-*HT* 3CD construct. Agrobacteria solutions carrying the respective P1 were prepared to an OD₆₀₀ of 0.8 and co-infiltrated with agrobacteria harbouring pEAQ-3CD with an OD₆₀₀ of 0.4. A picture was taken of the same infiltrated leaf for 10 days and compared to the control where pEAQ-*HT*-EV (empty vector) was expressed.

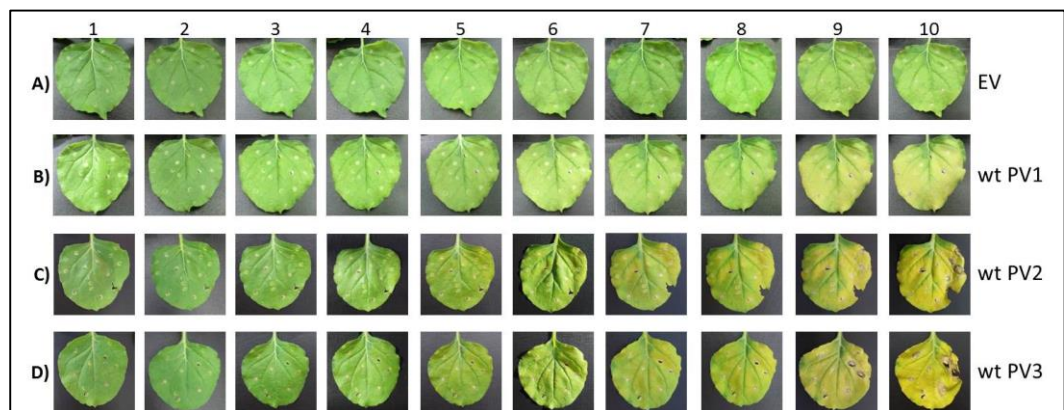


Figure 4.6-1: Effect of wt PV VLP expression over time on infiltrated *N. benthamiana* leaves. A) As a control agrobacteria harbouring pEAQ-*HT*-EV were infiltrated with an OD₆₀₀ of 0.4. Equal amounts of agrobacteria solutions containing B) pEAQ-*HT*-wt-PV1-P1 (OD₆₀₀ = 0.8) C) pEAQ-*HT*-wt-PV2-P1 (OD₆₀₀ = 0.8) D) pEAQ-*HT*-wt-PV3-P1 (OD₆₀₀ = 0.8) were mixed with pEAQ-3CD (OD₆₀₀ = 0.4) and co-infiltrated into leaves. A picture of the same infiltrated leaf was taken every day for 10 days.

Overall the assembly of pictures illustrates slight yellowing of the infiltrated leaf areas over time in all three serotypes though wt PV2 and wt PV3 show as stronger change of colour compared to wt PV1. However, also the control wears similar symptoms. Yellowing of agroinfiltrated leaves are a common sight and not to be mistaken for necrosis.

Summary of section 4.6: This section shows how the appearance of infiltrated leaves change over time when expressing wt PV1, 2 or 3 together with low levels of non-*HT* 3CD.

4.6.2 EXPRESSION OVER TIME

In order to obtain the highest possible yield of wt PV VLPs their expression over time was monitored and compared between the three serotypes. One plant each was agoinfiltrated with the respective P1 together with non-*HT* 3CD and leaf discs were sampled over a period of 10 days. The change in VP1 expression was analysed on a Western blot using anti-VP1 MAB8566 antibody which cross reacts with all three serotypes equally (Figure 4.6-2).

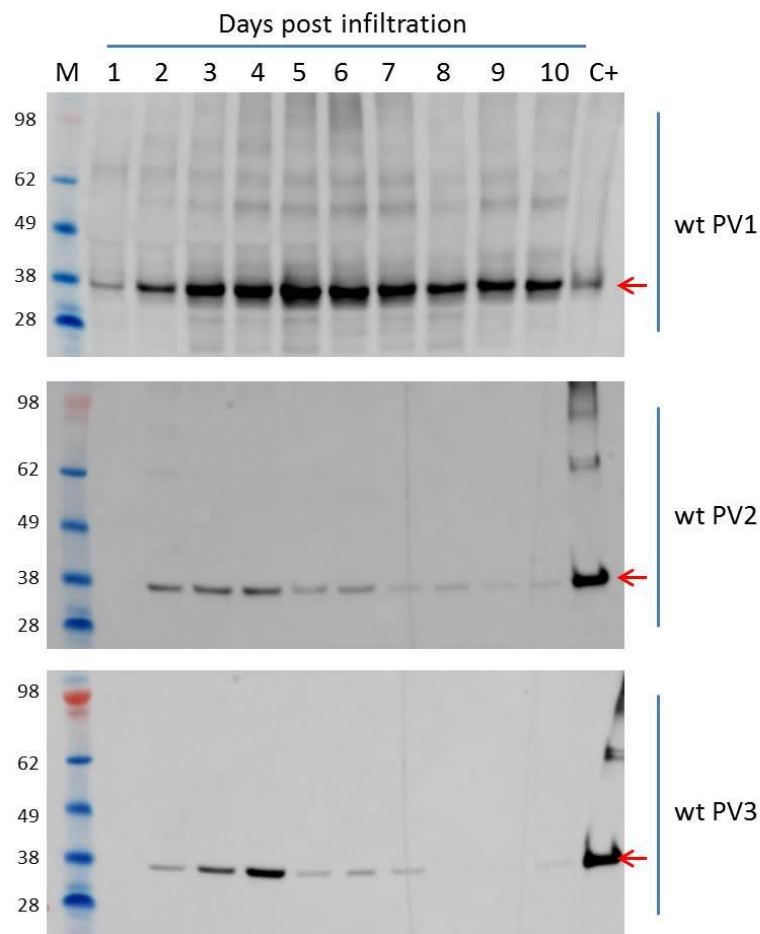


Figure 4.6-2: Expression of wt PV1, 2 and 3 VLPs over time in plants. A plant each was agoinfiltrated with the respective P1 together with non-*HT* 3CD and leaf discs were sampled for 10 days. The change in VP1 expression was analysed on a Western blot. The Western blots were probed with anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV1. Red arrows indicate position of VP1.

The expression of wt PV1 VP1 was already presented earlier but here it is used to emphasise the differences in expression between the three PV serotypes. Wt PV1 expresses very well in plants, with detectable levels of VP1 already 1 dpi. It then accumulates to the highest levels on 5-6 dpi after which it slowly decreases but even on

day 10 after infiltration VP1 is still detectable. In contrast wt PV2 shows the lowest expression levels of the three. In order to detect VP1 on the above Western blot the exposure time had to be 3-4-times longer than wt PV1 and wt PV3. The best yield of wt PV2 was achieved 4 dpi but afterwards the expression level dropped dramatically. Generally, wt PV3 expresses better than wt PV2 (confirmed in Figure 4.6-4) but it shows a similar pattern with the peak at 4 dpi followed by a striking decline in VP1.

Summary of section 4.6.2: Overall a striking difference in expression can be observed between the three PV serotypes with wt PV1 showing the highest levels of VP1 and it also accumulates for the longest period in plants. In direct contrast is wt PV2 which expresses the worst of the three, whereas wt PV3 ranges between wt PV1 and wt PV2.

4.6.3 EXPRESSING WT PV2 AND WT PV3 VLPS

As the expression of wt PV1 VLPs has been described earlier in this chapter, this section will focus on the characterisation of plant-made wt PV2 and PV3 VLPs. For this reason plants were infiltrated with either wt PV2 P1 or wt PV3 P1 together with non-*HT* 3CD and harvested 4 dpi. Because of the low expression level of these serotypes the crude plant extract was run through a sucrose gradient instead of a step gradient and each fraction was analysed for PV VLP content (Figure 4.6-3 A). The sucrose fraction containing the VLPs were combined, dialysed and then for further purification centrifuged through a Nycodenz gradient (Figure 4.6-3).

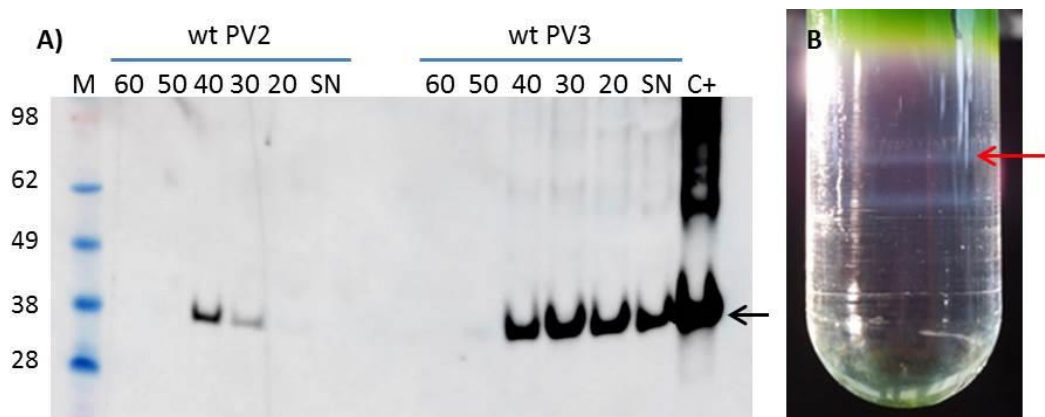


Figure 4.6-3: Purification of wt PV2 and wt PV3 VLPs. A) Plant extract harbouring either wt PV2 or wt PV3 VLPs was centrifuged through a sucrose gradient and the fractions were analysed for PV VP1 content using anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. SN= Supernatant. C+ = PV1. Black arrow indicates VP1. B) For further purification the samples were centrifuged through a Nycodenz gradient and the red arrow indicates the band containing the VLPs.

Figure 4.6-3 A shows the Western blot analysing each of the sucrose fraction for VP1 content. Most striking is the difference in VP1 expression: wt PV2 shows very small amounts compared to wt PV3. Also wt PV3 shows a signal for VP1 across many fractions and also in the SN, which indicates the presence of unassembled VP1, broken or incorrectly assembled particles. However, the strongest signal can be found in the 30% (w/v) and 40% (w/v) fraction where correctly assembled VLPs usually accumulate. The same is true for wt PV2 VLPs. Therefore, those fractions were combined and after dialysis centrifuged through a Nycodenz gradient after which two grey bands had formed as expected. The top band (indicated with a red arrow) contained the VLPs which was confirmed via immunodetection (data not shown) and visualised by TEM (Figure 4.6-4).

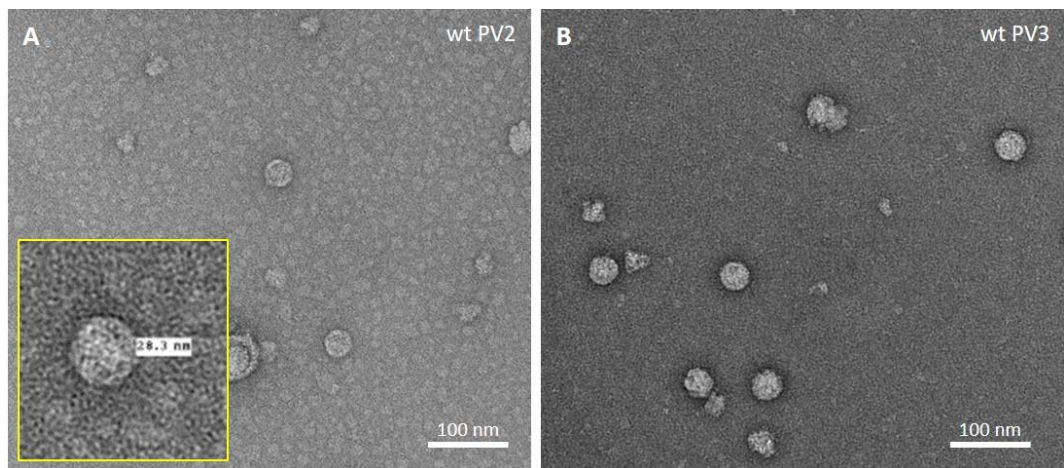


Figure 4.6-4: A) TEM image of wt PV2 VLPs. B) TEM image of wt PV3 VLPs.

On the left of Figure 4.6-4 wt PV2 VLPs are illustrated and on the right wt PV3 VLPs. In both images properly shaped and sized VLPs are visualised by TEM. Consistent with the results of the Western blot analysis (Figure 4.6-2, Figure 4.6-3) there appear to be fewer particles in the PV2 sample.

4.6.3.1 Quantitative analysis of wt PV1, 2 and 3 VLP expression

A quantitative comparison of the expression levels of wt PV1, 2 and 3 VLP obtained from plants was carried out using the Modified Lowry Assay (Thermo Fischer Scientific), following the manufacturer's instructions. The results are listed below:

- wt PV1 VLPs: ~70 mg/kg fresh weight
- wt PV2 VLPs: no data

- wt PV3 VLPs: ~60 mg/kg fresh weight

Of the three serotypes wt PV1 VLPs can be produced to the highest level in plants, with yields of up to 70 mg of purified particles/kg infiltrated plant tissue being obtained. This was closely followed by wt PV3 VLPs with yields of approximately 60 mg/kg infiltrated plant material. The analysis with wt PV2 was inaccurate presumably because the concentration was below the level of detection which reinforces the low yield to which wt PV2 accumulates in plants. The reasons behind this are not clear and one can only presume unstable mRNA or intrinsic problems leading to its degradation.

Summary of section 4.6.3: This section completes the aim of expressing all three PV serotypes in plants. Striking differences in yield were observed between the serotypes with wt PV2 showing the lowest concentration.

4.6.4 INSTABILITY OF WT PV VLPs

Figure 4.6-5 shows two electron micrographs that illustrate the problems which occurred when purifying the wt PV VLPs.

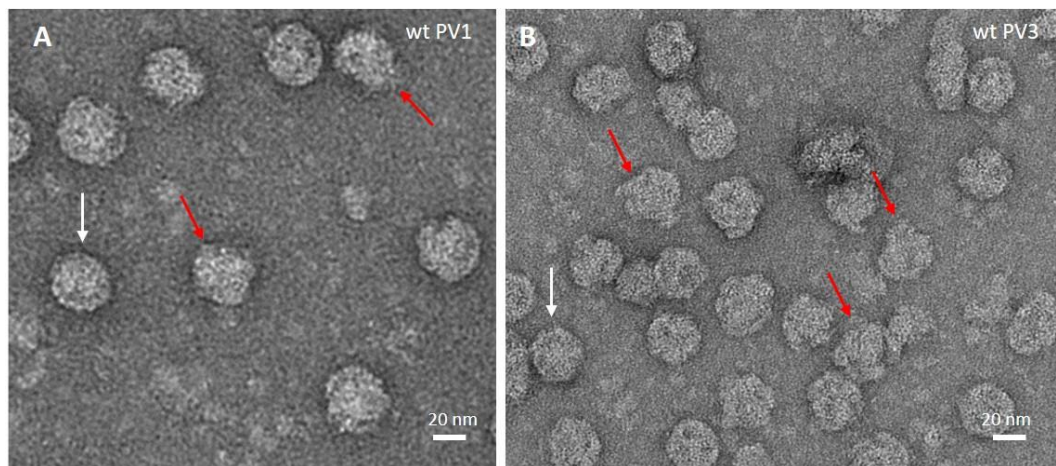


Figure 4.6-5: Example of structural instability of wt PV VLPs. A) TEM image of wt PV1 VLPs. **B)** TEM image of wt PV3 VLP. White arrows indicate correctly shaped VLPs, red arrows indicate broken, irregular particles.

In general, all three serotypes show signs of instability and when visualised by TEM as demonstrated by the presence of broken and irregularly shaped particles in preparations of plant-produced and purified wt PV1 and wt PV3 VLPs (see red arrows Figure 4.6-5). During this project no method could be developed to stabilise the wt

particles during extraction and purification. It is also possible that the VLPs deteriorate when they are still inside the plant cell cytoplasm.

These observations are in line with the findings of Guttman and Baltimore who clearly showed that particles in which VPO is uncleaved are much less stable than mature virions to a variety of conditions, including elevated temperature, detergents, high salt, and extremes of pH (Guttman and Baltimore, 1977a). Empty particles, similar to the VLPs, naturally occur during particle assembly and their outer surface and the protein shell are virtually indistinguishable compared to the virion but their role is not totally understood. Their N-terminal extension of VP1, as well as portions corresponding to VP4 and the N-terminal extension of VP2 are disordered, and many stabilising interactions that are present in the mature virion are missing (Basavappa et al., 1994). The disruption of the network eliminates extensive contacts between VP1, VP2, and VP3 within a protomer and between protomers in the pentamer (Hogle, 2002). The lack of these critical interactions may explain the decreased stability of the plant-made wt VLPs.

Two distinct antigenic forms of poliovirus exist, named D and C antigen. The D antigen is found on infectious virus whereas the C antigen is expressed on non-infectious particles. C particles do not display the antigens responsible for eliciting protective immune responses and are ineffective as vaccines. Although empty capsids are initially in the desirable D conformation they are easily converted to the C form during extraction and purification (Marongiu et al., 1981). A non-competitive sandwich ELISA assay was conducted by Helen Fox at NIBSC in order to measure the D antigen content of plant-produced wt PV VLPs type 1 and type 3 (Singer et al., 1989). Briefly, dilutions of antigen were captured with a serotype-specific polyclonal antibody, and then detected using serotype-specific, D antigen or C antigen-specific monoclonal antibodies followed by anti-mouse peroxidase conjugate. As a reference the European Pharmacopeia Standard for IPV was applied, which is a mixture of all three types (formulation for type 1, 2 and 3 is 320, 67 and 282 DAgU (Antigen Units) per ml respectively).

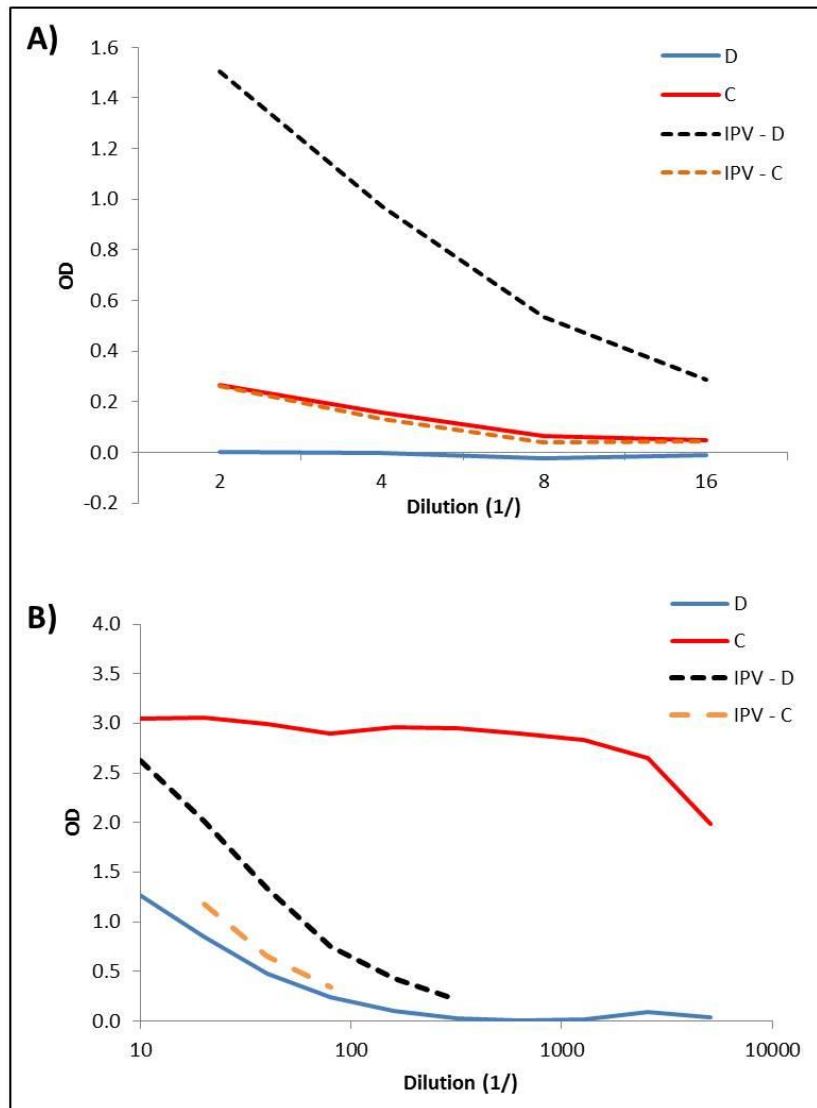


Figure 4.6-6: Non-competitive sandwich ELISA assay for the measurement of D antigen content
A) wt PV1 VLPs analysed for D/C antigen content using Mab 234 (D antigen) and Mab 1588 (C antigen). **B)** wt PV3 VLPs analysed for D/C content using MAb 520 (D antigen) and MAb 517 (C antigen). IPV = European Pharmacopeia Standard for IPV.

Part A of Figure 4.6-6 shows that no D antigen could be detected in the preparation of plant-made wt PV1 VLPs; only C antigen was identified. In Figure 4.6-6 B very high levels of C antigen were found in this sample of wt PV3 VLPs whereas the amount of D antigen is negligible. This confirms previous studies where PV VLPs were expressed in a different system (*Saccharomyces cerevisiae*) but also showed C antigenicity (Rombaut et al., 1994), meaning that they underwent the conformational change and are therefore unlikely to induce neutralizing and protective antibodies.

This was verified by Helen Fox at NIBSC who tested the ability of plant-produced wt PV3 VLPs to elicit the production of neutralising antibodies in rats. For immunogenicity

experiments in rats the European Pharmacopeia standard test for IPV batch release was followed. This involves immunising each rat intramuscular twice with 0.25 ml of the VLP sample. There are 4 serial dilutions, starting with the equivalent of a single human dose (here 32 DAgU for type 3) and 10 rats in each group. After 21 days the rats were bled, the serum collected and tested using a standard neutralising antibody assay for IPV batch release (European Pharmacopeia standard test). The serum sample from each rat was plated out in duplicate and titrated down the plate. The titrated serum samples were incubated with 100 TCID₅₀ of the appropriate serotype of Sabin virus per well for 3 hours. HEP2C (HeLa derivative) cells were added to the wells and incubated at 35 °C for 6 days. The endpoint dilution constitutes the neutralising antibody titre and is calculated using the Spearman Karber method. Across a group of 10 rats, the average geomean titre was obtained from the individual neutralisation titres. As a reference the European Pharmacopeia Standard for IPV was applied, which is a mixture of all three types (formulation for Type 1, 2 and 3 is 320, 67 and 282 DAgU/ml respectively).

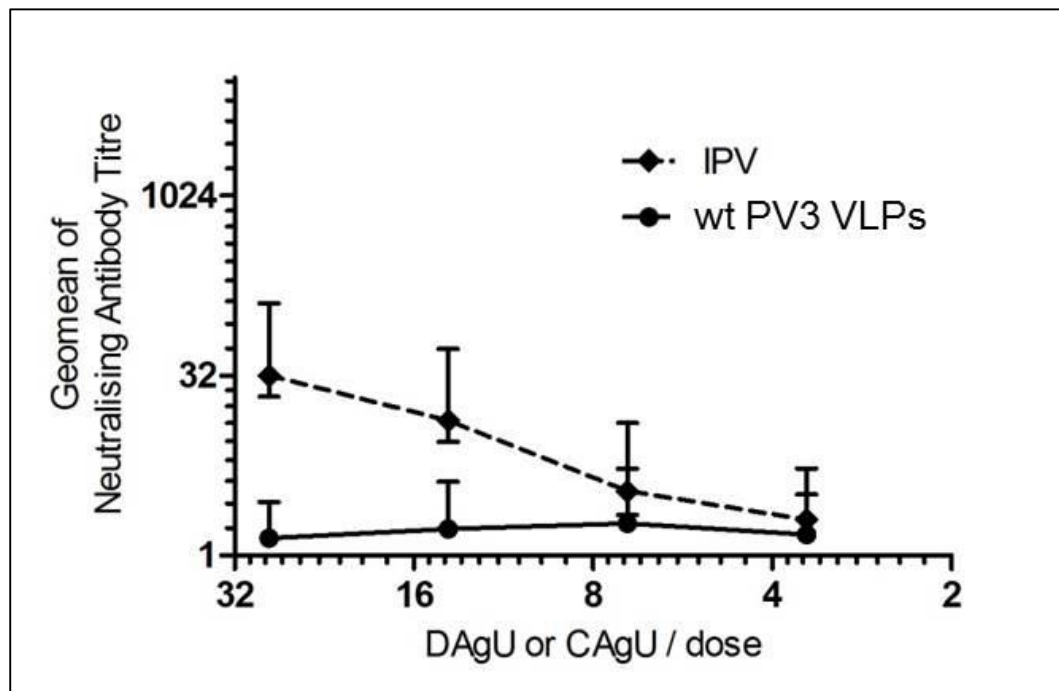


Figure 4.6-7: Neutralising antibody titre in rats after immunisation with wt PV3 VLPs. Each rat was immunised twice with wt PV3 VLPs. In total the sample was tested in 40 rats and the same number of rats was used for the control material IPV. After 21 days the rats were bled, the serum collected and tested using a standard neutralising antibody assay for IPV batch release (European Pharmacopeia standard test). IPV = European Pharmacopeia Standard for IPV.

They estimated the CAgU to an equivalent dose of DAgU for immunisation in rats but the immunogenicity was negligible compared to IPV.

Summary of section 4.6.4: Many of the plant-produced wt PV VLPs are irregular and not of icosahedral shape; they appear broken or incorrectly assembled. Empty PV particles are known to be labile due to the lack of critical interactions between the capsid proteins. When analysing the antigenicity, wt PV1 and wt PV3 VLPs show negligible to no D antigenicity but strong C antigenicity, confirming their structural instability. Wt PV3 VLPs do not induce neutralising antibodies in rats.

4.6.5 DO PV VLPs ENCAPSULATE REPLICATING RNA?

Instability of empty PV capsids is a well-known issue (Basavappa et al., 1994; Guttman and Baltimore, 1977b; Marongiu et al., 1981). The lack of critical internal interactions between the capsid proteins is most likely the main reason for the lability of the plant-made wt VLPs. Encapsulated genomic material can act as a stabilising force which VLPs lack (Da Poian et al., 1994). With the aim of increasing the stability of the PV VLPs their ability to take up foreign, harmless genomic material was investigated. This would also help to gain insight into the encapsulation procedure. Little is known about the genome encapsulation in PV and the process has been the subject of some controversy. Two possible models have been proposed: pentamers condense around the viral RNA during particle assembly to form a provirion. The alternate model proposes that pentamers first form an empty capsid, into which the viral RNA is inserted to yield the provirion; however more evidence supports the first model (Jiang et al., 2014; Putnak and Phillips, 1981; Verlinden et al., 2000).

So far the results of this work suggest that PV VLPs do not encapsidate plant RNA or DNA during assembly (Figure 4.5-1). However, the idea was to use replicating RNA from a distantly related virus to investigate if active replication might lead to encapsidation because encapsidation and replication is believed to be linked in PV (Saxena and Lomonosoff, 2014). For this purpose, CPMV was chosen; an extensively studied plant virus with a ss (+) RNA genome of two segments which is a member of the same order (the *Picornavirales*) as poliovirus. RNA-1 encodes the virus replication machinery and RNA-2 encodes two capsid proteins and the viral movement protein (Sainsbury et al., 2010a). RNA-1 is capable of replicating independently (Goldbach et al., 1980). Therefore, by deploying only RNA-1, replication takes place without the formation of CPMV particles. This was exploited in this experiment, where the constructs shown in Figure

4.6-8 were co-expressed in *N. benthamiana* with the aim of producing wt PV VLPs that encapsulate CPMV RNA-1 and which would thereby be stabilised:

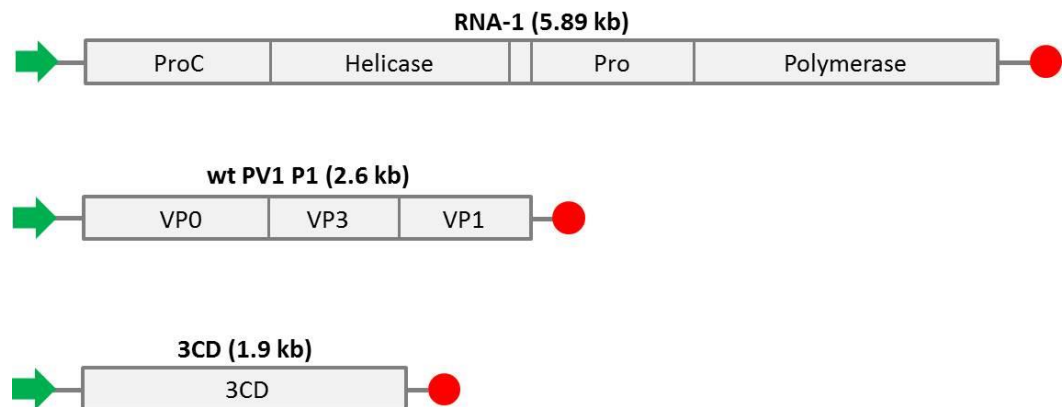


Figure 4.6-8: Schematic diagrams of constructs expressing RNA-1, wt PV1 P1 and 3CD. Relevant sections of pEAQ-HT-RNA-1, pEAQ-HT-wt-PV1-P1 and pEAQ-3CD are illustrated. RNA-1 encodes a 32 kDa proteinase co-factor (ProC); a 58 kDa helicase; a 2 kDa genome-linked viral protein (VPg); a 24 kDa viral proteinase (Pro) and a 87 kDa RNA-dependent RNA polymerase. The green arrow and red circle represent the CaMV 35S promoter and nos terminator respectively.

As a control VP60, which contains the large (L) and small (S) coat protein of CPMV, was co-expressed with RNA-1 to provide a positive control for encapsidation (Figure 4.6-9):

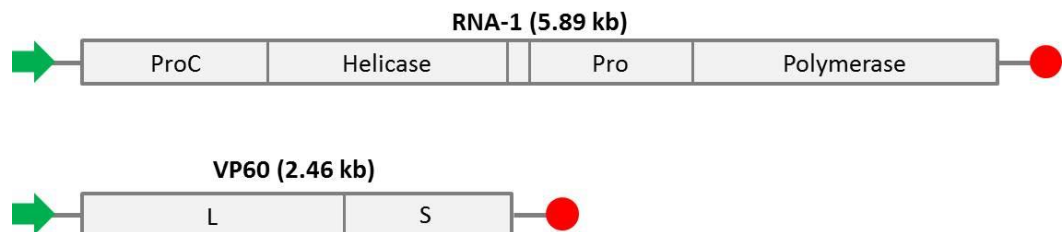


Figure 4.6-9: Schematic diagram of constructs expressed as control to demonstrate encapsulation of RNA-1. VP60 encodes the L and S coat proteins. The green arrow and red circle represent the CaMV 35S promoter and nos terminator respectively.

Furthermore, wt PV1 VLPs were expressed in the absence of CPMV RNA-1 as described earlier in this chapter as a negative control. The plants were harvested 6 dpi and purified over a sucrose cushion. In order to accommodate the needs of CPMV and PV, PBS buffer was used in this experiment. After dialysis the samples were visualised by TEM in order to assess particle appearance. The images are presented in Figure 4.6-10.

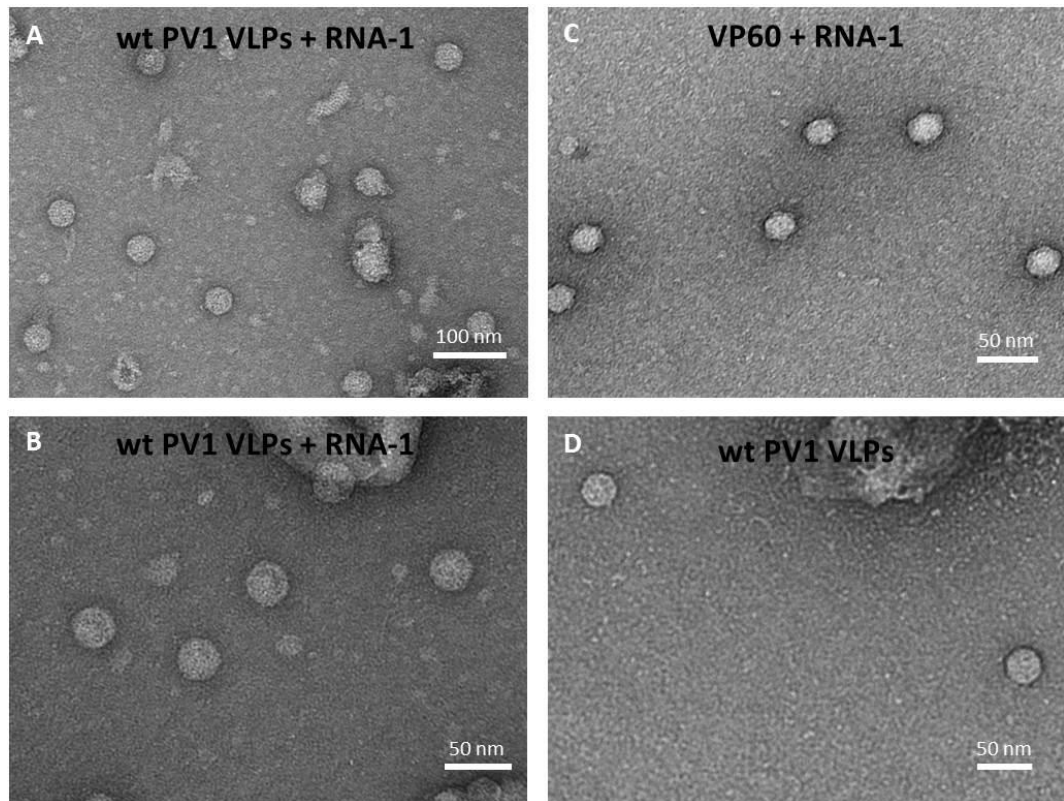


Figure 4.6-10: Monitoring particle assembly and appearance when co-expressing RNA-1 and wt PV1 VLPs. A), B) TEM image of wt PV1 VLPs co-expressed in plants with replicating CPMV RNA-1. C) Assembled CPMV particles visualised by TEM when VP60 was co-expressed (containing the L + S coat protein) with RNA-1. D) TEM image of standard wt PV1 VLPs.

Co-expressing RNA-1 with wt PV1 results in very evenly shaped particles with a slightly larger diameter than usual (Figure 4.6-10 A, B), but this could be explained by the buffer they are in (PBS) as already observed and described earlier in this chapter. However, the standard wt PV1 VLPs are of correct size (Figure 4.6-10 D) and in the same buffer. CPMV particles produced from co-expressing VP60 (L + S coat protein) are depicted in Figure 4.6-10 C. The TEM image show authentic particles of 28-30 nm in diameter. All particles in Figure 4.6-10 appear full because no stain has entered the capsid. Though, it has already been proven that wt PV VLPs do not always get penetrated by the negative stain even though they do not contain nucleic acid. Most CPMV particles are expected to show a full core as they will have encapsulated the co-expressed RNA-1.

From all samples RNA was extracted and analysed by NanoDrop spectrometer. As an additional control a sample of CPMV eVLPs was analysed. After adjusting the particle concentration, the same protocol for RNA extraction was followed, even though eVLPs do not contain RNA. The RNA was dissolved in exactly the same volume of sterile water and in the following table the RNA concentration of each sample is listed:

Table 4.6.1: RNA concentration of purified particles. RNA was extracted from purified particles and the concentration measured using the NanoDrop spectrophotometer.

Sample	RNA concentration
VP60 + RNA-1	173.4 ng/ μ l
Wt PV1 VLPs + RNA-1	118.7 ng/ μ l
Wt PV1 VLPs	39.1 ng/ μ l
eVLPs	55.0 ng/ μ l

Table 4.6.1 the measured RNA concentrations of all samples are listed and the highest amount of RNA was detected in the CPMV sample (VP60 + RNA-1). This was expected as CPMV is known to encapsidate its cognate RNA and thus serves as a positive control. The negative control in this experiment is the eVLP sample; as the name suggests eVLPs are empty and do not contain RNA. However, the NanoDrop apparently measured 55 ng/ μ l of RNA. This can be explained by the presence of impurities in the sample like phenol residues which can affect the reading of the machine. Interestingly PV VLPs that have been co-expressed in the presence of replicating RNA-1 show a higher RNA content than those produced in its absence. All together these results hint at the possible encapsulation of replicating CPMV RNA-1 by co-expressed wt PV1 VLPs.

To definitely demonstrate RNA-1 encapsidation by PV capsids it would be necessary to demonstrate the presence of full-length RNA-1 in RNA extracted from wt PV1 VLPs + RNA-1 by agarose gel electrophoresis. Unfortunately, all attempts at demonstrating this failed. Several reasons, such as low RNA concentration and RNA degradation, may account for this. However, the results obtained were tantalising and demand further investigation. Larger scale infiltrations are needed to produce more material for further experiments. In order to get a more definitive result it is worth considering northern blot or reverse-transcription (RT)-PCR analysis.

Summary of section 4.6.5: The preliminary data presented in this chapter suggest that wt PV1 VLPs may encapsidate replicating RNA-1 of CPMV when co-expressed in plants. However, the final proof is still to be made.

4.7 DISCUSSION

PV VLPs have been produced in various recombinant expression systems (e.g. vaccinia virus, baculovirus, *Saccharomyces cerevisiae*) (Ansardi, 1991; Brautigam et al., 1993; Jore et al., 1994), however, this work reports the first successful expression of wt PV 1, 2 and 3 VLPs in plants.

This chapter presents experiments that have been undertaken to optimise expression and purification of PV VLPs. For the synthesis of PV VLPs not only the polyprotein P1, which contains the sequences of the coat proteins, but also the proteinase 3CD are necessary; without the cleavage of P1 by the proteinase the mature capsid proteins do not form and VLPs do not assemble (Jore et al., 1988). Protein analyses showed that when only the P1 region is expressed, an antibody specific for VP1 reacts with a ~96 kDa product that is presumably the precursor to the capsid proteins. No evidence was obtained for the presence of mature VP1 in these extracts, indicating that, as predicted from other published reports, the poliovirus protease is required for capsid production (Hanecak et al., 1982; Toyoda et al., 1986). When co-expressing P1 and 3CD only mature VP1, and not the precursor molecule P1, was detected indicating that processing is highly efficient in plants. A very important step was reducing the level of 3CD expression as it causes severe necrosis in the infiltrated plant tissue. Reversal of the HT-mutation (AUG 161) on the 3CD construct and adjusting the P1 to 3CD ratio, the infiltrated tissue suffered less from necrosis leading to higher yields of PV VLPs.

Reversal of the HT-mutation (AUG 161) on the 3CD construct and adjustment of the P1 to 3CD ratio, resulted in the infiltrated tissue suffering less necrosis which, in turn led to higher yields of PV VLPs. The reduced 3CD levels proved to be adequate to achieve 100% processing of P1 and thus this lower level expression method was adopted for all subsequent work.

The first samples of PV VLPs contained large amounts of plant contaminants. Alternative extraction and purification techniques were therefore investigated to improve the purity

and concentration of the VLPs. Here, most beneficial proved to be increasing the duration of the Nycodenz ultracentrifugation step; longer spins separated the VLPs from plant contaminants with very similar densities. Furthermore, the effect of different buffers on the sample purity was tested. A combination of P-buffer for extraction and PIPES for the following purification steps had the most favourable outcome. However, more time could be invested in trying a buffer with an even lower pH, as PV naturally is acid stable and a lower pH could facilitate removal of more plant contaminants.

It was noted that in TEM images the wt PV VLPs looked full, which means their core does not allow stain penetration. This could indicate the presence of nucleic acid within the VLPs. However, analysis on non-denaturing agarose gels showed no evidence of nucleic acid within the particles as judged by ethidium bromide staining. This result is similar to that found with CPMV, a plant-infecting member of the order *Picornavirales* and may reflect similarity in the genome packaging mechanisms.

High yields of PV VLPs were achieved with approximately 70 mg/kg infiltrated leaf tissue for wt PV1. However, this could probably be even further improved with specific codon-optimisation or improvements to the protocol for extraction and purification. In comparison to other systems that successfully express PV VLPs the yield of ~70 mg/kg fresh weight is very good. In yeast for example, a yield of 0.1-1 mg of particles was obtained from 1 l of yeast culture (Jore et al., 1994).

The results also revealed differences in yield between the serotypes, especially type 2 proved difficult to be expressed in plants to the same concentration as the other two serotypes. Reasons behind these observations are only assumptions as generally the serotypes are structurally very similar. However, no known cases of wt PV2 have been reported since 1999 and therefore type 2 was declared eradicated by the WHO in September 2015. Intrinsic problems within the type 2 mRNA might lead to its fast degradation of the host cell defence mechanisms.

In the TEM images it became apparent that the plant-produced wt PV VLPs are unstable and undergo distortion and/or degradation during purification. Many broken particles are visible in the images even though the purification process was kept as gentle as possible. More importantly the antigenicity of the plant-produced wt PV1 and 3 VLPs was almost entirely of the C conformation. The same is true for PV VLPs generated in *Saccharomyces cerevisiae*, which were also shown to be C antigenic (Rombaut and Jore,

1997), implying that these particles are denatured. Empty capsids are known to be very unstable and that they readily switch antigenicity from the native form (D) to the non-native or heated form (C) (Basavappa et al., 1994). Thus, it came as no surprise that the plant-made VLPs could not induce neutralizing antibodies when tested in rats (Helen Fox, NIBSC). Again, the yeast-produced VLPs showed the same result. With the aim of stabilising their VLPs Rombaut and colleagues added pocket-binding compounds to the sample (Rombaut and Jore, 1997). The binding of `pocket factors` to the canyon in the virus capsid has been shown to positively influence stability of the virions and empty capsids (Grant et al., 1994; Rombaut et al., 1991). When Rombaut et al. added the compound prodavir they obtained D antigenic empty capsids which have the same immunogenicity as poliovirus virions (Rombaut and Jore, 1997). However, the use of such compounds is incompatible with commercial vaccine production (Katpally and Smith, 2007; Kelly et al., 2015). PV VLPs expressed in the baculovirus system induced only a very weak neutralizing response in mice (Urakawa, 1989); no data on the immunological response of the empty capsids isolated from the vaccinia virus expression system are available (Ansardi, 1991).

With the aim to stabilise the plant-made PV capsids and at the same time shed light into the encapsulation of nucleic acid it was investigated if replicating RNA would be taken up by the assembling PV VLPs. Very little is known about how *Picornavirales* encapsulate their genome and how they are able to distinguish foreign nucleic acid from their own genomic material. In this chapter I already discussed that plant-produced PV VLPs do not contain random host nucleic acid but potentially a replicating RNA from a related virus would be taken up. Hence CPMV was chosen; a plant virus with structural and genetic similarities with picornaviruses (Franssen et al., 1984). When only RNA-1 from its bipartite genome is deployed, replication takes place without the formation of CPMV particles (Goldbach et al., 1980; Van Bokhoven et al., 1993). Using a replicating RNA molecule was the key in this experiment as there is believed to be a linkage of encapsidation and replication for PV (Saxena and Lomonossoff, 2014). Preliminary data suggest that the RNA content of VLPs increases when VLPs are co-expressed with replicating RNA-1. Certainly, this experiment needs more thorough enquiry using more sensitive techniques to clarify if the RNA isolated from the PV VLPs actually is CPMV's RNA-1.

Overall this chapter demonstrates the first report of plant-made PV VLPs. However, these wt PV VLPs are labile and readily undergo the conformational change from D to C antigenicity, rendering them unsuitable as vaccines. Finding a way to improve their stability and to stop the conformational change from taking place without impacting their immunogenicity would create an inexpensive and safe vaccine candidate for the eradication and also post-eradication era. In the next chapter of this thesis I will introduce stabilised mutants developed by our collaborators at NIBSC and the University of Leeds that have increased thermostability and express to high levels in plants.

Chapter 5: STABILISED PV VLP MUTANTS

5.1 INTRODUCTION

The previous chapter describes the stability issues encountered with wt PV VLPs transiently expressed in plants. The purified VLPs do not induce neutralising antibodies when tested in animals, making them inappropriate as vaccines. The overall aim of the poliovirus work reported in this thesis was the generation of non-infectious PV VLPs that have the capability of eliciting a protective immune response against polio. Together with the WHO-funded consortium we developed stabilised mutant versions of the poliovirus gene P1, which keep the correct antigenicity during expression and purification. We showed that plant-based transient expression is an efficient method of producing these stabilised capsids. This work was built up on capsid stabilisation experiments on foot-and-mouth disease virus (FMDV) (Porta et al., 2013a; Porta et al., 2013b). In this chapter I describe the expression of the stabilised mutants in plants, analyse their characteristics and show their properties and potential.

As already mentioned in the earlier chapter, two distinct antigenic conformations of poliovirus exist, D and C, (Hummeler and Hamparian, 1958; Le Bouvier, 1955; Mayer et al., 1957) where D is the required conformation for the VLPs if they are to elicit an appropriate immune response. The virus undergoes the conformational change during cell infection, ending up as non-infectious C particles (see Figure 4.1-2) (Le Bouvier, 1955; Schwerdt and Fogh, 1957). C particles do not display the antigens responsible for eliciting protective immune responses and are therefore ineffective as vaccines. The D to C change is induced by binding the receptor CD155 where the N-terminal extension of VP1 and the myristoylated VP4 get externalised proposedly to anchor the virus in the cell membrane to facilitate the transport of viral components into the host cytoplasm (Danthi et al., 2003; De Sena and Mandel, 1977; Fricks and Hogle, 1990; Ren et al., 2013; Tosteson and Chow, 1997; Tosteson et al., 2004); an irreversible restructuring (Hogle, 2002).

Particles, like VLPs, with uncleaved VO are less stable than mature virions (Guttman and Baltimore, 1977a) and readily undergo the switch from D to C. They are also more

sensitive to elevated temperature, detergents, high salt and extremes of pH (Guttman and Baltimore, 1977a). Infectious PV has a complex internal protein network formed by VP4 and the N-terminal extensions of the other capsid proteins (Basavappa et al., 1994; Hogle, 2002). The failure of VLPs to form such a network interferes with critical internal interactions and may explain their decreased stability. Another element influences stability of PV capsids: the pocket factor (Grant et al., 1994).

On the surface of PV a prominent depression circumscribes the star-shaped mesa at the fivefold axes known as the canyon (see Figure 1.5-1). It is the site of receptor attachment (Belnap et al., 2000b; He et al., 2000). At the bottom of the canyon is an opening into a hydrophobic pocket between the two beta sheets of the VP1 capsid protein where the pocket factor binds (Filman et al., 1989). The pocket factor has been modelled as fatty acids (e.g. sphingosine, palmitate) based on the hydrophobic environment in the pocket and crystallographic studies (Hogle, 2002). The binding sites of the pocket factor and the CD155 receptor overlap and therefore it has been suggested that the pocket factor stabilises the capsid until a viable receptor competes for the binding site. Then the pocket factor is released which destabilises the virus and initiates uncoating (Grant et al., 1994; Rossmann, 1994; Rossmann et al., 2002). When antiviral drugs bind the pocket factor the PV particle becomes non-infectious (Rossmann, 1989). The reason behind this is thought to be overly strong stabilisation of the capsids which makes them resistant to uncoating. The binding of such drugs has also been shown to stabilise PV capsids against transitions from the native (D antigenic) state to the heated (C antigenic) state, changes that are required for productive cell entry (Grant et al., 1994; Hiremath et al., 1995; Rombaut et al., 1991). Hence, the presence of a ligand in the pocket plays a critical role in regulating particle stability. One pocket factor analogue which has been shown to bind polioviruses is termed GPP3 (De Colibus et al., 2015).

There are two differing approaches that could be used to produce stabilised VLPs: the use of structural information to identify mutations that could theoretically stabilised capsids, an approach used successfully with FMDV (Porta et al., 2013a) or the use of “forced evolution” to select for stabilising mutations by propagating the virus under restrictive conditions. Poliovirus, as well as other RNA viruses, is prone to mutations due to the non-proofreading RNA-dependent RNA polymerase (RdRp), which allows rapid evolution under selection pressure (Elde, 2012; Karakasiliotis et al., 2005). This property

can be exploited as a means of forced evolution to select for thermostable capsid mutants. Using both these approaches, our collaborators at the University of Leeds have attempted to generate stabilised empty PV capsids of PV1, while our collaborators at NIBSC worked on producing and identifying stabilised PV1, PV2 and PV3 empty capsids by forced evolution.

5.2 STABILISED PV1 VLPS

5.2.1 SELECTION AND CHARACTERISATION OF PV1 Q94C

As a first attempt in generating stabilised PV1 VLPs, our collaborators at the University of Leeds purposely changed amino acids in the capsid protein VP2 aiming towards stabilising the pocket. This was based on the work of Porta et al. who engineered a disulfide bond by mutating a single histidine residue at position 93 of VP2 located at the icosahedral 2-fold axis between adjacent pentamers in FMDV (Porta et al., 2013a). A number of mutant viruses have been constructed with substitutions designed to introduce side chains into the VP1 pocket – the location of the pocket factor present in infectious wt PV and the binding site of a number of compounds which increase the thermal stability of the particles. Some of the mutations prevented particle assembly but a single mutation allowed the formation of D antigenic empty PV1 particles with apparent enhanced stability.

In order to exchange Glutamine at position 94 with Cysteine, plasmid pEAQ-*HT*-wt-PV1-P1 was subjected to SDM using primer pair PV1 Q94C_F and PV1 Q94C_R (listed in Appendix 1) generating pEAQ-*HT*-PV1 Q94C. After verification of the incorporation of the mutation, pEAQ-*HT*-PV1 Q94C was co-expressed with 3CD in plants. Particles were purified following the standard protocol of a sucrose cushion followed by a Nycodenz gradient. The results are illustrated in Figure 5.2-1.

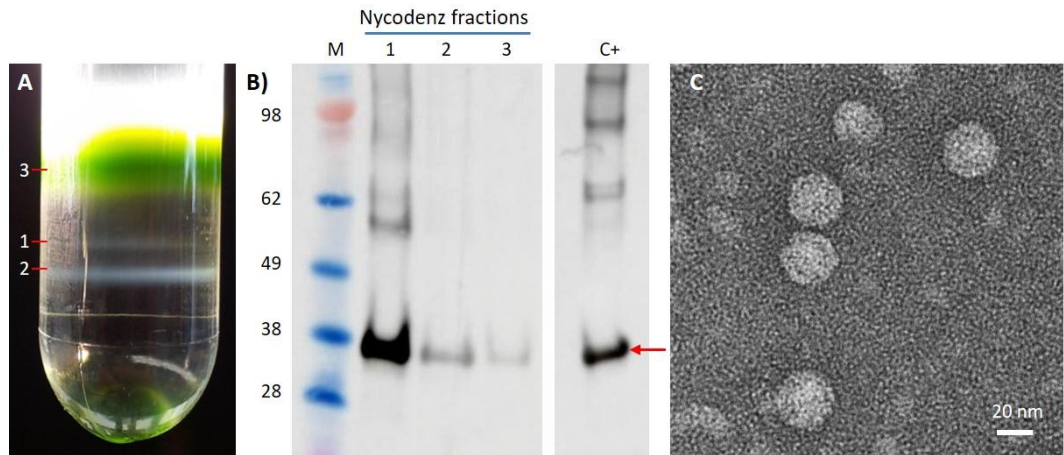


Figure 5.2-1: Purification of PV1 Q94C VLPs. A) The sample was purified through a Nycodenz gradient. Three bands formed within the gradient. B) The Nycodenz fractions were analysed on a Western blot for VP1 content using anti-VP1 MAB8566 antibody. Red arrow indicates VP1. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV3 SktSC8 VLPs. C) TEM picture of PV1 Q94C VLPs.

Expression and purification was simple because of the high expression level of this mutant. Figure 5.2-1 A shows the Nycodenz gradient which was used to purify the sample. Three bands formed within the gradient and band 1 was verified to contain the VLPs (Figure 5.2-1 B) which were visualised by TEM. The sample was sent to our collaborators at NIBSC who tested the VLPs for D/C antigen content using Mab 234 (D antigen) and Mab 1588 (C antigen) in a non-competitive sandwich ELISA (Singer et al., 1989). Briefly, two-fold dilutions of antigen were captured with a serotype-specific polyclonal antibody, then detected using serotype-specific, D antigen (Mab 234) or C antigen (Mab 1588) specific monoclonal antibodies followed by anti-mouse peroxidase conjugate.

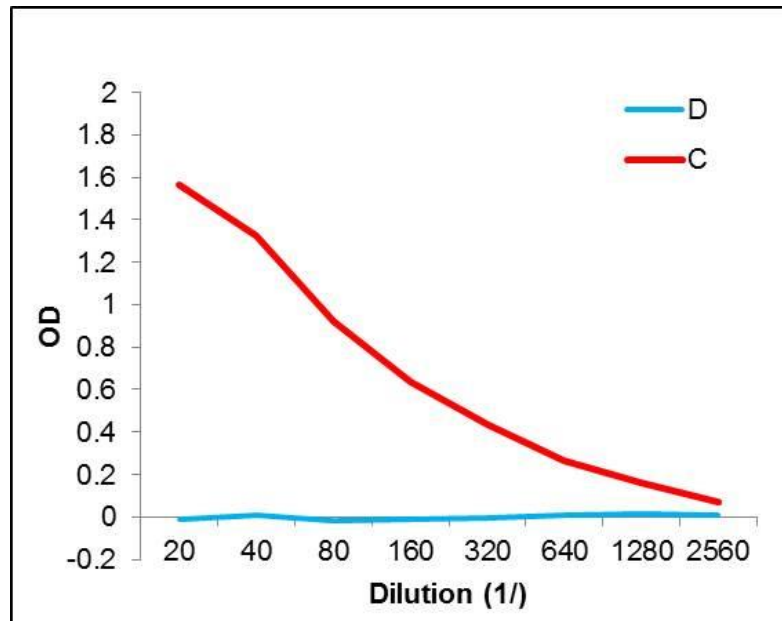


Figure 5.2-2: Potency of PV1 Q94C VLPs. PV1 Q94C analysed for D/C antigen content using Mab 234 (D antigen) and Mab 1588 (C antigen).

Unfortunately, the VLPs revealed only C antigenicity. At this point other data collected by my collaborators from thermostability experiments supported the evidence that the designed mutant PV1 Q94C does not fulfil the expected stability.

5.2.2 SELECTION OF THERMALLY-STABLE PV1 M2DELTA AND PV1 M3DELTA

Because the designed mutant PV1 Q94C did not appear to have enhanced stability, Oluwapelumi Adeyemi of the University of Leeds selected thermally-stable empty capsids of PV1. He identified mutations on the capsid surface and internal networks that are responsible for the stability (submitted for publication Adeyemi et al., 2016). His strategy is briefly described in the following paragraph.

Virus derived from an infectious cDNA clone (Mahoney strain) was passaged through rounds of thermal selection at increasing temperatures of 51 °C, 53 °C and 57 °C until there was a severe fitness cost. To identify the capsid mutations in thermally-selected virus pools, genomic RNA was extracted and the P1 coding region amplified by reverse transcription PCR and purified amplicons sequenced. Nucleotide sequence alignment of the thermally-selected viruses against wt PV-1 (Mahoney) reference sequence (NCBI GenBank locus V01149.1) revealed induced mutations. The wt PV1 empty capsids do not retain D antigenicity above 10 °C (Basavappa et al., 1994), however, the thermally-selected viruses were shown to have D antigenic empty capsids, with the switch to C

antigenicity occurring at 37 °C or higher. Together, these data demonstrate that both full virions and empty capsids of the thermally-selected viruses had an increased native antigenic tolerance for elevated temperatures. Two mutants were selected based on their performance in antigenic and particle stability tests, PV1 M2delta and PV1 M3delta, for recombinant expression in plants.

When aligned against wt PV1 (Mahoney), three changes could be detected in the P1 sequence PV1 M2delta. They are specified in Table 5.2.1.

Table 5.2.1: PV1 M2delta mutations and their location.

Mutation	Location
V1087A	B strand of the VP1 β -barrel; outer surface of the virus pocket
A1026T	Internal surface of the virus capsid; close to the N-terminus of VP1
F4046L	VPO

PV1 M3delta was aligned against wt PV1 (Mahoney) and five mutations were detected. They are listed in Table 5.2.2.

Table 5.2.2: PV1 M3delta mutations their location.

Mutation	Location
V1087A	B strand of the VP1 β -barrel; outer surface of the virus pocket
S1097P	Resides on B-C loop of the VP1 β -barrel
C3175A	Capsid surface residue, located between monomers within a pentamer
R4034S	VPO
D4045V	VPO

The following section characterises these stabilised mutants when expressed as VLPs in plants.

5.2.2.1 *Expression of PV1 M2delta and PV1 M3delta in plants*

The sequences for PV1 M2delta-P1 and M3delta-P1 were codon-optimised for the expression in *N. benthamiana* and cloned into pEAQ-HT via restriction digest. Plants were infiltrated with pEAQ-HT-PV1 M2delta-P1 or M3delta-P1 plus pEAQ-3CD and harvested 6 dpi. Due to time limitations only a sucrose cushion was carried out without further purification steps.

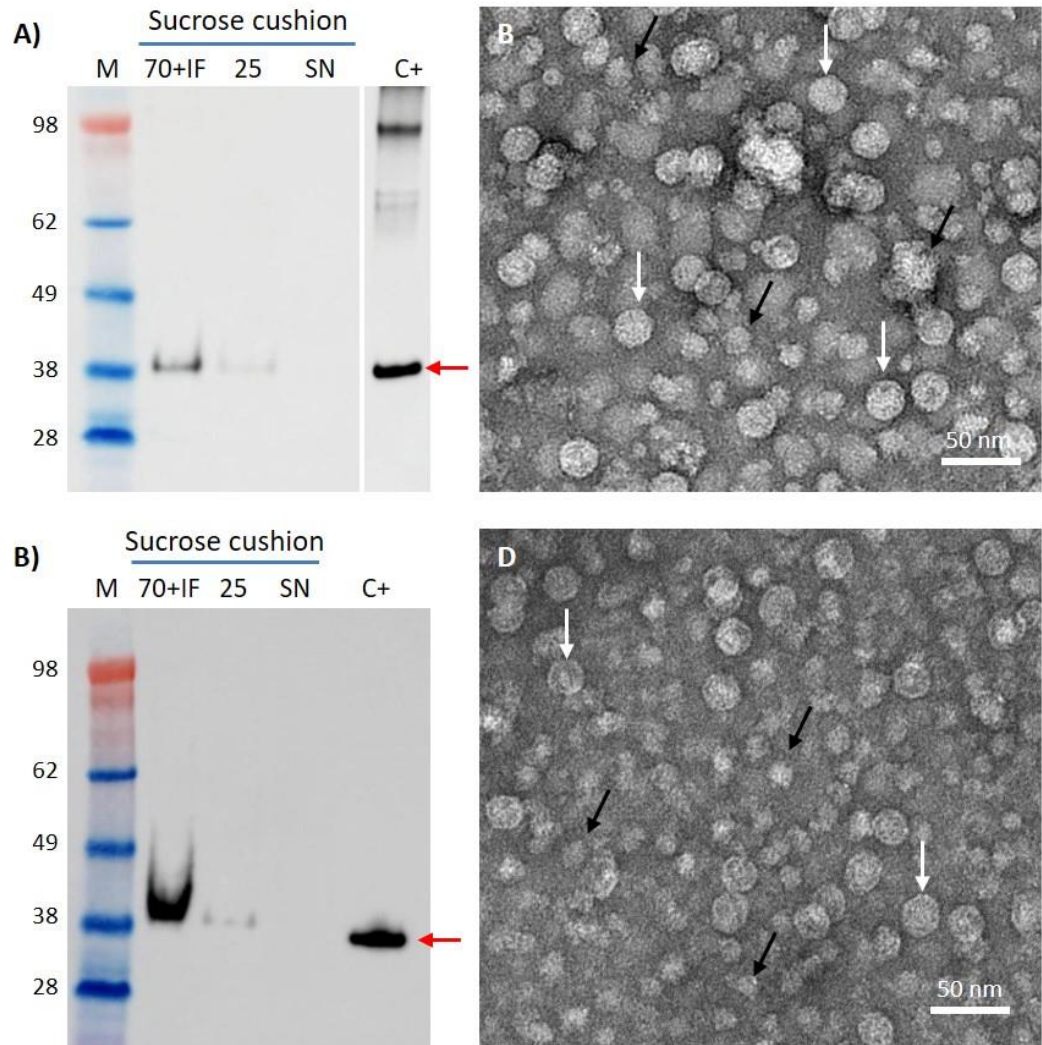


Figure 5.2-3: PV1 M2delta VLPs and PV1 M3delta VLPs roughly purified through a sucrose cushion. **A)** Sucrose fractions analysed for VP1 content on a Western blot with anti-VP1 MAB8566 antibody. **B)** TEM image of PV1M2delta VLPs. **C)** Sucrose fractions analysed for VP1 content via immunodetection on a Western blot using anti-VP1 MAB8566 antibody. **D)** TEM image of PV1M3delta VLPs. M = SeeBlue Plus 2 with molecular weights indicated. SN = Supernatant. C+ = PV3 SktSC8 VLPs. Red arrows indicate VP1. White arrows indicate VLPs and black arrows show contamination.

The 70% (w/v) sucrose fraction together with the interface (IF) contained the capsid protein VP1 as confirmed on the Western blots in Figure 5.2-3 A, C using the anti-VP1 MAB8566 antibody. After dialysis the samples were visualised by TEM (Figure 5.2-3 B, D). As expected both samples were still heavily contaminated with plant cell debris however; they also contained large amounts of correctly sized and apparently properly assembled VLPs.

For stability analyses both samples were sent to our collaborator at the University of Leeds who generated the mutants. The following results are preliminary data which

were gathered by Oluwapelumi Adeyemi at the University of Leeds and are presented here with his permission.

5.2.2.2 Thermostability analyses

For the purpose of determining the capsid integrity of the plant-made VLPs they were heat-treated at a range of temperatures for 30 minutes. Particle antigenicity was assayed by capture ELISA, using a purified polyclonal antibody (Rab 1A) as capture. Monoclonal PV1 antibodies Mab 234 and Mab 1588 were applied as primary antibodies to identify native and non-native particles, respectively (Osterhaus et al., 1983). An anti-mouse conjugated to HRP was applied as secondary antibody. ELISA was carried out according to standard protocols (Gan and Patel, 2013).

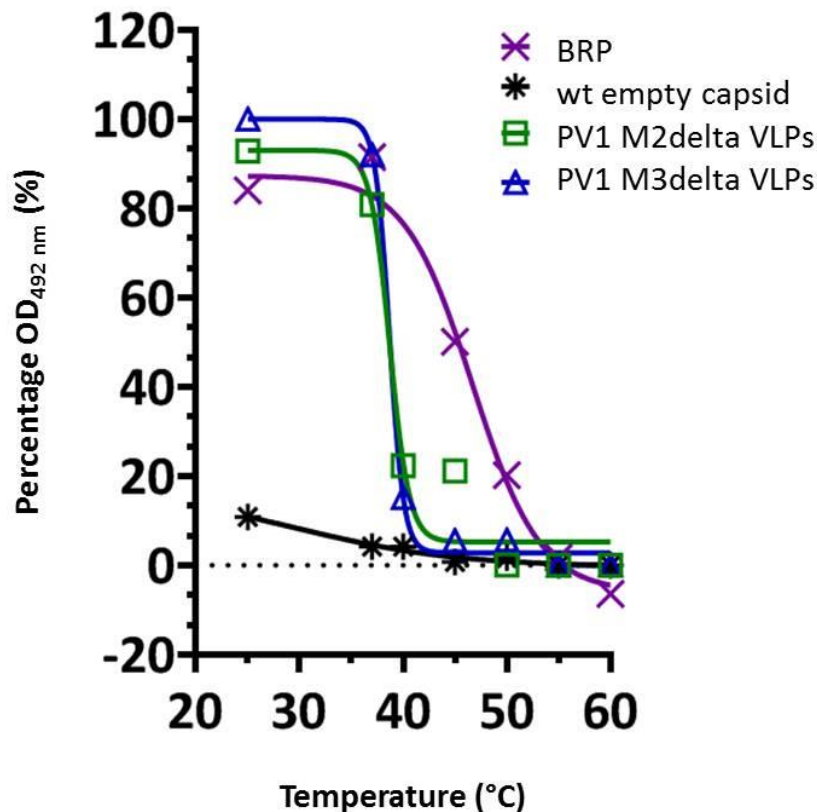


Figure 5.2-4: Thermal stability of plant-expressed PV1 M2delta and PV1 M3delta VLPs. VLPs were incubated at a range of temperature (from 25 °C to 60 °C) for 30 minutes. ELISA was carried using polyclonal (Rab 1A) antibody as capture. Monoclonal PV-1 antibodies Mab 234 and Mab 1588 were applied as primary antibodies to identify native and non-native particles. An anti-mouse conjugated to HRP was applied as secondary antibody. BRP = European Pharmacopeia Standard for IPV (Fuchs et al., 2003). (This figure was produced by Oluwapelumi Adeyemi and presented with his permission).

In comparison to wt empty capsids produced by Oluwapelumi Adeyemi in L-cells, the plant-made stabilised mutant VLPs (their purification is presented in 5.2.2.1) showed increased heat tolerance in this experiment. Both mutants lost their D antigenicity rapidly when 40 °C was reached. The antigenic shift for the corresponding mutant empty capsids occurred at 42 °C for PV1 M2delta and 45 °C for PV1 M3delta (submitted for publication Adeyemi et al., 2016); differences between the expression systems could explain the small variations in stability.

Oluwapelumi Adeyemi of the University of Leeds also conducted an immunoprecipitation experiment using single domain antibody fragments of camelid origin (nanobodies, or VHH). Purified plant-made VLPs and BRP, an inactivated polio vaccine control (Fuchs et al., 2003), were heat-treated at range of temperatures for 30 minutes and immunoprecipitated using anti-PV-1 D-specific and C-specific VHH nanobodies. Magnetic beads were added and incubated for 10 minutes at room temperature, under continuous shaking. Using a magnet, the beads were separated from the supernatant which was boiled in SDS-loading buffer (Schotte et al., 2012). Immunoprecipitated particles were immunoblotted against anti-VP0 rabbit polyclonal and anti-VP1 MAb antibodies (Figure 5.2-5).

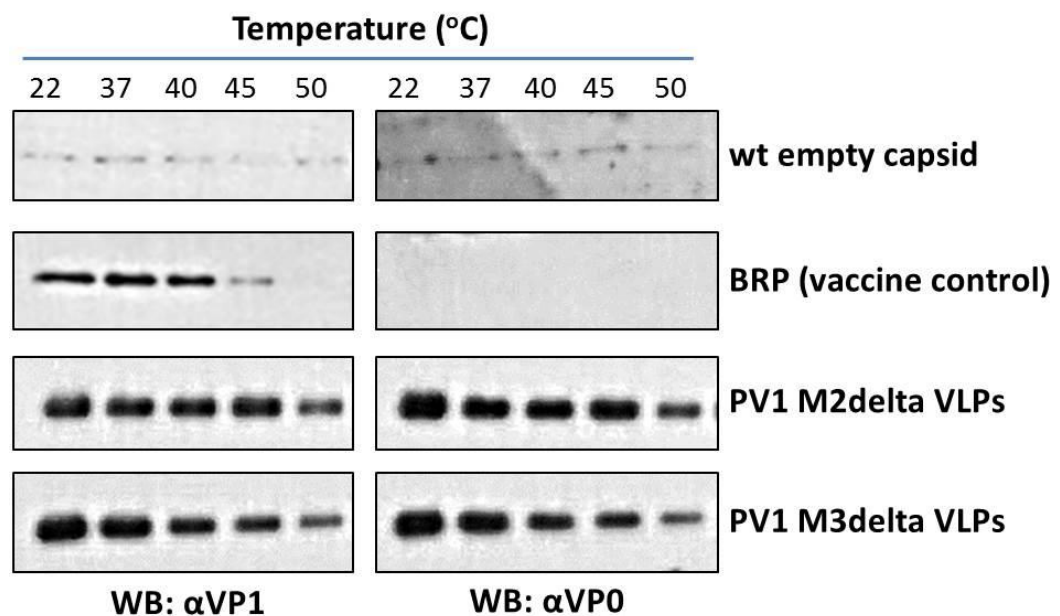


Figure 5.2-5: Immunoprecipitation using D antigenic VHH. VLP samples were incubated temperature range for 30 minutes, then cool to 4 °C. D antigenic VLPs were precipitated using specific VHH nanobodies (specific for D antigen). Western blots were incubated with anti-VP1 or anti-VP0 antibodies. (This figure was prepared by Oluwapelumi Adeyemi and presented with his permission).

In Figure 5.2-5 the samples were analysed for D antigen after incubation at a range of temperatures; testing the thermostability of the particles. Both plant-made mutant samples still contained D antigenic VLPs at 50 °C, whereas the vaccine control had lost most of the D antigenicity already at 45 °C. In the wt empty capsid sample D antigen was detected at 50 °C; an unexpected result because the wt is known to be highly instable.

Summary of section 5.2: Overall these preliminary results indicate successful capsid stabilisation in PV1 M2delta and PV1 M3delta. When expressed in plants both mutants showed increased thermostability compared to L-cell produced wt empty capsids however, the experiments need to be repeated and a more detailed analysis performed. The purposely designed mutant PV1 Q94C did not fulfil the expectations of increased capsid stability and was therefore not analysed further. The results suggest that forced evolution and selection of stable mutants is likely to be the most effective way of identifying stabilising mutations which can then be incorporated into VLPs.

5.3 SELECTION OF THERMOSTABLE MUTANTS

Our collaborators at NIBSC, Helen Fox and Dr. Andrew Macadam worked on the identification of mutations in the capsids of all 3 PV serotypes. This led to the identification of 3 stabilised mutants: PV1 MahSC6b, PV2 MEFSC6b and PV3 SktSC8. The P1 regions from these 3 mutants were expressed in plants in the presence of 3CD. The resulting VLPs were characterised as described in the following sections of this chapter.

The NIBSC group followed a slightly altered method for the generation of stabilised PV mutants compared to the method used by Oluwapelumi Adeyemi at the University of Leeds (Section 5.2.1). Their method is described in more detail in their most recent publication (submitted for publication Fox et al., 2016) . Briefly, for the development of PV1 MahSC6b, a mutation known to make Sabin type 3 vaccine strain temperature sensitive was introduced into the P1 region of a PV type 1 Mahoney strain. HEp2c cells were transfected with RNA transcripts from this construct, incubated at the non-permissive temperature of 39 °C and the resulting progeny examined by deep sequencing to identify stabilised mutants. They selected for the best mutants with thermostability and antigenicity tests, one of which was PV1 MahSC6b. The amino acid changes in this mutant are listed in the following table.

Table 5.3.1: PV1 MahSC6b mutations and locations.

Mutation	Location
R4018G	Internal network
T2025A	Pentamer interface
D2057E	Pentamer interface
L3119M	Internal
Q3178L	Promoter interface
H1248P	Promoter interface

For the development of PV2 MEFSC6b they followed the same strategy as they did with the type 1 mutant. Briefly, they inserted a destabilising mutation into MEF-2 and transfected HEp2c cells with the RNA transcripts from this construct, incubated at the non-permissive temperature of 39 °C and the resulting progeny was examined by deep sequencing to identify stabilised mutants. They selected for the best mutants with thermostability and antigenicity tests. PV2 MEFSC6b proved to be most stable. The amino acid changes in this mutant are listed in the following table.

Table 5.3.2: PV2 MEFSC6b mutations and locations.

Mutation	Location
I4057V	Internal network 3-fold
D2057A	Pentamer interface
Q3178L	Protomer interface
V1107I	Protomer interface
F1134L	Pocket
V1183L	Pocket

To generate stabilised type 3 mutants they started by growing type 3 Sabin vaccine strain in Hep2C cells at semi-permissive temperatures and identified eight mutations that suppress one of the main attenuating mutations which makes the virus temperature sensitive. Mutations identified as candidate capsid stabilising changes were introduced into Leon, the virulent precursor of the Sabin vaccine strain of type 3 in different combinations. Those mutants were grown at increasing temperature and further mutations emerged that have stabilising effects. The strain that is used for production of

inactivated serotype 3 polio vaccine, hence the appropriate target for VLP design, is the wt virus Saukett. Constructs were made in which amino acids were exchanged for the stabilising forms, resulting in PV3 SktSC8, with eight amino acid differences from the IPV Saukett sequence as shown in Table 5.3.3.

Table 5.3.3: PV3 SktSC6b mutations and their location.

Mutation	Location
T4067A	Internal network near three fold axis
L2018I	Beta sheet at pentamer interface
L2215M	Protomer interface
D2241E	VP2/VP3 interface, buried
H3019Y	Internal network, tube below 5 fold axis
L3085F	Beta sheet at pentamer interface
T1105M	North wall of canyon
F1132L	Pocket

Thermostability assays, D/C antigenicity tests and animal studies for these three mutants described in this section, were performed by Helen Fox and Dr. Andrew Macadam at NIBSC. The results are presented with their permission.

5.3.1 EXPRESSION AND PURIFICATION OF THERMOSTABLE MUTANTS

5.3.1.1 PV1 MahSC6b VLPs

To produce VLPs of mutant PV1 MahSC6b in plants, the P1 region of this mutant was inserted into pEAQ-*HT* and co-expressed with the 3CD proteinase. After 6 days the infiltrated tissue was harvested and any VLPs present were purified as described above. The results are summarised in Figure 5.3-1.

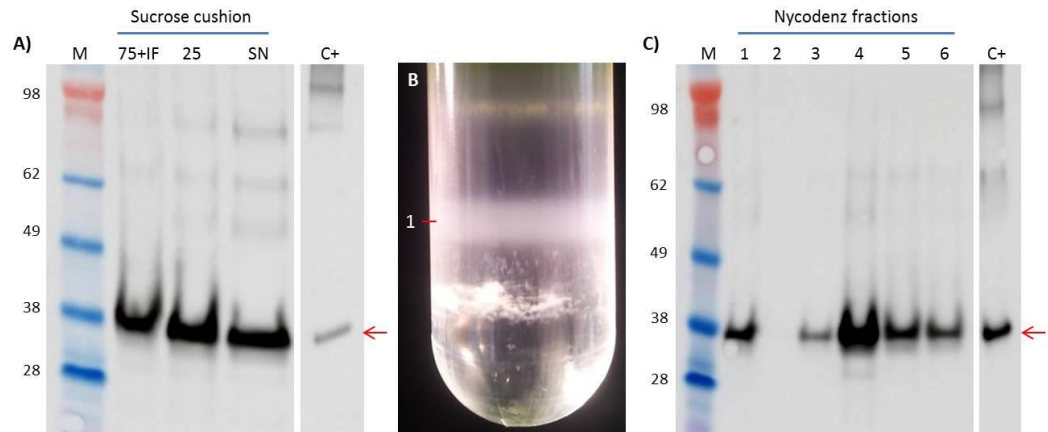


Figure 5.3-1: PV1 MahSC6b VLP purification from plants. **A)** The different fractions of the sucrose cushion were analysed on a Western blot using anti-VP1 MAB8566 antibody. **B)** The Nycodenz gradient after ultracentrifugation with an unusually wide and blurry grey band, indicated with the number 1. **C)** The Nycodenz fractions were retrieved and checked for VP1 content. First the grey band (1) was removed and then the gradient was fractionated from the bottom (2-6). Anti-VP1 MAB8566 antibody was used in both Western blots. SN = Supernatant. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV3 SktSC8 VLPs. Red arrows indicate position of VP1.

The Western blot probed with the anti-VP1 antibody showed strong signals in all three fractions retrieved from the sucrose cushion also the 25% (w/v) sucrose fraction and the SN contained VP1 (Figure 5.3-2 A). When the sample was purified through a Nycodenz gradient an unusually wide and blurry band appeared, which was extracted separately by piercing the side of the tube. Afterwards the gradient fractionated from the bottom (fractions 2-6). All were analysed for VP1 content via immunodetection (Figure 5.3-1 C). The grey band did contain VLPs but according to the Western blot the VLPs were spread across most of the gradient. Both results indicate that the sample contained particles of various sizes but it could also suggest instabilities in the capsid which caused the particles to break during purification.

Obtaining a pure sample of homogenous PV1 MahSC6b VLPs proved difficult as described above. However, the image below illustrates a clean sample of this plant-produced mutant received from a Nycodenz gradient after dialysis.

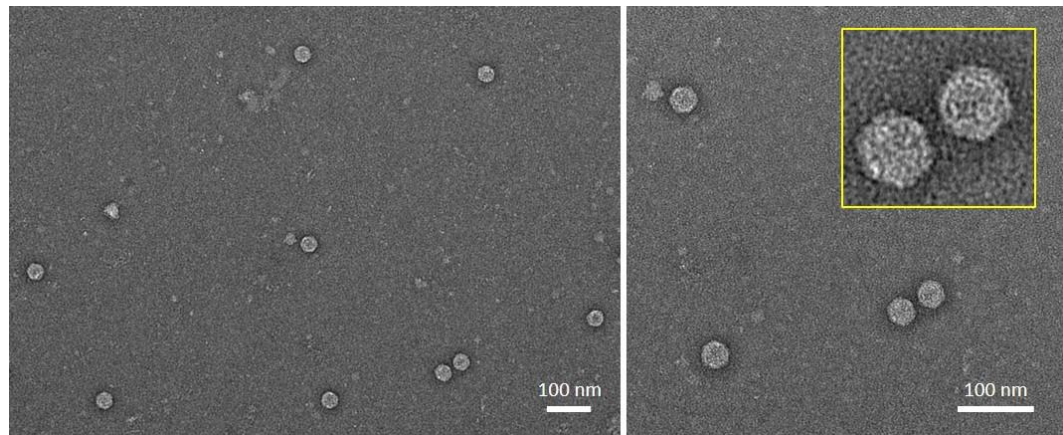


Figure 5.3-2: TEM images from PV1 MahSC6b VLPs. Visualised in negative stain.

The TEM images above visualise PV1 MahSC6b VLPs of typical PV size and shape. The yield of highly purified VLPs is negatively affected by the heterogeneity of this mutant but approximately 40 mg of purified particles/kg infiltrated plant tissue was achieved.

5.3.1.2 PV2 MEFSC6b VLPs

PV2 MEFSC6b was routinely co-expressed in plants with the necessary proteinase. From previous time course experiments I am aware of the early peak in protein expression of all type 2 VLPs and therefore harvest this mutant 4 dpi. Also, generally all type 2 VLPs have lower expression levels compared to the other serotypes when produced in plants. Therefore, a considerably larger number of plants had to be infiltrated in order to obtain sufficient amount of VLPs for subsequent analysis. In the following Figure 5.3-3 shows the last purification steps of PV2 MEFSC6b VLPs from plant material.

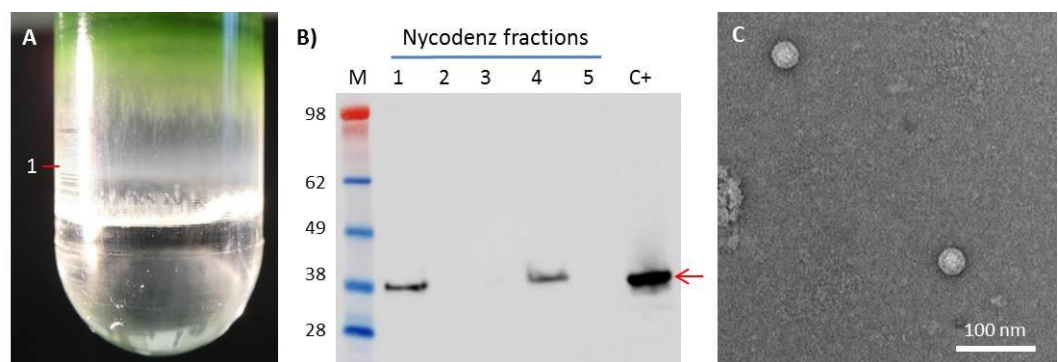


Figure 5.3-3: Purification of PV2 MEFSC6b VLPs. **A)** In the Nycodenz gradient the typical grey band formed during ultracentrifugation, labelled with 1. This band was extracted and then the gradient was fractionated by piercing the bottom of the tube (fractions 2-5). **B)** Each fraction was tested for VP1 content via immunodetection using anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV3 SktSC8 VLPs. Red arrow indicates VP1. **C)** VLPs were visualised in negative stain by TEM.

PV2 MEFSC6b VLPs were purified first through a sucrose cushion and then through a Nycodenz gradient as depicted in Figure 5.3-3 A. The grey band labelled with 1 was separately removed from the tube as it was expected to contain the VLPs. Afterwards the bottom of the tube was pierced and fractions 2-5 retrieved. Also fraction 4 tested positive for VP1 on the Western blot shown in part B; possibly material that was missed when the grey band 1 was retrieved. Fraction 1 and 4 were combined and visualised by TEM as presented in Figure 5.3-3 C. Approximately 5-8 mg purified PV2 MEFSC6b VLPs were prepared from 1 kg infiltrated plant material.

5.3.1.3 PV3 SktSC8 VLPs

With the aim to optimise VLP yield a time course experiment with PV3 SkSC8 was carried out. A leaf was infiltrated with the respective P1 and the proteinase 3CD and leaf discs were samples every day for 10 days. At the end of the experiment the samples were run on a PAGE gel before the protein got transferred on a nitrocellulose membrane. An anti-VP1 (MAB8566) was applied to detect the highest expression level of VP1 in the 10 samples (Figure 5.3-4).

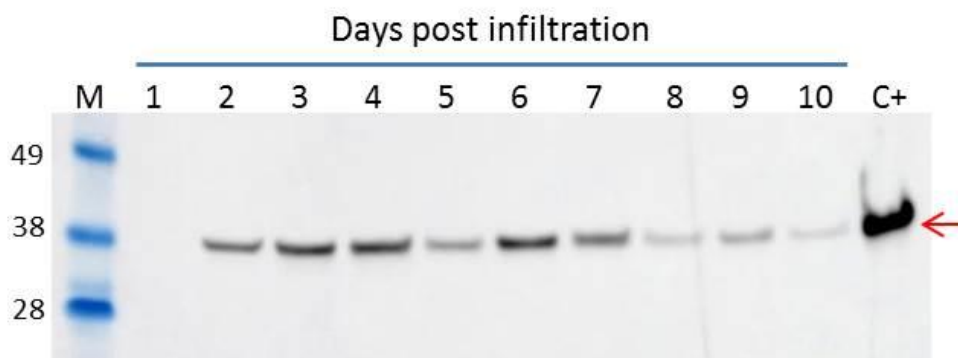


Figure 5.3-4: Expression of PV3 SktSC6b VLPs in plants over time. Equal amounts of agrobacteria solutions harbouring pEAQ-HT-PV3 SktSC8-P1 ($OD_{600} = 0.8$) or pEAQ-3CD ($OD_{600} = 0.4$) were mixed and infiltrated into leaves. A small scale protein extraction was conducted 1-10 dpi. Anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = PV3 SktSC8 VLPs. Red arrow indicates position of VP1.

The Western blot revealed detectable amounts of protein already 2 dpi and the highest yield at 6 dpi. Following this results, PV3 SktSC8 VLPs were always harvested on day 6 after infiltration.

PV3 SktSC8 VLPs were produced in plants by co-expressing the P1 region together with the proteinase. The infiltrated leaf tissue was harvested and the VLPs extracted and

purified as previously described. Below plant-made PV3 SktSC8 VLPs are visualised in negative stain.

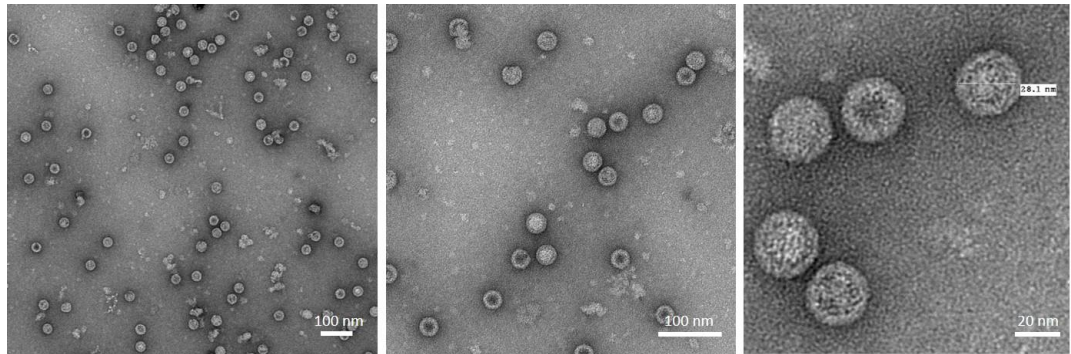


Figure 5.3-5: TEM images of PV3 SktSC8 VLPs.

The stabilised mutant PV3SktSC8 accumulates to very high levels in plants, with estimated concentration of 50-70 mg/kg infiltrated plant material. It is also a very durable and stable VLP which makes purification relatively simple. In the TEM images above one can see very evenly shaped particles of perfect size and form (Figure 5.3-5). Most particles appear empty with stain that has penetrated their core.

Summary of section 5.3.1: PV1 MahSC6b expresses well in plants, however its heterogeneity complicates the preparation of uniform VLP samples. PV2 MEFSC6b VLPs accumulate to very low concentration in plants, however up-scaling of the experiment produced sufficient material for further analyses. PV3 SktSC8 VLPs are very stable and highly expressed in plants.

5.4 IMMUNOLOGICAL DATA

5.4.1 D AND C ANTIGEN ELISA

Non-competitive sandwich ELISA assays were conducted by Helen Fox to measure the D antigen content of the plant-produced stabilised mutants (Singer et al., 1989). The analysed VLP samples were prepared following the routine protocol described in (see Figure 4.4-3); however, based on results obtained by collaborators, a new buffer was introduced which contains 20 mM EDTA (PBS + EDTA). Briefly, two-fold dilutions of antigen were captured with a serotype-specific polyclonal antibody, then detected using serotype-specific, D antigen or C antigen specific monoclonal antibodies followed by

anti-mouse peroxidase conjugate. The D antigen content of each test sample was evaluated against a reference of assigned D antigen content (Fuchs et al., 2003) by parallel line analysis (Combistats).

In the following Figure 5.4-1, plant-produced PV1 MahSC6b VLP samples were analysed, using D antigen (Mab 234) and C antigen (Mab 1588) specific monoclonal antibodies.

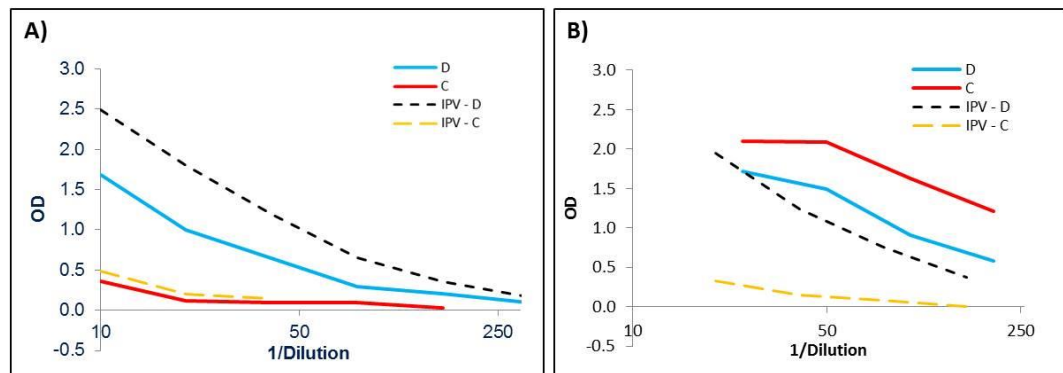


Figure 5.4-1: Non-competitive sandwich ELISA assay for the measurement of D antigen content in plant-produced PV1 MahSC6b VLP samples A) PV1 MahSC6b VLPs in PIPES buffer analysed for D/C antigen content using Mab 234 (D antigen) and Mab 1588 (C antigen). B) PV1 MahSC6b VLPs in PBS + EDTA buffer analysed for D/C content using MAb 234 (D antigen) and MAb 1588 (C antigen). IPV = European Pharmacopeia Standard for IPV.

The left graph in Figure 5.4-1 shows the D antigen content in the sample purified in PIPES buffer; a total of 140 DAgU/ml were detected in this sample. C antigen could also be detected but in very low amounts. Part B of Figure 5.4-1 illustrates that higher C than D antigen levels were obtained in the sample purified in PBS + EDTA buffer, though the total amount D antigen (530 DAgU/ml) was also higher. Both samples derived from very similar amounts of infiltrated plant tissue (90 leaves/ ~60 g) and were prepared the same way apart from the different buffers (see above).

In the following ELISA assay, two samples of PV2 MEFSC6b VLPs in either PIPES or PBS + EDTA buffer were analysed and compared (Figure 5.4-2). D antigen (Mab 1050) specific monoclonal antibody was utilised.

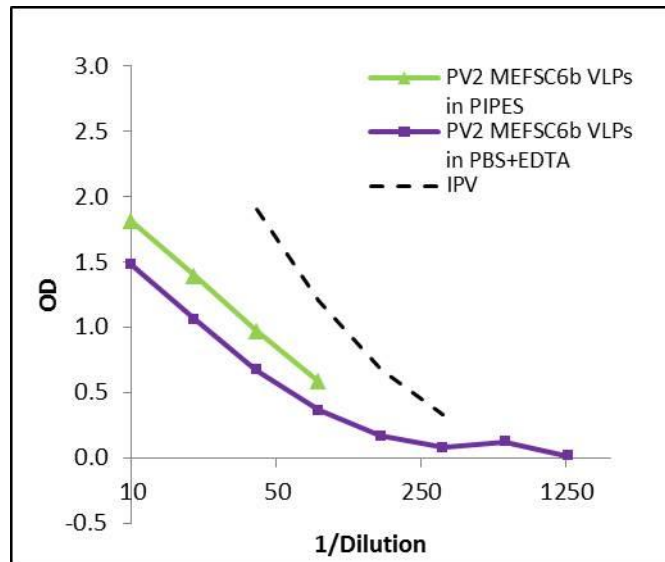


Figure 5.4-2: Non-competitive sandwich ELISA assay for the measurement of D antigen content in two PV2 MEFSC6b VLP samples. PV2 MahSC6b VLPs in PIPES buffer or PBS + EDTA buffer analysed for D antigen content using Mab 1050. IPV = European Pharmacopeia Standard for IPV.

In this ELISA the sample prepared in PIPES buffer contained slightly higher levels of D antigen with 45 DAgU/ml than the sample in PBS+EDTA buffer with 29 DAgU/ml. Both samples derived from very similar amounts of infiltrated plant material (150 infiltrated leaves/ approximately 300 g) and were prepared following the same routine protocol. However, these small differences can be due to the plants growing at varying speeds depending on the time of the year, changes in temperature or lighting conditions in the glasshouse. The C antigen was not tested because there is currently no C specific antibody available for type 2. Therefore, it is difficult to conclude which buffer stabilises the VLPs best and produces the sample with the favourable D to C antigen ratio. This result is in strong contrast to the findings with PV1 MahSC6b, where the PBS + EDTA buffer produced a sample with significantly more DAgU than PIPES buffer.

The following graph shows a non-competitive sandwich ELISA assay analysing a PV3 SktSC8 VLP sample prepared from plant material, using D antigen (Mab 520) or C antigen (Mab 517) specific monoclonal antibodies.

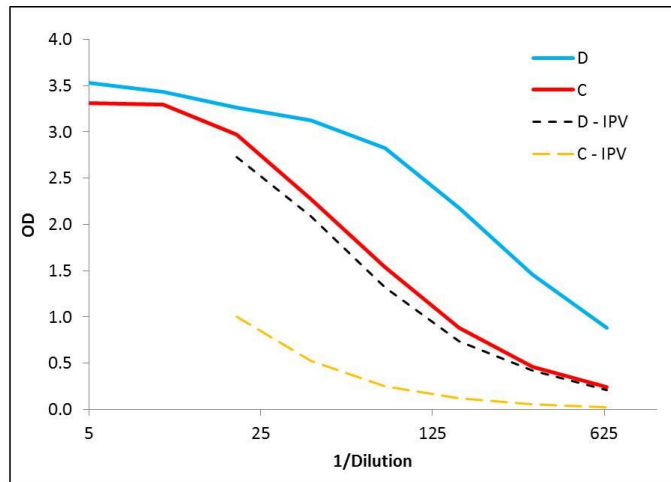


Figure 5.4-3: Potency plant-made PV3 SktSC8 VLPs. Non-competitive sandwich ELISA assay for the measurement of D/C antigen content using Mab 520 (D antigen) and Mab 517 (C antigen). IPV = European Pharmacopeia Standard for IPV.

An estimate of 2200 DAgU was measured in this sample derived from 90 infiltrated leaves (~60 g plant tissue). It represents the high potency of this specific type 3 mutant.

Summary of section 5.4.1: PV1 MahSC6b VLPs show great amounts of D antigen when using EDTA enriched buffer, but also high levels of C antigen. As already mentioned PV2 MEFSC6b expresses comparably weak in plants, however useful amounts of DAgU could be obtained. PV3 SktSC8 VLPs produces very large amounts of DAgU.

5.4.2 THERMOSTABILITY ASSAY

The plant-expressed stabilised VLPs (produced following the routine protocol described in Figure 4.4-3) were exposed to a range of temperatures for ten minutes and assayed for D antigen and C antigen content in order to test their thermostability. As a control empty capsids of the same mutant produced by infection in mouse L cells, were analysed in parallel. IPV's maximum heat tolerance is known and used here as a performance comparison.

In Figure 5.4-4, plant-made PV1 MahSC6b VLPs prepared in two different buffers were analysed using D antigen (Mab 234) and C antigen (Mab 1588) specific monoclonal antibodies.

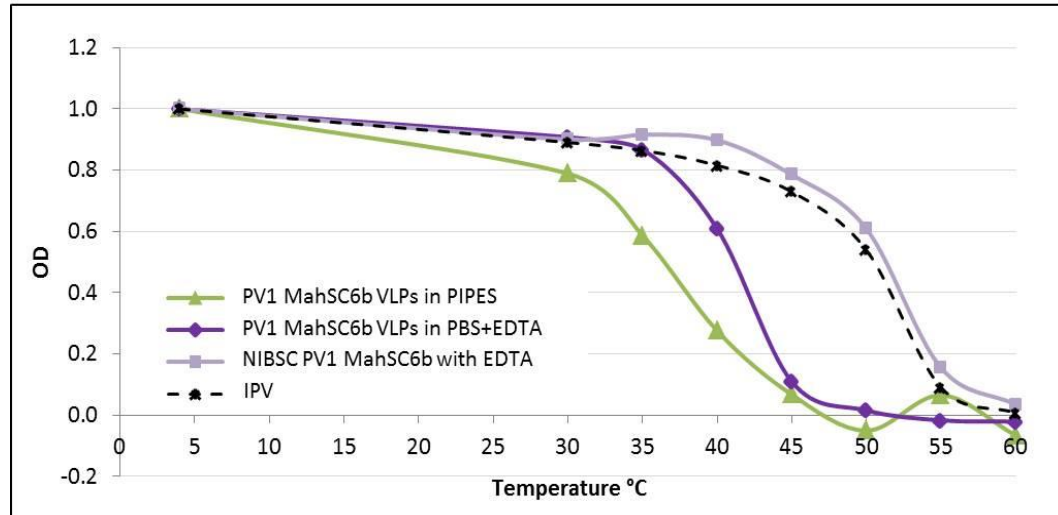


Figure 5.4-4: Thermostability PV1 MahSC6b VLPs. Reactivity of plant-produced VLPs compared to empty capsids and IPV with Mab 234 (D specific) in ELISA after incubation at different temperatures for 10 minutes.

In this experiment two samples of plant-made PV1 MahSC6b VLPs were analysed; these are the same samples that were tested for their D/C antigenicity above (Figure 5.4-1). The VLPs kept in PIPES were least tolerant to the temperature increase and changed D to C antigenicity at 30-35 °C. In direct comparison the VLPs in PBS + EDTA had slightly increased thermostability. Overall it is apparent that the plant-produced PV1 MahSC6b VLPs are less stable than the empty capsids made in L cells. The empty capsids produced by infection of L cells were more tolerant to the increased temperature than the currently marketed IPV, which converts from D to C antigen at 49-52 °C.

In order to determine their thermostability, two samples of PV2 MEFSC6b VLPs in either PIPES or PBS + EDTA buffer were assayed for D antigen (Mab 1058) content (Figure 5.4-5).

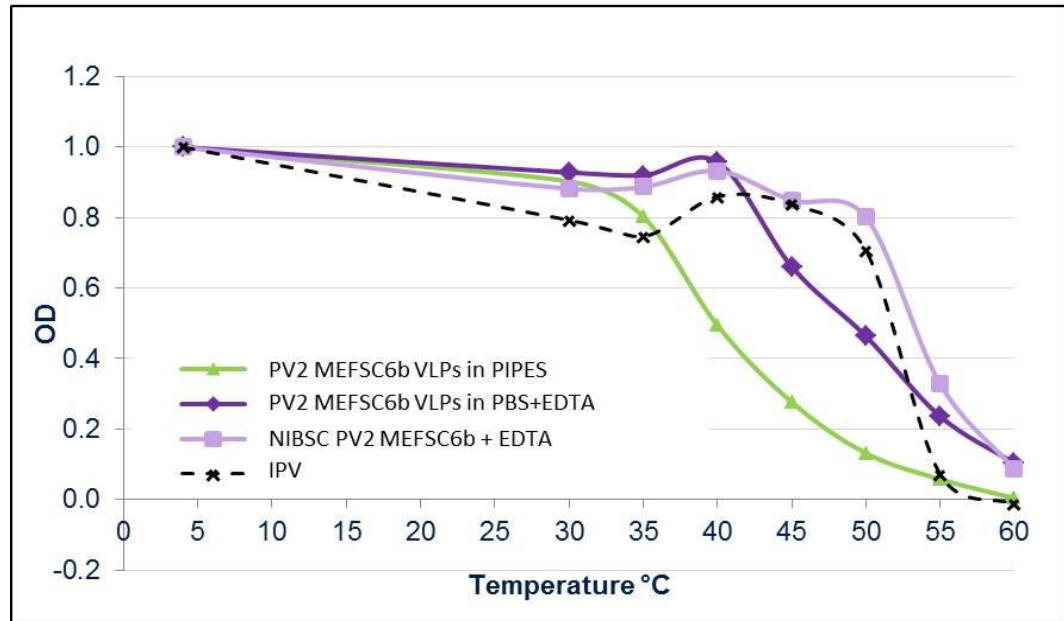


Figure 5.4-5: Thermostability of PV2 MEFSC6b VLPs. Reactivity of plant-produced VLPs compared to empty capsids and IPV with Mab 1050 (D specific) in ELISA after incubation at different temperatures for 10 minutes.

The outcome of this experiment is very similar to the one of PV1 MahSC6b VLPs shown in Figure 5.4-4. VLPs made in PBS + EDTA buffer were more thermostable than VLPs kept in PIPES. And again VLPs produced in plants seem less thermostable as those produced in L cells.

5.4.2.1 Influence of EDTA on particle stability

This experiment was conducted after differences in stability were observed in samples of the same mutant that was kept in different buffers (see Section 5.4.2). We were able to identify the chelating agent EDTA to play a key role in the stability of VLPs. Here, a non-competitive sandwich ELISA assay was used to observe the D to C antigen switch under the influence of EDTA. A sample of PV3 SktSC8 VLPs in PBS buffer was split in half and to one part EDTA was added to a final concentration of 20 mM. The samples were slowly heated from 4 °C to 60 °C and serotype-specific polyclonal antibody was utilised to capture antigen, then detected using serotype-specific, D antigen (Mab 520) or C antigen (Mab 517) specific monoclonal antibodies followed by anti-mouse peroxidase conjugate.

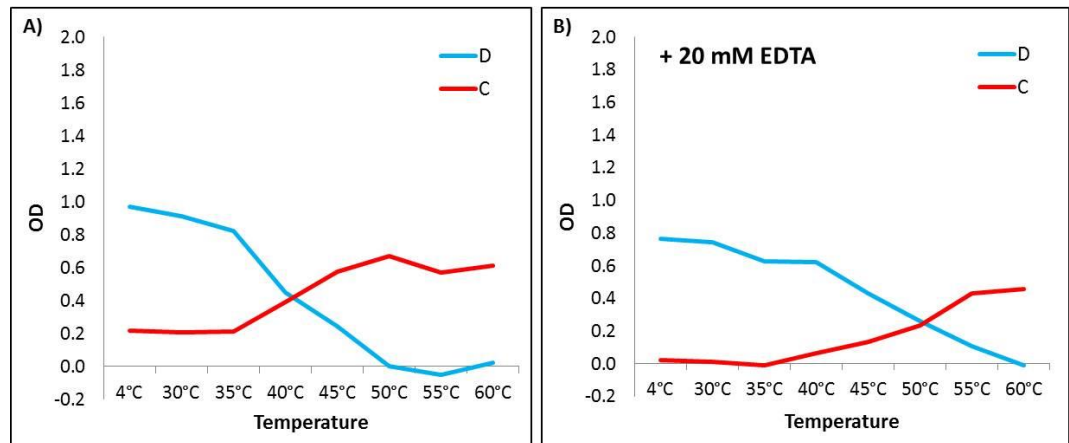


Figure 5.4-6: Influence of EDTA on VLP integrity. Non-competitive sandwich ELISA assay for the measurement of D to C antigen conversion in PV3 SktSC8 VLPs using Mab 520 (D antigen) and Mab 517 (C antigen) when heated. **A)** PV3 SktSC8 VLPs in PBS buffer. **B)** PV3 SktSC8 VLPs in PBS and 20 mM EDTA. IPV = European Pharmacopeia Standard for IPV.

This ELISA illustrates a clear role of EDTA in keeping the VLPs in the desired D conformation at elevated temperatures. Without the added EDTA the switch from D to C started at 35 °C, whereas with added EDTA the change in conformation was slower and did not start until 40°C was reached. In order to analyse if a lower concentration of EDTA would also affect the stability of the VLPs several concentrations were tested and the results presented in the following graph (Figure 5.4-7).

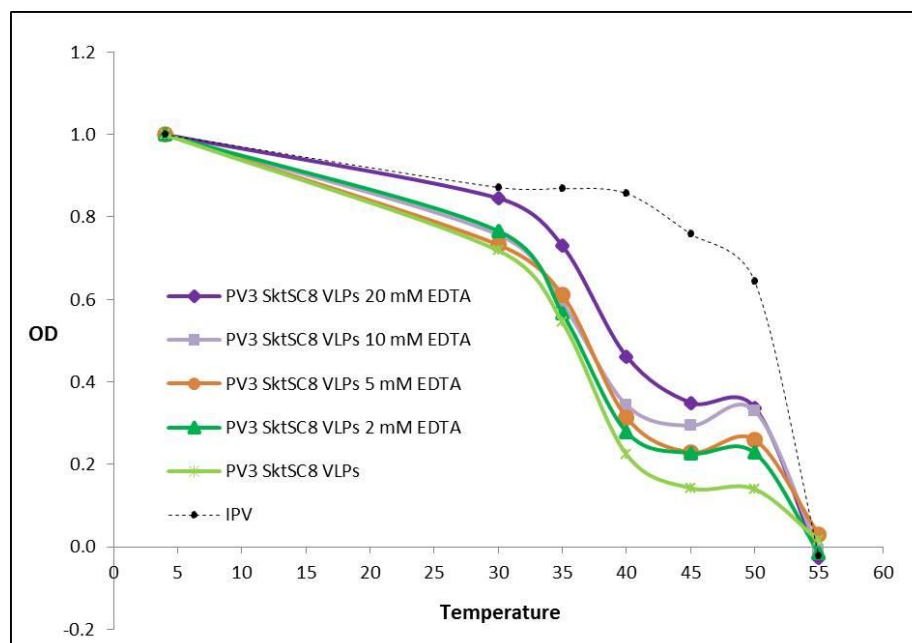


Figure 5.4-7: Effect of different EDTA concentrations on VLP thermostability. Non-competitive sandwich ELISA assay for the measurement of D in PV3 SktSC8 VLPs using Mab 520 when heated under the influence of increasing EDTA concentrations. IPV = European Pharmacopeia Standard for IPV.

Several concentrations were tested in order to determine if a lower amount than 20 mM EDTA would also stabilise the VLPs. However, the concentration of 20 mM EDTA achieved the best result in this experiment; the higher the concentration of EDTA the later the shift from D to C antigenicity occurred. Based on these results 20 mM EDTA was added to all stabilised mutant VLPs produced in plants. It was also investigated if removing divalent cations from the buffer would increase the thermostability of PV3 SktSC8 VLPs (data not shown). The routinely used PBS + EDTA buffer contains 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺. Therefore, the VLPs were transferred into a PBS buffer without divalent cations present but no difference was found (loss of 50% DAgu at 40 °C). In future experiments it should be investigated if even higher concentrations of EDTA in the buffer can increase particle stability.

Summary of section 5.4.2: All mutant VLPs retain their D antigenicity better when 20 mM EDTA is added. Though, when the mutants are expressed as VLPs in plants they are less thermostable than the empty capsid version of themselves made in L cells.

5.4.3 NEUTRALISING ANTIBODY RESPONSE AND PROTECTION IN ANIMALS

The neutralising antibody response of plant-produced PV1 MahSC6b VLPs and PV3 SktSC8 VLPs was analysed in either mice or rats by ELISA. Furthermore, their potency to elicit a neutralising antibody response was compared to empty capsids produced in L cells. As a reference the European Pharmacopeia Standard for IPV was applied and the animals were also immunised with PBS as a negative control. The animals were immunised intramuscular and after 21 days they were bled, the serum collected and tested using a standard neutralising antibody assay for IPV batch release (European Pharmacopeia standard test). The overall outcome of these experiments was that the plant-produced mutant PV VLPs do elicit neutralising antibodies but less than IPV and the empty capsids produced in L cells. However, comparing an immune response of either L cell produced empty capsids with plant-produced VLPs is not very meaningful. The empty capsids were produced by infection which is very different to the recombinant expression of VLPs. Other factors that are necessary for virus infection could be present in the sample and influence the antibody response in the animals.

Transgenic mice carrying the human receptor for poliovirus are susceptible to infection and paralysis. Groups of eight animals were immunised with one dose of PV1MahSC6b VLPs either produced at NIBSC in L cells or in plants, PBS as a negative control or the

European reference IPV (Fuchs et al., 2003). The mice were then challenged with infectious wt poliovirus and monitored for any signs of paralysis for 14 days. This experiment was also carried out with PV3 SktSC8 VLPs. Both plant-made stabilised mutants (PV1 MahSC6b and PV3 SktSC8) gave 100% protection against wt virus in mice. No difference was detectable between the empty capsids produced in L cells or the plant produced VLPs.

Summary of section 5.4.3: Both plant-expressed mutants, PV1 MahSC6 VLPs and PV3 SktSC8 VLPs elicit 100% protection in transgenic mice against infectious virus; even though their immune response in rats or mice is comparably weak.

5.5 STRUCTURAL ANALYSIS OF PV3 SKTSC8 VLPs

The structural analysis was carried out by our collaborators at the University of Oxford using samples provided by myself. The data in this thesis are presented with the kind permission of Dr. Mohammad Bahar, Prof. David Stuart and Dr. Elizabeth Fry.

Cryo-EM analysis demands highly concentrated and pure samples. The mutant PV3 SktSC8 was selected because of its stability and high expression levels in plants. In order to obtain a suitable sample for cryo-EM analysis, PV3 SktSC8 VLPs were purified from approximately 200 g infiltrated leaf tissue. Expression and purification was carried out following the routine protocol of two successive density gradients (see Figure 4.4-3). For this analysis the stabilised VLPs were extracted using P-buffer and further purified in PIPES buffer. Structures of the stabilised plant-expressed PV3 SktSC8 VLP were determined by cryo-EM in the absence and presence of the GPP3 (3-(4-pyridyl)-2-imidazolidinone) pocket-binding compound (De Colibus et al., 2015). 4046 particles of PV3 SC8 and 2060 particles of PV3 SktSC8 + GPP3 yielded structures at 3.6 Å and 4.1 Å resolution respectively (Figure 5.5-1). The models were refined (Afonine et al., 2012) and structure validation was performed. The polypeptide main chain and side chains were well resolved for most of the capsid (Figure 5.5-2), allowing an atomic model for the majority of the three capsid proteins (VP0, VP1 and VP3) to be manually built into the cryo-EM density maps.

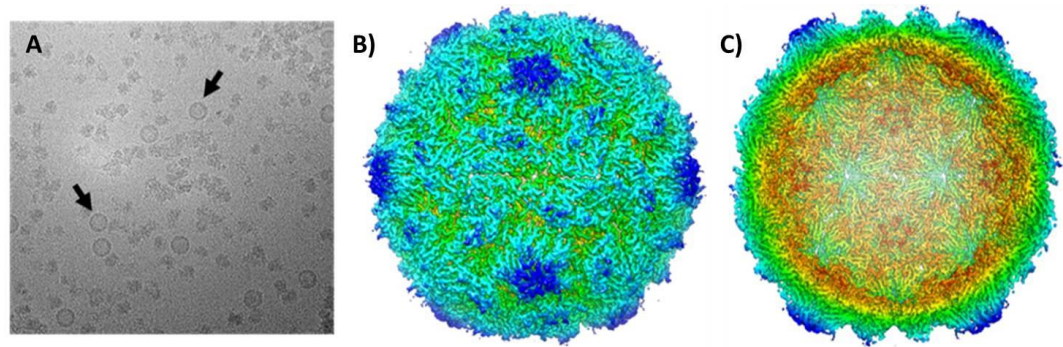


Figure 5.5-1: Cryo-EM structure of PV3 SktSC8 VLP at 3.6 Å. **A)** Cryo-EM image of PV3 SktSC8 VLPs in vitreous ice. Examples of some VLPs are highlighted with black arrows. **B)** 3D reconstruction of PV3 SktSC8 VLP at 3.6 Å resolution, viewed along the 2-fold axis. The surface is coloured by radius from the centre of VLP from blue through green to red from the highest to the lowest radius. **C)** A central slice through the VLP to show the empty internal surface, viewed along the 2-fold axis and coloured as in **(B)**.

The overall architecture of the plant expressed PV3 SC8 VLP preserves structural features characteristic of native conformation (D form) particles such as the canyon depression around the fivefold vertex of the capsid (Figure 5.5-1 B). It revealed that the atomic structure of PV3 SktSC8 VLPs closely resemble the native wild-type poliovirus (see Figure 1.5-1) (Hogle et al., 1985).

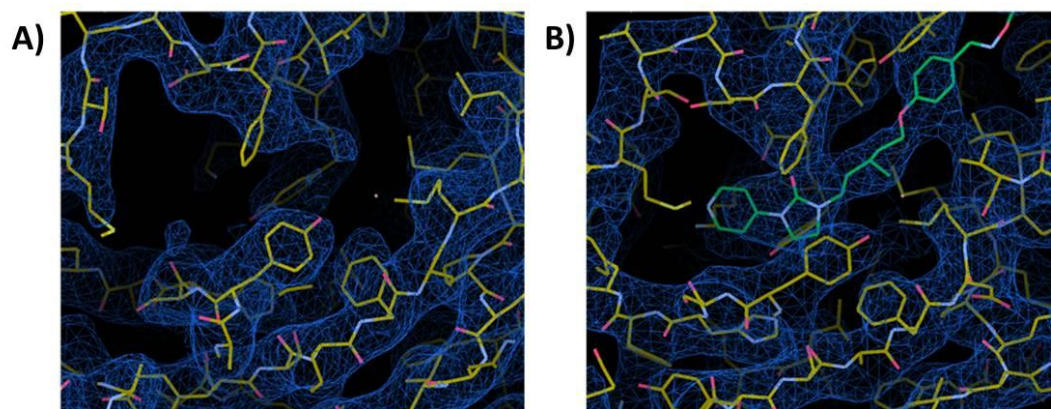


Figure 5.5-2: Cryo-EM density for PV3 SktSC8 and PV3 SktSC8 + GPP3 VLPs. **A)** Cryo-EM density map at 3.6 Å resolution of PV3 SktSC8 VLP looking at the VP1 protein pocket. VP1 is shown as sticks with carbon atoms coloured yellow, nitrogen atoms in blue and oxygen in red. **B)** Cryo-EM density map at 4.1 Å resolution of the PV3 SktSC8 VLP with GPP3 pocket factor compound bound to the VP1 protein pocket. VP1 is shown as sticks and coloured as in **(A)**. The GPP3 molecule is coloured in green. Cryo-EM density maps are contoured at 1.0 σ .

Cryo-EM density is not observed for a pocket factor molecule in the VP1 protein for the PV3 SktSC8 structure at 3.6 Å resolution (Figure 5.5-2). However, the structure of the PV3 SktSC8 VLP mixed with the GPP3 pocket binding molecule determined at 4.1 Å resolution shows unambiguous density for GPP3 bound to the VP1 protein pocket (Figure 5.5-2 B). The binding mode of GPP3 to VP1 is very similar to structures of other D

conformation polio capsids, suggesting that the plant-expressed PV3 SktSC8 VLP assembles in an authentic D form competent to bind pocket factor molecules.

Summary of section 5.5: Cryo-EM analysis revealed that the atomic structure of stabilised PV3 SktSC8 VLPs closely resemble the native wt poliovirus.

5.6 DISCUSSION

This chapter presents the results for potential vaccine candidates. It reflects the ultimate aim in generating stabilised PV VLPs that could potentially displace the currently available vaccines. In this chapter results were illustrated of those stabilised VLPs produced in plants and I will discuss their outcome.

Initial work with the PV1 Q94C mutant quickly suggested that the deliberate design of PV mutants did not result in the desired increase in capsid stability. Therefore, an alternative strategy of applying selection pressure during propagation of the virus was adopted with the aim of obtaining empty capsids with enhanced stability. Directed evolution is a classical approach in virology and generated a vast amount of mutants with potentially great vaccine characteristics (submitted for publication Fox et al., 2016). However, the aim of the consortium is to develop a recombinantly expressed VLP vaccine, which would have the advantage of not requiring the growth of infectious virus, as is necessary for current vaccines. This would be a major advantage for the final eradication of poliovirus. For this reason, several expression systems have been proposed for the production of stabilised VLPs: insect cells, yeast and *E. coli*. Plants however, have so far proved to be the most successful and it was possible to express mutant PV VLPs from all three serotypes. These have been characterised as described in this chapter.

The type 1 mutants produced by forced evolution, developed at the University of Leeds, have not been deeply analysed yet due to time limitations. The data presented in this work are preliminary and only give an indication towards their potential. So far PV1 M2delta and PV1 M3delta VLPs produced in plants seem to have slightly increased capsid stability but further tests have to be carried out to confirm this. Both are very well expressed in plants, so such analyses should be possible.

The third type 1 mutant that was expressed and analysed was PV1 MahSC6b developed at NIBSC. Time allowed more complete analysis of this stabilised VLP expressed in plants and promising data were collected. In particular, it fully protected mice carrying the human PV receptor against challenge with a paralysing dose of infectious virus. This is a very positive result and certainly the most important one. The mice were also bled before the challenge and the serum tested for the presence of neutralising antibodies. It was noticeable that the plant-made VLPs triggered a significantly lower neutralising response than empty capsids made in mouse L cells. This discrepancy between L cell produced empty capsids and plant-produced VLPs has been observed with every stabilised VLP so far. The empty capsids were produced by electroporating L cells, which lack the receptor for poliovirus, with full-length RNA transcripts encoding the stabilised mutants to give a single cycle of infection. The particles produced were purified on sucrose gradients (submitted for publication Fox et al., 2016). Obviously there are differences between generating empty capsids in L cells via infection compared to the recombinant expression of VLPs in plants. During infection non-structural proteins are also expressed and these could play a role in the immune response in animals if they co-purify with the empty particles. These proteins are not present in the plant-made samples. This could be an explanation for the weaker antibody response in animals. Furthermore, the uncharacteristic pocket factor of the plant-made VLPs could also have an influence on the antibody response in animals.

The D/C antigen ELISA assay of PV1 MahSC6b VLPs revealed high D antigen levels in a sample that was purified in PIPES buffer. When PBS + EDTA buffer was utilised much more DAgU could be purified from the plant material; however, increased amounts of C antigen were also present. There could be plenty of reasons behind these discrepancies, one of which could be variations in the plant growth conditions or accidental changes in the purification. Generally, purification of this mutant caused trouble as it seemed to produce heterogeneous VLPs which could be a reflection of stability issues. This was noticed during the density gradients where the PV1 MahSC6b VLPs were spread over several fractions. Also cryo-EM revealed flexibility or heterogeneity in this VLP (Dr. Mohammad Bahar, University of Oxford, personal communication). Therefore, this mutant needs careful evaluation and further studies to finalise a verdict about its suitability as a vaccine candidate.

Working with type 2 has been most challenging as its expression or accumulation levels are very low in plants. PV2 has also proved to be the most difficult to express in other heterologous systems (Prof. Ian Jones, University of Reading, personal communication). One can only assume unstable mRNA or intrinsic instabilities of the capsid proteins lead to its early degradation. However, PV2 MEFSC6b was the most successful type 2 stabilised mutant and therefore chosen to be represented in this thesis. The results so far indicate that the VLPs purified from plants are in the desired D conformation. As already mentioned above they are less thermostable than IPV or the empty capsids produced in L cells even with added EDTA. No protection studies have been carried out with the type 2 VLPs yet, because of the recent change in the composition of OPV vaccine. In April 2016 the WHO switched from trivalent OPV (tOPV) to bivalent OPV (bOPV) by removing the type 2 component (OPV2) from immunisation programmes. The absence of a type 2 vaccine means that any research using virulent type 2 requires higher containment facilities than the other serotypes with consequences for challenge experiments. As soon as those facilities are available the type 2 mutant will be tested in animals.

PV3 SktSC8 has been the most reliable and successful sVLP so far with very positive and promising results. It accumulates to high concentrations in plants, is very robust and therefore easy to purify. It keeps its D antigenicity very well during purification and from 90 infiltrated leaves ~2200 DAgU could be extracted. Even though the neutralising antibody response in rats was weak it provided complete protection for transgenic mice against infectious virus.

We noticed variations not only in the antibody response in animals but also in the thermostability between L cell produced empty capsids and the plant-made VLPs in our studies. There are several reasons behind this which I already discussed above. Another factor however, was quickly identified. My collaborators at NIBSC used a PBS based buffer containing 20 mM of the chelating agent EDTA when purifying their empty capsids. A rather unusual addition as magnesium chloride is known to stabilise Sabin polio vaccine (Mirchamsy et al., 1978). We analysed its effect on the antigenic switch during increased temperature and it became clear that EDTA supports the PV3 SktSC8 VLPs in their D form but concentrations less than 20 mM did not increase capsid stability. Also type 1 and type 2 mutants showed increased thermostability when 20 mM EDTA was present. Preliminary data, which are not presented in this thesis, showed that

EDTA has a stabilising effect only on the mutants but not on the wt VLPs. It is therefore hard to say what exactly the key stabilising factor is. We assumed the removal of Ca^{2+} by EDTA could possibly have a positive effect on the stabilised VLPs but when they were transferred into a buffer without divalent cations no increased stability was observed. Possibly useful could be adjusting the concentration of divalent cations in the buffer, especially Ca^{2+} , which might result in even more resistant particles. Also I would like to experiment with even higher concentrations of EDTA and see if that would further increase capsid integrity.

High resolution cryo-EM resolved the structure of the PV3 SktSC8 VLPs to 3.6 Å resolution illustrating its similarity to wild-type polio 3 Sabin strain and demonstrating that recombinant plant-expression of polio VLPs produces authentic particles with the correct antigenic features. The PV3 SktSC8 structure does not show strong density for a pocket factor in the VP1 pocket. However, it is unlikely that the VP1 pocket is empty; because the pocket has not collapsed (structure is native antigenicity). Weak density is observed at very low cryo-EM density map contour levels. This suggests that the VP1 pocket is occupied with a heterogeneous mixture of pocket factors, acquired from the plant cell expression system. Indeed, when the PV3 SktSC8 VLP is mixed with a well-known pocket binding compound (GPP3), a complex can be formed. The structure of PV3 SktSC8 + GPP3 shows clear, unambiguous density for the pocket-binding molecule in the VP1 pocket. The structure of PV3 SktSC8 + GPP3 is unchanged in other aspects compared to PV3 SktSC8 without GPP3, and both structures display a native antigenic conformation. It is possible to soak pocket factor compounds into the VP1 pocket of recombinant plant expressed polio VLPs. This suggests an additional method for further, enhanced stabilisation of synthetic polio vaccines.

In summary, plants successfully express stabilised mutants VLPs of all three serotypes of PV. The presented data suggest effective production of stabilised VLPs in plants which demonstrate promising results. However, more work has to be invested in generating uniform samples. Furthermore, the reasons behind the difference between L cell produced empty capsids and the plant-produced VLPs have to be further examined.

Chapter 6: AN ALTERNATIVE PRODUCTION SYSTEM

6.1 INTRODUCTION

The previous chapter described the successful synthesis and purification of immunogenic, stabilised PV VLPs in whole *N. benthamiana* plants using transient expression, underlining the great potential of this expression platform. However, critics complain about the need of large greenhouses and unusual techniques and methods when using whole plants as a production system, despite the fact that companies like Medicago or Kentucky BioProcessing, Inc. (KBP) have proved the feasibility of industrial production of pharmaceuticals using this approach. The currently used systems for large-scale manufacturing like mammalian cells, yeast or bacteria cell cultures grow in bioreactors and their handling is backed by years of cumulative experience and process optimisation. Yet working with plant-based systems does not necessarily demand big investment in greenhouses or adopting completely new strategies if plant cell suspension cultures are used. The handling of plant cell suspension cultures is very similar to the other more established systems and therefore possibly more attractive to industry. With the licensed PMT Protalix's Elelyso, plant suspension cultures have proven their capability of producing a commercially available pharmaceutical.

Plant suspension cultures combine the advantages of whole plants with those of traditional fermenter systems. They grow in contained, controlled and sterile environment with medium costs (as defined in Table 6.1.1). The cultivation strategies are versatile with almost no limitation for mass culture (Santos et al., 2016). For a comparison between whole plants, plant cell suspension cultures and mammalian cell cultures see Table 6.1.1 below.

Table 6.1.1: Comparison among the available systems for biopharmaceutical production. Cost: Low, \$20–100/g; Medium, \$50–1000/g; High, \$1000–10,000/g (Santos et al., 2016).

System	Contamination risk	Scalability	Cost
Whole plant systems	Low	Very high	Low
Plant cell suspension	Very low	High	Medium
Mammalian cells	High	Medium	High

Bright Yellow-2 (BY-2) is a widely used tobacco cultivar which was developed in 1968 by the Japan Tobacco Company. It can multiply up to 100-fold within 7 days with a doubling time of 16-24 h under ideal conditions (Santos et al., 2016). They are grown in the dark which reduces energy costs for production. Transformation with *A. tumefaciens* has proven to be highly efficient (Nagata et al., 1992); allowing the synthesis of several biopharmaceuticals. Some are listed in the following Table 6.1.2:

Table 6.1.2: Biopharmaceuticals produced in BY-2 cell suspension culture. FW = fresh weight, TSP = total soluble protein (Santos et al., 2016).

Protein	Indication	Yield
Hepatitis B Surface Antigen (HBsAg)	Hepatitis B vaccine	6.5 µg/g FW
Human Growth Hormone	Growth hormone	Up to 35 mg/l
EPO	Tissue protective function	Low
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	Production of white cells	Up to 250 µg/l
IL-4	Immunoregulation	0.18 µg/l
IL-12	Immunoregulation	Up to 160 µg/l
α -HBsAg Mab	Hepatitis B antibody	Up to 15 mg/ l
Norwalk virus capsid protein	Acute gastroenteritis vaccine	Up to 1.2% TSP

The Table above demonstrates the versatility of this expression system and its potential. All these positive factors attracted my attention looking for an alternative production platform for the PV VLPs. Furthermore, BY-2 cells had already been successfully transformed with the pEAQ system and used to produce human serum albumin (Sun et al., 2011). At the VTT Technical Research Centre of Finland, Dr. Anneli Ritala and Jaana Rikkinen introduced me to the stable expression of recombinant proteins in BY-2 cells. With their help I tested the system with the relatively simple NNV and successfully expressed NNV VLPs in a transgenic line of BY-2 cells. To my knowledge this was the first time assembled VLPs were expressed in a plant suspension culture. The results are presented in detail later in this chapter. However, the main aim was the expression of PV VLPs in these cells. In a workshop run by the Fraunhofer IME (Aachen, Germany) in Cape Town, South Africa (2015) I was taught by Markus Sack and his colleagues how to transiently express proteins in BY-2 cells; a technique developed and patented by Dr. Thomas Rademacher (WO2013113504). The fast and simple technique of casting BY-2 cell packs or “cookies” seemed to address some of the problems critics have with whole plants as expression hosts and therefore appeared worth investigating as a means

for the production of PV VLPs. The following Figure 6.1-1 illustrates the simplicity of the technique.



Figure 6.1-1: Making a cookie. BY-2 cells are poured into a Büchnerfunnel, which is laid out with sterile filter paper, and a vacuum is applied to remove the media. When a solid cell pack has formed it gets transferred with the filter paper into a petri dish where agroinfiltration takes place. The cell packs are incubated in a lightproof box at 28 °C with a water reservoir to keep the humidity high.

The cells are grown in Erlenmayer flasks on a shaker at 28 °C in the dark. However, cultivation strategies are versatile and range from stirred tank reactors (Doran, 1999), wave reactors (Eibl et al., 2010) to bubble columns (Terrier et al., 2007). After 3-4 days the cells have grown and multiplied and are ready to be used. Under sterile conditions the cells are poured into a Büchner funnel, which is laid out with sterile filter paper, and a vacuum is applied. When the liquid media is completely removed and the cell pack has formed it gets transferred into a petri dish. Agrobacteria harbouring the gene of interest are carefully dropped onto the cell pack until it is saturated. The cell packs are stored in a lightproof box at 28 °C with a water reservoir for the purpose of keeping the humidity high.

This chapter is about the exploration of BY-2 cells and their potential as a suitable alternative production platform for whole plants. VLPs were expressed in a transgenic BY-2 cell line but also via transient expression. Characterisation of expression is presented in the following sections as well as properties of the BY-2 cell made VLPs.

6.2 STABLE EXPRESSION OF NNV VLPs IN BY-2 CELLS

For the generation of stable transgenic BY-2 cells the same construct used for transient expression of NNV VLPs in plants (pEAQ-*HT*-NNV) was sent to Jaana Rikkinen, VTT Finland. She prepared 2-3 days old BY-2 cell cultures with 0.25 mM acetosyringone and set up agrobacteria solution carrying pEAQ-*HT*-NNV to an OD₆₀₀ of 1.0. Both were mixed

together and left to incubate at 28 °C for 2-3 days. Afterwards the BY-2 cells were transferred onto solid MS media with appropriate antibiotics (25 ppm kanamycin, 500 ppm carbenicillin and 500 ppm vancomycin) and left in the darkness at 28 °C. After 2-3 weeks small calli had grown which were tested via PCR using the primer pair C1/C3 (listed in Appendix 1) for the gene of interest. When I arrived at VTT we tested in total 15 calli positive for the NNV coat protein gene and therefore further analysed them for protein expression. Small scale protein extraction was carried and analysed on a Western blot using anti-NNV antibody.

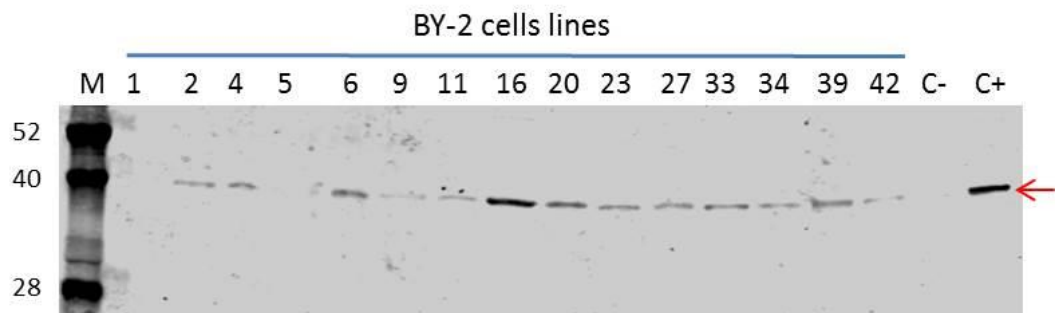


Figure 6.2-1: Analysing BY-2 cell lines for NNV coat protein expression. Small scale protein extraction was carried out on 15 BY-2 cell lines and checked for NNV coat protein content via immunodetection using anti-NNV (ab26812) antibody. C- = BY-2 cells. C+ = plant-made NNV VLPs. Red arrow indicates position of the coat protein.

All but cell line 1 and 5 showed detectable levels of NNV coat protein in the Western blot above (Figure 6.2-1). The cell lines showed differences in expression level of the desired protein. BY-2 cell line 16 displayed the highest concentration of NNV coat protein and thus was chosen for larger scale VLP production.

To check for assembled NNV VLPs, 500 ml of MS media was inoculated with BY-2 cell line 16 and after 3.5 days the cells were harvested. They were homogenised using a mortar and pestle and after a clarification spin the VLPs were purified through a discontinuous sucrose cushion. Each sucrose fraction was retrieved after ultracentrifugation and analysed on a Western blot using anti-NNV (ab26812) antibody. The Western blot is displayed in Figure 6.2-2 A.

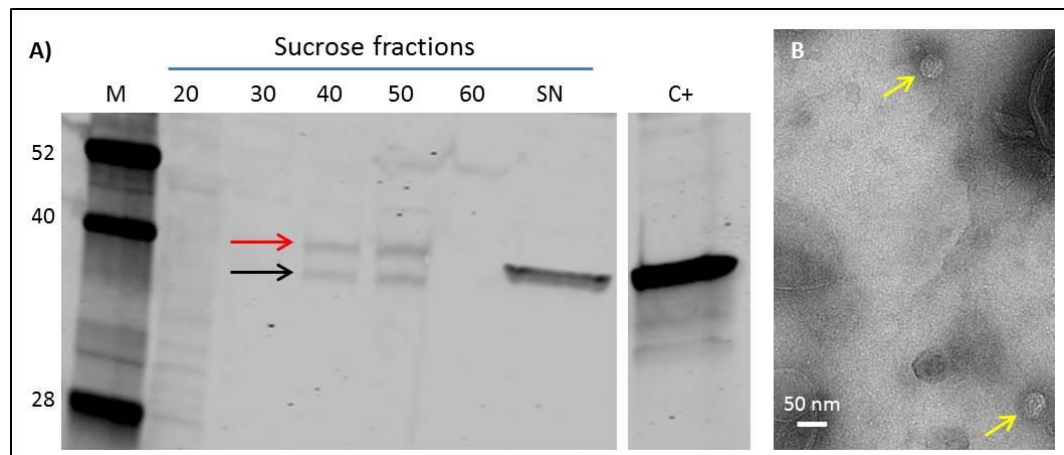


Figure 6.2-2: NNV VLP purification from transgenic BY-2 cell line. **A)** The VLPs were purified through a discontinuous sucrose gradient of 20%-60% (w/v), each fraction was retrieved and analysed on a Western blot. SN = Supernatant. C+ = plant-made NNV-VLPs. Anti-NNV (ab26812) antibody. M = Protein ladder, molecular weights indicated. C+ = plant-made PV3 SktSC8 VLPs. Red arrow indicates NNV coat protein alpha. Black arrow indicates mature NNV coat protein beta. **B)** TEM image of NNV VLPs purified from transgenic BY-2 cells. Yellow arrows indicate NNV VLPs.

The Western blot showed a weak signal for the NNV coat protein in the 40% (w/v) and 50% (w/v) sucrose fractions. Specifically, two bands could be seen (indicated by a red and black arrow in Figure 6.2-2 A), an observation I made occasionally when expressing NNV VLPs transiently in plants; a possible explanation for this has been discussed earlier in this thesis (see section 3.4). There was also a strong signal in the SN, which suggests the presence of non-assembled coat protein. The 40% (w/v) and 50% (w/v) sucrose fractions were combined and dialysed overnight. TEM analysis showed the first NNV VLPs made in transgenic BY-2 cells (Figure 6.2-2 B).

Summary of section 6.2: Generating a transgenic BY-2 cell line is relatively simple, but nonetheless time-consuming technique compared to transient expression. A transgenic BY-2 cell line successfully produced NNV VLPs.

6.3 PROOF OF EXPRESSION FOR PV VLPs

In order to express all three wild-types and mutants, agrobacteria were prepared exactly the same way as for the infiltration of plants (see section 2.3.2). Agrobacteria harbouring the respective P1 gene were diluted to an OD₆₀₀ of 0.8 and agrobacteria

carrying the gene for the proteinase were diluted to an OD_{600} of 0.4 in MMA buffer. Both solutions were mixed at equal ratios and dropped onto the cell pack. After 5 days incubation at 28 °C a small scale protein extraction was carried out and after separation on a SDS-PAGE gel the protein was transferred onto a nitrocellulose membrane.

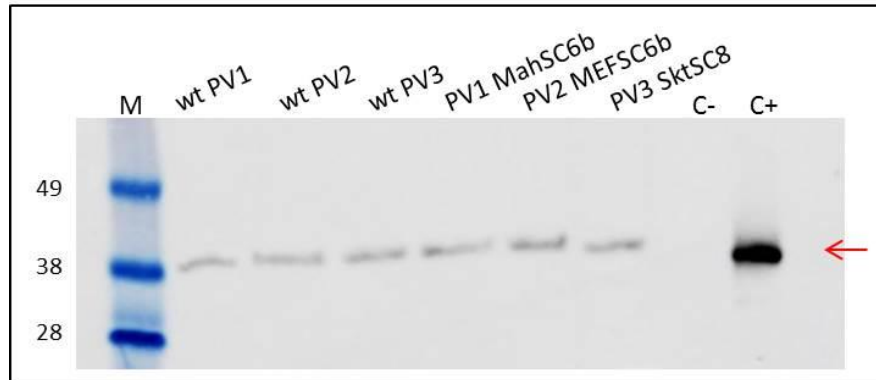


Figure 6.3-1: Proof of VP1 expression in BY-2 cells. Agrobacteria carrying the respective P1 gene were mixed with agrobacteria containing the proteinase. They were dropped onto the cell pack and incubated for 5 days at 28 °C in a lightproof box. Small scale protein extraction was carried out and the results were analysed via immunodetection. Anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C- = pEAQ-*HT-EV*. C+ = plant-made PV3 SktSC8 VLPs. Red arrow indicates VP1.

The Western blot above (Figure 6.3-1) demonstrates the successful expression and proteolytic processing of the P1 regions of all the wt PV serotypes and their respective stabilised mutants. VP1 appeared equally strong expressed in all six samples.

With the aim of identifying the time of peak PV protein expression in the cells and to compare it to plants, a time course experiment was carried out with all three serotypes and their respective stabilised mutant. A BY-2 cell pack was formed and suspension of Agrobacterium containing plasmids encoding P1 and 3CD added. Equal amounts of cells were sampled every day using a cork borer. As a negative control Agrobacteria containing pEAQ-*HT-EV* (empty vector control) were added to the BY-2 cells while the positive control was a sample of PV3 SktSC8 VLPs purified from plants. The protein content was analysed on a Western blot using anti-VP1 MAB8566 antibody.

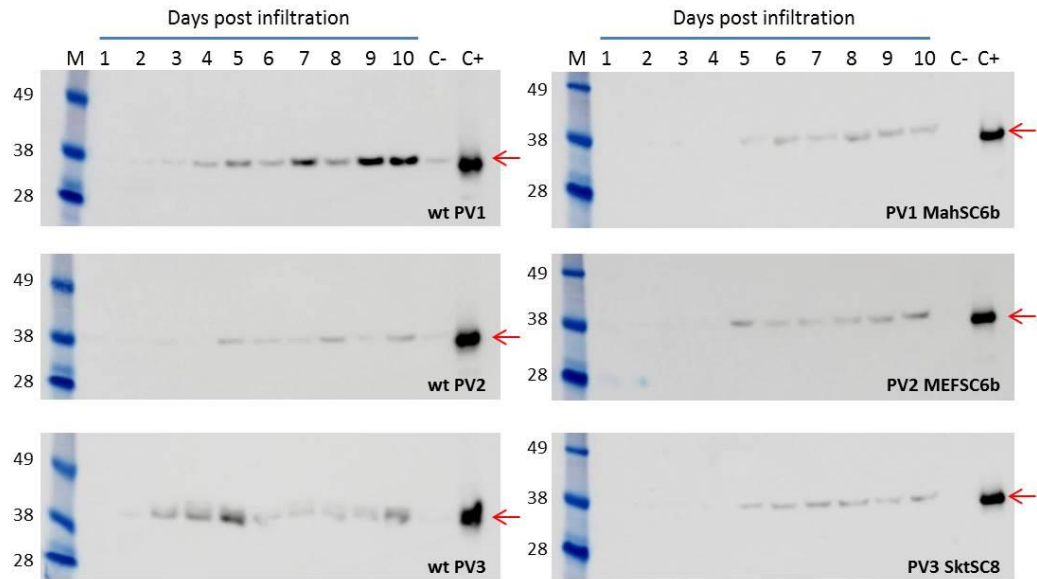


Figure 6.3-2: Expression of PV VLPs in BY-2 cell packs over time. All three serotypes and the respective mutants were expressed in BY-2 cell packs. With a cork borer the same amount of cells was sampled for 10 days and the protein content analysed for VP1 via immunodetection. Anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C- = pEAQ-HT-EV. C+ = plant-made PV3 SktSC8 VLPs. Red arrows indicate position of VP1.

The Western blots showed VP1 expression starting at 5 dpi, only wt PV1 and wt PV3 displayed detectable levels of VP1 2-3 days after infiltration. In the wt PV1 sample the level of VP1 increased during the time of incubation whereas with the other types the amount of VP1 stayed relatively constant (after day 2). Based on these results it is difficult to determine if longer incubation times would increase the protein/VLP amount in the cells. It would be worth repeating this time course experiment and also increase the time over which sampling took place to confirm these results. In most types the concentration of protein stayed constant from day 5 to day 10 after infiltration which is in contrast to the expression pattern in whole plants, where the protein level reduces on day 7-8 after infiltration.

In order to see if VLPs are able to assemble in BY-2 cells the experiment was scaled up and a VLP-purification was carried out. So far only the three stabilised mutants below have been analysed for VLP assembly.

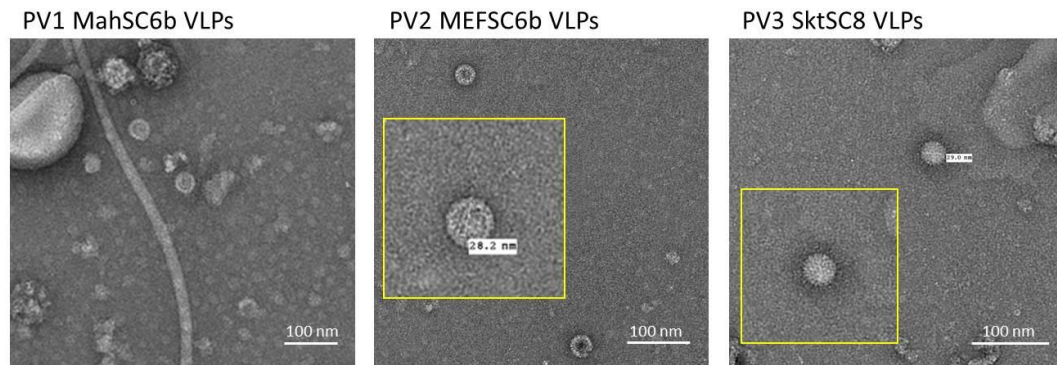


Figure 6.3-3: TEM images of stabilised VLPs made in BY-2 cells. PV1 MahSC6b VLPs, PV2 MEFSC6b VLPs and PV3 SktSC8 VLPs visualised by TEM in negative stain.

The TEM images above show that correctly assembled VLPs of PV1 MahSC6b, PV2 MEFSC6b and PV3 SktSC8 can be produced using the cell pack technology. The VLPs were of expected size and shape; no obvious differences were visible compared to plant-produced particles. However, in the following section plant-made VLPs are compared in more detail to those produced in BY-2 cells and the differences are highlighted.

Summary of section 6.3: Transient expression of PV VLPs in BY-2 cells is successful and correct assembly was proven.

6.4 WHOLE PLANTS VS. BY-2 CELLS

During extraction and purification abnormalities in sedimentation behaviour were identified in the cell pack produced VLPs which triggered a direct comparison between the PV VLPs produced in *N. benthamiana* leaves and those expressed in BY-2 cell packs. As an example PV3 SktSC8 was chosen because of its stability and reliability. For the purpose of this experiment BY-2 cell packs were formed and infiltrated with pEAQ-HT-PV3 SktSC8-P1 and pEAQ-3CD; the same constructs were co-infiltrated into *N. benthamiana* leaves. The cell packs and plants were harvested 6 dpi and homogenised using either a mortar and pastel for the BY-2 cells or a blender for the infiltrated leaf material. For the purification an identical protocol was followed. The samples were either purified over a sucrose cushion made up out of 5 ml 25% (w/v) sucrose and a 1 ml bottom fraction containing 70% (w/v) sucrose or through a discontinuous sucrose gradient (20-60% w/v) as shown in Figure 3.2-1. Each fraction was analysed for VP1 on a Western blot using anti-VP1 MAB8566 antibody.

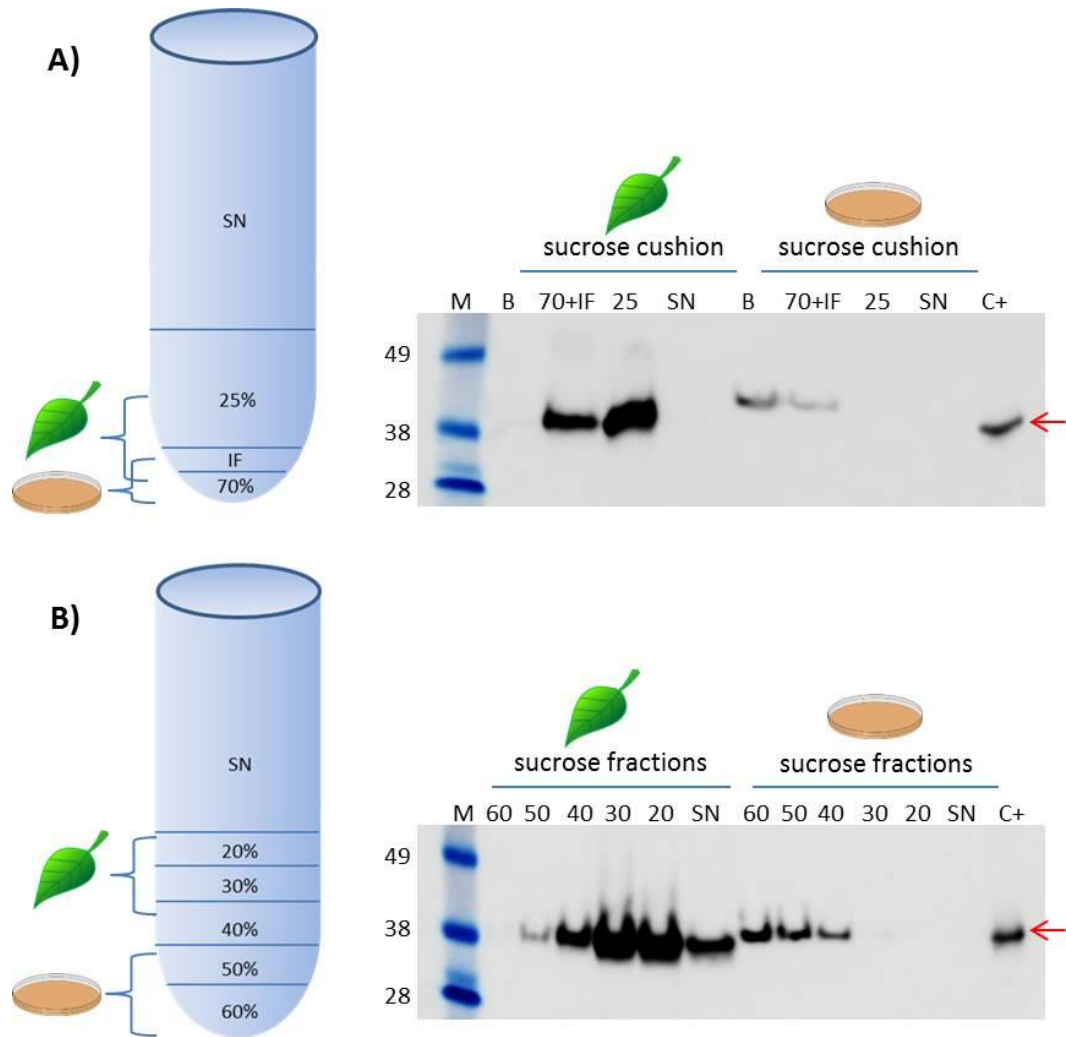


Figure 6.4-1: Comparison of plant-produced- with BY-2 produced PV VLPs. PV3 SktSC8 VLPs were extracted from plants or BY-2 cells and separately purified. On the left a schematic drawing represents the make-up of the gradient and where the VLPs were located; either produced in plants (represented by the leaf) or expressed in BY-2 cell packs (illustrated by the coloured petri dish). **A)** The VLPs were purified through a sucrose cushion, the fractions retrieved and analysed on a Western blot: the bottom 500 µl fraction (B), the 70% and interface (IF), the 25% and the supernatant (SN). **B)** The VLPs were purified through a discontinuous sucrose gradient of 20%-60% (w/v), each fraction was retrieved and analysed on a Western blot. SN = Supernatant. Anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = plant-made PV3 SktSC8 VLPs. Red arrows indicate VP1.

The results above illustrate slight differences in the sedimentation behaviour between plant-made and BY-2 cell-made PV VLPs (Figure 6.4-1). The sketch of the gradients show where most VLPs were located, either expressed in plants (represented by a leaf) or in BY-2 cells (represented by a petri dish). In both the sucrose cushion and the sucrose gradient, the VLPs expressed in BY-2 cells sedimented further in the gradients than those produced in whole plants. Additionally, when expressed in cell packs, the VLPs were of higher consistency: the signal of VP1 detected in Western blots was spread over fewer fractions in the sucrose gradient (Figure 6.4-1 B). It is also noticeable that in the

sucrose gradient analysis of the BY-2 cell-made VLPs (Figure 6.4-1 B), no signal could be detected in the SN. Signal in this fraction can be an indication of non-assembled protein or broken particles. In order to check particle shape and size they were visualised by TEM. No yield comparison has been carried out yet.

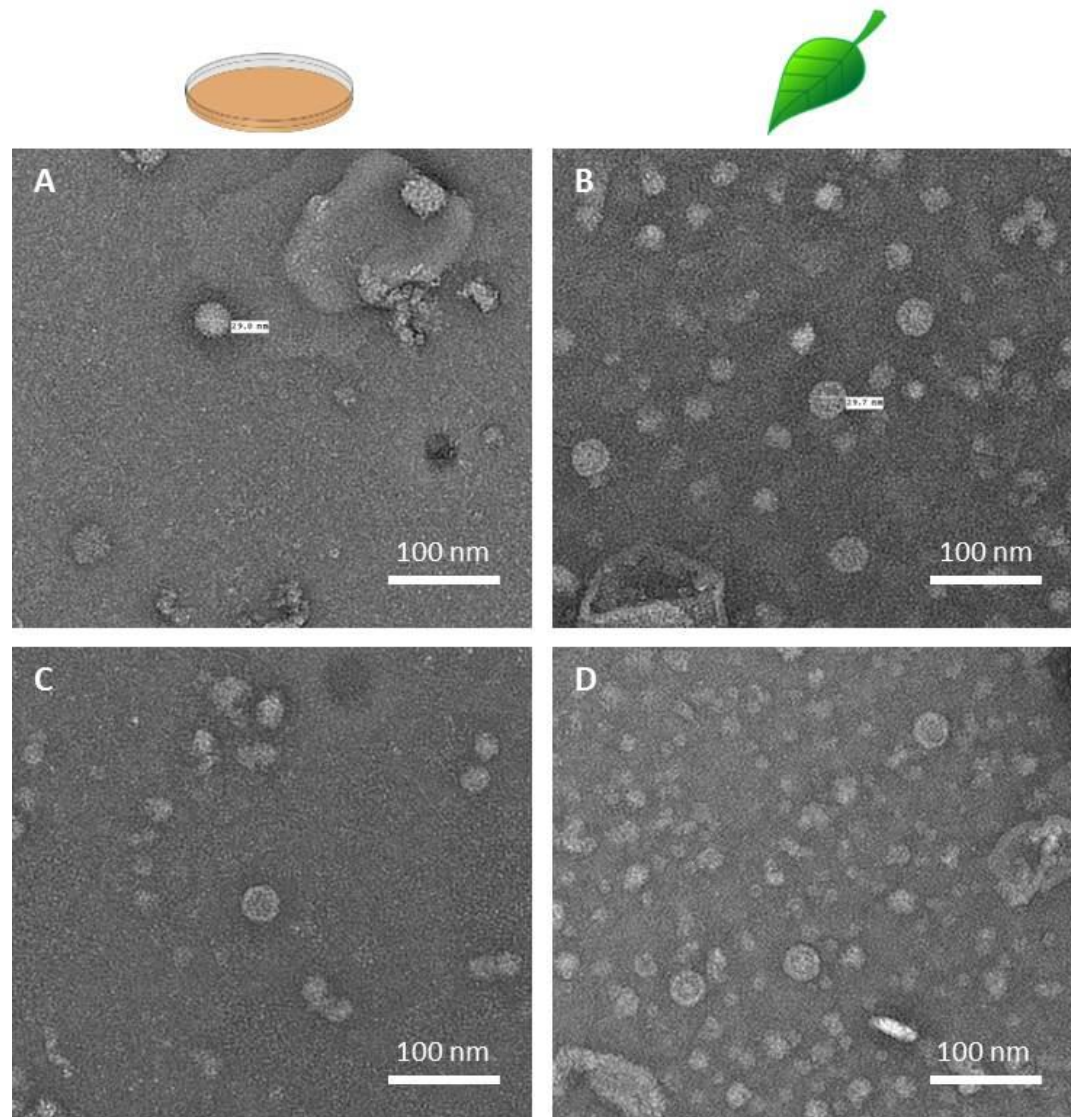


Figure 6.4-2: TEM images of PV3 SktSC8b VLPs made in either BY-2 cells or whole plants. A+B) VLPs were purified by sucrose cushion. **C+D)** VLPs were purified by sucrose gradient. On the left the VLPs were produced in BY-2 cells (represented by the petri dish) and on the right in plants (represented by the leaf).

Even though the VLPs produced in the two systems showed differences in sedimentation behaviour, they appeared even and identical under the TEM. The VLPs were of the right size (27-30 nm in diameter) and the usual shape. Again, it is hard to tell if the VLPs were empty or if they encapsulated host nucleic acid.

Summary of section 6.4: PV VLPs expressed in BY-2 cells sediment more quickly than those produced in whole plants and they are also more uniform. When visualised by TEM, no differences in shape and size could be detected implying the BY-2 cell-produced particles are denser.

6.5 IMMUNOLOGICAL PROPERTIES OF BY-2 CELL PRODUCED PV2 MEFSC6B VLPs

For a slightly larger scale production, cell packs were formed out of 300 ml of BY-2 cells and inoculated with PV2 MEFSC6b and the 3CD proteinase. After 9 days incubation at 28 °C the cells were homogenised and purified through a sucrose cushion followed by a Nycodenz gradient. Based on previous experiments and the high density of BY-2 cell-made VLPs (section 6.4), the sucrose cushion was slightly altered to start with a 35% (w/v) sucrose layer instead of 25% (w/v) with the aim to improve sample purity.

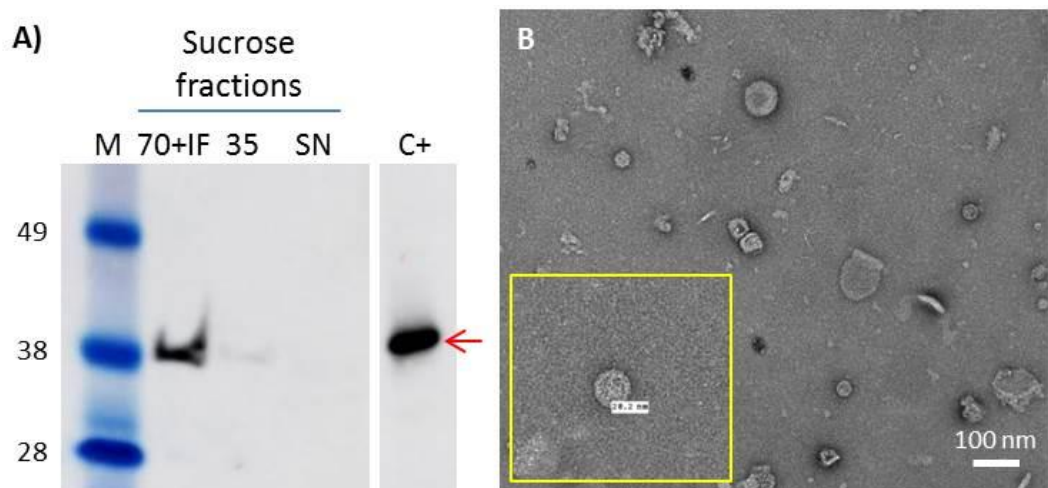


Figure 6.5-1: Purification of PV2 MEFSC6b VLPs from BY-2 cells. A) The clarified cell extract was purified through a sucrose cushion made out of 5 ml of a 35% (w/v) sucrose solution and a 1 ml 70% (w/v) sucrose bottom fraction. Each fraction was analysed for VP1 content on a Western blot using anti-VP1 MAB8566 antibody. M = SeeBlue Plus 2 with molecular weights indicated. C+ = plant-made PV3 SktSC8 VLPs. Red arrow indicates VP1. B) PV2 MEFSC6b VLPs made in BY-2 cells visualised by TEM in negative stain.

The Western blot showed a strong signal in the bottom part of the sucrose cushion. This fraction was dialysed and further purified through a standard Nycodenz gradient (as described in section 2.6.3). The PV2 MEFSC6b VLPs were visualised by TEM in negative stain (Figure 6.5-1 B). The sample was still contaminated even though the VLPs have been through two purifying gradients. Obtaining a clean sample of BY-2 cell-made VLPs

will clearly require alterations and improvements to the extraction and purification strategy.

Helen Fox (NIBSC) used a non-competitive sandwich ELISA assay to measure the D-antigen content of the BY-2 cell made PV2 MEFSC6b VLPs (Singer et al., 1989). Briefly, two-fold dilutions of antigen were captured with a serotype-specific polyclonal antibody, then detected using serotype-specific, D antigen (Mab 1050)-specific monoclonal antibody followed by anti-mouse peroxidase conjugate. No C specific type 2 antibody was available. The D antigen content of each test sample was evaluated against a reference of assigned D antigen content (Fuchs et al., 2003) by parallel line analysis (Combistats).

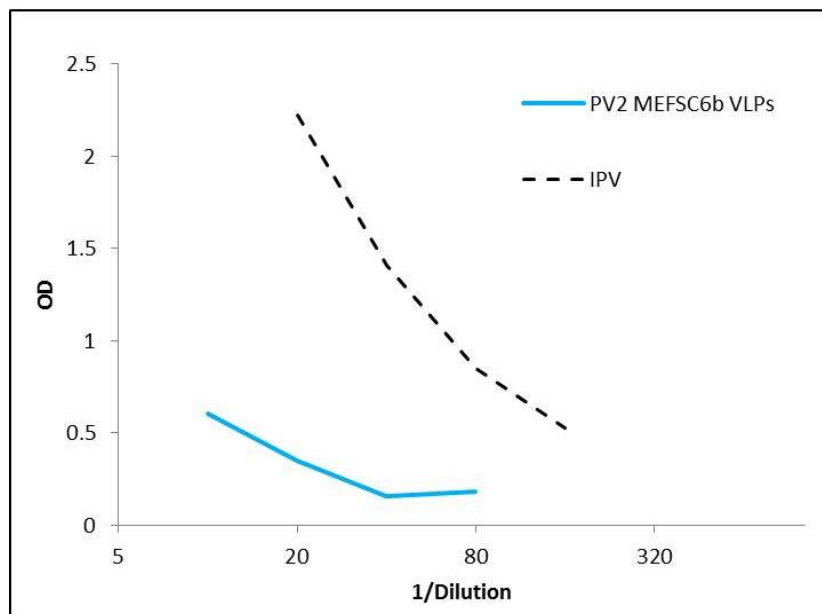


Figure 6.5-2: Potency of PV2 MEFSC6b VLPs made in BY-2 CELLS. Non-competitive sandwich ELISA assay for the measurement of D antigen content in PV2 MEFSC6b VLP sample made in BY-2 cell packs using Mab 1050 (D antigen). IPV = European Pharmacopeia Standard for IPV.

In the ELISA a small amount of D antigen was measured. The sample, which derived from 300 ml BY-2 cell culture, contained approximately 4 DAgU/ml. A satisfying result especially because type 2 expresses to only a very low level in comparison to the other types but still a small amount of D antigen could be produced.

Summary of section 6.5: D antigenic PV2 MEFSC6b VLPs can be produced in BY-2 cells.

6.6 DISCUSSION

The results presented in this chapter are still preliminary but point towards the possibilities that BY-2 cells offer as an alternative expression system for pharmaceuticals including, in this specific case, for VLP-based vaccines. This chapter demonstrates the successful expression of all PV serotypes and correct particle assembly of the stabilised mutants PV1 MahSC6b, PV2 MEFSC6b and PV3 SktSC8 in BY-2 cells. The PV VLPs are expressed over a long period of time at a constant level, compared to transient expression in plants where the concentration decreases after 7-8 days: possibly an advantage of BY-2 cells over whole plants.

A larger scale production has only been attempted with PV2 MEFSC6b VLPs and the sample tested positive for D antigen. In this experiment 300 ml of MS media was inoculated with BY-cells, a volume that could easily be increased. There is almost no limitation for mass culture with BY-2 cells. Also casting the cell packs can be scaled up and experiments are currently ongoing at the Fraunhofer IME Aachen on improving this large scale technique. Quite simply with the use of bigger cookie casting equipment larger cell packs could be formed.

In case the cell pack technique appears too elaborate one could generate a transgenic BY-2 cell line which constantly expresses the desired protein or VLP. Once a stable line has been established the workflow is simple, quick and easily scalable. Working with transgenic BY-2 cells is very similar to other commonly used tissue cultures and for that reason referred to as the “HeLa-cells in the biology of higher plants” (Nagata et al., 1992). In this chapter successful expression of NNV VLPs in transgenic BY-2 cells is demonstrated. Future work will include larger scale expression in order to do in depth analysis of these transgenically produced VLPs, comparison to plant-produced NNV VLPs and for animal studies. A transgenic BY-2 cell line could also be generated for PV VLPs. Combining the polyprotein P1 with the proteinase in a single expression cassette would facilitate the successful creation of a line expression both proteins.

Interestingly, differences in density could be observed between BY-2 cell-produced VLPs and plant-made VLPs. When purified through density gradients the VLPs, made in the BY-2 cell culture, sedimented much further down the gradient than those expressed in plants. Their density appeared of higher consistency whereas plant-expressed PV VLPs

usually spread over several fractions in the density gradients. The reason behind this is still not understood but possibly encapsulation of host nucleic acid could lead to increased density. Previous experiments showed that plant-made PV VLPs do not take up host RNA or DNA, however in TEM images mixtures of empty and full looking particles can be seen. Also high resolution microscopy revealed that the plant-made particles are loaded to various degrees with unknown material. The environment in BY-2 cells might promote uptake of random host nucleic acid or even protein by the PV VLPs. High resolution microscopy could help solving this uncertainty. It would also be interesting to see in high resolution images if BY-2 cell-made PV VLPs carry pocket factors. The existence of a pocket factor could have an impact on density.

In order to promote BY-2 cell suspension cultures, improvements to the extraction and purification methods will have to be undertaken. The BY-2 cells are surprisingly tough and therefore breaking them open is a challenge. Using mortar and pestle is suitable only to a limited extent but alternatives like cell disruptors could facilitate the extraction of VLPs from the cells. Experiments involving freezing the cells with liquid nitrogen prior to attempting extraction have been conducted and this approach improved the VLP yield slightly. The stabilised PV VLPs withstood the harsh treatment of liquid nitrogen (data not shown). Purification has also been an issue and more work has to be invested in this area in the future.

When aiming for commercial production of pharmaceuticals the yield of the product is of course of very high importance. The BY-2 cell line has been utilised as an expression host for numerous recombinant proteins mostly because of its exceptional growth rate. However, the yields in the BY-2 cells are still generally low (see Table 6.1.2), especially when compared to mammalian cell cultures which produce yields of 5 g/l (Walsh, 2010). Low protein productivity remains a major obstacle that limits extensive commercialisation of plant cell suspension cultures as production platforms. High levels of contaminants in the samples have hindered credible measurements of VLP concentration and therefore no VLP yield has been determined yet. Further development of plant cell cultures is needed in order to meet the general industrial demand of grams per litre product titre.

Chapter 7: FINAL DISCUSSION

Overall, the work presented in this thesis demonstrates the potential of plant-based production of VLP vaccine candidates. First, the comparatively simple NNV VLP was expressed, which has never been done in plants before. The capsid of this virus consists of a single type of coat protein that self-assembles into a stable T = 3 icosahedral capsid when expressed in a variety of heterologous systems, including, as demonstrated by the work reported in this thesis, plants. Structural studies carried out by Daniel Hurdiss at the University of Leeds, revealed authentic looking plant-made particles when compared to wild-type NNV. Currently ongoing are further improvements of the structure to resolve the P-domains. Immunological studies of this VLP vaccine candidate are currently in preparation in collaboration with Dr. Richard Paley at CEFAS, Weymouth, UK. A positive outcome of the immunological studies would be a step towards the final aim of developing an oral vaccine against VNN. Possibilities like pressing plant material harbouring NNV VLPs into pellets and feeding it to the animals should be investigated in the future. This basic NNV VLP was a great starting point for the expression of the more complex PV VLPs in plants. Correct particle assembly of PV requires processing of the capsid precursor polyprotein, which was provided by the co-expression of the proteinase. The work in this thesis represents the first report of the production of immunologically effective non-infectious poliovirus-like particles in plants. The expression level of the plant-produced stabilised VLPs vary between the serotypes but levels of up to 70 mg purified VLPs per kg infiltrated plant tissue were obtained. All analysed plant-expressed stabilised mutants showed improved capsid stability and those that were tested protected transgenic mice from infectious virus. The approach of stabilised VLP-based vaccines could be adopted for related picornaviruses e.g. foot-and-mouth disease virus (FMDV) or hepatitis A virus (HAV). In summary, this work demonstrates the potential of plants as a production platform for various VLP-based vaccines. Furthermore, I could show the successful expression of all wild-types and three stabilised PV VLPs, as well as stable expression of NNV VLPs in BY-2 cells, an alternative expression system to whole plants.

Despite the many advantages of plants as expression systems (discussed in section 1.1.1) they are still overshadowed by other production platforms like mammalian cell

cultures, yeast or *E. coli*; mostly because these systems are backed by decades of experience and their simple growth conditions compared to the demands of plants. However, whole plants compared with traditional biomanufacturing platforms that grow in bioreactors, demand significant less capital investment and show >50% reduction in cost of goods compared with published values at similar production scales (Nandi et al., 2016). Slowly change is happening and companies realise the potential lying within plants as production hosts for pharmaceuticals (Lomonossoff and D'Aoust, 2016).

Plant suspension cultures are a potentially attractive alternative to whole plants or even traditional microbial or animal cell cultures as production platforms for pharmaceutical production. In comparison with whole plants grown in greenhouses, cell suspension cultures offer tighter control over the growth conditions in standardised bioreactors, therewith enabling higher batch to batch consistency and a greater opportunity of adhering to cGMP. Also, they do not require light which reduces the cost of growing the cultures. In contrast to mammalian cells, the plant cell cultures grow at lower temperatures (20-28 °C vs. 37 °C), yet another cost reducing factor. Important also is the feasibility of downstream processing which is simplified in plant cell suspension cultures by the lack of waxes in plant cell cultures (Reuter et al., 2014).

Plant cell suspension cultures have been utilised as an expression host for numerous recombinant proteins (listed in Table 6.1.2). I have already mentioned Protalix's Eleyso, the first PMT protein approved by the FDA for human use (see section 1.1.2). Yet another great example for successful commercial production of pharmaceuticals in plant-cell culture is Phyton Biotech, utilising the Plant Cell Fermentation (PCF®) platform technology. Phyton is the largest supplier of high quality Paclitaxel and Docetaxel (chemotherapy medication) in the world, producing nearly 500 kg Paclitaxel annually under strict GMP conditions. They propagate their plant cell cultures in reactors that can hold between 80 and 7500 litres with a total capacity of 220 000 litres, especially designed for large scale plant cell cultivation (Phyton, 2016).

The feasibility of large scale plant-cell culture cultivation has been confirmed by the success of Phyton Biotech's Paclitaxel and Docetaxel. In order to estimate costs of this production system and to compare it to whole plants I contacted Dr. Tanja Holland (IME Fraunhofer Aachen, Germany) who is an expert in the plant-cell pack technology. The MS media used for the propagation of BY-2 cells costs 0.50-1.00 € per litre. When utilising a 30 litre continuous fermentation reactor, which needs daily addition of 15

litres fresh media, for the propagation of plant suspension cells the costs for media come to approximately 450 € per month and it takes 16 h per month of man power to keep the process going. From this reactor 200 litres or 22 kg cell culture wet weight can be harvested per month; this correlates with 250-500 *N. benthamiana* plants. However, for the growth of 250-500 plants an estimate of 25-50 m² are needed, whereas the fermenter requires only a base of 3-4 m². Furthermore, getting plants from seed to a mature plant ready to be infiltrated takes 5-6 weeks (2 weeks germination and 3-4 weeks growing), whereas BY-2 cells multiplying 80- to 100-fold over 1 week in optimal conditions (Nagata et al., 1992). In summary plant cell cultures are inexpensive to grow and need less space and time than whole plants. However, comparing costs of whole plants and plant cell suspension cultures is not straight-forward because the yields of recombinantly produced proteins differ between both systems. In general, the obtainable yield of proteins produced in the BY-2 cell line is low (see Table 6.1.2). Thus, further development is needed in order to meet the general industrial demand of grams per litre product titre.

In conclusion, plants, whole or as cell suspension culture, are a promising platform for the production of VLP-based vaccines. The work presented in this thesis provides evidence for the potential of the plant-expressed stabilised PV mutants. However, more work has to be invested in getting 3 potent and reliable stabilised PV VLPs. Especially type 1 and type 2 have to be further analysed and optimised. Getting this project towards commercial production is the main future goal. It would be a great achievement to replace the current polio vaccines with plant-produced stabilised VLPs. It would also be a big step forwards for plant-based production of biologics and a great advertisement for this platform. The production of this VLP-based polio vaccine would be a non-profit venture, but nevertheless a big honour. Unfortunately, traditional expression hosts are still dominating the pharmaceutical market but I hope that in future plants will convince big pharmaceutical industries to invest into this technique. The results presented in this thesis are certainly a step into the right direction.

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APPENDIX 1: LIST OF PRIMERS

Details of the primers used for amplification of inserts, verification of clones and introduction of mutations in the work described in this thesis are presented below.

For verification of inserts cloned in between the 5`UTR and 3`UTR of pEAQ-*HT*:

C1	AACGTTGTCAGATCGTGCTTCGGCACC
C3	CTGAAGGGACGACCTGCTAAACAGGAG
5`UTR	GTTTTCCCGTGGTTTTCGAACTTG
3`UTR	GCACACCGAATAACAGTAAATTCAAATAAAG

For SDM of P19 to generate P19m:

P19-R43W-SDM-F	CGAGTTGGACTGAGTGGTGGCTACATAACGATGAG
P19-R43W-SDM-R	CTCATCGTTATGTAGCCACCACTCAGTCCAACCTCG

For SDM of AUG 161 to generate non-*HT* pEAQ:

Rev AUG161_F	GCTTCGGCACCAGTACAATGTTTTCTTTCACTG
Rev AUG161_R	CAGTGAAAGAAAACATTGTACTGGTGCCGAAGC

For SDM of PV1-P1 to generate pEAQ-*HT*-PV1 Q94C:

PV1 Q94C_F	CTCTACCATCACCCTGCGAGGCTGCTAACTCTGTTG
PV1 Q94C_R	CAACAGAGTTAGCAGCCTCGCAAGTGGTGATGGTAGAG

APPENDIX 2: SEQUENCES

Sequences utilised in this work, codon-optimised for the expression in *N. benthamiana* and synthesised by GeneArt, are listed below. Start codons are highlighted in **green**, stop codons are highlighted in **red**. The restriction sites used for cloning into pEAQ-HT, namely AgeI and XhoI, are underlined in orange and blue, respectively.

3CD

TCAAGCCGCATACCGGTAACA**ATG**GGTCCTGGTTTCGATTACGCTGTTGCTATGGCTAAGAGG
AACATCGTGA CTGCTACCACCTCTAAGGGAGAGTTCACTATGCTTGGTGTGCACGATAACGTGG
CAATTCTTCTACTCAGCTAGCCCTGGTGAGAGCATTGTGATCGATGGTAAAGAGGTTGAGAT
CCTGGATGCTAAGGCTCTTGAAGATCAGGCTGGTACTAACCTTGAGATCACCATCATCACCTGA
AGAGGAACGAGAAGTTCAGGGATATCAGGCCTCACATCCCTACCCAGATTACCGAGACTAACG
ATGGTGTGCTGATCGTGAACACCAGCAAGTACCCTAACATGTACGTTCCAGTGGGTGCTGTTAC
TGAGCAGGGTTACCTTAATCTTGGTGGTAGGCAGACTGCTAGGACCCTGATGTACAATTTCCCT
ACTAGAGCTGGTCACTGCGGTGGTGTGATTACCTGCACTGGTAAGGTGATCGGTATGCACGTT
GGTGGTAATGGTTCTCACGGTTTCGCTGCTGCTCTGAAGAGGTCTTACTTCACCCAATCTAGCCA
GGGTGAGATCCAATGGATGAGGCCTTCTAAGAGGTTGGGATACCCTATCATCAACGCTCCATCT
AAGACCAAGCTTGAGCCTAGCGCTTTCCACTACGTTTTCGAGGGTGTGAAAGAGCCTGCTGTTT
TGACCAAGAACGATCCTAGGCTTAAGACCGATTTCTGAAGAGGCTATCTTCTCCAAGTACGTGGG
TAACAAGATCACCGAAGTGGATGAGTACATGAAGGAAGCTGTGGATCACTACGCTGGTCAGCT
GATGTCTCTGGATATTAACACCGAGCAGATGTGCCTTGAGGATGCTATGTACGGAACCGATGGT
CTTGAGGCTCTGGATCTTTCTACCTCTGCTGGTTACCCTTATGTGGCTATGGGTAAGAAGAAGAG
GGATATCCTGAACAAGCAGACCAGGGATACCAAAGAGATGCAAAAAGCTGCTGGATACCTACGG
TATCAACCTTCTCTTGACCTACGTGAAGGATGAGCTGAGGTCAAAGACCAAGGTGGAACAG
GGAAAGTCTAGGCTGATCGAGGCTAGCTCTCTGAACGATTCAGTGGCTATGAGGATGGCTTTTCG
GTAACCTGTACGCTGCTTTCCACAAGAACCCTGGTGTGATCACTGGTTCTGCTGTTGGTTGCGAT
CCTGATCTGTTCTGGTCTAAGATCCCTGTGCTGATGGAAGAGAAGCTGTTTCGATTTCGATTACAC
CGGATACGATGCTAGCCTTAGCCCTGCTTGGTTTGAGGCACTTAAGATGGTGCCTTGAGAAGATC
GGTTTCGGTGATAGAGTGGATTACATCGATTACCTGAACCACAGCCACCACCTGTACAAGAACA
AGACCTACTGCGTGAAGGGTGGTATGCCTTCTGGTTGCTCTGGAACCAGCATCTTCAACAGCAT
GATCAACAACCTGATTATCAGGACCCTGCTGCTGAAAACCTACAAGGGTATCGATCTGGATCAC
CTGAAGATGATCGCTTACGGTGTGATGTTATCGCTAGCTACCCTCACGAAGTTGATGCTTCTCT
GCTTGCTCAGAGCGGTAAGGATTACGGTCTTACTATGACCCCTGCTGATAAGAGCGCTACCTTC
GAGACTGTGACTTGGGAGAATGTGACCTTCTGAAGAGATTTTTTCAGGGCAGATGAGAAGTAC
CCTTCTGATCCATCCTGTGATGCCTATGAAGGAAATCCACGAGTCTATCAGGTGGACCAAGG
ATCCTAGGAATACCCAGGATCACGTGAGATCTCTGTGCCTTCTGCTTGGCATAACGGTGAGGA
AGAGTACAACAAGTTCCTGGCTAAGATCAGATCCGTGCCTATTGGTAGGGCTCTGCTTTTGCCT
GAGTACAGCACCTTTACAGAAGATGGCTGGATAGCTT**TAG**CTCGAGCTGGGCCTCATGGGCC
TTCCGCTCACTGCCGCTTTCCAG

PV1 MahSC6b

AGGCCGCATACCGGTAAACAATGGGTGCTCAGGTGTCATCTCAGAAGGTTGGAGCACACGAGAA
 CAGCAATGGTGCTTACGGTGGTAGCACCATCAACTACACCACCATTA ACTACTACAGGGATAGC
 GCTAGCAACGCTGCTTCCAAGCAGGATTTAGCCAGGATCCTAGCAAGTTCACCGAGCCTATTA
 AGGATGTGCTGATCAAGACCGCTCCTATGCTGAACTCTCCTAACATTGAGGCTTGC GGTTACAG
 CGATAGGGTGTGCAACTTACCCTGGGAACTCTACCATCACCGCTCAAGAGGCTGCTAATTCT
 GTTGTGGCTTATGGTAGGTGGCCTGAGTACCTTAGAGATAGCGAGGCTAATCCTGTGGATCAGC
 CTA CTGAACCTGAAGTTGCTGCTTGCAGGTTCTACACCCTTGATACTGTGAGCTGGACCAAAGA
 AAGCAGAGGATGGTGGTGGAAAGCTGCCTGATGCTTTGAGAGATATGGGACTGTTCCGGTCAGAA
 CATGTACTACCACTACCTGGGAAGGTCTGGTTACACTGTT CATGTGCAGTGAACGCTTCCAAGT
 TCCATCAAGGTGCTCTTGGTGTTCGCTGTGCCTGAGATGTGTCTGGCTGGTGATTCTAACACT
 ACCACCATGCACACCAGCTACCAGAA TGCTAATCCAGGTGAGAAGGGTGGAACTTTACTGGAA
 CTTTCACCCCTGATAACAACCAGACCAGTCTGCTAGAAGGTTCTGCCCTGTGGATTACCTTCTT
 GGTAAACGGAACCCTTCTGGGAAACGCTTCGTTCCCTCACCAGATTATCAACCTGAGGACCA
 ACAACTGCGCTACCCTGTGCTTCTTACGTGAACAGCCTGAGCATCGATAGCATGGTGAAGCA
 CAACA ACTGGGTATCGCTATTCTTCTCTGGCTCCTCTGAACTTCGTTCCGAATCTTCTCCAGA
 GATCCCTATCACCCCTGACCATTGCTCCTATGTGCTGCGAGTTCAACGGTCTGAGGAACATTACCT
 TGCCTAGGCTT CAGGGTCTGCCTGTGATGAATACCCCTGGTTCTAACCACTGACCGCTGAT
 AACTTCCAGTCTCCATGTGCTCTGCCTGAGTTCGATGTGACCCCTCCTATTGATATCCCTGGTGA
 GGTGAAGAACATGATGGAATTGGCTGAGATTGATACCATGATCCCTTCGATCTGAGCGCTACC
 AAGAAAAACACCATGGAAATGTACAGGGT GAGGCTGAGCGATAAGCCTCATACCGATGATCCT
 ATCCTGTGCCTGTCTGTCTCCTGCTTCTGATCCTAGGCTTTCTCACACCATGCTTGGTGAGATC
 CTGAATTACTACCCACTGGGCTGGTAGCCTGAAGTTCAC TTTTATGTTCTGCGGTAGCATGAT
 GGCTACCGGAAAGCTTCTTGTGCTTACGCTCCTCCTGGTGTGATCCTCCTAAAAAAGAAAAG
 AAGCTATGCTGGGAACCCACGTGATCTGGGATATTGGTCTGCAGTCAAGCTGCACTATGGTGGT
 GCCTTGGATTTCCAATACCACCTACAGGCTGACCATCGATGATAGCTTACCGAGGGAGGTTAC
 ATCAGCGTGTCTACCAGACTAGGATTGGTGGCCACTTAGCACCCCTAGAGAGATGGATATTC
 TGGGTTTCGTGAGCGCTTGAACGATTTCTCTGTGAGGCTTCTTAGGGATACCACCCACATTGAG
 CAGAAGGCTCTTGCTCAAGGTCTTGGTCAGATGCTTGAGAGCATGATCGATAACACCGTGAGG
 GAAACTGTGGGTGCTGCTACTTCTAGAGATGCTCTGCCAAATACCGAGGCTTCTGGTCTACTCA
 CAGCAAAGAGATTCCAGCTTACCCTGTTGAGACTGGT GCTACTAATCCTCTGGTGCCTTCTG
 ATACTGTT CAGACTAGGCATGTTGTGCAGCACAGGTCTAGGTCCGAGTCTCTATTGAGTCATTC
 TTCGCTAGAGGAGCTTGCCTGACCATCATGACTGTGGATAACCCTGCTAGCACCACCAACAAGG
 ATAAGTTGTTTCGCTGTGTGGAAGATCACTTACAAGGATACCGTGCAGCTGAGAAGAAAGCTTGA
 GTTCTTCACTTACAGCAGGTTTCGATATGGA ACTGACCTTCGTGGTGACCGCTAACTTCACCGAGA
 CTAACAACGGTCACGCTCTGAATCAGGTGTACCAAATCATGTATGTGCCTCCAGGTGCTCCTGTT
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PV1 M3delta:

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PV1 M2delta:

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PV1 M3delta:

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APPENDIX 3: PUBLICATIONS

During the course of this thesis, two reviews were published:

Marsian, J., and Lomonossoff, G.P. (2016). Molecular pharming-VLPs made in plants. *Curr Opin Biotechnol* 37, 201-206.

Steele, J.F., Peyret, H., Saunders, K., Castells-Graells, R., Marsian, J., Meshcheriakova, Y., and Lomonossoff, G.P. (2017). Synthetic plant virology for nanobiotechnology and nanomedicine. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*.