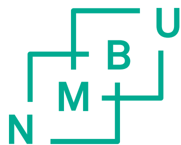

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Analysis of antibody responses in Atlantic salmon (*Salmo salar* L) vaccinated against Piscine Reovirus using antigens produced in tobacco (*Nicotiana benthamiana*)

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Plant Sciences

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Abstract

Aquaculture is emerging to be one of the most important food industries in the world, and will be a crucial part of securing food supply of fish protein for a population expected to reach close to 10 billion by 2050. Norway produced 1.3 million tons of Atlantic salmon in 2015, with a sales value of 44.4 billion NOK.

In 1999, an until then unrecorded disease emerged in Atlantic salmon held in seawater, and in 2010 the disease was linked with a novel virus Piscine Reovirus (PRV). There are currently no cell lines commercially available for *in vitro* cultivation of the virus, making it difficult to produce the virus in bulk for vaccine production. Hence, the use of plant biotechnology is being sought as an alternative for large scale production of PRV antigens for use in vaccine development.

This thesis explores the possibility of producing antigens for a vaccine against PRV using lettuce and tobacco as plant bioreactors. This is done by transient transformation via agroinfiltration. This study shows that vaccines based on PRV Sigma-1 and Mu-1 antigens produced in *Nicotiana benthamiana* were able to induce antibody responses in vaccinated Atlantic salmon (*Salmo salar* L). Therefore, future studies should seek to evaluate the efficacy of these plant based vaccines to produce long-term protective immunity in vaccinated fish.

The Sigma-1 and Mu-1 antigens produced in *N. benthamiana* and *E. coli*. used in the salmon vaccination study were provided by supervisor Prof. Jihong Liu Clarke's group at NIBIO.

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1. Introduction

1.1 Aquaculture

Aquaculture is emerging to be one of the most important food industries in the world. In 2014 the total consumption of fish was at 146.3 million tons, with more than 56 million people working in the primary sector of the industry (FAO 2016). The increase is projected to continue with a consumption of 196 million tons by 2025 of which 57% will be from aquaculture (OECD/FAO 2016).

The total volume of wild–caught fish from commercial fisheries has remained relatively stable since the late 1980s, and in 1974 aquaculture only provided 7% of the global fish consumption (FAO 2016), while 2014 was the first year that fish produced from aquaculture surpassed traditional fisheries, and this continued in 2015 (OECD/FAO 2016). This shows that aquaculture will be crucial for securing food supply of fish protein for a world population that is expected to reach close to 10 billion by 2050. In 2014, the total harvest from aquaculture was 73.8 million tonnes with a value of 160 billion US$ (FAO 2016).

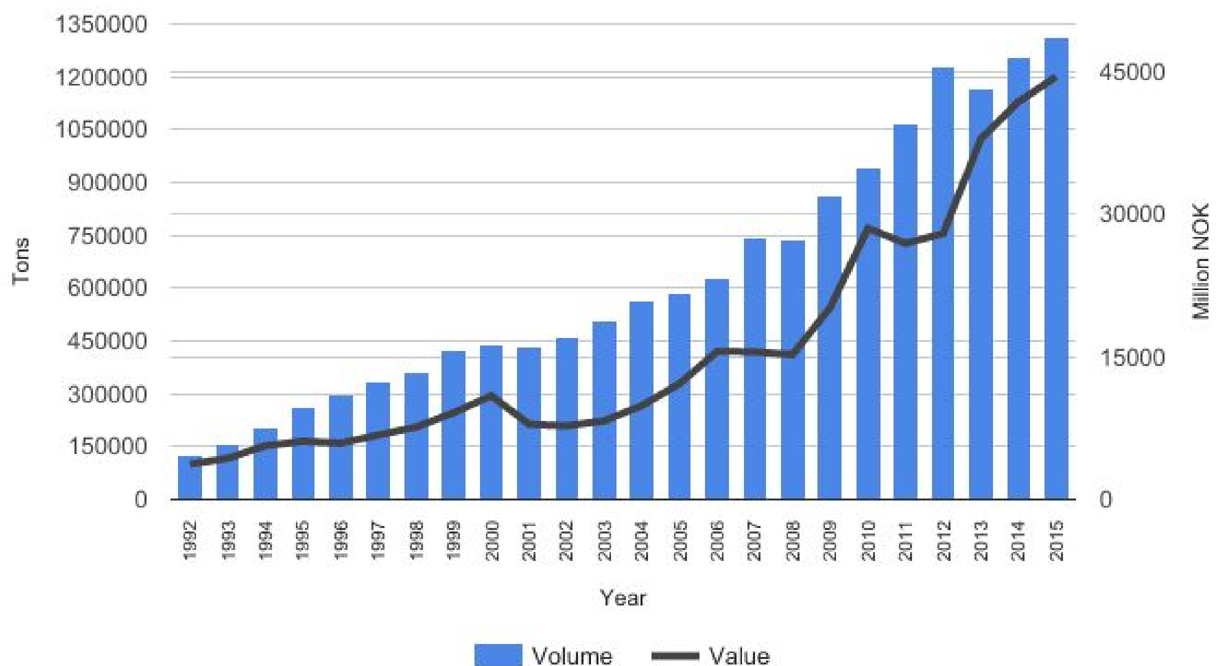


Figure 1. Atlantic salmon production in Norway.
Total production and value of Atlantic salmon (*Salmo salar* L.) in Norway from 1992-2015. Source: SSB 2016.

1.2 Atlantic salmon

Farmed Atlantic salmon (*Salmo salar* L.) plays an important role in the global production of fish in aquaculture, in tonnage and value (OECD 2016). Norway produced 1.3 million tons of salmon in 2015, with a sales value of 44.4 billion NOK (SSB 2016). In Norwegian aquaculture, Atlantic salmon is by far the most important species representing 94.5% of the total aquaculture harvest in 2015 (SSB 2016). The production in Norway has more than doubled only the last ten years (Figure 1).

1.3 Fish health in aquaculture

This intense nature of fish farming increases the risk of infectious diseases (Krkosek 2010). Fish farming has increased markedly not only in tonnage, but also in the different types of species farmed and locations used, and this expansion has led to a higher risk of spreading infectious agents (Crane et al. 2011). Diseases in aquaculture cause economic losses and can also represent a source of infection for wild species, thus having a potential impact on natural biodiversity (Morton et al. 2016, Crane et al. 2011).

Domestication of fish is relatively recent compared to domestication of animals for land-based farming, although carp farming in China dates some 4000 years back. Emergence of disease is inevitable with intense farming which results in use of antibiotics and chemotherapy for treatment. Vaccination at commercial scale as a means of disease control was not applied until the 1970s (Gudding et al. 2013).

In the wild most diseases cause no significant threat to a population, but this changes with the high density in fish farming. Disease prevention and control through vaccination is thus a logical approach (Gudding et al. 2013). In the late 70s and early 1980’s, a new disease causing substantial economic losses in salmonid sea cage farming at Hitra in Norway emerged. It was concluded that it was caused by the pathogenic bacterium *Vibrio salmonicida* (Egidius et al. 1986). Since then all salmon were vaccinated against this disease before being transferred to sea. The disease is today named cold-water vibriosis (Gudding et al. 2013). There are now many different vaccines for Atlantic salmon, most of them being water-in-oil based vaccines and administered via injection (Brudeseth et al. 2013). In 2012, more than 95% of the smolts in Norwegian salmon aquafarming were vaccinated against six different pathogens (PHARMAQ 2012). Large scale vaccination was primarily developed for the high value production of salmonids, but vaccines are now available for 17 different species, against 6 viral diseases and more than 22 bacterial diseases (Brudeseth et al. 2013). The development and use of vaccines has been one of the most important factors that has driven the success of the modern salmonid farming (Gudding et al. 2013, Brudeseth et al. 2013).

1.4 Heart and skeletal muscle inflammation

In 1999, an until then unrecorded disease emerged in Atlantic salmon held in seawater (Kongtorp et al. 2004). The affected fish showed abnormal swimming behaviour and inappetence (Kongtorp et al. 2004b). Because of the characteristic lesions found in heart and muscle tissue the disease was named Heart and skeletal muscle inflammation (HSMI). Typical

pathological autopsy findings include pale heart, yellow liver, ascites and petechiae in visceral fat (Kongtorp et al. 2004).

HSMI is seen throughout the year but occurs more frequently during spring and summer (Kongtorp et al. 2004). Salmon show symptoms 5-9 months after sea transfer, but has been reported as early as 14 days after transfer (Kongtorp et al. 2004b). In 2002, fish from 41 different Norwegian salmon farms were diagnosed with HSMI (Kongtorp et al. 2004). An outbreak with similar symptoms and high mortality among second year salmon was reported in Scotland in June 2004, as the first possible incident outside Norway (Ferguson et al. 2005).

In 2015, 135 different locations were diagnosed with HSMI in Norway (Fiskehelserapporten 2015), 101 cases in 2016 (Fiskehelserapporten 2016). Close to 100% of the fish in a caged population will have pathological changes in heart and skeletal muscle tissues (Watanabe et al. 2006), and mortality can be as high as 20% (Kongtorp et al. 2004b). The possibility of infection spreading from one cage to another has been documented (Watanabe et al. 2006). The stress resulting from management can prolong recovery and increase mortality (Kongtorp et al. 2004). The fact that salmon can handle less stress with an HMSI infection makes it less capable to handle treatments against salmon louse and other factors required to maintain a healthy population (Fiskehelserapporten 2010).

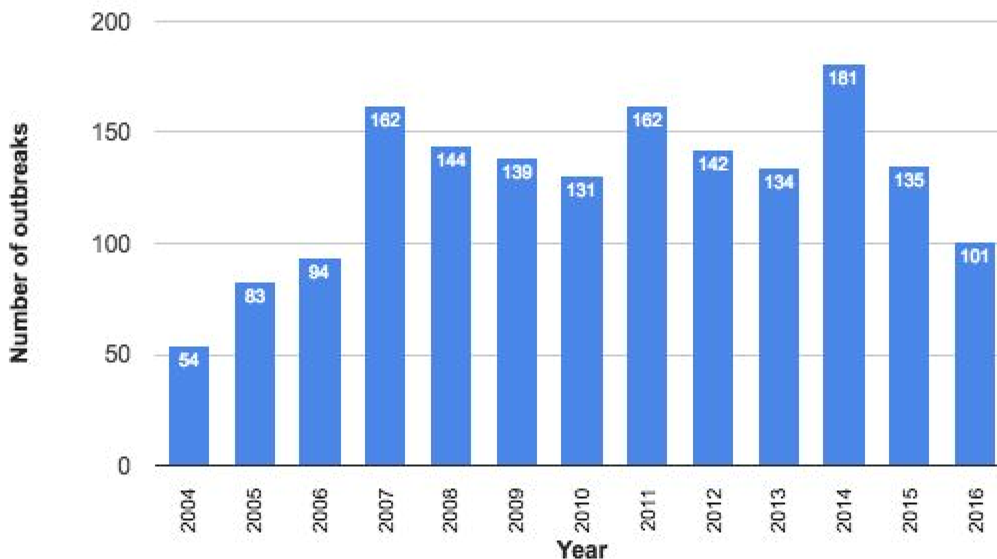


Figure 2. Number of HSMI outbreaks.
Number of locations with HSMI outbreaks in Norway from 2004-2016. Source: Fiskehelserapporten 2016.

1.5 Piscine reovirus

In 2010, Palacios et. al. established the connection between HSMI and the novel virus Piscine Reovirus (PRV). This was done by testing for different pathogens of HSMI infected fish (Palacios et al. 2010). Kibenge et. al presented in 2013 the first genomic analysis of PRV in North and South America, confirming the extended geographical distribution of the virus. PRV is

ubiquitous in Norway and was reported to be found in 13.4% of wild Atlantic salmon. But there seems to be no correlation between registered PRV in wild populations and HSMI outbreaks in sea farmed salmon (Garseth et al. 2013). The amount of PRV increases significantly during an HSMI outbreak (Løvoll et al. 2012). PRV may be present in the host both before and in a period after the outbreak (Wiik-Nielsen et al. 2012).

PRV belongs to the *Reoviridae* family within the *Spinareovirinae* subfamily. *Reoviridae* consists of 15 different genera of virus with a variety of hosts including fish, reptiles, birds, mammals, insects and plants (Attoui et al. 2011). The two genera found in *Reoviridae*, *Orthoreovirus* (ORV) and *Aquaorthoreovirus* (AqRV), show a common evolutionary origin (Attoui et al. 2002). Although ORV and AqRV have a varied host range, they share homology in most of the 10 or 11 genome segments (Kim et al. 2004). PRV most likely belongs to a new reovirus genus related to both ORV and AqRV (Palacios et al. 2010). There are currently no cell lines commercially available for *in vitro* cultivation of the virus. The cell line used previously, GF-1, is covered by intellectual property rights (Mikalsen et al. 2012).

Table 1. Piscine reovirus proteins

The table shows the different PRV protein segments, the length and assumed theoretical weight. Modified from Markussen et al. 2013.

Segment	Protein name	Length (nt)	Theoretical weight (kDa)
L1	λ3	1286	144.2
L2	λ2	1290	143.7
L3	λ1	1282	141.5
M1	µ2	760	86.0
M2	µ1	687	74.2
M3	µNS	752	83.5
S1	σ3	330	37.0
S2	σ2	420	45.9
S3	σNS	354	39.1
S4	σ1	315	34.6

The PRV genome has a weight of 23.3Kb and consists of 10 dsRNA segments (Markussen et al. 2013). They are labelled based on size, with three large (L1, L2, L3), three medium (M1, M2, M3) and four smaller segments (S1, S2, S3, S4). The proteins coded by the large segments are named λ (λ1, λ2, λ3), proteins coded by the medium sized segments are named µ (µ1, µ2, µNS) and proteins from the smaller segments are named σ (σ1, σ2, σ3, σNS). Homologues of proteins λ1, λ2, λ3, µ1, µ2, µ3, σ2 and σNS are found in ORV and AqRV (Palacios et al. 2010).

Palacios et. al. reported in 2010 that the proteins coded from S1 and S4 seemed unique to PRV, but Key et. al. concluded in 2013 that the product of the ORF1 in segment S1 is an outer clamp protein analogue to σ3 in mammalian orthoreovirus (MRV) and that the product of segment S4 is an homologue to an outer fibre protein in MRV.

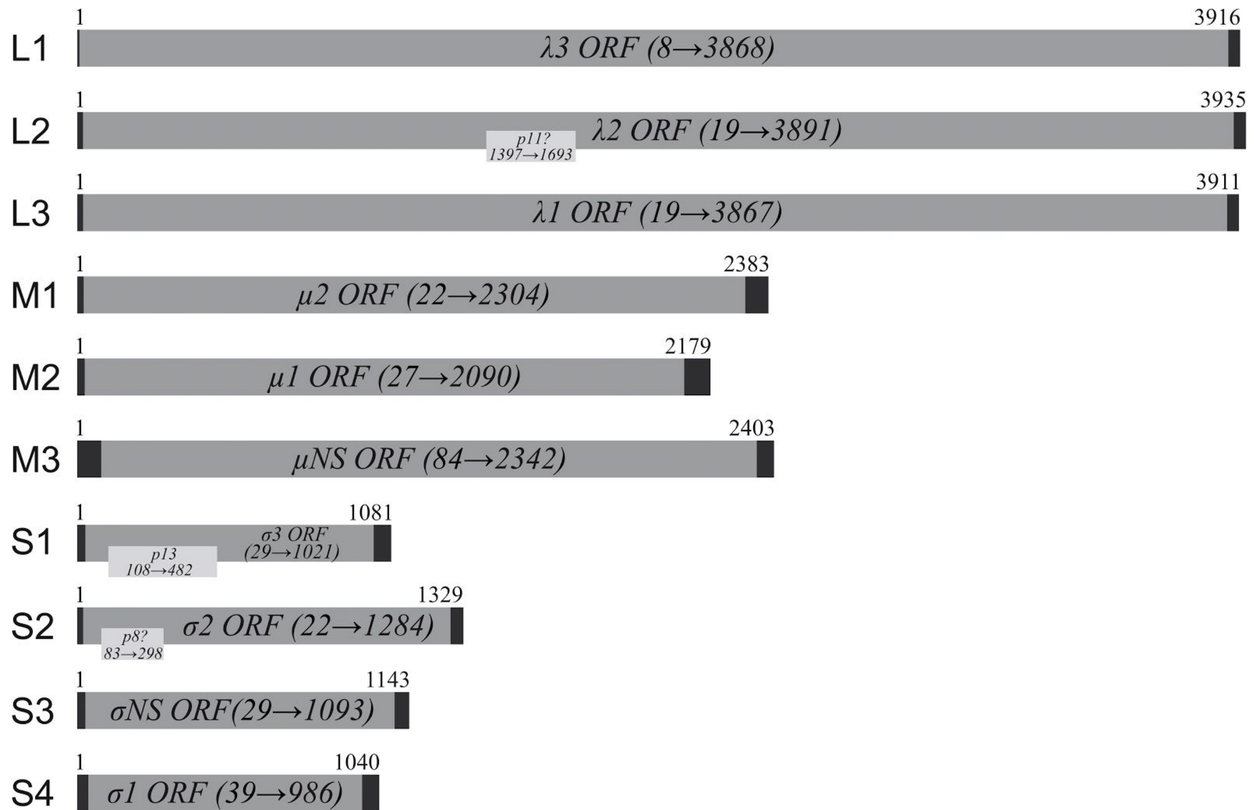


Figure 3. PRV genome.

PRV gene segments are assigned according to mammalian reoviruses. Gene segments L2, S1 and S2 are possibly polycistronic. Source: Markussen et al. 2013.

The PRV genome has 10 ORFs, but possibly 13 as S1, S2 and L2 may be polycistronic. Eight of the ten segments are, based on comparison with MRV, assumed to be structural proteins. The segments M3 and S3 are nonstructural proteins. The segments M2 and S4, coding for μ1 and σ1 respectively, are part of the outer capsid proteins (Markussen et al. 2013). μ1 and σ1 are the outer capsid proteins of PRV that were used in this thesis.

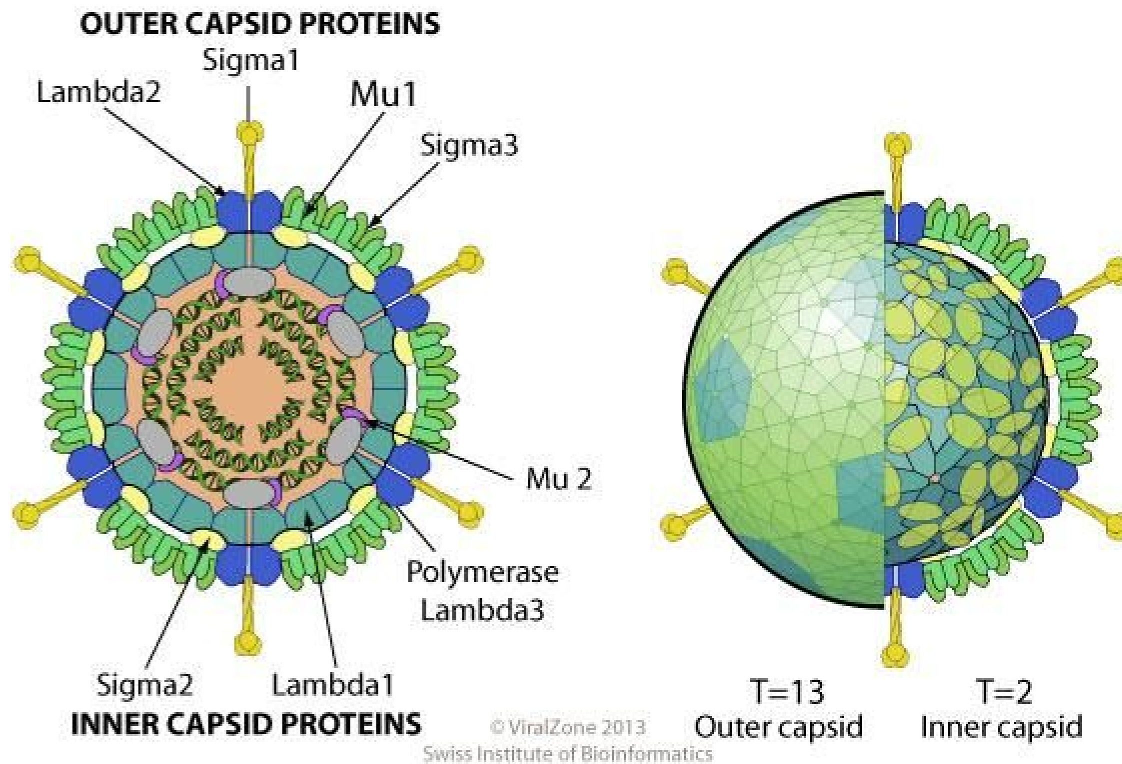


Figure 4. MRV protein structure.
Inner and outer capsid protein structure of MRV. Source: Viral Zone, Swiss Institute of Bioinformatics.

1.6 Plant biotechnology

Plants have through history been a great resource for natural medical products, and many of today's widely used drugs are originally derived from plants. The advances in molecular biology during the last few decades has now made it possible for plants to contribute to this industry in a new way via molecular farming. Molecular farming is the use of genetically engineered plants for the production of a specific protein. This technique is now a promising new method for production of many different biopharmaceutical products (Ma et al. 2012).

Up until now most recombinant proteins have been produced using mammalian, fungal and bacterial cells such as *Escherichia coli*. In addition, some proteins produced by these hosts will not have the correct folding, disulfide bridges or glycosylation of the proteins (Daniell et al. 2001, Gomord et al. 2004, Ma et al. 2003). Plant bioreactors are a potent alternative, with multiple advantages over the more established systems (Ma et al. 2012).

Plant based systems can in contrast to prokaryotic systems perform eukaryotic post translational modifications, which often are essential for the products´ bioactivity (Ma et al. 2003, Gomord et al. 2004). Also, the plant bioreactor systems do not present any risk of pathogen contamination as there are no known pathogens shared by plants and animals (Tremblay et al. 2010). Pathogen contamination is always a concern when using mammalian cells (Ma et al. 2012). The seemingly most important argument why plants serve as bioreactors is a most

promising way forward for production of recombinant proteins, is the simplicity of the system and cost efficiency. This makes molecular farming with plants scalable and at low cost. In addition the plants can use light as its main energy source which also reduces costs. Plants are also hardy and inert, making them an easy to grow and a readily manageable system (Tremblay et al. 2010).

Table 2. Recombinant protein production systems
Comparison of cost, production time and quality for different production systems for recombinant human pharmaceutical proteins. Modified from Ma et. al. 2003.

System	Overall cost	Production time	Scale-up capacity	Product quality	Glycosylation	Contamination risks
Bacteria	Low	Short	High	Low	None	Endotoxins
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk
Mammalian cell culture	High	Long	Very low	Very high	Correct	Viruses, prions and oncogenic DNA
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, prions and oncogenic DNA
Plant cell cultures	Medium	Medium	Medium	High	Minor differences	Low risk
Transgenic plants	Low	Long	Very high	High	Minor differences	Low risk

There are a few alternative strategies to achieve expression of homologous proteins in plants. Most widely used is stable transformation, while other well known techniques are chloroplast transformation and transient expression (Ma et al. 2012).

1.7 Transgenic plants

Transgenic plants are plants with a foreign gene implemented in the nuclear DNA (Ma et al. 2012). Transgenic plants are very good options both for scale-up and long term production as the foreign gene is permanently implemented in their nuclear DNA (Tremblay et al. 2010). The major drawback with this technique is that the production cycle requires quite some time, about 6-9 months (Ma et al. 2012). In addition there is the risk of transgenic material escaping the system and spreading into the wild and cause ecological problems (Pilson et al. 2004).

Two types of methods can be used to introduce the foreign gene into the host, biolistic transformation and *Agrobacterium*-mediated transformation (Ma et al. 2012). *Agrobacterium*-mediated transformation is exploiting the unique characteristics of the soil bacterium *Agrobacterium tumefaciens*, which is able to introduce a foreign gene into a plant host. This is done via a tumor inducing plasmid (Ti), that introduces a section of the plasmid (T-DNA) into the plant's chromosome. By introducing the gene of interest that will express the recombinant protein into the T-DNA, the gene of interest will be introduced into the plants nuclear DNA (Gelvin et al. 2003).

Not all plants are natural hosts of *Agrobacterium tumefaciens*, and an alternative technique for introduction of a foreign gene is biolistics. By shooting the plant cells with naked DNA attached to a heavy metal, pieces of the DNA will be introduced in the plant's genome. This particle bombardment is performed with a gene gun, and can also be applied to other genomes than the nuclear (Shrawat et al. 2006).

Chloroplast transformation is a technique that can yield high amounts of the wanted recombinant protein because of the polyploid nature of the chloroplast genome as well as the sheer amounts of chloroplasts in a plant cell. As there can be up to 100 chloroplasts with 100 copies of the genome each, you could in theory reach a 10.000-fold amplification of protein production using chloroplast transformation (Chebolu et al. 2009). Chloroplast genomes are inherited maternally in most plants (Zhang et al. 2003), and this minimises the risk of contamination of wild crops with transgenic pollen (Chebolu et al. 2009).

Transient expression is achieved by infiltrating plant tissue with modified *Agrobacterium*, which will then introduce a specific gene into the plant cell with help of the Ti-plasmid. Janssen and Gardner showed in 1990 that the expression of genes introduced by *Agrobacterium* happens both in a stable and a transient fashion. The expression of proteins from the stable transformation usually increase 10-14 days post inoculation, while the expression of proteins from the transient transformation can peak just few days after inoculation (Janssen et al. 1990). This suggests that the *Agrobacterium* introduces a lot more copies of T-DNA than just those who are incorporated in the nuclear DNA (Janssen et al. 1990), and that the expression from the non integrated T-DNA will diminish days after the peak because of instability (Lacroix et al. 2013). Compared to the stable transformation of plants using *Agrobacterium*, the transient transformation is both faster and simpler, and therefore is a very good method for screening different variables in a green bioreactor system (Yang et al. 2000). This can present a very important means to acquire e.g. a vaccine against a rapidly spreading pandemic within reasonable time (Tremblay et al. 2010).

1.8 Plant material

Nicotiana benthamiana is the plant most widely used for experiments for production of recombinant proteins (Chen et al. 2016), and thus the protocols are well developed for this species (Fischer et al. 2004). But *N. benthamiana* contains disadvantageous substances like nicotine and other toxic alkaloids that makes the downstream processing difficult and renders the plant unsuitable for production of an oral vaccine (Fischer et al. 2004).

Lettuce contains no harmful substances, thus it represents a good option as a producer of recombinant proteins for an oral vaccine (Pniewski et al. 2011). Lettuce also contains less substances that can impede the purification process of the homologous protein (Sohi et al. 2005). Concerning lettuce as a host to express recombinant proteins using transient transformation with *Agrobacterium*, there are a number of publications supporting that lettuce is a viable option for this type of expression. Lettuce produced virus like particles (VLPs), human

growth hormone, monoclonal antibodies and human interferon are all examples of this (Lai et al. 2012, Joh et al. 2005, Negrouk et al. 2005, Li et al. 2007).

1.9 The pEAQ vector series

The pEAQ vector series emerged from the development of a virus based expression system using the Cowpea Mosaic Virus (CPMV) (Peyret et al. 2013). The CPMV - *HT* was a non replicating system developed from the CMPV expression system (Sainsbury et al. 2008), and this system was then further refined into the pEAQ vector series (Sainsbury et al. 2009).

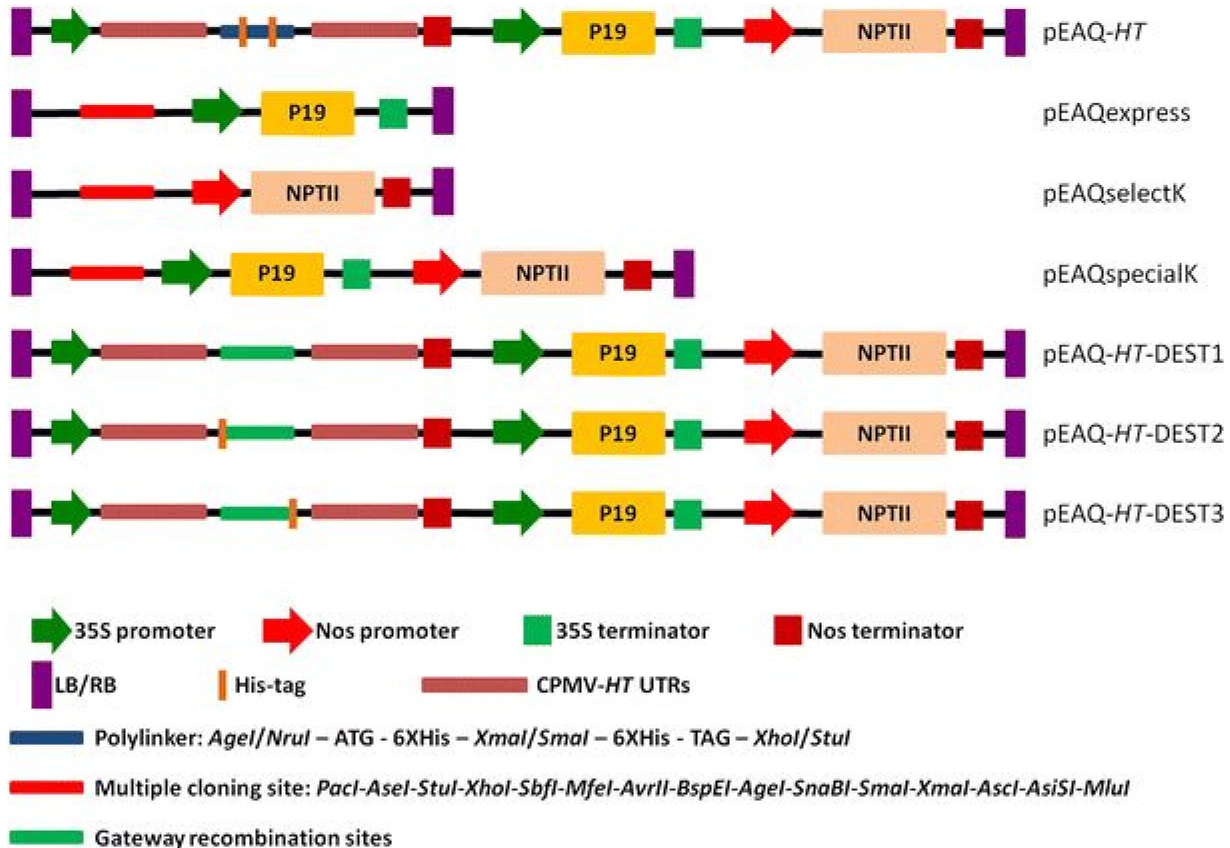


Figure 5. The pEAQ vectors

The figure shows the different versions of pEAQ vectors available. Source: Peyret et al. 2013

The pEAQ vectors are available with multiple cloning sites as well as Gateway cloning sites. All versions are featuring the P19 gene silencing suppressor, and most include the NPTII kanamycin resistance gene (Figure 5). The possibility of two CPMV-*HT* expression cassettes can enhance expression as two different proteins can be produced in the same cell (Montague et al. 2011). The DEST2 and DEST3 versions of the pEAQ vectors has a hexahistidine tag included (Figure 5). The most preferred *Agrobacterium* strain to combine with the pEAQ vectors is LBA4404 (Sainsbury et al. 2009).

The production of the core antigen of Hepatitis B (HBcAg) was one of the first successes using the pEAQ vectors to express a viral capsid. This was done in *N. benthamiana* with a yield of 200-500 mg/kg fresh plant tissue (Thuenemann et al. 2013).

Other viral proteins and VLPs have also successfully been expressed using the pEAQ vectors, including the L1 major coat protein from Human papillomavirus type 8 (Matic et al. 2012) and VP3, VP7, VP2 and VP5 proteins from the Bluetongue virus forming a VLP (Thuenemann et al. 2013).

The Sigma-1 and Mu-1 antigens produced in *N. benthamiana* plants by supervisor Prof. Jihong Liu Clarke's group were used in the salmon vaccination study together with *E. coli* derived Sigma-1 and Mu-1.

2. Aim of the thesis

The aim of this thesis was to express the outer capsid proteins Sigma-1 and Mu-1 from the PRV virus in plants using the pEAQ vector transient expression system, and further test the immune response of these two plant produced antigens compared with a salmonid alphavirus-based replicon vaccine and an inactivated whole virus vaccine, in a vaccination trial using Atlantic salmon parr.

The general aim of this research project was to develop a low-cost, safe and efficacious vaccine against HSMI.

3. Materials and methods

3.1 Agroinfiltration

Agrobacterium tumefaciens strain LBA4404 were already transformed by electroporation using pEAQ-*HT*-DEST2 (Figure 5) expression vectors harbouring the PRV Sigma-1 and Mu-1 genes respectively in separate cultures. Both constructs had a N-terminal his-tag adding the following residues: MHHHHHHPITSLYKKAGLENLYFQG. The transformed *Agrobacterium* stocks were supplied by supervisor Prof. Jihong Liu Clarke's research group at NIBIO. A few cells from the *Agrobacterium* stocks, stored in glycerol at -80°C, were put in mini cultures containing 5 ml lysogeny broth (LB) with 5 µl 50 mg/ml kanamycin and left to incubate overnight at 28°C with shaking. 1 ml of the 5 ml mini cultures were then transferred to midi cultures containing 50 ml LB with 50 µl 50 mg/ml kanamycin, 10 µl 100 mM acetosyringone and 500 µl pH 5.7 2(*N*-morpholino) ethanesulfonic acid (MES)-KOH, and left to incubate overnight at 28°C with shaking. The midi cultures were then precipitated at 4,000 rpm at room temperature (RT) for 60 minutes and then resuspended in 50 ml infiltration buffer containing 500 µl 1 M MgCl2, 50 µl 1M pH 5.7 MES-KOH and 100 µl 100 mM acetosyringone in 50 ml H2O. The 50 ml infiltration buffer was then diluted further with infiltration buffer until it reached OD_{600} = 0.5 and left overnight at RT. The same infiltration buffer was also prepared without *Agrobacterium* to be used as a negative control.

For expression of the recombinant protein in plant hosts, two different plant species were tested; Lettuce (*Lactuca sativa*) var. *veronique*, and *N. benthamiana*. The plants were infiltrated at 3-4 weeks of age, depending on size, using the youngest fully expanded leaves for infiltration, avoiding the cotyledons.

Two different techniques for the actual agroinfiltration were used; syringe-infiltration and vacuum-infiltration. For the syringe-infiltration a 10 ml syringe was aspirated with the *Agrobacterium* suspension. Then the leaves were pricked with a sterile needle and the syringe placed over the wounds to carefully force the suspension into the intercellular space in the leaves. The infiltrated area appears darker than the rest of the leaves, and as much as possible of the leave surface was infiltrated.

For vacuum-infiltration the base of the pots were covered to prevent soil from falling into the *Agrobacterium* suspension. The plants were then turned upside down and as much as possible of the plants were submerged into the *Agrobacterium* suspension in a bowl placed inside a vacuum desiccator. The lettuce was treated for 2x30 seconds at 0.8 bar and *N. benthamiana* for 2x60 seconds at 0.5 bar, making sure the vacuum was released carefully to make sure a best and most gentle infiltration possible. All non-infiltrated leaves were removed and the plants were left in greenhouse with artificial lighting, until they were ready for harvest. The *N. benthamiana* was kept at 21°C with 70% RH and 150-200 µmol, while the lettuce was kept at the same parameters except with temperature at 20°C.

The harvest was done by freezing the leaf material immediately with liquid nitrogen, trying at best to harvest only the leaf plates and not the veins, and stored at -80°C until further analysis.

The agroinfiltration was repeated in four separate rounds. Plants were vacuum-infiltrated by a more experienced member of the research group with the *Agrobacterium* buffer prepared by the author, as a measure to eliminate the infiltration procedure as a variable. Also plants were infiltrated by the author with *Agrobacterium* buffer that was prepared by a more experienced member of the research group, as a measure to eliminate the preparation of the *Agrobacterium* infiltration buffer as a variable. The negative controls were un-infiltrated plants and plants infiltrated using buffer without the *Agrobacterium*.

3.2 Protein extraction

Protein extraction was done by phenol extraction based on protocols from Faurobert et al. 2007.

The frozen leaf material was grounded to a fine powder while still frozen, this was done in mortar while adding liquid nitrogen to keep it frozen. Then 0.2 g of each different sample to be tested was added to 0.6 ml extraction buffer, containing 0.5 M tris(hydroxymethyl)aminomethane (Tris) pH 9.4, 50 mM EDTA, 0.7 M Sucrose, 0.1 M KCl, 2% 2-Mercaptoethanol and cOmplete$_{TM}$ EDTA-free Protease Inhibitor Cocktail (Roche), in eppendorf tubes. The tubes were then vortexed and incubated on ice for 10 minutes with intermittent mixing. Then 0.6 ml of phenol saturated with citrate buffer 4.2 pH was added and the tubes were mixed well and left on a shaker for 10 minutes. The tubes were then centrifuged for 10 minutes at 5,500 g and 4°C. The phenol solution gathered at the top layer in the tubes and was pipetted into clean tubes. 0.6 ml of extraction buffer was then added followed by 10 minutes on a shaker. The tubes were then centrifuged for 10 minutes at 5,500 g (4°C), and the top layer was pipetted into clean tubes. Four times the final volume in the tubes of cold precipitation buffer containing 0.1 M NH4 in methanol was then added and left overnight at -20°C.

The following day the precipitated proteins were pelleted by centrifugation for 10 minutes at 10,000 g and 4°C, after which the supernatant was removed and 0.1 ml precipitation buffer was added and the pellet was dissolved using a pipette tip. Additional 0.9 ml of precipitation buffer was then added and tubes were centrifuged for 10 minutes at 10,000 g and 4°C, and supernatant removed. This step with breaking up pellet, adding a total of 1 ml of precipitation buffer, centrifugation and removal of the supernatant, was then repeated before the pellets were left to air dry for 20-30 minutes or until a visual inspection confirmed that the pellet was dry.

For the re-solubilisation of the pellet 50 µl of 1% SDS was added and the pellet was broken up using a pipette tip. Additional 50-150 µl of 1% SDS was then added to each tube depending on the size of the final protein pellet. The tubes were then left for 1-2 hours at RT with intermittent mixing. The tubes were then centrifuged for a few seconds to pellet the undissolved material, and the supernatant was pipetted into clean tubes.

3.3 Protein quantification

Quantification of the protein content from the phenol extraction was done by Bicinchoninic acid assay (BCA), using Pierce BCA Protein Assay Reagent Kit. Six different protein dilutions with Bovine Serum Albumin (BSA) and 1% SDS was made at 2, 1.33, 0.67, 0.33, 0.2 and 0.067 mg/ml to make up the standard curve. 48 µl of BCA reagents (A+B) at a 50:1 ratio was added to 6 µl of sample and left to incubate at 37°C for 30 minutes. The absorbance of the samples at 562 nm and the corresponding protein content was then measured with a Thermo Scientific NanoDrop 200c UV-Vis Spectrophotometer.

The protein quantification was repeated four times, once for every repetition of the agroinfiltration.

3.4 Protein separation

The separation of proteins was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Based on the results from the BCA analysis, the correct amount of sample solution was distributed in eppendorf tubes to have close to equal amounts of protein in each tube, and mixed with 3,5 µl 4x LDS-PAGE sample loading buffer at pH 8.5 containing 106 mM Tris HCl, 141 mM Tris Base, 2% lithium dodecyl sulfate, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250 and 0.175 mM Phenol Red. 1.5 µl of 100 nM dithiothreitol (DTT) was added as reducing agent in each tube. Total target volume was 15 µl, and the amount protein was 20 µg. Tubes were then placed in a heat block at 70°C for 10 minutes, followed by a brief centrifugation and storage at -20°C until further use.

The NuPAGE SDS-PAGE setup from Invitrogen was assembled following the manufacturer's instructions, and the inner chamber was filled with freshly prepared 1X MES SDS-PAGE running buffer. The outer chamber was filled with previously used running buffer. 15 µl of the samples were then loaded in the NuPAGE 4-12% Bis-Tris gel with 17 wells together with 4 µl of Thermo Scientific Spectra Multicolor Broad Range Protein Ladder. The gel was then run with constant voltage at 175 V for 45 minutes.

The protein separation was repeated four times, once for every repetition of the agroinfiltration.

3.5 Western blot

Blotting was done with the iBlot semi-dry blotting system from Life Technologies Inc. on a nitrocellulose membrane and ECL detection was done with an Azure c400 imaging system.

The plastic plates holding the gels were opened with a gel knife, and the “teeth” on the gels were removed. The gels were then transferred to a tray with distilled water to rinse off excess SDS. The blotting sandwich was then assembled according to the manufacturer's instructions, with the gels on top of the nitrocellulose membrane, making sure there were no air bubbles

trapped between the layers. Program number 3 on the iBlot system, which supplies 20 V of constant voltage, was then run for 7 minutes, after which the blot sandwich was removed from the machine and disassembled. The gels were then stained with Coomassie staining to confirm that the SDS-PAGE and blotting procedure was successful.

The blotting membrane was placed in a blocking buffer containing 50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TBST) + 5% BSA, and left on a rocking table for one hour at RT. When the blocking of the nitrocellulose membrane was complete the membrane was cut into smaller segments to make possible the correct antibodies to be applied to the correct parts of the membrane. The segments were then placed in the primary antibody solution containing TBST + 1% BSA and rabbit polyclonal antibodies raised against Mu-1 and Sigma-1 correspondingly in a 1:5000 dilution, and left overnight at RT on a rocking table.

The following day the primary antibody solution was removed and stored at 4°C for later use, and the membranes were washed for 30 minutes total in six rounds of five minutes each in TBST. The solution containing the secondary antibodies were then applied, containing TBST + 1% BSA and Promega goat anti-rabbit IgG Horseradish Peroxidase(HRP) conjugate in a
1:50 000 dilution, and left incubating for 2-3 hours under agitation. The secondary antibody solution was then poured off and stored for later use, and the membranes were once again washed for 30 minutes total in six rounds of five minutes each in TBST. Finally the membranes were rinsed once in TBS.

Development was done by chemiluminescent detection using the GE Amersham ECL Prime western blotting detection reagent and an Azure Biosystems Axure C400 system.

The western blot was repeated four times, once for every repetition of the agroinfiltration.

3.6 Fish experiment

Table 3. Overview of the different groups included in the vaccination study.
Table 3 shows the number of salmon parr allocated to each group as well as the abbreviation for the each group, sampling time-points and the number of fish sampled for blood and tissue collection after vaccination and challenge. Only the 60 fish were sampled at 6 weeks post vaccination (wpv), and 10 fish from the control group sampled at day one were used in this study.

Vaccine Groups		Sampling time post							Total # fish
		Weeks Post vaccination (wpv)			Weeks post challenge (wpc)				
		Day-1	T=6 wpv	T=10 wpv	T=4 wpc	T=6 wpc	T=8 wpc	T=10 wpc	
Replicon	Gr1		10	10	10	10	10	10	60
Escherichia coli	Gr2		10	10	10	10	10	10	60
N. benthamiana antigens	Gr3		10	10	10	10	10	10	60
Inactivated whole virus	Gr4		10	10	10	10	10	10	60
Unvaccinated control	Gr5	10	10	10	10	10	10	10	70
Total # fish		10	50	50	50	50	50	50	310

Atlantic salmon parr with an average weight of 60-70 grams were vaccinated using four different vaccines as shown in Table 3 as well as the negative control group. Each group was allocated a total of 60 fish while the control group was allocated 70 fish.

The *E. coli* produced antigens were formulated as a water-in-oil (w/o) vaccine using oil adjuvant Montanide™ ISA 763 VGA (Seppic, France) based on manufacturer's recommendations. Briefly antigen and oil adjuvant were mixed at a 30:70 ratio (w/w) basis corresponding to 74:26 at volume basis. The *E. coli* produced antigens were provided by supervisor Prof. Jihong Liu Clarke's research group at NIBIO. The antigens were expressed in *E. coli* from a pET14b expression vector in which the antigen-encoding genes were inserted. The antigen formed inclusion bodies which were isolated and washed before use. The formulation was administered using the intraperitoneal route at a dosage of 50 µg/fish, injection volume of 0.1 ml.

The *N. benthamiana* antigens were produced by transient agrofitration as described above, and subsequently extracted and purified using IMAC. The *N. benthamiana* produced antigens were provided by supervisor Prof. Jihong Liu Clarke's research group at NIBIO. The vaccine was formulated as a water-in-oil (w/o) vaccine using SEPPIC ISA 763 VG adjuvant based on manufacturer's recommendations. Briefly antigen and oil adjuvant were mixed at a 30:70 ratio (w/w) basis corresponding to 74:26 at volume basis. The formulation was administered using the intraperitoneal route at a dosage of 30 µg/fish, injection volume of 0.1 ml.

The inactivated whole virus vaccine was made by using a heart homogenate from PRV positive fish, followed by inactivation using 0.5% formalin for 48 hours and dialysis for 48 hrs to remove the formalin. The concentration of the antigen was determined by quantitative RT-PCR to be Ct-18.20. The vaccine was formulated as a water-in-oil (w/o) vaccine using SEPPIC ISA 763 VG adjuvant based on manufacturer's recommendations. Briefly antigen and oil adjuvant were mixed at a 30:70 ratio (w/w) basis corresponding to 74:26 at volume basis. The formulation was administered using the intraperitoneal route at a dosage of 0.1 ml/fish.

The replicon vaccine included a salmonid alphavirus backbone expressing the PRV structural proteins, Mu-1 and Sigma-1. Briefly antigen and oil adjuvant were mixed at a 30:70 ratio (w/w) basis corresponding to 74:26 at volume basis. The formulation was administered by the intramuscular route at a dosage of 50 µg/fish, injection volume of 0.1 ml. The replicon vaccine was prepared by Dr. T.C. Guo, NMBU as part of the Vivafish project (RCN project no. 237315) according to methods previously described (Guo et al. 2015).

Figure 6. Fish experiment tank setup.
Left: Fish tank setup. Right: Atlantic salmon parr after vaccination.

At day one all the 60 fish in each group were vaccinated with the corresponding vaccine.
Blood and tissue samples were collected from 10 fish on the day of vaccination from the unvaccinated control group. The tissue samples collected were from the heart, muscle, pancreas, head kidney and spleen. Thereafter samples were collected after 6 weeks post vaccination as shown in in Table 3 above, also from 10 fish.

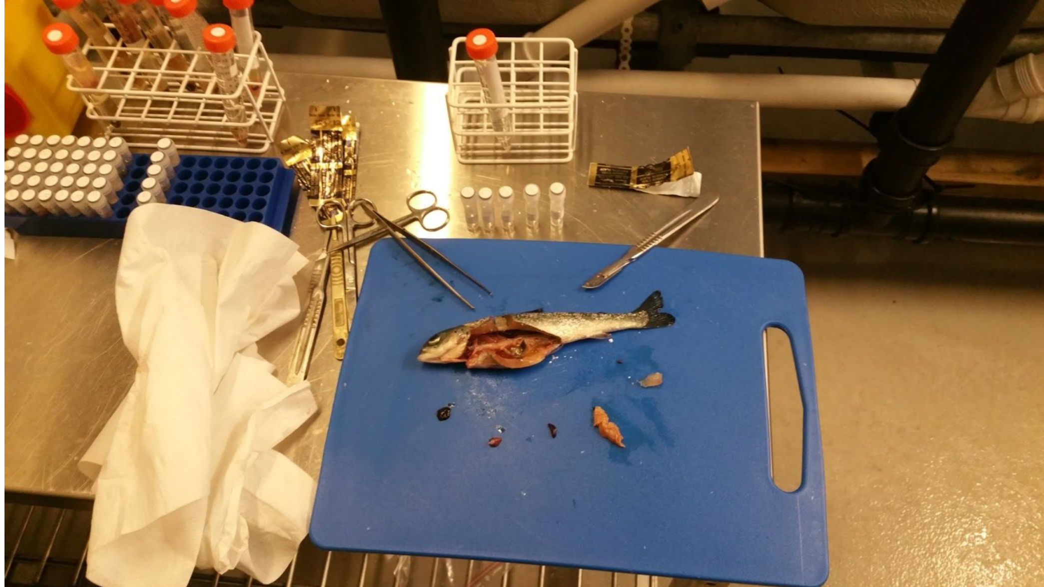


Figure 7. Sampling of salmon parr tissues.
Tissue and blood samples were collected 6 weeks post vaccination.

3.7 ELISA for detection of antibodies against PRV in salmon serum

Five 96 well microtiter plates for each antigen (Sigma-1 and Mu-1), were coated with 100 µl of each antigen (produced in *E. coli*, as described above) in coating buffer made of bicarbonate buffers (Na_2CO_3 + $NaHCO_3$ + distilled water). The catch-antibody coated on the plates prior to adding the antigens (from *E. coli*, see Figure 8 below) were polyclonal anti-PRV made against Sigma and Mu in rabbit, used at a 1:2000 dilution. After adding the catch antibody, the plates were incubated overnight at 4°C for attachment to occur.

The plates were then washed three times using 250 µl of phosphate buffered saline plus Tween 20 (PBST) (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4 + 0.1% (v/v) Tween 20) for each well before adding 250 µl blocking buffer, containing PBS and 5% fat free dry milk. After adding the blocking buffer the plates were left for incubation for two hours at room temperature (RT). After blocking, the plates were then washed three times using 250 µl of PBST. Thereafter, PRV antigens made from *E. coli* as described above were added at 1:2000 dilution, and left overnight at 4°C.

The following day the plates were once again washed three times with 250 µl of PBST for each well and 100 µl of serum samples diluted in diluent buffer (PBST plus 1% dry milk) were added to each well. The serum dilutions were added to the ten microtiter plates in duplicates using dilutions of 1:50, 1:100, 1:200 and 1:400 for all the different individuals for each treatment, and the plates were left incubating overnight at 4°C (Table 4). All vaccine groups had 10 fish while the control group had seven fish. Each serum sample was diluted in duplicate.

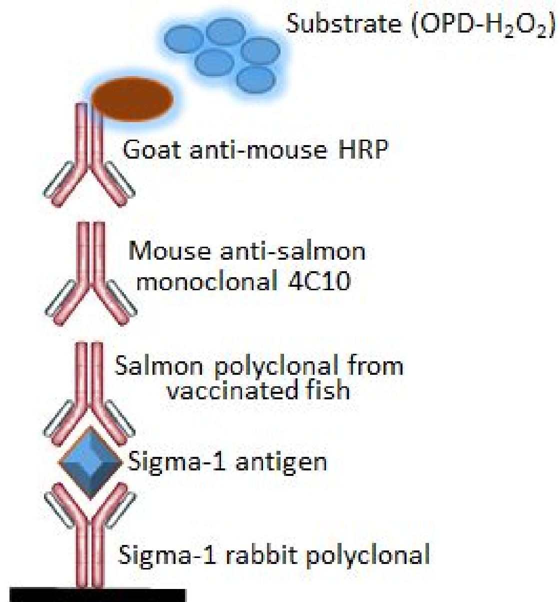
Table 4. Overview of the different treatments, individuals and dilutions in the ELISA test. Sigma-1 and Mu-1 antigens were coated onto five microplates each representing the five different treatments; inactivated whole virus, *E.coli*, *N. benthamiana*, replicon and negative control. The test was run using duplicates for each individual with 1:50, 1:100, 1:200 and 1:400 dilutions of the salmon serum.

	Sigma/Mu + Inactivated whole virus/*E.Coli*/*N. benthamiana*/Replicon/Negative control									
	1	2	3	4	5	6	7	8	9	10
A	Fish 1 1:50	Fish 1 1:50	Fish 2 1:50	Fish 2 1:50	Fish 3 1:50	Fish 3 1:50	Fish 4 1:50	Fish 4 1:50	Fish 5 1:50	Fish 5 1:50
B	Fish 1 1:100	Fish 1 1:100	Fish 2 1:100	Fish 2 1:100	Fish 3 1:100	Fish 3 1:100	Fish 4 1:100	Fish 4 1:100	Fish 5 1:100	Fish 5 1:100
C	Fish 1 1:200	Fish 1 1:200	Fish 2 1:200	Fish 2 1:200	Fish 3 1:200	Fish 3 1:200	Fish 4 1:200	Fish 4 1:200	Fish 5 1:200	Fish 5 1:200
D	Fish 1 1:400	Fish 1 1:400	Fish 2 1:400	Fish 2 1:400	Fish 3 1:400	Fish 3 1:400	Fish 4 1:400	Fish 4 1:400	Fish 5 1:400	Fish 5 1:400
E	Fish 6 1:50	Fish 6 1:50	Fish 7 1:50	Fish 7 1:50	Fish 8 1:50	Fish 8 1:50	Fish 9 1:50	Fish 9 1:50	Fish 10 1:50	Fish 10 1:50
F	Fish 6 1:100	Fish 6 1:100	Fish 7 1:100	Fish 7 1:100	Fish 8 1:100	Fish 8 1:100	Fish 9 1:100	Fish 9 1:100	Fish 10 1:100	Fish 10 1:100
G	Fish 6 1:200	Fish 6 1:200	Fish 7 1:200	Fish 7 1:200	Fish 8 1:200	Fish 8 1:200	Fish 9 1:200	Fish 9 1:200	Fish 10 1:200	Fish 10 1:200
H	Fish 6 1:400	Fish 6 1:400	Fish 7 1:400	Fish 7 1:400	Fish 8 1:400	Fish 8 1:400	Fish 9 1:400	Fish 9 1:400	Fish 10 1:400	Fish 10 1:400

The following day the plates were washed three times with 250 µl of PBST for each well and 100 µl mouse anti-salmon monoclonal antibody (4C10) at a dilution of 1:30 in diluent buffer was added to each well. Thereafter, the plates were left to incubate for one hour at RT. The plates were then washed following the same procedure as described above, but this time the washing was repeated four times. Then, 100 µl of goat anti-mouse monoclonal antibody conjugated with horseradish peroxidase (HRP) (DAKO; Glostrup, Denmark) at a concentration of 1:1000 in diluent buffer was added to each well, and left incubating in RT for one hour.

Once again the plates were washed four times with 250 µl of PBST for each well and 100 µl of the substrate solution, containing 0.1 mL OPD substrate (O-Phenylenediamine dihydrochloride, DAKO; Glostrup, Denmark) containing 30% H_2O_2 was added to each well before the plates were left to incubate at RT for 15 minutes.

After 15 minutes 50 µl of stop solution, containing 50 ml 1M H_2SO_4 in 945 ml ddH_2O, was added to each well and the optical density (OD) was measured at 492 nm wavelength using a GENios ELISA spectrophotometer (TECAN, Genios, Boston, USA).



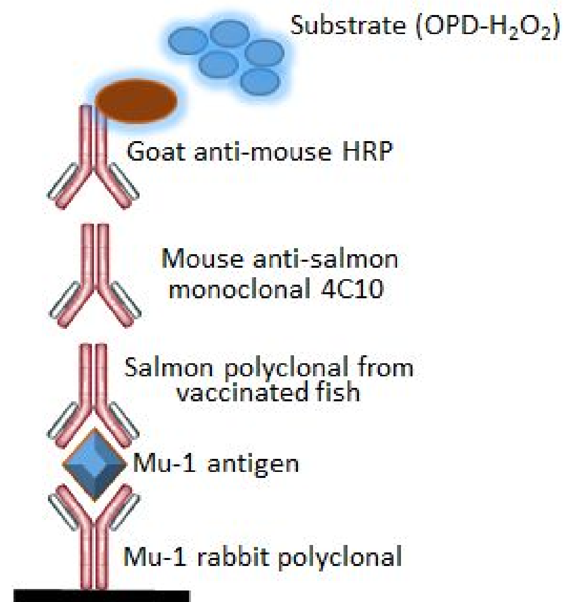


Figure 8. ELISA layout.
This figure shows ELISA layout plan for the Sigma1 and Mu1 antigens.

4. Results

4.1 Agroinfiltration

The pictures below (Figure 9) are of lettuce leaves from the fourth round of agroinfiltration at 6 days post infiltration (dpi). The aim of this part of the experiment was to express the Sigma-1 and Mu-1 antigens from PRV using *Agrobacterium* mediated transient agroinfiltration containing pEAQ vectors harbouring the genes for the PRV antigen proteins Sigma-1 and Mu-1.

Figure 9. Lettuce leaves 6 dpi.
The pictures above show lettuce leaves from the plants infiltrated with *Agrobacterium* containing Sigma-1 and Mu-1 genes, as well as negative controls with and without infiltration buffer, after harvest at 6 dpi. The Sigma-1/Mu-1 Control Buffer leaves were from plants infiltrated with infiltration buffer and *Agrobacterium* prepared by a separate member of the research group. These lettuce plants were all infiltrated manually by syringe.

All of the lettuce leafs from the four treatments with *Agrobacterium* show chlorosis compared with the negative control. The lettuce leaf with the “Lettuce Sigma-1” treatment also shows some necrosis. The negative control infiltrated with only infiltration buffer also shows a darker green color than the leaves infiltrated with *Agrobacterium* (Figure 9).

4.2 Protein quantification

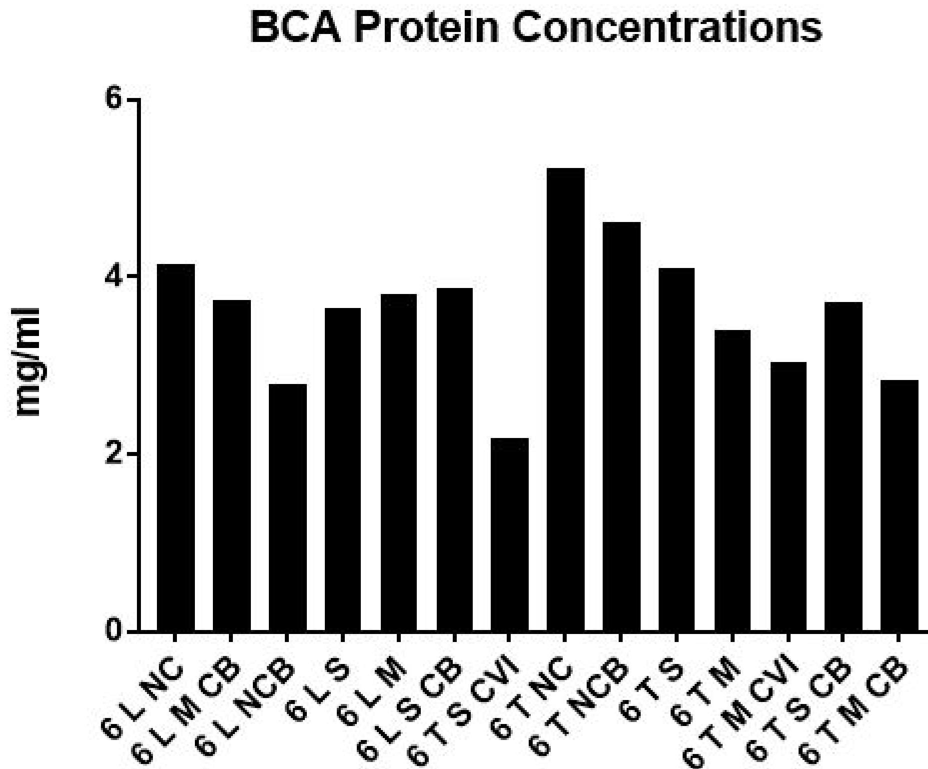


Figure 10. BCA protein concentrations.
The graph is presenting the different protein concentrations from the protein extraction of the leaf samples of lettuce and *N. benthamiana*. 6=6 dpi, L=Lettuce, NC=Negative Control, M=Mu-1, CB=Control Buffer, NCB=Negative Control Buffer, S=Sigma-1, T=*N. benthamiana* and CVI=Control Vacuum Infiltration.

The protein concentrations were between 2.2 and 5.3 mg/ml.

4.3 SDS-PAGE and Western blot

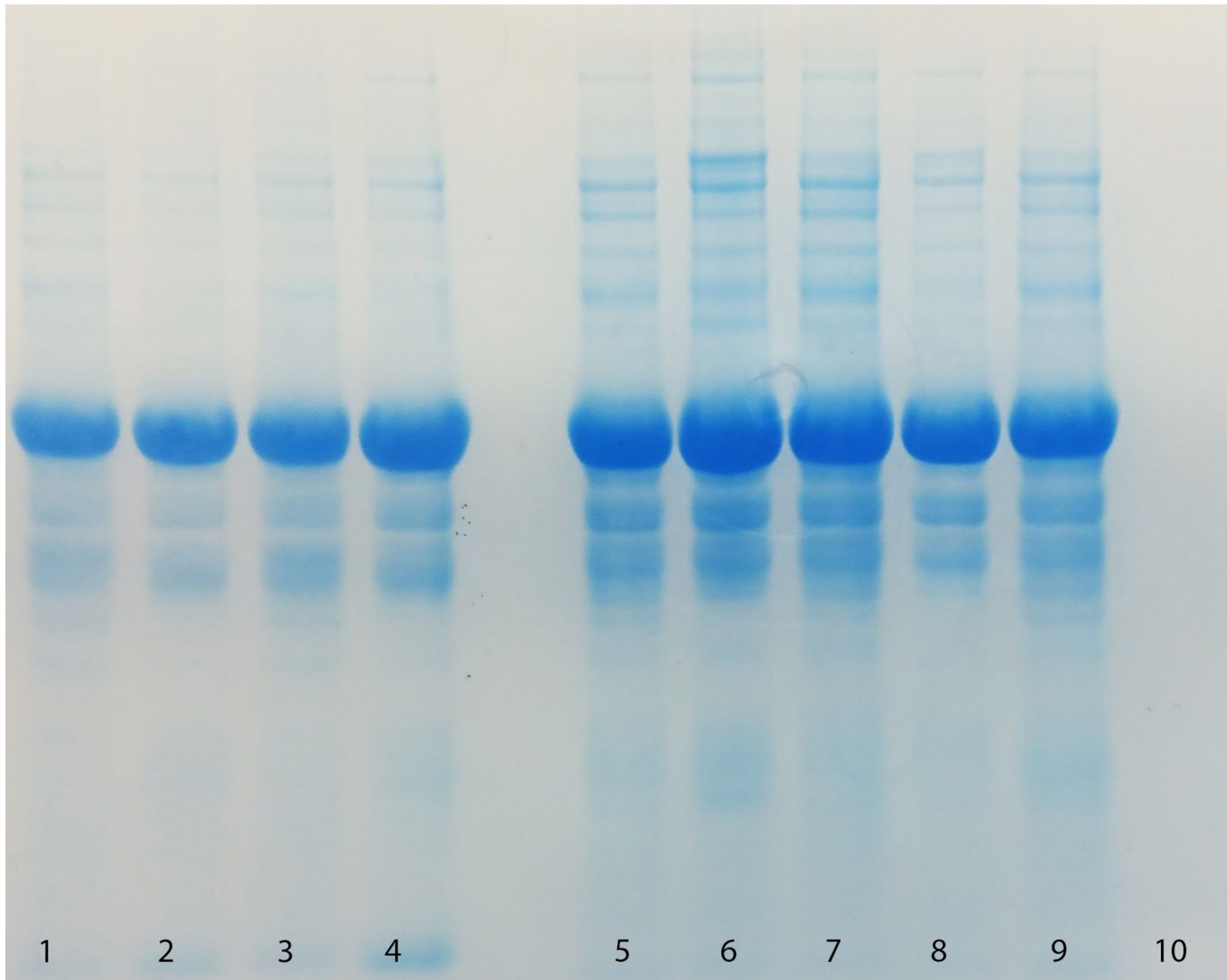


Figure 11. SDS-PAGE Sigma-1.
Image shows the NuPAGE gel after the SDS-PAGE protein separation and the transfer of proteins to the nitrocellulose membrane via blotting, for the samples containing the Sigma-1 antigens, stained with Coomassie staining. All samples collected 6 dpi. Lane 1=Lettuce Sigma-1, 2=Lettuce Negative Control, 3=Lettuce Sigma-1 Control Buffer, 4=Lettuce Negative Control Buffer, 5=*N. benthamiana* Sigma-1, 6=*N. benthamiana* Negative Control, 7=*N. benthamiana* Sigma-1 Control Buffer, 8=*N. benthamiana* Negative Control Buffer, 9=*N. benthamiana* Sigma-1 Control Vacuum Infiltration and 10=Ladder.

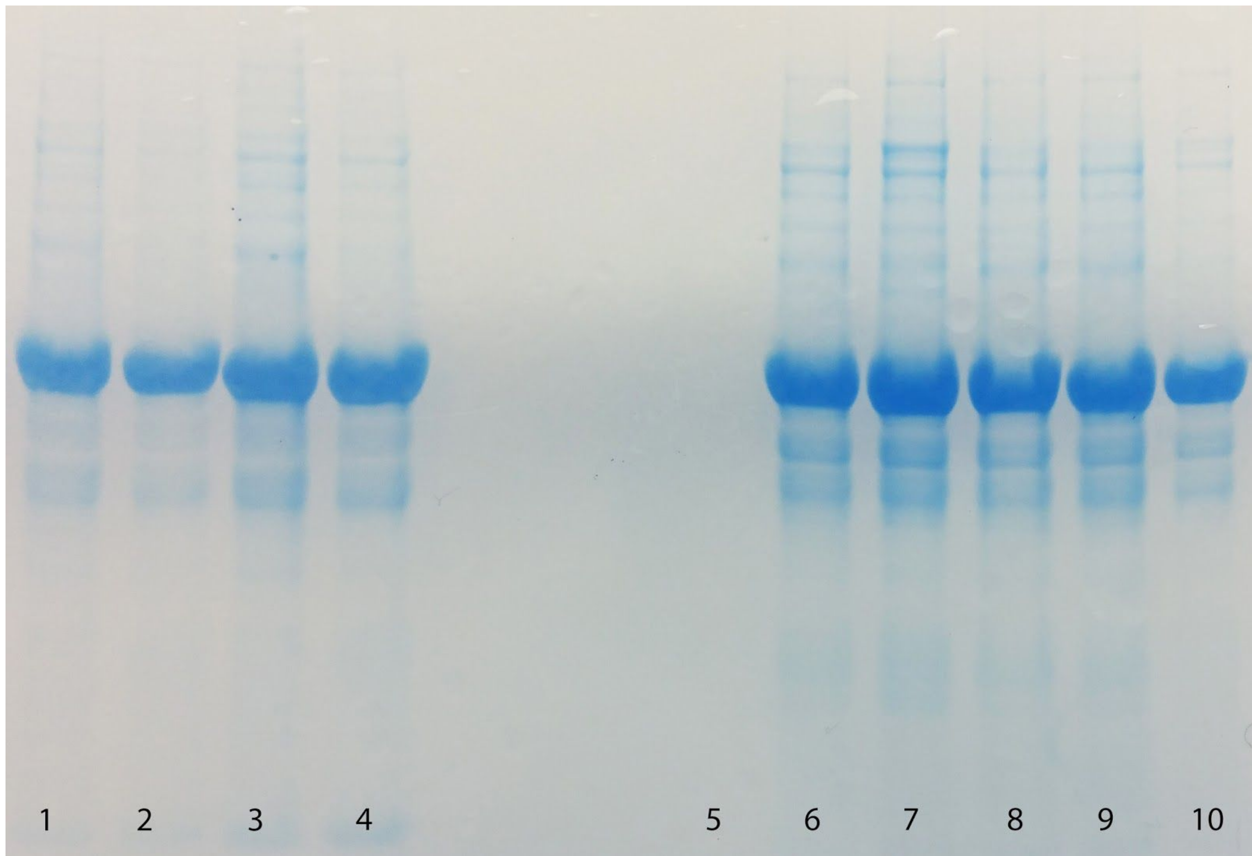


Figure 12. SDS-PAGE Mu-1.
Image shows the NuPAGE gel after the SDS-PAGE protein separation and the transfer of proteins to the nitrocellulose membrane via blotting, for the samples containing the Mu-1 antigens, stained with Coomassie staining. All samples collected 6 dpi. Lane 1=Lettuce Mu-1, 2=Lettuce Negative Control, 3=Lettuce Mu-1 Control Buffer, 4=Lettuce Negative Control Buffer, 5=Ladder, 6=*N. benthamiana* Mu-1, 7= *N. benthamiana* Negative Control Buffer, 8=*N. benthamiana* Mu-1 Control Vacuum Infiltration, 9=*N. benthamiana* Mu-1 Control Buffer and 10=*N. benthamiana* Negative Control Buffer.

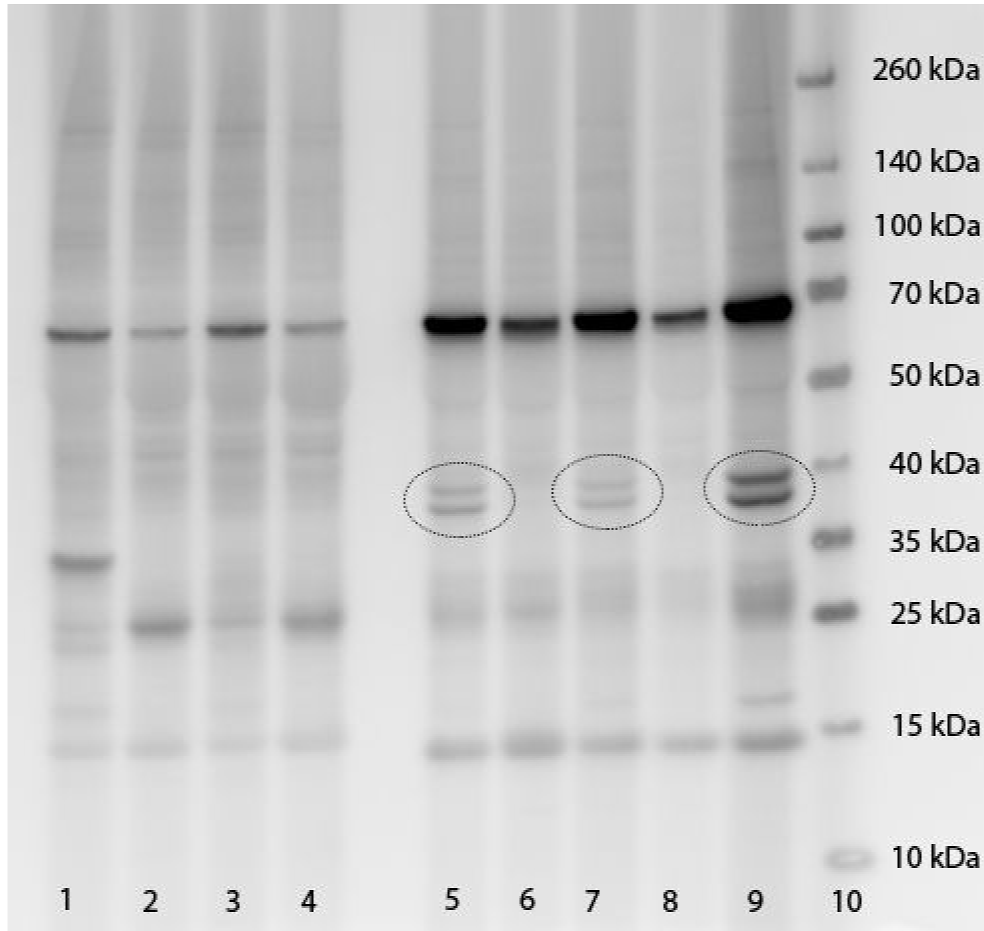


Figure 13. Western blot Sigma-1.
Western blot of samples from the Sigma-1 agroinfiltration at 6 dpi. Lane 1=Lettuce Sigma-1, 2=Lettuce Negative Control, 3=Lettuce Sigma-1 Control Buffer, 4=Lettuce Negative Control Buffer, 5=*N. benthamiana* Sigma-1, 6=*N. benthamiana* Negative Control, 7=*N. benthamiana* Sigma-1 Control Buffer, 8=*N. benthamiana* Negative Control Buffer, 9=*N. benthamiana* Sigma-1 Control Vacuum Infiltration and 10=Ladder. The circles indicate the Sigma-1 bands position.

The western blot of samples from the plants transformed to express the Sigma-1 antigens shows bands in lane 5=*N. benthamiana* Sigma-1, lane 7=*N. benthamiana* Sigma-1 Control Buffer and lane 9=*N. benthamiana* Sigma-1 Control Vacuum Infiltration. The bands are present between the ladder bands indicating 40 kDa and 35 kDA, which corresponds with the theoretical weight of the Sigma-1 protein at 34.6 kDa. The Sigma-1 bands in lane 5, 7 and 9 appears in a double fashion, with one band showing a weight closer to 40 kD while the second is band is lighter. There are no evident Sigma-1 bands in the lanes from the lettuce samples.

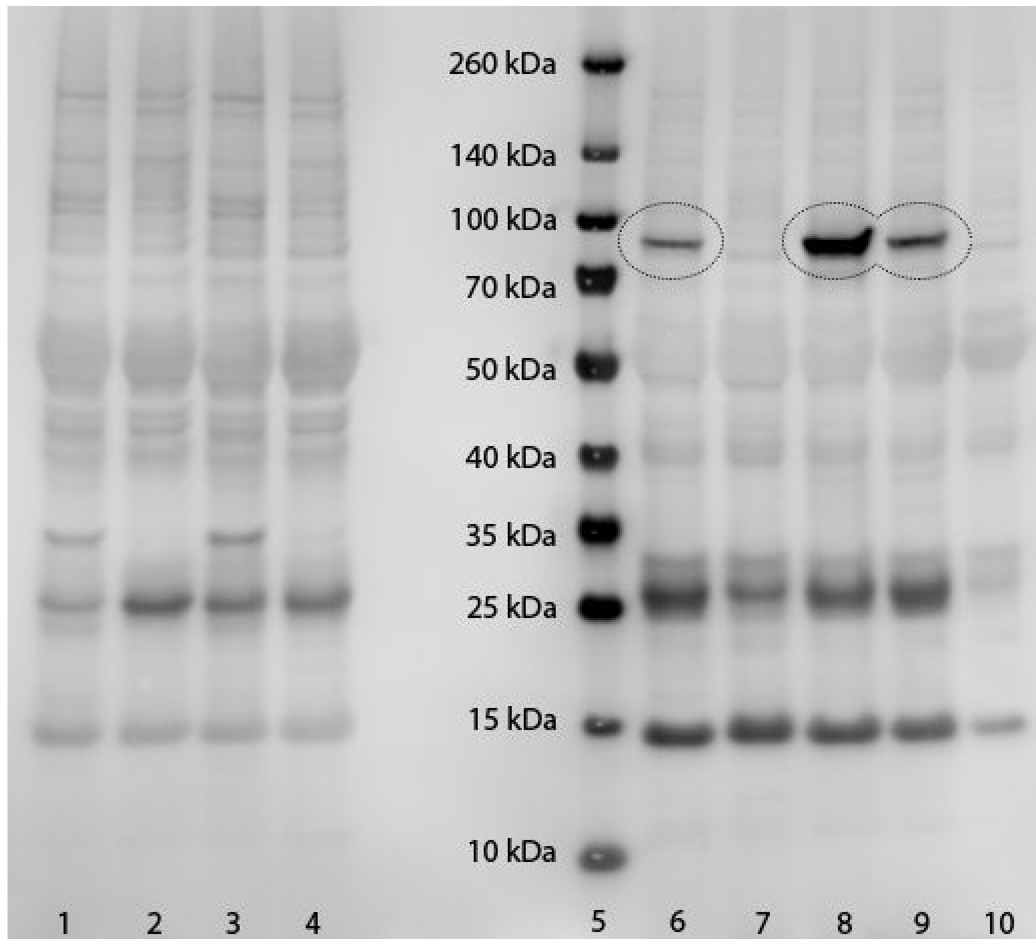


Figure 14. Western blot Mu-1.
Western blot of samples from the transformation with Mu-1 genes at 6 dpi. Lane 1=Lettuce Mu-1, 2=Lettuce Negative Control, 3=Lettuce Mu-1 Control Buffer, 4= Lettuce Negative Control Buffer, 5=Ladder, 6=*N. benthamiana* Mu-1, 7=*N. benthamiana* Negative Control Buffer, 8=*N. benthamiana* Mu-1 Control Vacuum Infiltration, 9=*N. benthamiana* Mu-1 Control Buffer and 10=*N. benthamiana* Negative Control Buffer. The circles indicate the Mu-1 bands position.

The western blot of samples from the plants transformed to express the Mu-1 antigens shows bands in lane 6=*N. benthamiana* Mu-1, lane 8=*N. benthamiana* Mu-1 Control Vacuum Infiltration and lane 9=*N. benthamiana* Mu-1 Control Buffer. The bands are present between the ladder bands indicating 100 kDa and 70 kDA, which corresponds with the theoretical weight of the Mu-1 protein at 74.2 kDa. There are no evident Mu-1 bands in the lanes from the lettuce samples.

4.4 Enzyme linked immunosorbent assay

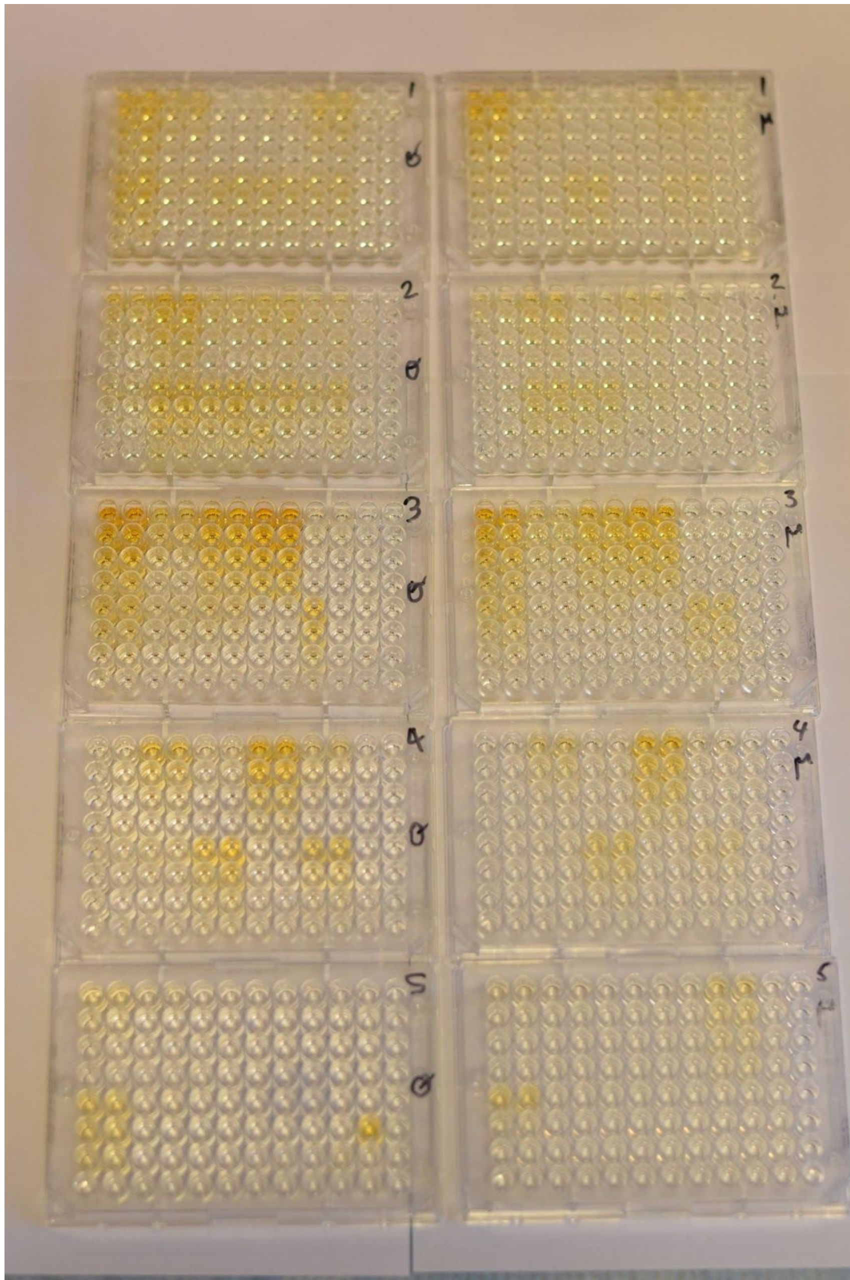


Figure 15. ELISA Results.
The picture shows the actual ELISA test reaction results. The left row is the plates tested against Sigma-1 antigens, and the right row are tested against Mu-1. They are organised from 15 from top to bottom, with 1 = Replicon , 2 = *E. coli*, 3 = *N. benthamiana*, 4 = inactivated whole virus and 5 = negative control group.

The coloration shows clear variance between the different groups, and the strongest color is present on the Sigma-1 *N. benthamiana* group. There is a background coloration in a few of the wells in the negative control groups (Figure 15).

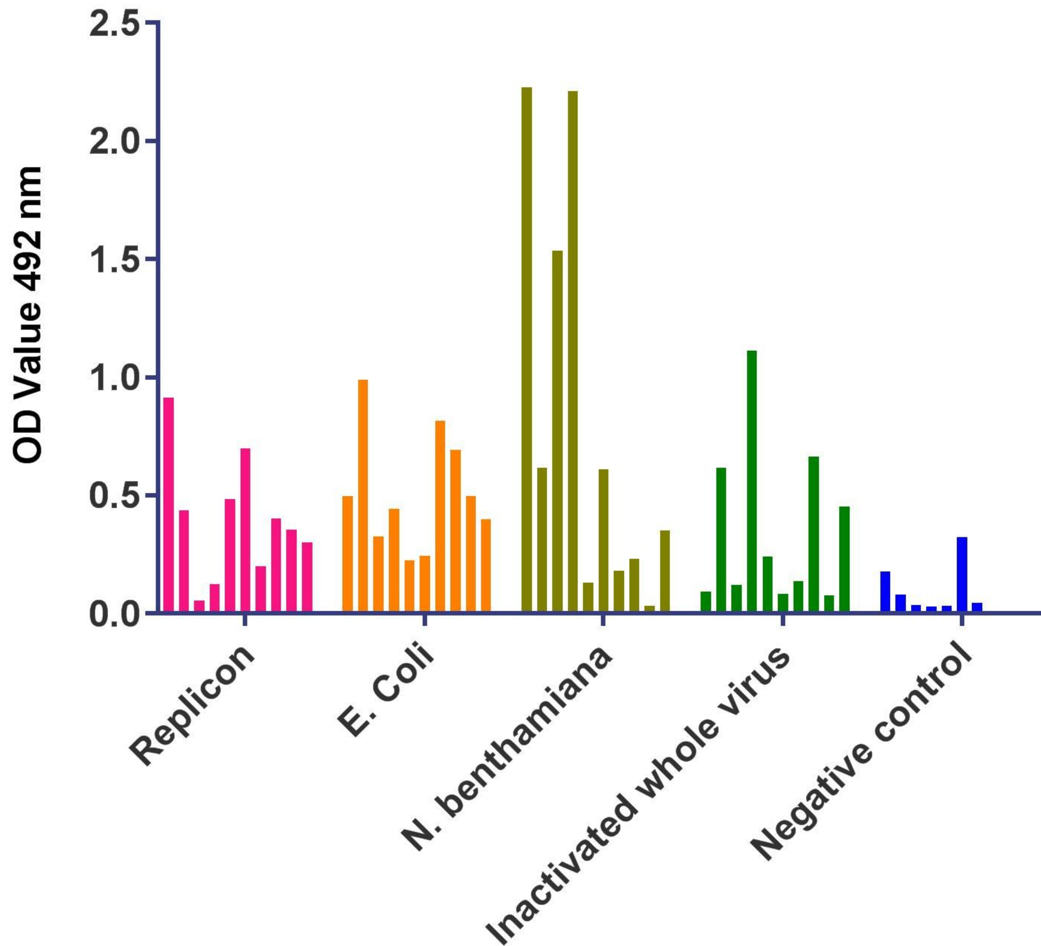


Figure 16. Sigma-1 Antibody levels (ELISA).
The graph shows the OD_{492} nm values from the ELISA test for individual fish (each bar represents one fish) in the five different vaccination groups as well as the negative control group.

Table 5. Statistics for the Sigma-1 data.
This table shows obtained OD values, average, SD, and max and min values for the Sigma-1 ELISA data.

	Replicon	***E. Coli***	***N. benthamiana***	**Inactivated whole virus**	**Negative Control**
Mean	0.3965	0.513	0.8132	0.3603	0.1038
Std. Deviation (SD)	0.2598	0.2499	0.8548	0.3463	0.1107
Minimum	0.05595	0.2248	0.03425	0.0782	0.03125
Maximum	0.9128	0.9915	2.226	1.113	0.3247
Number of values	10	10	10	10	7

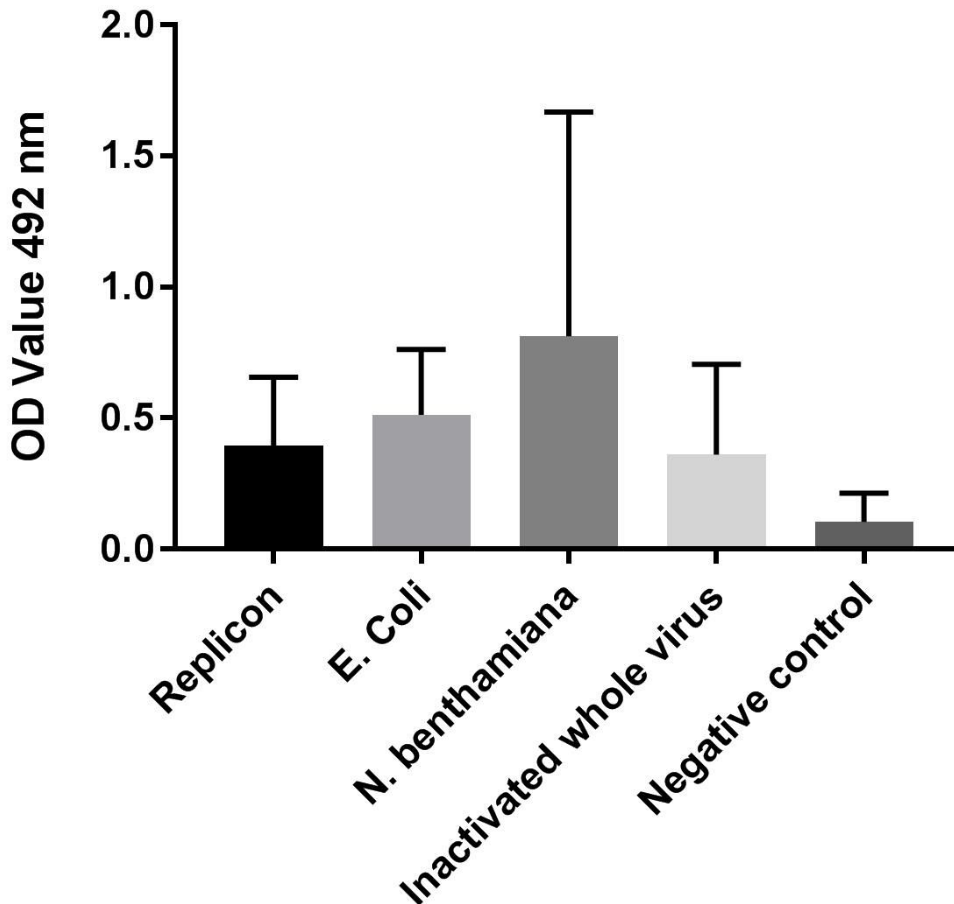


Figure 17. Sigma-1 average antibody levels (ELISA).
The graph shows the average OD (+ standard deviation) values at 492 nm from the ELISA test for the four different vaccination groups as well as the negative control.

The data from the ELISA test of serum from the fish in the Sigma-1 vaccinated fish shows a significant increase in antibody level for the *E. coli* (p=0.001) *N. benthamiana* (p=0.023) and Replicon (p=0.001) groups compared to control. The *N. benthamiana* group has the highest values with maximum at 2.226 OD at 492 nm and a mean of 0.8132. The SD is 0.8548, because of high variation between fish (Figure 17 above). The antibody response in the replicon, *E. coli* and inactivated whole virus groups all show lower variation than the *N. benthamiana* group.

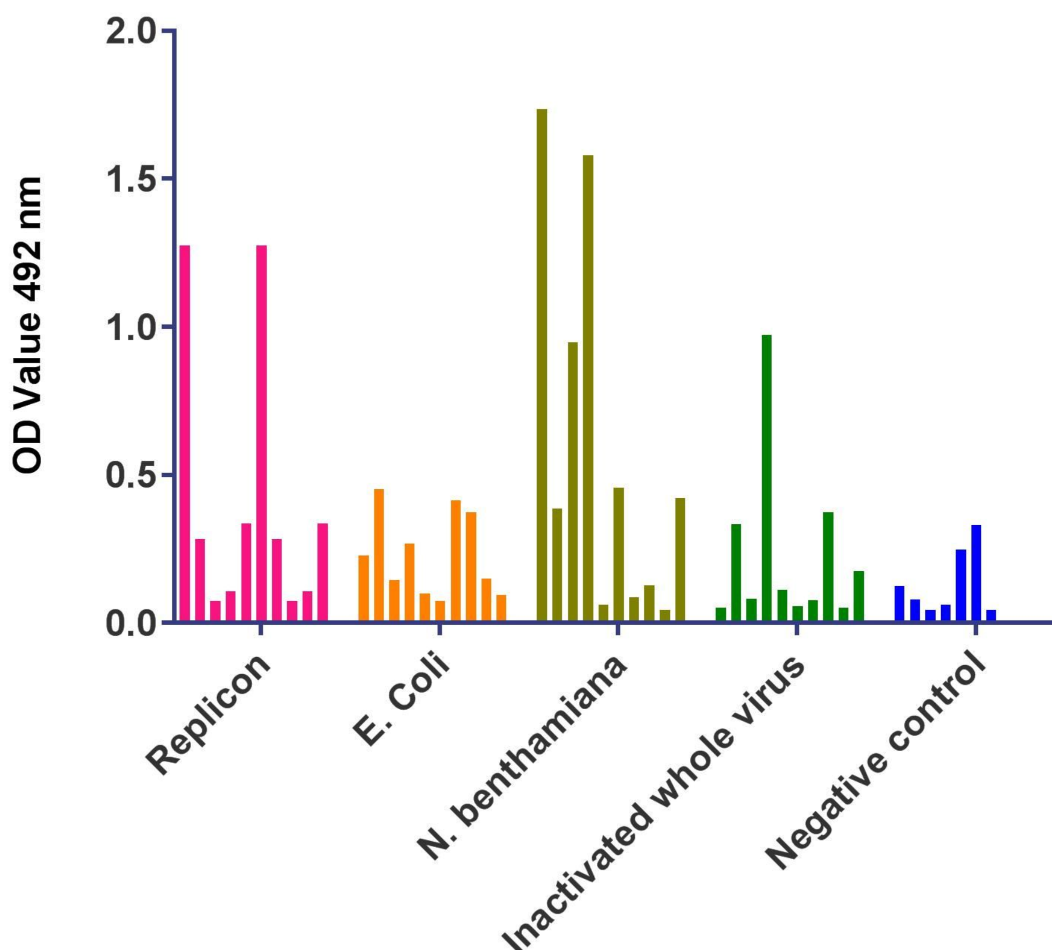


Figure 18. Mu-1 Antibody levels (ELISA).
The graph shows the OD values at 492 nm, from the ELISA test for individual fish (each bar represents 1 fish) in the four different vaccination groups as well as the negative control group.

Table 6. Data for the Mu-1 vaccinated groups
This table shows obtained OD values, average, SD, and max and min values for the Mu-1 ELISA data.

	Replicon	*E. Coli*	*N. benthamiana*	Inactivated whole virus	Negative Control
Mean	0.4149	0.2297	0.5842	0.2286	0.1331
Std. Deviation (SD)	0.4654	0.1409	0.6284	0.2866	0.1123
Minimum	0.0734	0.07315	0.04355	0.0521	0.04435
Maximum	1.275	0.451	1.737	0.973	0.33
Number of values	10	10	10	10	7

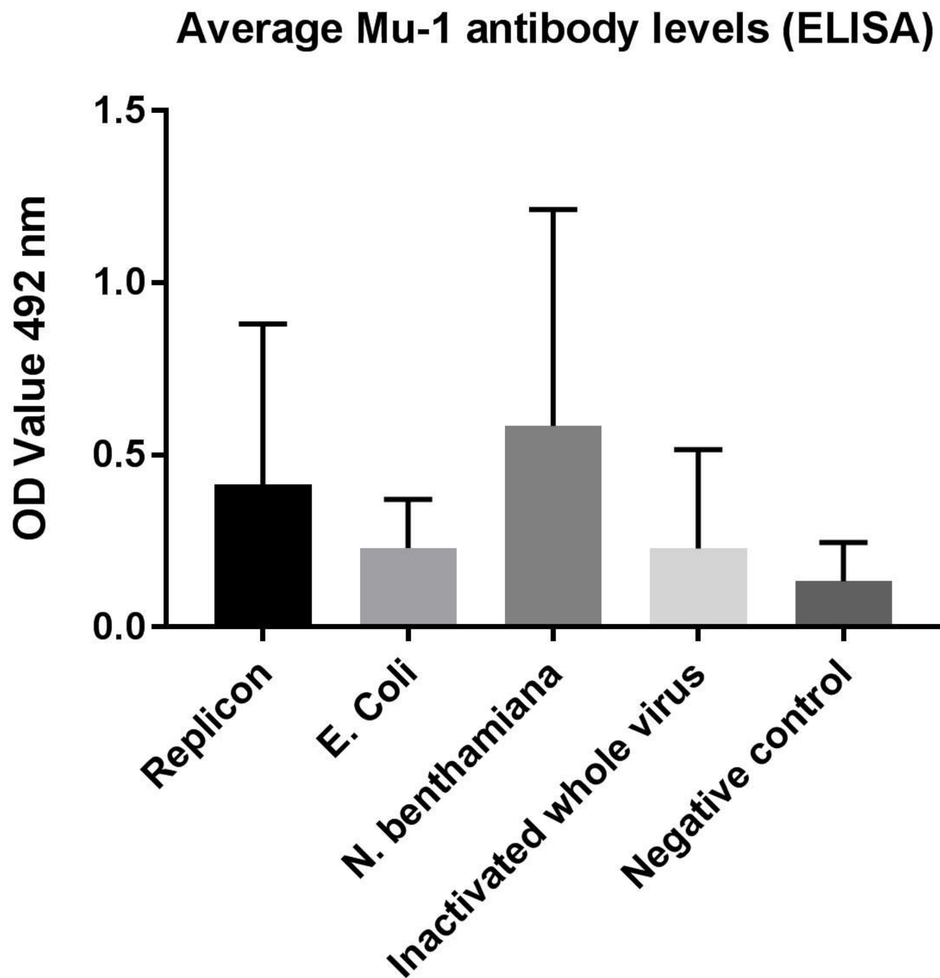


Figure 19. Mu-1 average antibody levels (ELISA).
The graph shows the average OD values (+ standard deviation) at 492 nm for the ELISA test for the four different vaccination groups as well as the negative control.

The data from the ELISA test with the Mu-1 antigen shows no significant increase in vaccinated groups compared to controls (all p values >0.05).

5. Discussion

5.1 Aquaculture and HSMI/PRV

In 1999 a disease that affected heart and skeletal muscle tissue (HSMI) was first recorded in Norwegian Atlantic salmon, and in 2010 the disease was linked with a novel virus named Piscine Reovirus (PRV) (Kongtorp et al. 2004, Palacios et al. 2010). While use of vaccines has been an important factor in the success of modern salmonid farming (Brudeseth et al. 2013) there is as of now no available vaccine against HSMI on the market, nor is there any treatment available against this disease (Fiskehelserapporten, 2015). As the trend in number of outbreaks of HSMI in Norway is increasing (Figure 2), and the disease recently also has been reported in other parts of the world (Kibenge et al. 2013), the development of an efficacious vaccine against the disease is important.

Vaccines used to prevent disease in salmon farming are delivered by injection. Although this is the most effective method, it is cumbersome and expensive. A vaccine administered orally can be an alternative solution (Gudding et al. 2013). *N. benthamiana* contains substances like nicotine and other toxic alkaloids (Fischer et al. 2004) that makes it unsuited for producing an oral vaccine. As lettuce contains no harmful substances (Pniewski et al. 2011), it represents a good alternative when looking for a substitute for *N. benthamiana* as a source of antigen production for an oral vaccine.

5.2 Agroinfiltration

In this thesis *Agrobacterium* stocks was used with the pEAQ-*HT*-DEST2 vector encoding the Sigma-1 and Mu-1 genes, which are part of the PRV outer capsid proteins. Outer capsid proteins are used as a source of antigen for the vaccine preparation because inactivated vaccines primarily induce antibody responses and capsid antigens are prime targets for binding with antibodies. The *Agrobacterium* stocks were provided by supervisor Prof. Jihong Liu Clarke's research group at NIBIO.

Four separate rounds of transient transformation using agroinfiltration was done with the goal being to express the PRV outer capsid Sigma-1 and Mu-1 proteins. All four rounds of lettuce infiltration and subsequent molecular analysis showed, similar to the result presented from the fourth round of agroinfiltration (Figure 13 and Figure 14), no bands on western blot at the expected size for Sigma-1 and Mu-1 antigens.

The leaves infiltrated with *Agrobacterium* showed chlorosis and even some necrosis compared to both the negative controls (Figure 9). This indicates that even though there were no Sigma-1 or Mu-1 antigen found in the lettuce leaves the transformed *Agrobacterium* was successfully infiltrated into the leaves.

The fact that the positive controls in *N. benthamiana* produce the antigens and that the very same infiltration buffer and *Agrobacterium* was used for both the lettuce and *N. benthamiana* (Figure 13 and Figure 14) indicates that the factor that hinders the expression of the

recombinant protein from lettuce lies in the combination of the Sigma-1 and the Mu-1 proteins and lettuce as the host plant. The recombinant proteins could be too toxic for the lettuce host.

Another factor that could have effect on the expression of the Sigma-1 and Mu-1 antigens in lettuce is the choice of vector. The pEAQ vector series is not very explored in combination with transient transformation in lettuce. Actually, the only other recombinant protein expressed using transient transformation in lettuce with the pEAQ vector system is the production the hepatitis C virus E1E2 heterodimer, which was done by supervisor Prof. Jihong Liu Clarke's research group at NIBIO (unpublished).

In an effort to further develop the idea of an oral vaccination against PRV using the Sigma-1 and Mu-1, other types of vectors could be tested to see if they could yield a better result. Another approach could be to screen other possible plant hosts with the same system. Other PRV capsid proteins could also be tested.

Another aspect to be considered in the effort to develop an oral vaccine with recombinant proteins expressed in plants is the fact that the leafy types of crops, like *N. benthamiana* and lettuce, give the expressed protein an unstable nature and needs to handled thereafter (Fischer et al. 2004). An alternative to consider could be to express the protein in a more stable plant tissue, and in that way facilitate the distribution of the antigens. Many cereal plants have been successfully used for this purpose (Stoger et al. 2000). This would of course require to move towards a stable transformation, but it is interesting as a lot of fish feed is plant cereal based.

5.3 SDS-PAGE and Western blot

The Sigma-1 bands in lane 5,7 and 9 in the Western blot in Figure 13 appears in a double fashion, with one band close to 40 kDa and the other band appearing 2-3 kDa lighter. Both the Sigma-1 and Mu-1 constructs are adding 25 amino acids in a N-terminal his-tag. The calculated molecular weight of the amino acids of the his-tag is 2996.4 Da. This makes the total expected calculated weight of the protein expressed by the Sigma-1 construct 37.6 kDa, and the Mu-1 expressed protein 77.2 kDa.

The two separate Sigma-1 bands shown in Figure 13 could be a result of posttranslational modifications (PTMs). Most proteins will go through multiple PTMs, and if not all of the expressed proteins undergo the same modifications, the proteins would achieve different molecular weights. PTMs like glycosylation or other modifications adding residues can will increase the molecular weight, while proteolytic processing can cleave the protein and reduce size (Gomord et al. 2004).

5.4 Fish experiment

5.4.1 Vaccination and blood sampling

The vaccination experiment part of this thesis was run as part of a larger vaccination trial at Faculty of Veterinary Medicine, Oslo, where four different PRV vaccines were tested in Atlantic

salmon. As described in Table 3 there was one vaccine based on PRV replicon, one based on inactivated whole PRV virus as well as two subunit vaccines with antigens produced in *E. coli* and *N. benthamiana*. The subunit vaccines were based on Sigma-1 and Mu-1 antigens.

The initial plan was to also test the Sigma-1 and Mu-1 antigen produced in lettuce in parallel, but as the effort to express these two recombinant proteins in lettuce failed, this was not possible. Instead *N. benthamiana* produced antigens were used, which were compared with other vaccine preparations. The Sigma-1 and Mu-1 antigens, both produced in *E. coli* and *N. benthamiana,* were provided by supervisor Prof. Jihong Liu Clarke's research group at NIBIO.

The vaccination trial went as planned, with sampling of ten fish from the negative control group at day one and ten fish from each of the five groups at six weeks post vaccination. Blood sampling from such small fish can be difficult. This is the reason why we did not get enough blood from all the fish in the control group and ended up with only seven samples compared to ten from the vaccination groups.

5.4.2 Enzyme linked immunosorbent assay

Some of the wells testing the negative control group in the ELISA test showed results above background levels, although none of the fish in this groups had been vaccinated with Sigma-1 and Mu-1 antigens (Figure 15). The reaction could be due to improper washing of the plates during the protocol of the ELISA test but could also result from a persistent infection of the fish with PRV as it is known that the virus is widespread in Norwegian aquaculture (Fiskehelserapporten 2016).

The ELISA test results from the Sigma-1 vaccinated fish shows a significant increase in antibody levels for *E. coli* ($p=0.001$) *N. benthamiana* ($p=0.023$) and replicon ($p=0.001$) vaccinated groups compared to control. The vaccine based on *N. benthamiana* antigens showed the highest antibody levels compared to other groups (Figure 17) which open prospects of using plant-derived antigens for immunization against PRV infection. The data from the ELISA test for the Mu-1 antigen shows no significant increase of antibody titers in vaccinated fish compared to the control groups ($p>0.05$) (Figure 19).

Some of the findings for the Sigma-1 groups warrant additional comments. The main characteristic of a replicon vaccine is its ability to mimic virus replication and in this way provide several rounds of replication. This can result in an elevated amount of antigen being produced compared to traditional protein based or other non-replicating vaccines (Lundstrom 2016). However, level of immune responses (antibody levels) will depend on antigen dose or rather amount of plasmid given and the success of uptake of plasmid into muscle cells after injection. Further, alphavirus replicons are not the most efficient 'drivers' of antigen expression (Evensen, personal communication). For the *E. coli*-based antigens, there were for the main part inclusion bodies and here conformation of inclusion bodies might play a role for the immunogenicity of the vaccine.

Although we see antibodies as a response to the different vaccines it is not certain that these will give any actual protection when challenged with the disease. This would have to be tested with in a challenge study of the vaccinated fish followed by assessment of protection against pathology in target organs (skeletal muscle and heart).

Yet another observation is the variation in antibody levels in the *N. benthamiana* group. The reason for such variation is not easy to explain. This could be a result of uneven distribution of antigens in the vaccine formulation or variation in injection volume of the vaccine but this is less likely. Technical issues have to be considered like washing and incubation time.

6. Conclusions and future perspectives

This thesis has shown that *N. benthamiana* can be used to produce Sigma-1 and Mu-1 antigens of PRV by transient expression via agroinfiltration. The plant produced Sigma-1 PRV outer capsid protein was able to produce antibodies against PRV in vaccinated Atlantic salmon parr. The group of salmon vaccinated with the Sigma-1 antigen produced in *N. benthamiana* resulted in antibody responses when compared to the replicon, *E. coli* and inactivated whole virus based vaccines. This suggests that plants can be used for production of viral antigens for fish vaccination.

Future studies should focus on evaluating the efficacy of these vaccines by challenge studies of vaccinated fish. Different combinations of vector systems and other plant species should also be explored as *N. benthamiana* contains substances disadvantageous for purification and oral administration.

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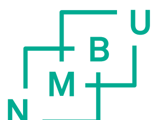

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