SUBCELLULAR LOCALIZATION OF DNAJC14 PROTEIN CHAPERONE IN THE PRESENCE AND ABSENCE OF 

POTATO VIRUS A (PVA) INFECTION

ANGANA BORAH
Master's Thesis
University of Helsinki
Department of Agricultural Sciences
Master's Degree in Biotechnology (Plant Biotechnology)
2017
Potato virus A (PVA) is a single stranded RNA virus, belonging to genus Potyvirus. It is commonly found in potato growing countries and it can decrease potato yield up to 40%. PVA replicates in the cytoplasm of the plant cell, but some of its proteins are targeted to the nucleus. Dnajc14 is a protein chaperone found in potato plants and it plays a role in protein folding, complex assembly and molecular disaggregation. These functions might also help RNA virus infection. When nuclear proteome analysis of potato plants was done, it was found that Dnajc14 protein was present in the nucleus of healthy potato cells, while it was absent from the nucleus of PVA infected cells. The objective of this research work was to confirm the subcellular localization of Dnajc14 in healthy and PVA-infected plants. In this research work, Dnajc14 protein was cloned in fusion with Green Fluorescent Protein (Dnajc14-GFP and GFP-Dnajc14) and the localization of the protein was checked in both healthy Nicotiana benthamiana leaves and N. benthamiana leaves systemically infected with PVA using epifluorescence microscope. PVA was tagged with Red Fluorescence Protein in order to identify the infected cells under the microscope. It was observed that in healthy cells, Dnajc14 protein was localized to the nucleus. When various views of the cells in different leaves were checked, it was found that in PVA-infected cells, the number of cells showing nuclear fluorescence was less than in the healthy cells. The decrease in the amounts of Dnajc14 protein in the nucleus of PVA-infected plants was calculated to be about 31%. The results suggest that there is an alteration in the level of this protein chaperone during PVA infection. This change may be beneficial for the viral infection. This report confirms the localization of Dnajc14 in the nucleus of healthy potato plants.
TABLE OF CONTENTS:

1. Introduction........................................................................................................................................6

2. Review of literature
   2.1 Potyvirus..........................................................................................................................................6
   2.2 Potyvirus Infection.........................................................................................................................8
   2.3 Molecular chaperones and viral infection.....................................................................................8
   2.4 Nuclear proteome analysis............................................................................................................10
   2.5 Nuclear cytoplasmic trafficking.....................................................................................................12
   2.6 Agrobacterium transformation......................................................................................................12
   2.7 Agroinfiltration.............................................................................................................................13

3. Objective of the study.........................................................................................................................13

4. Materials and Methods
   4.1 Plasmids used for GFP fusion protein cloning..............................................................................14
   4.2 RNA Extraction using Trizol..........................................................................................................14
   4.3 RT-PCR............................................................................................................................................15
   4.4 Restriction digestion of restricted vector.......................................................................................17
   4.5 Purification of restricted vectors and the inserts...........................................................................17
   4.6 Ligation Reaction..........................................................................................................................18
   4.7 Preparation of selection plates......................................................................................................18
   4.8 E.coli transformation.....................................................................................................................18
   4.9 Colony PCR....................................................................................................................................18
   4.10 Control digestion of the clones....................................................................................................19
   4.11 Sequencing.....................................................................................................................................20
4.12 Agrobacterium transformation................................................. 20
4.13 Agroinfiltration......................................................................... 21
4.14 Localization of Dnajc14 protein.................................................. 22

5. Results

5.1 Comparison of the two infiltration methods .................................. 23
5.2 Subcellular localization of Dnajc14 in healthy and PVA-infected Nicotiana cells using epifluorescence microscopy ........................................... 24
5.3 Number of cells showing GFP fluorescence (Dnajc14 localization) in healthy cells versus PVA-infected cells ....................................................... 25

6. Discussion...................................................................................... 26

7. Conclusion.................................................................................... 29

8. Acknowledgement....................................................................... 30

9. References................................................................................... 31
1. INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are grown all over the world; Asia and Europe are the major producers. They are infected by various viruses, which causes a decrease in their yield and quality. One of the most widespread potato infection is from *Potato virus A* (PVA), which can decrease the yield of potatoes by 40%. It is very common in potato growing areas of the world, and is transmitted by aphids. The symptoms of PVA include mosaic patterns on leaves, stunting of the plant, necrosis and leaf malformations. The replication mechanism of PVA is quite complex and it also interferes with the host proteins.

Plant viruses replicate in the cytoplasm, but some of their proteins are targeted in the nucleus. This nuclear cytoplasmic trafficking of plant host proteins during viral infection in unknown. In earlier work, the nuclear proteome of the leaves of systemically infected (*Potato virus A*) potato plants was analyzed and compared with healthy leaves. According to the analysis, Dnajc14 protein was only found in the nuclei of healthy potato leaves, but was absent from nuclei samples of PVA-infected leaves (Rajamäki et al., unpublished data). This analysis needs to be confirmed and it might suggest that PVA infection changes the protein localization, which may be helpful in the viral replication.

2. REVIEW OF LITERATURE

2.1 Potyvirus

The genus *Potyvirus* of viruses is the largest known group of plant RNA viruses. It belongs to the family *Potyviridae*. They can cause significant economic loss by decreasing the yield and/or quality of a wide range of crops and is widespread all over the world. For this reason, this group of viruses is studied much more than any other genera of plant pathogenic viruses. They are commonly transmitted by aphids in a non-persistent manner, and some of them are transmitted through seeds. In the last decade, there have been many advances in research regarding the structure,
taxonomy, evolution, epidemiology, diagnosis, functional characterization of viral proteins and molecular interaction with hosts and vectors (Revers and Garcia, 2011).

The virion particles of potyviruses are non-enveloped, filamentous of 720-850 nm long and 12-15 nm in diameter. They have helical symmetry. The genome structure is monopartite, linear and ssRNA strand, which is 10 kb in size. The 3’ end of the genome has a poly-A tail. At the 5’ terminal, potyviruses contain a specific gene called Viral Genome-linked protein (VPg), which has an important role in the infection cycle of the virus, including viral multiplication and movement in plants. (Rajamäki and Valkonen, 2003). By various studies, it is found that VPg can even suppress the sense mediated RNA silencing (Freire, 2014). The central and carboxyl terminus of potyviruses have conserved regions that encode for viral proteins, Protein 1 - Helper Component Proteinase (HCPro) - Protein 3 - first 6kDa protein (6K1) - Cylindrical Inclusion (CI) - second 6kDa protein (6K2) - VPg – Nuclear Inclusion protein A (Nla) – Nuclear Inclusion protein B (Nlb) – Capsid Protein (Revers & Garcia, 2011). Figure 1 shows the genome structure of potyvirus. These proteins have various functions: P1 is a serine protease. HC is a protease that has multifunctional ability. It is involved in aphid transmission, and also in cell-to-cell and long movement and symptom development. It also has the ability to suppress viral induced gene silencing (VIGS) (Anandalakshmi et al., 1998). P3 is a peptide protein and is a viral symptom determinant in plant host. 6K1 helps in the replication cycle of the virus at the beginning of the infection, by forming replication vesicles. (Danci et al., 2009). Cylindrical Inclusion protein (CI) has a role in the virus replication and cell-to-cell movement (Jiménez et al., 2005). 6K2 protein helps the virus in its long-distance movement and symptom induction (Spetz and Valkonen, 2003). Nla and Nlb proteins have nuclear targeting signals. The capsid protein (CP) of potyvirus helps in the disassembly of virus for infection, during initial entry to the plant cell and helps in other functions during the infection cycle like translation of RNA, movement of the virus, display of symptoms and virulence (Danci et al., 2009). Most of these proteins have a role to play in its infection cycle.
Figure 1: Genome structure of potyvirus containing a poly A-tail at the 3’ end and VPg protein at the 5’ end of this linear 10 kb ssRNA strand. The ORF is translated into a polyprotein and is cleaved into 10 functional proteins.

2.2 Potyvirus infection

Like all other plant RNA viruses, potyviruses replicate in the cytoplasm of the host plant. Once the potyviral particles enter the plant cell, the viral genome is uncoated and RNA is released to the cytoplasm, virion disassembly occurs and it gets translated. After infecting a cell, the virus moves to new cells through plasmodesmata and then enters the vascular bundles of the plant. The virus is transported to distant cells through phloem sap, to spread throughout the whole plant (Revers and Garcia, 2011).

Various proteins are associated with the viral infection cycle, which has been studied using fluorescent tags. Different host factors have also been recognized that interact with potyviral protein and has a role in its infection. Studies show that, during PVA infection, a multifunctional nuclear inclusion protein, Nla, accumulates in the nucleus of the infected cell. (Rajamaki and Valkonen, 2009).

Some molecular chaperone proteins are also involved in the viral infection cycle.

2.3 Molecular chaperones and viral infection

Molecular chaperones belong to a protein family that help other proteins in their folding or unfolding and assembly or disassembly. They are found in both plants and animal cells. They are also known as heat-shock proteins (HSP). These are often ATP dependent reactions and are triggered by various kinds of stresses, like heat, cold, UV etc. (Park and Seo, 2015).

After the amino-acid sequence of the proteins is encoded, the chaperones assist proteins to fold into functional structures. Molecular chaperones promote efficient
folding of the proteins to gain functional activity (Hartl et al., 2011). They also help in protein assembly, translocation and degradation. Under stress conditions, molecular chaperones help in stabilizing proteins and refolding them.

Molecular chaperones in plants can be classified into five major families, according to their molecular weight; HSP100, HSP90, HSP70, HSP40 and small HSP (sHSP). They can be present in cytoplasm, nucleus, endoplasmic reticulum, chloroplast and mitochondria (Park and Seo, 2015).

HSP100 proteins offer heat tolerance in plants. HSP90 responds to various stress conditions and is a core component of various protein complexes. It also has a role in the normal growth and development of some plants (eg. Arabidopsis). HSP70 is the most abundant class of heat-shock proteins and helps in protein disaggregation and folding. It also controls the viral replication cycle and movement of the virus. HSP40/DnaJ proteins are a co-chaperone of HSP70 system and has a role in viral infection cycle and viral intracellular and intercellular movement. sHSP helps in protein binding and refolding during thermal stress. They are also involved in viral infection (Verchot, 2012).

In this research work, the molecular chaperone Dnajc14 was studied. Dnajc14 belongs to HSP40 group of molecular chaperones. DnaJ proteins are found in a wide range of organisms from bacteria to humans. DnaJ proteins contain a N-terminal conserved domain called J domain, that interacts with HSP70 and C-terminal region of 120 to 170 residues (Verchot, 2012). Dnajc14 is of size 1122 nucleotides / 373 amino acids.

Molecular chaperones are also known to help in potyviral infection (Hofius et al., 2007). DnaJ plays a major role in protein folding, complex assembly and molecular disaggregation during RNA virus infection (Verchot, 2012). DnaJ proteins contribute to membrane bound events relating to virus intercellular movement. Two homologous NSm-binding proteins (movement proteins) of DnaJ family from Nicotiana and Arabidopsis were identified by Soellick et al., 2000.

A study on Potato virus Y infection in tobacco plants showed that the coat protein of the virus interacts with a novel subset of DnaJ-like proteins, named NtCPIPs, of tobacco. It was identified that the coat protein core region is essential for
plasmodesmal trafficking. They suggested that NtCPIPs act as important susceptibility factors during PVY infection, possibly by recruiting heat shock protein 70 chaperones for viral assembly and/or cellular spread. (Hofius et al, 2007).

Another study on PVA infection in *N. benthamiana* D. shows HSP70 along with its co-chaperone CPIP can affect the function of viral coat protein, ultimately in potyviral infection. They found that HSP70 and CPIP have a major role in the translational activity of the virus that is associated with potyviral replication (Hafren et al, 2010).

To know about the proteins or chaperone proteins present inside a cell, nuclear proteome analysis could be done.

2.4 Nuclear proteome analysis

A nucleus is a complex cell organelle and the determination of its protein content can be useful in any systematic characterization. The nuclear proteome analysis can detect the protein content of the nucleus. The results of this method provide direct evidence that the nuclear proteome consists of at least 14% of the entire proteome (Fink et al, 2008).

Nowadays, there are optimized methods for analysing the nuclear proteome. The nucleus of the plant cell is isolated and the proteins present are identified. Plant tissues are homogenized and by filtering, pelleting and eliminating debris/other cell-organelles, the nucleus is separated using density gradient centrifugation. Heavy nuclei are collected at the bottom through this.

After isolating nuclei, proteins are isolated using methods like two-dimensional protein electrophoresis (which separates the proteins based on their isoelectric point and molecular weight). These protein molecules are then digested by specific protease (for example: pepsin, trypsin etc.) to form peptides. Peptides are then allowed to separate using Liquid chromatography or Electrospray ionization mass-spectrometry, or matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry or combination of these techniques, and thus, they are identified. The identified proteins function can then be classified by performing database search.

The overview of the process of nuclear proteome analysis is mentioned in the figure below (*Figure 2*):
In 2004, Khan and Komatsu analyzed the nuclear proteome of rice (*Oryza sativa*) and identified 190 proteins. It was found that most of the identified proteins have a role in gene expression and regulation of the plant cell.

In 2008, Casati *et al.* found that in the nuclear proteome of maize (*Zea mays*) when the plants were exposed to UV-B light, there was a difference in the amount of protein present, than those plants which were not exposed to UV-B light.

While analyzing the nuclear proteome of potato plants, it was found that the nucleus of the healthy plants contains Dnajc14 protein chaperone but it was absent in the nuclear proteome of the PVA infected plants. (Rajamäki *et al.*, unpublished data).

Some of the nuclear proteins may be transported to other cell organelles under stress conditions. This transport takes place through nuclear pores and the mechanism is known as nuclear-cytoplasmic trafficking.
2.5 Nuclear-cytoplasmic trafficking

Nuclear-cytoplasmic trafficking means the transport of various proteins into or out of the nucleus through nuclear pore complexes (NPCs). These transports are triggered by nuclear targeting signals. The proteins that need to be targeted into the nucleus carry nuclear localization signal (NLS) and the proteins that need to be exported from the nucleus carry nuclear export signal (NES). The transport of the proteins through the NPCs is facilitated by soluble transport receptors called importins and exportins, which recognizes the signals and bind to the proteins for transportation (McPherson et al., 2015).

There have been recent studies on subcellular localization of protein during viral infection. The localization is determined by using several techniques, like the expression of viral proteins fused to fluorescent tags in plants (particularly by agro-infiltration in N. benthamiana) and tracking them in the cells using epifluorescence microscopy (Revers and Garcia, 2011). The reason behind this nuclear trafficking is still a matter of research.

2.6 Agrobacterium transformation

*Agrobacterium tumefaciens* is a gram-negative, non-sporing, motile, rod-shaped bacterium which causes crown gall disease of a wide range of dicotyledonous (broad-leaved) plants. It causes infection by transferring T-DNA part of its Ti (tumour-inducing) plasmid to the plant, and this DNA integrates into the plant’s genome, causing the production of tumours and associated changes in plant metabolism. This unique mode of action of *A. tumefaciens* has enabled this bacterium to be exploited by many plant biologists in molecular and genetic studies to introduce DNA into plants (Gelvin, 2003).

T-DNA binary vectors revolutionized the use of Agrobacterium to introduce genes into plants. These plasmids are small and easy to manipulate in both *Escherichia coli* and Agrobacterium and generally contain multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned. Many vectors were designed for specialized purposes, containing different plant selectable markers, promoters, and poly (A) addition signals between which genes of interest could be inserted, translational enhancers to boost the expression of transgene, and protein-
targeting signals to direct the transgene-encoded protein to locations within the plant cell. Since the initial discovery of its mode of action in the early 1980s, scientists are attempting to improve this “natural genetic engineer” for biotechnology purposes. (Gelvin, 2003).

2.7 Agroinfiltration
Agroinfiltration is a versatile, rapid and simple technique that is widely used for transient gene expression in plants. Transient expression of a gene is that the gene is transcribed and translated but it is not integrated to the genome of the plant. So, this is an easy method of gene delivery and the gene can be detected within a short time.

In this research work, the binary vector, pA is used for Agrobacterium transformation with Dnaj-GFP and GFP-Dnaj inserts.

3. OBJECTIVE OF THE STUDY
The aim of this study is to confirm the subcellular localization of Dnajc14 protein chaperone in healthy plants and PVA-infected plants.

Dnajc14 is a protein chaperone found in potato plants. In an earlier unpublished study where nuclear proteome analysis was done of PVA infected potato leaves and healthy ones, it was observed that there is a difference in localization of Dnajc14 protein in the two cases. In healthy plant, Dnajc14 was localized to the nucleus, while in the PVA-infected plants Dnajc14 was absent from the nucleus of the cells. Thus, the aim of this study was to confirm the sub-cellular localization of this protein chaperone, Dnajc14.

To carry out the research the Dnajc 14 protein was cloned in fusion with Green Fluorescent Protein (GFP) and examined under epifluorescence microscope for its localization, in healthy Nicotiana benthamiana plants and PVA-infected N.benthamiana plants. PVA was tagged with Red Fluorescent Protein (RFP) to identify the cells containing infection, under the microscope.
4. MATERIALS AND METHODS:

4.1 Plasmids used for GFP fusion protein cloning:

In this research work, two plasmid vectors were used for cloning.

i) pRT-GFP-TGBpX plasmid, containing *Bam*HI and *Xba*I restriction sites, was used for GFP-gene fusion, and

ii) pRT-GFP plasmid, containing *Eco*RI and *Nco*I restriction sites was used for gene-GFP fusion.

4.2 RNA extraction using Trizol:

RNA was extracted from leaves of potato variety, Pentland Crown, according to Trizol protocol. For this method, leaves were ground in liquid nitrogen and transferred to 2 ml Eppendorf tubes and 1.5 ml of Trizol was added and vortexed. The samples were then incubated at room temperature for 5 mins. Centrifugation at 12000 g for 10 mins at 4°C was done. The supernatant was transferred to a new 2 ml tube. 0.4 ml of chloroform was added and shaken vigorously by hand for 15 sec. All these steps mentioned above were done in the fume-hood. The samples were then incubated for 5 min at room temperature. Again, centrifugation was done for 15 mins at 12000 g at 4°C. The upper aqueous phase was collected in a fresh tube and 1 ml of isopropanol was added and mixed. After that, centrifugation for 20 min at 12000 g was done at 4°C. The pellet was washed using 1 ml of 75% ethanol and vortexed, followed by centrifugation at 12000g for 15 mins at 4°C to collect the pellet. The pellet was then dried by keeping the tubes upside down on paper towels, until the pellet became clear. The pellet was then dissolved in 70µl of nuclease-free water by heating at 55-60°C for 10 mins.

The isolated RNA was then purified using Lithium Chloride (LiCl) precipitation method. For this, 35µl of 8 M LiCl was added to the samples and kept in -20°C overnight. After overnight incubation, the tubes were centrifuged for 30 mins at 13000g at 4°C. Then the pellet was washed with 75% ethanol and centrifuged for 5 min at 13000g. The pellet was dried and dissolved in 20µl of nuclease-free water.

The concentration and purity of the isolated RNA samples were determined using nanodrop spectrophotometer the wavelength of 260 nm and the absorbance ratio at
260 nm/280 nm (A260/280) and 260nm/230 nm (A260/230). RNA absorbs UV light most strongly at 260 nm and thus, the ratio 260 to 280 is used to determine the purity of the RNA samples. An absorbance ratio ~2.0 is considered to be "pure".

4.3. RT-PCR:

Reverse transcriptase PCR was performed to amplify the Dnajc14 protein gene from the RNA samples. The following steps were performed for this:

4.3.1 DNase treatment of RNA samples:

The isolated RNA samples were given DNase treatment to remove any DNA contamination. For this, 1µg of each RNA sample was mixed on ice, with 1µl of RNase-free DNase 10X Reaction buffer, 1µl of RNase-free DNase (1U/µl) and the final volume was made to 10µl. The mixture was then incubated at 37°C for 30 minutes and then 1µl of DNase Stop Solution was added to terminate the reaction, followed by incubation at 65°C for 10 minutes to inactivate the DNase.

4.3.2 Reverse-transcription reaction:

After the DNase treatment, the 11µl of the RNA samples were mixed with 1µl of random hexamers and heated to 70°C for 10 minutes and cooled down quickly on ice. The contents were then spun down, and these were added: 4µl of 5X M-MLV reverse transcriptase buffer, 2µl of Dithiothreitol (0.1 M), 1µl of dNTPs, 0.5µl of Rnasin (Promega) and 1µl of M-MLV reverse transcriptase (200 units/µl; Promega). The contents of the tubes were then mixed well and incubated at 37°C for 1 hour. Then reaction was then stopped by heating it at 70°C for 10 mins. The cDNA samples were then stored in –20°C for later use in PCR.

4.3.3 Primers for RT-PCR:

The primers that were designed are as follows. The underlined sequences refer to the site of restriction of each specific restriction enzyme.
For cloning of *Solanum tuberosum* Dnaj14 to pRT-GFP-TGBpX vector (>pRT-GFP-Dnaj14):

<table>
<thead>
<tr>
<th>Gdnaj14-pRTBamHI-F (BamHI)</th>
<th>5’-ataagatccATG GAACATCAATACTACAAG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gdnaj14-pRTXbaI-R (XbaI)</td>
<td>5’-aattctagaTTA GTTACTTCTAGGCCTAC-3’</td>
</tr>
</tbody>
</table>

For cloning of *Solanum tuberosum* Dnaj14 to pRT-GFP vector (>pRT-Dnaj14-GFP):

<table>
<thead>
<tr>
<th>dnaj14G-pRTEcoRI-F (EcoRI)</th>
<th>5’-ttagatccATG GAACATCAATACTACAAG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaj14G-pRTNcoI-R (NcoI)</td>
<td>5’-aattctaggcggcGTATACTTCTAGGCCTACC-3’</td>
</tr>
</tbody>
</table>

For sequencing/colony-PCR:

As Dnajc14 protein gene is 1122nt, 373 aa long, a random sequence was taken from the middle of the Dnajc14 sequence and a primer was designed, so that we get the full-length DNA sequence after cloning.

| dnaj14-F                     | 5’-GTCAGAGGAGGTATCATAGC-3’ |

**4.3.4 PCR conditions and reaction:**

Phusion DNA polymerase (Thermo Fisher Scientific) was used in the PCR reaction to amplify the Dnajc14 protein gene using the specific designed primers (as mentioned above in 4.3.3). The following PCR reaction and conditions were followed:

- Reaction:

<table>
<thead>
<tr>
<th>10x Phusion HP buffer</th>
<th>10μl</th>
<th>10x Phusion HP buffer</th>
<th>10μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM dNTP</td>
<td>1μl</td>
<td>10mM dNTP</td>
<td>1μl</td>
</tr>
<tr>
<td>BamHI forward</td>
<td>2 μl</td>
<td>EcoRI forward</td>
<td>2 μl</td>
</tr>
<tr>
<td>XbaI reverse</td>
<td>2μl</td>
<td>NcoI reverse</td>
<td>2μl</td>
</tr>
<tr>
<td>Phusion (2U/μl)</td>
<td>0.5μl</td>
<td>Phusion (2U/μl)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>cDNA</td>
<td>2μl</td>
<td>cDNA</td>
<td>2μl</td>
</tr>
<tr>
<td>MQ water</td>
<td>32.5μl</td>
<td>MQ water</td>
<td>32.5μl</td>
</tr>
</tbody>
</table>
• **Conditions:**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initiation</strong></td>
<td>98°C</td>
<td>3'</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>98°C</td>
<td>15''</td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
<td>58°C</td>
<td>15''</td>
</tr>
<tr>
<td><strong>Elongation</strong></td>
<td>72°C</td>
<td>40''</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>98°C</td>
<td>15''</td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
<td>64°C</td>
<td>15''</td>
</tr>
<tr>
<td><strong>Elongation</strong></td>
<td>72°C</td>
<td>40''</td>
</tr>
<tr>
<td><strong>Final</strong></td>
<td>72°C</td>
<td>10''</td>
</tr>
</tbody>
</table>

4.4 **Restriction digestion of the vectors and the insert:**

The vectors (pRT-GFP-TGBpX and pRT-GFP) and the PCR products (inserts containing *dnajc14* gene) were restricted using restriction enzymes specific for each. Plasmid pRT-GFP-TGBpX was restricted at *BamH* I and *Xba* I restriction sites and plasmid pRT-GFP was restricted at *EcoR* I and *Nco* I sites. The digestion mixture was prepared and incubated in heat block at 37°C for 1 hour 30 mins. 1 µl Calf Intestine Alkaline Phosphatase (CIAP) was added to the vector after this period and incubated for 30 minutes. CIAP catalyzes the hydrolysis of 5' phosphatase groups from the restricted sites of the vector and prevents self-ligation.

4.5 **Purification of the restricted vectors and the inserts:**

The vectors (pRT-GFP-TGBpX and pRT-GFP) and PCR products were purified using kit: VWR Omega Gel Extraction Kit and then analyzed on the gel.
### 4.6 Ligation Reaction:

T4 ligation enzyme (Promega) was used for the ligation reaction and the protocol was followed according to the standard.

### 4.7 Preparation of the selection plates:

The pRT plasmids contain ampicillin resistance gene. Thus, LB agar medium containing ampicillin were prepared to select the ligated samples. For this, 7.5 g of Bactor Agar was added in 500ml water and autoclaved at 121°C under 1 atm for 25 minutes. To this media, 150 μg/ml Ampicillin was added for selection.

### 4.8 *E. coli* transformation: Cloning

The cloning of vector DNA is a key step in genetic engineering, gene studies and many other applications in plant research. A cloning vector is a piece of DNA from a virus, plasmid or other organism in which a foreign DNA is inserted and which can be transferred to other organisms, e.g. *E. coli*.

In this research work, pRT-GFP-TGBpX plasmid and pRT-GFP plasmid were used for cloning GFP-Dnaj insert and Dnaj-GFP insert respectively.

Competent cells of *E. coli*, DH5α were used for transformation using heat-shock protocol. In this protocol, competent cells were thawed on ice and to 5 μl of chilled ligation mixture was added to 50 μl of competent cells. It was mixed gently by flicking the tube 4-5 times. Then, the mixture was placed on ice for 30 minutes, followed by heat shock at 42°C for 90 seconds. After heat-shock, the samples were transferred and incubated on ice for 2 minutes, to which 600 μl of room temperature LB was added. Then the tubes were placed at 37°C for 60 minutes in shaker and centrifuged at 600 rpm for 2 minutes. The selection plates were warmed to room temperature and 100 μl of the ligation mixture is spread onto the plates. The plates were incubated overnight at 37°C.

### 4.9 Colony PCR:

The clones that showed growth in the ampicillin containing selection plates were picked up for colony PCR. Colony PCR was done to check whether the transformed
plasmids contain our specific insert or not, and the products were analysed in the gel. F-501 Dynazyme II (Thermo Fisher Scientific) enzyme was used to amplification.

The PCR reaction and conditions followed were:

- **Reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x optimized Dynazyme buffer</td>
<td>2.5μl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>0.5μl</td>
</tr>
<tr>
<td>EcoRI forward primer</td>
<td>1 μl</td>
</tr>
<tr>
<td>NcoI reverse primer</td>
<td>1μl</td>
</tr>
<tr>
<td>F-501 Dynazyme II (2U/μl)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>MQ water</td>
<td>19.5μl</td>
</tr>
</tbody>
</table>

- **Conditions**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation</td>
<td>94°C</td>
<td>5'</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30''</td>
</tr>
<tr>
<td>Annealing</td>
<td>52°C</td>
<td>30''</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>1'</td>
</tr>
<tr>
<td>Final</td>
<td>72°C</td>
<td>5'</td>
</tr>
</tbody>
</table>

4.10 Control Digestion of the Clones:

The samples giving positive bands in the colony PCR were then used for plasmid isolation. Plasmids were then isolated using kit Gen Elute Plasmid Miniprep Kit (Sigma). Then, to check the integration of the insert to the plasmid, digestion is done using EcoRI and NcoI for Dnaj-GFP clones and BamH I and Xba I for GFP-Dnaj clones.
4.11 Sequencing of clones

The clones were sent for sequencing to Haartman Institute, Helsinki for sequencing.

4.12 Agrobacterium transformation:

4.12.1 Transfer to binary vector:

For Agrobacterium transformation, the expression cassette was transferred to a binary vector, pA plasmid which is about 10kb in size and has spectinomycin resistance gene. The region between the two HindIII restriction sites is the whole expression cassette. So, restriction digestion of these two sites was done and the region was transferred to the binary vector, pA. A binary vector contains two different plasmids: binary plasmid and helper plasmid. Helper plasmid contains vir genes and it is already inside Agrobacterium cells. It contains the T-DNA region which is transferred during transformation process and other one contains vir gene that facilitate the transduction of the T-DNA. The same steps were followed as mentioned above for the cloning of the binary vector.

4.12.2 Preparation of Agrobacterium competent cells:

Agrobacterium cells were grown in LB-rifampicin (25µg/ml), carbenicillin (100µg/ml) media and left for shaking overnight at 28°C. The next morning, 1ml of culture is taken and grown in fresh LB-rif(25µg/ml), carb(100µg/ml) media until the OD(600nm) becomes 0.5. The cells were then chilled on ice for 5 mins and then centrifuged at 3000g for 5 mins at 4°C. The cells were then resuspended in 1ml of 20 mM Calcium Chloride solution with 15% glycerol. This was then transferred to Eppendorf tubes and frozen in liquid nitrogen to –80°C.

4.12.3 Transformation:

The Agrobacterium competent cells were thawed in ice and 50ng of the plasmid DNA was added to the tubes, followed by freezing it in liquid nitrogen for 5 mins. Again, the cells were thawed at 37°C for 5 mins, to which LB solution was added and incubated at 28°C for 1-2 hours with gentle shaking. The cells were then centrifuged and most of the supernatants were discarded. The cells were then plated in selection plates containing LB-rifampicin (25µg/ml), carbenicillin (100µg/ml), spectinomycin (100µg/ml) and grown at 28°C for 2 days.
4.12.4 Colony PCR:

Colony PCR was performed to check whether the colonies have the correct insert. For colony PCR, the samples were boiled for 3-5 minutes at 100°C in 50 µl water. Then 2µl of the samples were used for the PCR reaction and the products were then analyzed in the gel. The same PCR conditions and reaction was used as mentioned before in 4.9.

4.13 Agro Infiltration:

The positive clones screened through colony PCR were then used for agroinfiltration. In 5 ml of LB-rif(25µg/ml), carb(100µg/ml), sm(100µg/ml) media was used to grow the transformed Agrobacterium and incubated overnight at 28°C. The next day the cells were collected by centrifugation for 10 minutes at 4000 rpm, room temperature. The pellet was re-suspended in a 5ml solution of 10mM MgCl₂ and 20µM acetosyringone and vortexed. The solution is then kept for 3 hours at room temperature. The Optical Density (OD)₆₀₀ was adjusted to 0.6. The OD is adjusted at 600 because this wavelength is optimum to measure Agrobacterium growth.

Nicotiana benthamiana plants were used for infiltrating. Half of the Nicotiana plants were healthy and half of them were Potato Virus A infected. PVA was tagged with RFP to identify the cells with infection under epifluorescence microscope. The PVA infection was done in two different ways:

I) PVA infected at the same time as agroinfiltration:

N. benthamiana plants were allowed to grow till 5 to 6 leaves stage and the middle-aged leaves of the plants are chosen for infiltration. The cloned constructs along with PVA-RFP is infiltrated in the ratio of 1:1 to leaves of half of the plants. To the other half, cloned constructs along with RFP were infiltrated.

II) PVA infected plants (10 days infection) used for agroinfiltration:

In this method, when the N. benthamiana were at three leaves stage, the lower leaves were infiltrated with PVA-RFP and then allowed to grow. After 10 days of PVA inoculation, the cloned constructs were infiltrated to the upper leaves of the infected plants. To the other healthy plants, cloned constructs along with RFP were also infiltrated to those.
4.14 Localization of Dnajc14 protein chaperone:

After Agroinfiltration, plants were allowed to grow for three days. Then on the third day, Zeiss epifluorescence microscope (Axioimager M2) was used to detect the localization of the Dnajc14 protein chaperone in the healthy and the PVA infected *Nicotiana* leaves. Filters Alexo Fluor 555 and Alexo Fluor 488 was used to check RFP and GFP fluorescence respectively, and photographs were taken at exposure 190 seconds and 260 seconds respectively.
5. RESULTS

5.1 Comparison of the two infiltration methods

The experiments were carried out in healthy *N. benthamiana* plants and PVA infected *N. benthamiana* plants. There were two means of infiltrating the constructs containing GFP-tagged Dnajc14 gene. In one method, the constructs and the PVA-RFP was infiltrated simultaneously to the leaves of *N. benthamiana*. However, this method did not allow for the spread of infection in the leaves evenly, which caused difficulty in checking the results under microscope after three days of inoculation. The other method was to infect young *N. benthamiana* leaves with PVA-RFP and then after 10 days, infiltrate the constructs to the upper leaves of the systemically infected plants. After ten days, the initial PVA inoculated leaves showed symptoms of PVA infection like yellowing of leaves, as seen in Fig 3. When the constructs were infiltrated, it was allowed to grow for three days and the leaves were then observed under epifluorescence microscope for fluorescence. Several pieces of different leaves were checked to identify the sub-cellular localization.

Fig 3: PVA-RFP inoculated leaves after 10 days showing symptoms: yellowing of the leaf.
5.2 Subcellular localization of Dnajc14 in healthy and PVA-infected *Nicotiana* cells using epifluorescence microscopy

In both methods, after three days of infiltration the fluorescence was checked under Zeiss epifluorescence microscope (Axioimager M2). PVA was tagged with RFP, so that infected cells could be recognized under the epifluorescence microscope. Thus, cells showing red fluorescence are the PVA infected cells. It was observed that the cells showed fluorescence in the nucleus. However, the amount of nuclear fluorescence was less in the PVA-infected cells than healthy cells. (Fig. 4 and 5).

*Fig.4: Fluorescence of healthy and PVA-infected cells when GFP is tagged to the C-terminus of Dnajc14 protein (Dnaj-GFP). Top: Healthy Nicotiana cells under 10X magnification, PVA-infected cells under 10X; Bottom: healthy cells under 20X magnification, PVA-infected cells under 40X magnification. A difference in the abundance of nuclear fluorescence can be observed between healthy and infected cells.*
Fig. 5: Fluorescence of healthy and PVA-infected cells when GFP is tagged to the N-terminus of Dnajc14 protein (GFP-Dnaj). Top: Healthy Nicotiana cells under 10X magnification, PVA-infected cells under 10X; Bottom: healthy cells under 20X magnification, PVA-infected cells under 40X magnification. The fluorescence was faint in this experimental setup.

5.3 Number of cells showing GFP fluorescence (Dnajc14 localization) in healthy cells versus PVA-infected cells

There appeared to be a difference in the number of cells showing nuclear fluorescence between healthy leaves and PVA-infected leaves. Therefore, the number of nuclei were counted, using four different views of four different leaf pieces, under 20X magnification of the epifluorescence microscope. The infected cells had less number of fluorescent nuclei than the healthy cells, in Dnaj-GFP constructs; i.e., constructs containing GFP at the C-terminus of the protein Dnaj14.
In GFP-Dnaj constructs, the number could not be calculated as they gave faint fluorescence. The number of cells showing fluorescence in Dnaj-GFP is mentioned in Table 2.

Table 2: Number of cells showing nuclear fluorescence in 4 different views of different leaves under 20X magnification

<table>
<thead>
<tr>
<th>Microscopic views</th>
<th>No. of fluorescent nuclei in healthy plants</th>
<th>No. of fluorescent nuclei in PVA-infected plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnaj-GFP 1</td>
<td>49</td>
<td>31</td>
</tr>
<tr>
<td>Dnaj-GFP 2</td>
<td>48</td>
<td>35</td>
</tr>
<tr>
<td>Dnaj-GFP 3</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>Dnaj-GFP 4</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Dnaj-GFP(Total)</td>
<td>155</td>
<td>108</td>
</tr>
<tr>
<td>GFP-Dnaj</td>
<td>faint</td>
<td>faint</td>
</tr>
</tbody>
</table>

In the cells having constructs containing GFP at the C-terminus of Dnaj14, the number of cells giving nuclear fluorescence in both healthy versus PVA-infected cells, we can say that, there is a decrease of approx. 31% of nuclear fluorescence in infected cells. (as seen from the table 2 above).
6. DISCUSSION

By checking the fluorescence in healthy leaves and PVA infected leaves, various observations can be made. Firstly, it was observed that among the two methods of infiltration, when we infected the plants with PVA and then later infiltrated our constructs after few days (as mentioned in 3.13 I), the infection was more evenly distributed in the leaves and it was easier to observe under the microscope, than with the method where the construct and PVA was infiltrated simultaneously (as described in 3.13 II). In the first mentioned method, the leaves showed more red fluorescence, meaning that the infection had spread evenly throughout the leaves. So, this method was used to observe the results and count the number of nuclei.

It was also observed that GFP tagged to N-terminus and the C-terminus of the protein Dnajc14, gave a different result. GFP tagged to the C-terminus gave proper fluorescence under microscope. While, when GFP was tagged to the N-terminus of the protein, then the fluorescence was faint. This may be due to that different folding of the protein Dnajc14 to form tertiary structures, which might have been less successful when N-terminus GFP tag was used.

In a previous study, fluorescent tagging analysis was used to study the protein expressed by full-length gene of Arabidopsis thaliana by Tian et al., in 2004. From this study, the subcellular localization and expression of proteins within the cells was observed to characterize the Arabidopsis proteome.

A protein End Binding 1 (EB1), which regulates microtubule (MT) dynamics, was studied using GFP tags and it was found that there is a difference in localization of EB1 in N-terminal tags and C-terminal tags. GFP tagged to C-terminal had same response and the same localization as their untagged counterparts, they had MT-binding ability, whereas, N-terminal tagged protein significantly had lower ability of MT binding. (Skube et al., 2011).

Through this current research work, it was shown that the Dnajc14 protein chaperone is localized to the nucleus of healthy potato plants.

There are some previous studies where the localization of other DnaJ like proteins has been mentioned. Vitha et al. (2003) had found that a chloroplast-targeted DnaJ like protein, encoded by ARC6 gene is localized to the plastid envelope membrane,
in Arabidopsis. This protein is closely related to a prokaryotic cell division protein, Ftn2 found in cyanobacteria.

Another DnaJ protein, LeCDJ1, which is responsible in the response of plants to abscisic acid, is targeted in the chloroplast of tomatoes (Lycopersicon esculentum). Transgenic plants over-expressing LeCDJ1 protein showed heat tolerance (Kong et al., 2013).

Moreover, this study shows that the PVA-infected plants showed decreased number of fluorescent nuclei as compared to the number of nuclear fluorescence in healthy plants, i.e. in infected plants Dnajc14 protein was less localized to the nucleus than in healthy plants (Table 2).

It was expected that the protein Dnaj14 would be absent in the PVA infected cells, based on previous nuclear proteome analysis. However, through this research work, it was observed that the protein was present in the nuclei of the PVA-infected cells but less in number as compared to the healthy ones. Based on previous studies on amount of protein in healthy and virus infected plants, it can be said that this decrease might be because PVA infection is causing a drop in the amount of Dnajc14 protein in the plants to help in its infection cycle and express its symptoms. The amount of decrease in Dnajc14 protein/gene can be checked using protein quantification methods like Bradford Assay. During further research, Viral Induced Gene Silencing (VIGS) method can be used to determine the function of this protein, Dnajc14.

A study conducted on Tobacco mosaic virus infected tobacco leaves showed that there is a decrease in the level of Filamentation, temperature sensitive Heat-shock protein (ftsH), encoded by DS9 gene, which maintains the quality of certain cytoplasmic and membrane proteins, as compared to the healthy leaves. This decrease in level affected the functioning of the chloroplast of tobacco leaves, and they ultimately formed necrotic lesions (Seo et al., 2000).

Another protein ferrodoxin I, showed decreased levels in Nicotiana tabacum, when infected with Tobacco mosaic virus. This decrease also led to the expression of mosaic symptoms of the virus. (Ma et al., 2008).
Chatterjee and Ghosh (2008) found that there is a decrease in the amount of total soluble protein in *Yellow vein mosaic virus* infected mesta plants (*Hibiscus cannabinus* L.) than in healthy mesta plants. This was confirmed by SDS-PAGE profiling of the total soluble protein content of the plants.

7. CONCLUSION

It can be concluded that the localization of Dnajc14 protein is in the nucleus of healthy potato plants. The number of cells showing nuclear fluorescence was less in PVA-infected plants than healthy plants. This suggests that, in the infected plants the nucleus containing Dnajc14 protein chaperone was less than the healthy cells. This decrease in the level of Dnajc14 protein might have a major effect on the plant viral infection cycle or expression of symptoms of the virus. Total protein content can also be checked to quantify the amount of decrease in the amount of Dnajc14 protein in the infected plants. Further studies can be done using these results to learn about the role of Dnajc14 in the PVA infection cycle in potato plants, using methods like RNA silencing. This study confirms the localization of Dnajc14 in the nucleus of the healthy cells.
8. ACKNOWLEDGEMENTS

Firstly, I wish to place my sincere thanks to my supervisor, Minna Rajamäki who helped me to learn various techniques by being a constant source of well needed guidance throughout this research work. Despite her busy schedule, she was always there to look at the progress of my project work and provide various useful suggestions for improvement.

I would like to express my utmost sincere gratitude to Prof. Jari Valkonen for offering me an opportunity to carry out this research work and helping and advising me during the entire research period and thesis writing.

I am also extremely grateful to my KPAT group members for being there for me while I was carrying out my various experiments to show me the correct technique and for helping me to handle various equipment.

I take this opportunity to extend my heartiest gratitude to Erasmus Mundus BRAVE scholarship programme, because of which I got an opportunity to undergo my Master’s study at University of Helsinki, Finland and work on this thesis work as a part of the curriculum, which has proved to be one of the most significant academic challenges I ever had to face and gain valuable experience.

Finally, I am highly thankful to my parents and my brother, and my friends for their never-ending support and encouragement during my entire study life.
9. REFERENCES


