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## **Molecular testing of raspberry plants infected with *Tomato black ring virus***



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of Plant Biology and Forest Genetics, Swedish University of  
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## **Molekylär testning av hallonplantor infekterade med *Tomato black ring virus***

## **Molecular testing of raspberry plants infected with *Tomato black ring virus***

**Keywords:** *Tomato black ring virus*, raspberry, *Rubus idaeus*, coat protein, cloning, sequencing

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## SAMMANFATTNING

Växtvirus som överförs via nematoder (bl.a. nepovirus) orsakar stora ekonomiska förluster i kommersiellt viktiga grödor, såsom tomater, vinranka och hallon över hela världen. För att undvika virus spridning har intresset för kartläggning av virusen ökat. Ett av de viktigaste nepovirusen som drabbar hallon är *Tomato black ring virus* (TBRV). TBRV har tidigare bekräftats i hallonprover från Vitryssland, med hjälp av ELISA. Syftet med detta kandidatarbete var att bekräfta dessa resultat genom att amplifiera, klonera och sekvensera virusets kapsidproteinet (*CP*).

Totalt amplifierades och sekvensbestämde åtta virala cDNA från två olika hallonprov. Tyvärr matchade ingen sekvens TBRV, utan alla analyserade sekvenser visade hög identitet med växtgener och är förmodligen från hallon.

Anledningen till att amplifieringen misslyckades flera gånger kan vara att den vitryska TBRV-stammen skiljer sig från tidigare kända TBRV-isolat

## ABSTRACT

Nematode-transmitted plant viruses (such as nepoviruses) cause great economically losses in commercially important plants such as tomatoes, grapevines and raspberries all over the world. To avoid spread of the viruses the interest in mapping the viruses has increased. One of the important nepoviruses infecting European red raspberries (*Rubus idaeus*) is *Tomato black ring virus* (TBRV). TBRV has earlier been confirmed in raspberry samples from Belarus by ELISA and the aim of this bachelor degree project was to confirm these results by amplifying, cloning and sequencing the *coat protein* (*CP*) gene of the virus.

In total eight viral cDNA samples, from two different raspberry samples, were amplified and sequenced. Unfortunately, no sequence matched TBRV, and all analyzed sequences showed a high identity to plant genes and are probably from raspberry.

The reason why the amplification failed several times could be because the TBRV strain from Belarus differs from previously known TBRV isolates.

## TABLE OF CONTENTS

1. INTRODUCTION.....	6
1.1 Nepoviruses .....	6
1.2. <i>Tomato black ring virus</i> .....	6
1.3. Nematodes .....	6
1.4. Methods .....	7
1.5. Aim .....	8
2. MATERIALS AND METHODS .....	8
2.1. Buffer and media .....	8
2.2. Primers.....	8
2.3. cDNA synthesis .....	8
2.4. PCR.....	9
2.5. PCR fragment purification.....	11
2.6. Cloning - ligation.....	11
2.7. Cloning - transformation .....	11
2.8. Cloning - confirmation .....	12
2.9. Sequencing preparations.....	12
2.10. Sequencing and sequence analysis .....	12
3. RESULTS.....	12
3.1. PCR.....	12
3.2. Cloning .....	15
3.3. Sequencing and sequence analysis .....	18
4. DISCUSSION .....	19
5. CONCLUSIONS .....	21
ACKNOWLEDGEMENTS .....	21
REFERENCES.....	22

# 1. INTRODUCTION

## 1.1 Nepoviruses

*Nepovirus* (Nematode-transmitted Polyhedral viruses) is a genus of positive single-stranded RNA plant viruses of the family *Secoviridae* and sub-family *Comoviridae*, which causes economical losses in commercially important plants such as tomato, grapevines and raspberries (Jończyk *et al.* 2004a, ViralZone). The genome of nepoviruses consists of two RNAs: RNA1 contains genes for replication and protein processing and RNA2 contains genes for the coat protein (CP) and virus movement. The *CP* gene is very variable and is usually species specific, which makes it suitable for identifying and distinguishing virus species (Le Gall *et al.* 2004). Nepoviruses are divided into three subgroups; a, b and c depending on the size of the RNA2 (Steinkellner *et al.* 1992). The virus particles are icosahedral and 28-30 nm in diameter (ViralZone).

## 1.2. Tomato black ring virus

*Tomato black ring virus* (TBRV) is one of the important nepoviruses of subgroup b infecting raspberry (*Rubus idaeus*), causing small crumble berries and lower fruit yield, which reduces the value of the crop. A chronically infected plant produces few berries and will usually die within 4-5 years after infection compared to uninfected plants that can be productive for 20 years or more. Other symptoms may be chlorotic spots and ringspots on the leaves of the raspberry plants (Jończyk *et al.* 2004b, EPPO).

TBRV has been shown to have a wide host range and has spread all over the world. The hosts include important berry and fruit plants (*Rubus*, *Ribes*, *Fragaria* and *Prunus*), sugar beet, potatoes and different vegetables (*Allium*, *Brassica*, *Solanum* and *Phaseolus*). The virus has been confirmed in many European countries for example France, Finland, Russia, Sweden, Germany, UK and Poland, but also in Asian countries like India, Japan and Turkey. TBRV has been found in USA, Canada, Kenya and Brazil which makes the virus widespread over the world (EPPO).

There are no previously known sequences of TBRV from raspberries; therefore it would be very interesting to see if the sequences differ from isolates of other hosts.

## 1.3. Nematodes

TBRV is transmitted by plant-parasitic nematodes of the genus *Longidorus* (*L. attenuatus* and *L. elongates*) (Murant 1970). The nematodes are 2-12 mm long and feed via a 60-250 µm long stylet piercing the root (Brown *et al.* 1995). It starts its feeding process by secreting saliva in which the virus is present. Thus, when a nematode feeds on a healthy plant after feeding on an infected plant, the healthy plant is inoculated the virus. However, the virus particles do not remain in the nematode during the three molts of its life cycle nor are they passed on to the eggs. Therefore, a newly molted nematode must feed on an infected plant to be able to transmit the virus. As a result, crop rotation may be used to avoid the spread of TBRV (Murant 1970, EPPO).

The virus can be transmitted through seeds, which makes it possible for the virus to spread over wide areas. On the other hand, transmission by nematodes alone will only spread the

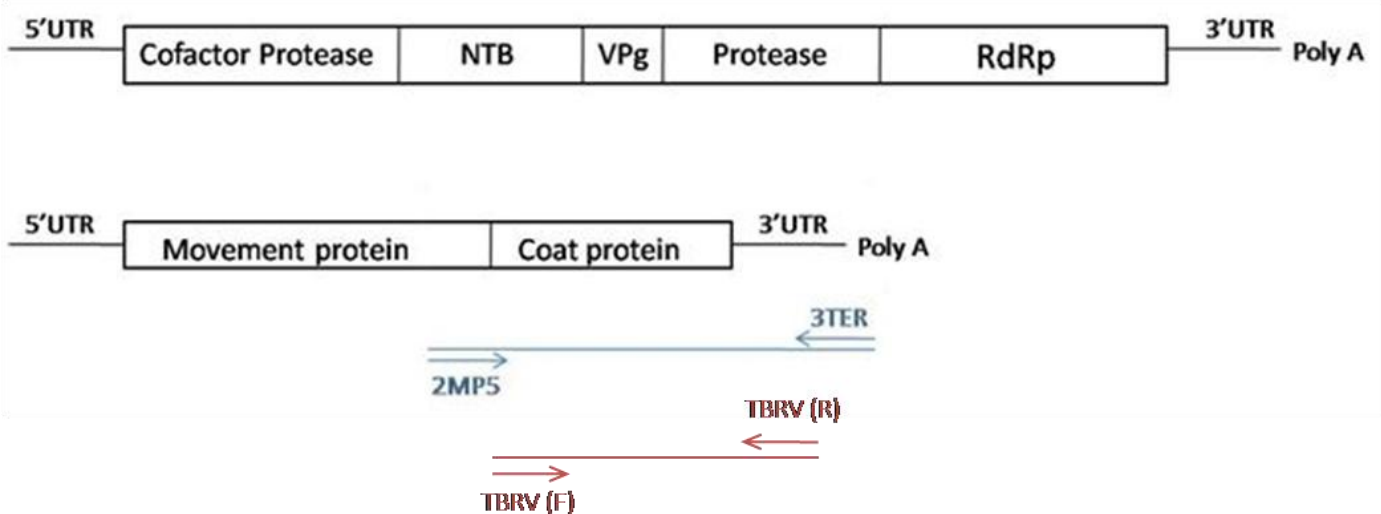
virus over smaller areas. Raspberries, and some other perennial plants, are propagated through stem cuttings, which means that if the mother plant is infected the stem cuttings will also be infected causing problems on their new growing spot (Lister & Murant 1967). This can be avoided by using certified planting material in which the viruses have been eliminated in an *in vitro* cultivation step (Kvarnheden, 2010-05-28).

#### 1.4. Methods

In this study the aim was to amplify, clone and sequence the *CP* gene of TBRV in order to confirm presence of TBRV in European red raspberry plants from Belarus and to study viral diversity. All raspberry samples were collected in Belarus but many of the cultivars had other origin, i.e. Russian or Polish. Viral RNA extraction had already been done as well as the cDNA synthesis.

This project was therefore started by amplifying the *CP* region of the TBRV genome by PCR. The *CP* region is located on RNA 2 of TBRV and was the target of the PCR amplification since it usually is very species specific and therefore suitable for this project where one wants to confirm the presence of a specific virus. The genome organization of TBRV and primer binding sites can be seen in Figure 1.

When the *CP* region has been successfully amplified the next step is to insert the fragment into a vector and later transform the vector with insert into competent *Escherichia coli* cells of the strain DH5 $\alpha$ . The vector that will be used in this case is the pGEM<sup>®</sup>-T Easy vector system (Promega) with a size of 3015 bp. The vector is prepared by cutting with EcoRV and adding a 3' thymidine to both ends. These thymidine overhangs make the insertion and ligation more effective. The vector contains two recognition sites for the restriction enzyme Eco RI (GAATTC) which will release the insert after digestion. This makes it possible to confirm the size of the insert by digesting the vector with Eco RI and then visualizing the restriction products by gel electrophoresis.



**Figure 1.** TBRV genome organization and binding sites for the two primer pairs used. 2MP5 with initial annealing at nucleotide 2316 and 3TER with initial annealing at 4654. TBRV (F) begins the annealing at nucleotide 2579 and TBRV (R) at 4352. The upper strand represents RNA 1 and the lower strand represents RNA 2. NTB stands for NTB-binding protein and RdRp for RNA-dependent RNA polymerase. The figure is modified from Jończyk *et al.* (2004).

The next step is to purify the plasmid and send it for sequencing to confirm that the correct fragment was amplified in the PCR. By analyzing the sequence it is possible to confirm which region that was amplified and which organism it originated from by comparing the sequence to the GenBank database of NCBI using BLAST.

### **1.5. Aim**

Twelve cDNA samples possibly containing TBRV were provided: 2-4, 7, 12, 14-18 and 20-21. According to previous ELISA results these samples contain TBRV. The aim of this bachelor project is to confirm these ELISA results and to study genetic diversity.

## **2. MATERIALS AND METHODS**

### **2.1. Buffer and media**

For gel electrophoresis 0.5x TBE buffer was used. It was prepared by diluting 5x TBE buffer that was made according to the following instructions: 54 g Tris, 27.5 g boric acid and 3.7 g EDTA (Triplex III) were mixed and water was added to a final volume of 1 L.

LB agar for plates was made by mixing 4 g Bacto™ Tryptone, 2 g Bacto™ yeast extract, 4 g NaCl in 400 ml milliQ H<sub>2</sub>O. The pH was measured (allowed pH was 7-7.5) and 6 g Bacto™ agar was finally added. The solution was autoclaved and 400 µl ampicillin was added to a final concentration of 100 µg/ml before pouring into the plates. Agar plates were stored at 4°C.

Liquid LB medium was made by mixing 4 g Bacto™ Tryptone, 2 g Bacto™ yeast extract, 4 g NaCl in 400 ml milliQ H<sub>2</sub>O. The pH was measured (allowed pH is 7-7.5) and the solution was autoclaved.

### **2.2. Primers**

The primer pair used for amplification of cDNA was according to Jończyk *et al.* (2004): 2MP5 5'-ACT TCA GGG CTT TCC GCT-3' was used as forward primer and 3TER 5'-TTG CTT TTT GCA GAA AAC ATT-3' was used as reverse primer for PCR number 1-11. The expected size of the fragment is 2.3 kb.

For PCR number 12, new primers were designed using the TBRV RNA2 sequence (GenBank accession number AY157994) as template: TBRV (F) 5'-TTT TGG GGA AGA GAA ACA AC-3' was used as forward primer and TBRV (R) 5'-TAA GAA ATG CCT AAG AAA CTA-3' as reverse primer. The expected size of the fragment is 1.7 kb.

All primers were ordered from Invitrogen.

### **2.3. cDNA synthesis**

The provided RNA samples were reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Eight µl 0.1% DEPC-treated water, 1 µl random primers, 1 µl dNTP and 3 µl RNA eluate (352µg/ml) were mixed and incubated in a 65°C water bath for 5 min and then for 10 min on ice. Four µl first strand buffer, 2 µl DTT and 1 µl



SuperScript III Reverse Transcriptase (Invitrogen) were added to the mix and incubated according to the following profile: 25°C 5 min, 50°C 60 min, 70°C 15 min. cDNA template was stored at -20°C.

## 2.4. PCR

Several attempts were done to amplify the *CP* gene of TBRV from cDNA samples. PCR programs and master mix compositions are shown in Tables 1-4. Samples used in each PCR and their origin and cDNA concentrations are shown in Tables 5 and 6. All PCR products were visualized on 1% agarose gels in 0.5x TBE buffer together with a MassRuler™ DNA ladder (Fermentas).

Since the wanted fragment of 2.3 kb from the first primer pair is considered as rather long, two kinds of DNA polymerases (DreamTaq™ DNA polymerase and *Taq* DNA polymerase) were used in PCR 4, in order to find out whether one was better than the other.

In PCR 11, PCR products from PCR 10 were used as DNA template in order to increase the concentration of DNA to facilitate subsequent cloning steps. In this case, only 20 cycles were needed. However, this PCR was not successful and the PCR product from PCR 10 was used for the following steps.

PCR 12 was done as a gradient PCR with the second pair of primers to find an optimal annealing temperature for the primers. The gradient covered 43-55°C, more specifically 43, 43.8, 45.3, 47.4, 50.3, 52.5, 54 and 55°C. Samples representing temperatures 47.4°C and 45.3° were run on an additional agarose gel. A very weak band for the 45.3°C sample was cut out for further purification.

**Table 1.** Programs for all PCRs done with the first primer pair

PCR step	PCR 1		PCR 2		PCR 3		PCR 4-11*	
	T (°C)	Time	T (°C)	Time	T(°C)	Time	T(°C)	Time
Initial denaturation	95	2''	95	2''	95	2''	95	2''
Denaturation	95	30'	95	30'	95	30'	95	30'
Annealing	45	30'	43	30'	43	30'	45	30'
Elongation	72	2'' 20'	72	2'' 20'	72	2'' 20'	72	2'' 30'
Final elongation	72	10''	72	10''	72	10''	72	10''
(Denaturation-Annealing-Elongation) x 40 cycles								

\* In PCR 11, only 20 cycles were run

**Table 2.** Programs for all PCRs done with the second primer pair

<b>PCR 12</b>		
<b>PCR step</b>	<b>T (°C)</b>	<b>Time</b>
Initial denaturation	95	2''
Denaturation	95	30'
Annealing	45	30'
Elongation	72	2''
Final elongation	72	10''
(Denaturation-Annealing-Elongation) x 40 cycles		

**Table 3.** Master mix (1x) composition for all PCRs done with the first primer pair.

<b>Reagent</b>	<b>PCR 1-2 volume (µl)</b>	<b>PCR 3 volume (µl)</b>	<b>PCR 4-11* volume (µl)</b>
DreamTaq™ buffer	5	2.5	2.5
10 µM 2MP5 forward primer	1	1	1
10 µM 3TER reverse primer	1	1	1
10 mM dNTP	0.5	0.5	0.5
DreamTaq™ DNA polymerase (5u/µl)	0.25	0.4	0.25
cDNA template (1183-3489µg/ml)	1	3	1
milliQ water		to a final volume of 25µl	

\*In PCR 4, two master mixes were prepared using two different DNA polymerases: DreamTaq™ DNA Polymerase (Fermentas) and *Taq* DNA Polymerase (Fermentas). For *Taq* DNA Polymerase (0.3 µl), 2.5 µl 10X *Taq* Buffer with KCl, 1.5 µl MgCl<sub>2</sub> were used instead of DreamTaq™ buffer.

**Table 4.** Master mix (1x) composition for all PCRs done with the second primer pair

<b>Reagent</b>	<b>PCR 12 volume (µl)</b>
DreamTaq™ buffer	2.5
10 µM TBRV (F) forward primer	1
10 µM TBRV (R) reverse primer	1
10 mM dNTP	0.5
DreamTaq™ DNA polymerase	0.25
cDNA template	0.5
milliQ water	to a final volume of 25µl

**Table 5.** Samples used in each PCR. Twelve cDNA samples tested positive for *Tomato black ring virus* with ELISA were provided and all of them were used in PCR at least once

<b>PCR</b>	<b>Samples used*</b>
1	2-4, 7, 14-15, 17-18
2	3
3	20
4	3
5	7, 12
6	2, 14
7	2, 21
8-10	16, 21
11	PCR products from PCR 10
12 (gradient)	3

\* Origins of the samples are summarized in Table 6

**Table 6.** Cultivars of virus-infected raspberry plants used for testing of *Tomato black ring virus* by reverse transcription PCR. All samples were collected in Belarus, but some cultivars originated from Russia and Poland. The table does also show the cDNA concentration of each sample used

Sample	cDNA concentration (µg/ml)	Cultivar
2	1321	Heracl, Russia
3	3373	Abrikosovaya, Russia
4	1440	Polana, Poland
7	3489	Meteor, Belarus
12	1494	Alyonushka, Belarus
14	1355	Zoloty Kupola, Russia
15	1267	Alyonushka, Belarus
16	1292	Beskid, Poland
17	1360	Alyonushka, Belarus
18	1608	Zeva Herbsternt, Belrus
20	3226	Babye Leto, Russia
21	1183	Porana Rosa, Poland

## 2.5. PCR fragment purification

Bands of the correct size, 2.3 kb or 1.7 kb depending on which primer pair that was used, were cut out using a scalpel and purified using GeneJET™ Gel Extraction kit (Fermentas) according to manufacturer's instructions. Note that this step was done only for PCR 4 and 12 since the visualization revealed additional unspecific products (Figures 2 and 4).

In all other cases PCR products were purified using GeneJET™ PCR purification Kit (Fermentas) according to manufacturer's instructions.

## 2.6. Cloning - ligation

To ligate the vector and the amplified cDNA, 5 µl 2x Rapid Ligation Buffer (Promega), 4 µl purified PCR product, 1 µl 50 ng/µl pGEM-T Easy Vector (Promega) and 1 µl 3 u/µl T4 DNA Ligase (Promega) were mixed. The mix was left on the bench for 1 h or at 4°C overnight. The ligation mix was used for transformation and stored at -20°C.

Since PCR products of PCR 10 had low concentrations (Figure 10), 8 µl purified PCR product was used.

## 2.7. Cloning - transformation

Competent *E.coli* cells of the strain DH5α (100 µl) were mixed with 5 µl of the ligation mix and put on ice for 30 min to transform the vector into the competent cells. The cells were put in a 42°C water bath for 50 s and then on ice for 2 min. LB medium (200 µl) was added and the cells were incubated at 37°C for 1 h 30 min on a shaker.

Two LB agar plates were used for each transformation culture. Before the cultures were spread, 40 µl IPTG and 40 µl X-Gal were added to each plate. On one of the plates, 40 µl of the culture was spread and on another one the rest of the culture was spread (approximately 265 µl). The plates were sealed and incubated at 37°C overnight. White colonies were picked (4-8, depending on how many available) and re-suspended in 30 µl milliQ water.

Clones from the transformation were mixed with 4 ml liquid LB media and 4 µl ampicillin and incubated at 37°C overnight on a shaker.

For storage at -70°C, 800 µl of an overnight culture was mixed with 200 µl 99.5% glycerol.

### **2.8. Cloning - confirmation**

A PCR was done in order to confirm that the correct insert had been cloned. Two µl Dream Taq buffer (Fermentas), 0.1 µl of each primer, 0.4 µl dNTP (10 mM) and 0.1 µl Dream Taq polymerase (Fermentas) were mixed with 1 µl DNA template (colony in 30 µl milliQ water from the transformation step). The cycle settings for the first primer pair were the same as for PCR 4 (Table 1). The settings for the second primer pair were the same as for PCR 12 (Table 2). PCR products were visualized on a 1% agarose gel in 0.5x TBE buffer together with a MassRuler™ DNA ladder (Fermentas) (Figures 5, 7 and 9).

The plasmid DNA was purified from bacterial cultures using GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions. Digestion of the plasmid DNA was done in order to see that the insert was present. The digest was carried out by mixing 15 µl milliQ water, 2 µl 10x FastDigest Buffer (Fermentas), 2 µl purified plasmid DNA and 1 µl FastDigest Eco RI enzyme (Fermentas). The mix was incubated at 37°C for 15 min and then visualized on a 1% agarose gel in 0.5x TBE buffer using MassRuler™ DNA ladder (Fermentas) (Figures 6, 8 and 10).

### **2.9. Sequencing preparations**

Twenty µl of purified plasmid was transferred to a 1.5 ml micro centrifuge tube and the DNA concentration was measured with a NanoVue spectrophotometer (GE Healthcare). Each sample was diluted to 100 ng/ml and 50 µl of the solution was transferred to a new 1.5 ml micro centrifuge tube and sent for sequencing.

In total eight clones were sent for sequencing originating from cDNA samples 3 and 16.

### **2.10. Sequencing and sequence analysis**

The sequencing was performed by Macrogen Inc., Korea, using M13 F universal primer 5'- (GTA AAA CGA CGG CCA GT)-3'.

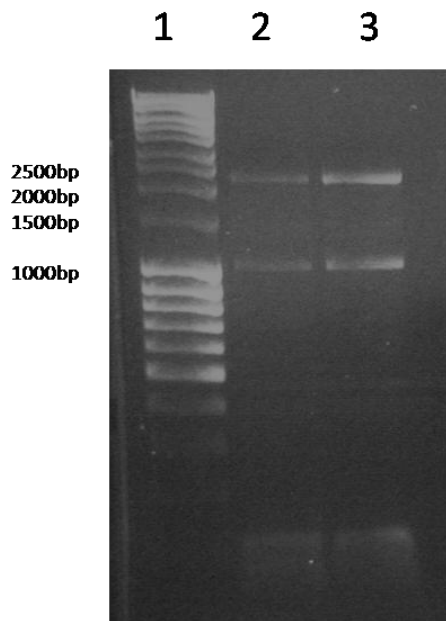
Sequences were compared to the GenBank database of NCBI using BLAST.

## **3. RESULTS**

### **3.1. PCR**

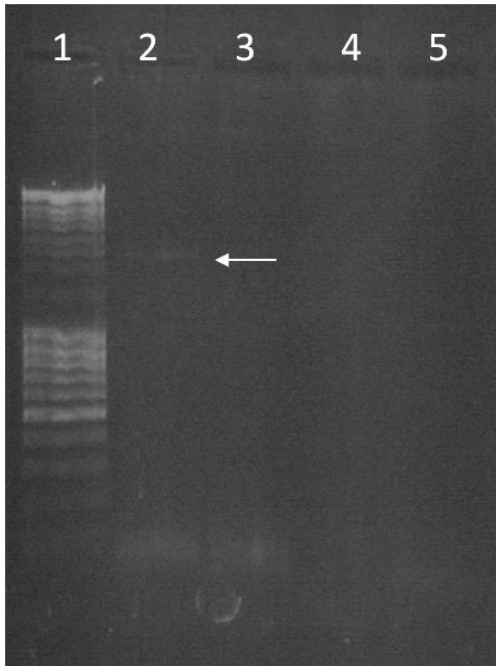
Successful amplifications were obtained for PCR 4, 10 and 12, where a PCR product of the expected size was obtained for cDNA samples 3 and 16 (Figures 2-4). For PCR 4 and 12 unspecific products were formed in addition to the right-sized product, 2.3 kb for the first primer pair and 1.7 kb for the second primer pair. In those two cases the bands of the correct size were cut out and further purified instead of purifying the complete PCR.

In PCR 4, cDNA sample 3 was used as a template with the first primer pair and two different DNA polymerases, DreamTaq™ DNA Polymerase and *Taq* DNA Polymerase, both from Fermentas (Figure 2). This amplification confirmed that both the polymerases had the capability of amplifying the rather long fragment of 2.3 kb. From now on only DreamTaq™ DNA Polymerase was used.



**Figure 2.** Gel of PCR 4. Lane 1 is DNA ladder, lane 2 is cDNA sample 3 amplified with *Taq* DNA polymerase and lane 3 is cDNA sample 3 amplified with DreamTaq™ DNA Polymerase. An unspecific product can be seen at 1 kb. The band at 2.3 kb, in lane 3 was cut out and purified for further analysis.

In PCR 10, cDNA sample 16 was used as a template but due to a low DNA concentration only a weak band at 2.3 kb could be seen on the agarose gel (Figure 3).



**Figure 3.** Gel of PCR 10. Lane 1 is DNA ladder, lane 2 is cDNA sample 16, lane 3 is cDNA sample 21, lane 4 is empty and lane 5 is negative control. Only cDNA sample 16 was positive but the band was very weak. The white arrow points out the band at 2.3 kb.

In PCR 12, the second primer pair was used for the first time and a temperature gradient was performed to find the optimal annealing temperature for the primers. As can be seen in Figure 4, the optimal annealing temperature is around 45°C, where the best amplification took place with a band at 1.7 kb in lane 7. Amplifications of samples representing the temperatures 47.4°C and 45.3°C (lanes 6 and 7) were run on an additional agarose gel. A very weak band for the 45.3°C reaction was obtained and cut out for further purification. There is no figure attached for the second agarose gel since the fragment of interest was cut out quickly, avoiding as much UV radiation as possible.

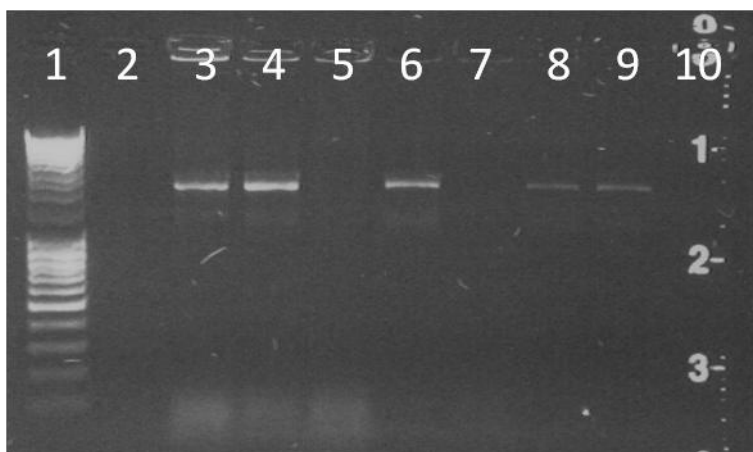


**Figure 4.** Gel of PCR 12. Lane 1 is DNA ladder, lanes 2-9 are cDNA sample 3 used for amplification in a temperature gradient spanning 43-55°C (55, 54, 52.5, 50.3, 47.4, 45.3, 43.8 and 43°C, respectively). Reactions with 50.3, 47.4 and 45.3°C (lanes 5, 6 and 7, respectively) have the expected product of 1.7 kb. Products from annealing at 47.4 and 45.3°C were used for further analysis.

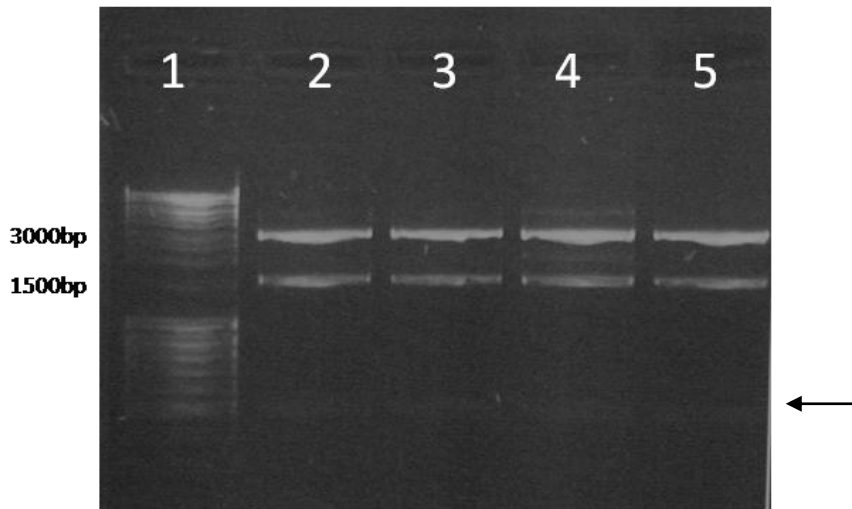
### 3.2. Cloning

The cloning always resulted in few white colonies ( $\leq 8$ ) and a lower total number of colonies ( $\leq 20$ ) than expected.

After the cloning a PCR was run as a confirmation. In Figure 5, the confirmation of clones for cDNA sample 3 is shown. Successful cloning was confirmed for five of the white colonies picked from the overnight plates, which is indicated by a band at 2.3 kb. Four of these clones were further analyzed with Eco RI digestions (Figure 6). All of the clones were digested resulting in bands at 1.5 kb and 0.3 kb corresponding to the insert (smaller than expected) as well as at 3.0 kb corresponding to the vector. Clones 2, 5 and 7 were sequenced.

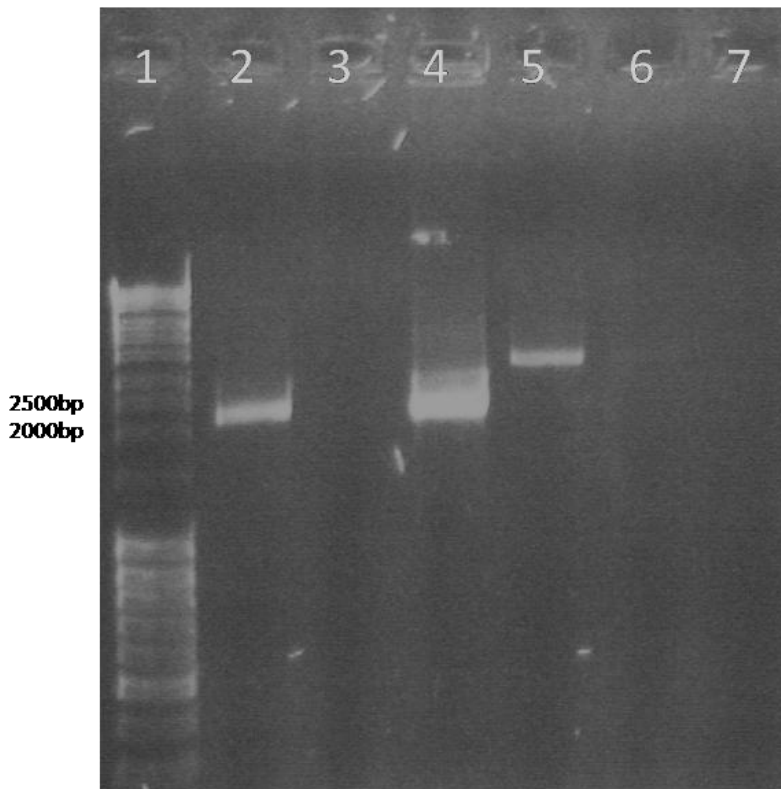


**Figure 5.** PCR-confirmation of clones from cDNA sample 3 (first time). Lane 1 is DNA ladder, lanes 2-9 are clones 1-8 and lane 10 is negative control. Clones 2, 3, 5 and 7, which gave the expected band at 2.3 kb, were used for further analysis with restriction enzyme digestion.



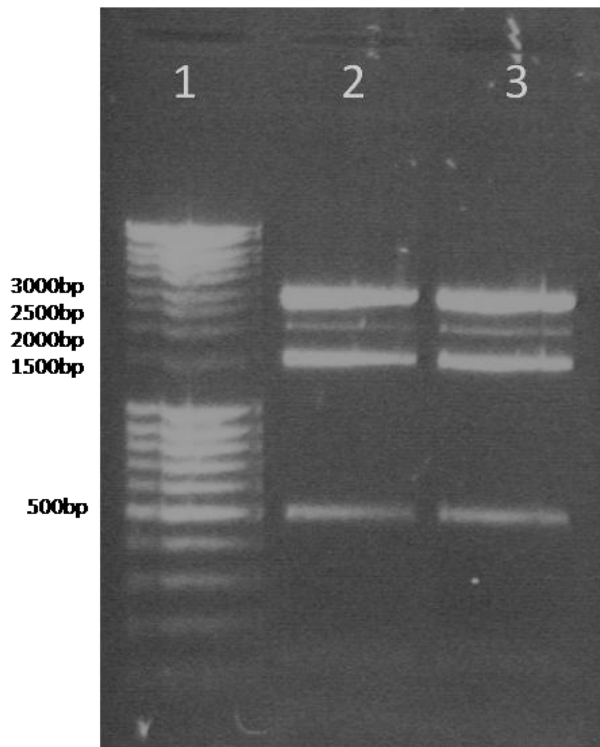
**Figure 6.** Eco RI digest to confirm cloning of PCR product from cDNA sample 3 (first time). Lane 1 is DNA ladder, lane 2 is clone 2, lane 3 is clone 3, lane 4 is clone 5 and lane 5 is clone 7. The bands just above 1.5 kb and at 0.3 kb (black arrow) are the insert and the band at 3 kb represents the vector. Clones 2, 5 and 7 were prepared and sent for sequencing.

For the cloning of the product from cDNA sample 16, two clones out of five white colonies were confirmed by PCR to contain the expected insert of 2.3 kb (Figure 7). Both clones were digested with Eco RI showing the vector at 3 kb and the insert just above 1.5 kb and at 0.5 kb (Figure 8).



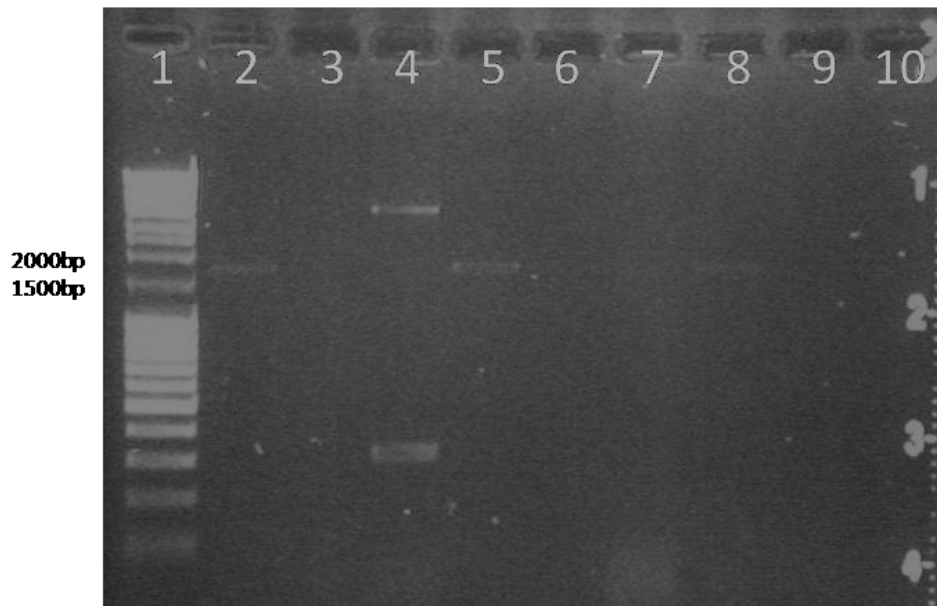
**Figure 7.** PCR-confirmation of clones for cDNA sample 16. Lane 1 is DNA ladder, lanes 2-6 are clones 9-13, respectively, and lane 10 is negative control. Clones 9 and 11, which gave the expected band at 2.3 kb, were used for further analysis with restriction enzyme digestion.



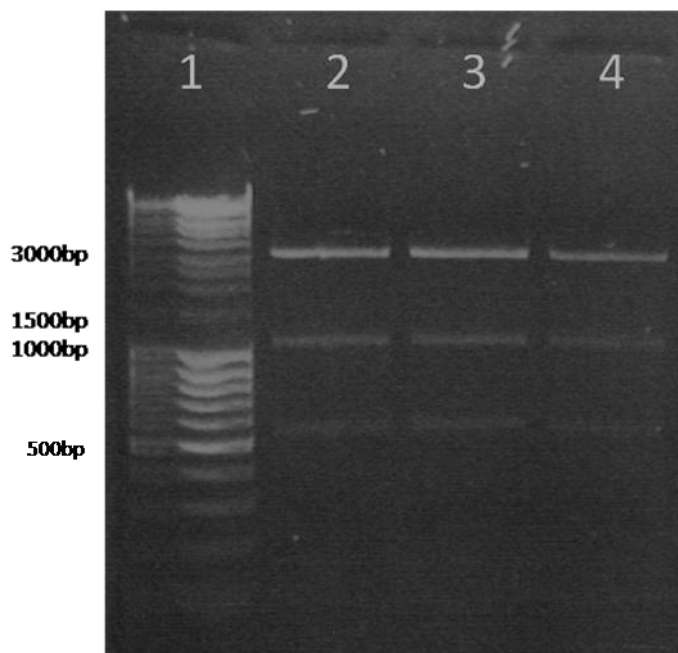


**Figure 8.** Eco RI digest to confirm cloning of PCR product for cDNA sample 16. Lane 1 is DNA ladder, lane 2 is clone 9 and lane 3 is clone 11. The bands just above 1.5 kb and at 0.5 kb are the insert and the band at 3 kb represents the vector. Both clones were sequenced.

The cloning was repeated for cDNA sample 3 and three clones were confirmed with PCR giving the expected band at 1.7 kb (Figure 9). All three clones were digested with Eco RI visualizing the vector at 3 kb and the insert at 1.2 kb and 0.5 kb (Figure 10).



**Figure 9.** Confirmation of clones from cDNA sample 3 (second time). Lane 1 is DNA ladder, lanes 2-9 are clones 1-8, respectively, and lane 10 is negative control. Clones 1, 4 and 7, which gave the expected band of 1.7 kb, were used for further analysis with restriction enzyme digestion.



**Figure 10.** Eco RI digest to confirm cloning of PCR product for cDNA sample 3 (second time). Lane 1 is DNA ladder, lane 2 is clone 1, lane 3 is clone 4 and lane 4 is clone 7. The bands at 1.2 kb and 0.5 kb correspond to the insert and the band at 3 kb represents the vector. All three clones were sequenced.

### 3.3. Sequencing and sequence analysis

Clones 2, 5 and 7 of cDNA sample 3 were sent for sequencing. All three clones showed a significant identity to the pentatricopeptide gene of *Ricinus communis* (castor bean) with a query coverage of 90-91% and maximum identity of 76% (Table 7). The second hit for all those sequences were not significant because of low query coverage (6%). Clones 9 and 11 of cDNA sample 16 showed a high sequence identity to the pentatricopeptide gene of *Ricinus communis* (Table 7). In addition, they shared a high sequence identity at 75% with

chromosome 2 of *Solanum lycopersicum* (tomato, [AC215486.2](#)) with a query coverage of 83% and e-value of 7e-95.

Clones 1, 4, and 7 from the second cloning attempt for cDNA sample 3 were also sequenced. All three sequences matched the oligopeptide transporter gene of *Populus trichocarpa* (black cottonwood) with high query coverage and low e-values. The maximum identity for the sequences was 77% (Table 7).

**Table 7.** Summary of the sequence hits when compared to the GenBank database of NCBI using BLAST

Clone	Sample	Hit	Accession	Query coverage	Max identity	E-value
2	3	<i>Ricinus communis</i> , pentatricopeptide repeat-containing protein, putative, mRNA	<a href="#">XM_002532665.1</a>	91%	76%	5e-126
5	3	<i>Ricinus communis</i> , pentatricopeptide repeat-containing protein, putative, mRNA	<a href="#">XM_002532665.1</a>	90%	76%	8e-124
7	3	<i>Ricinus communis</i> , pentatricopeptide repeat-containing protein, putative, mRNA	<a href="#">XM_002532665.1</a>	90%	76%	5e-126
9	16	<i>Ricinus communis</i> , pentatricopeptide repeat-containing protein, putative, mRNA	<a href="#">XM_002532665.1</a>	91%	76%	1e-127
11	16	<i>Ricinus communis</i> , pentatricopeptide repeat-containing protein, putative, mRNA	<a href="#">XM_002532665.1</a>	91%	76%	4e-127
1	3	<i>Populus trichocarpa</i> , oligopeptide transporter OPT family, mRNA	<a href="#">XM_002305653.1</a>	89%	77%	8e-134
4	3	<i>Populus trichocarpa</i> , oligopeptide transporter OPT family, mRNA	<a href="#">XM_002305653.1</a>	89%	77%	8e-134
7	3	<i>Populus trichocarpa</i> , oligopeptide transporter OPT family, mRNA	<a href="#">XM_002305653.1</a>	88%	77%	5e-131

#### 4. DISCUSSION

During this project much time was spent on optimizing the PCR conditions. In total, 12 PCR runs were performed of which three were successful and possible to continue with. Optimization implies changing of PCR mix composition, cycle conditions and primers.

In the early PCR attempts, where there were problems getting the fragment amplified, there were concerns that the CP fragment might be too long for the DreamTaq™ DNA polymerase.

As a result, *Taq* DNA Polymerase was also used in PCR 4 in order to find the most suitable polymerase. In PCR 4, cDNA sample 3 was amplified with both DNA polymerases (Figure 2). Evidently, the *CP* fragment was not too long for either of the polymerases.

The reason that PCR 1-3 did not work was probably because of incorrect PCR mixes. In the first two attempts, the buffer volume was too large by mistake. In the third PCR, the buffer volume was corrected, but polymerase and cDNA volumes were increased. For this PCR no amplification of the *CP* fragment took place. The reason can be either because of the increased volumes of template and polymerase or because of absence of TBRV in cDNA sample 20. All samples were not tried out for all different PCR conditions, which could have been done if this would not have been a short, time-limited project.

For all the unsuccessful PCRs, primer dimers could be seen on the gels (results not shown). This at least suggests that all reagents of the PCR mix were added and that no component was forgotten.

The most used cycle condition was the one used in PCRs 4-11 (Table 1) for cDNA 3 and 16. Unfortunately, TBRV was not confirmed in those samples.

In PCR 12, the second primer pair was used. The primers gave the best amplification at 45.3°C. This primer pair also resulted in many unspecific bands, which resulted in that the bands had to be cut out and purified instead of purifying the PCR amplification mix directly.

There were some problems with the cloning that resulted in few colonies. The reason may be that the cells had lost competence or that the cells were exposed to the warm spreading tool which might have killed them. However, there were always some white colonies even if the total number of colonies maybe was smaller than expected.

Regarding the digestion, which is done to confirm the insert, two bands are expected: one band at 3015 bp for the vector and a second band of 2.3 kb or 1.7 kb depending on primer pair. The digestions were visualized on agarose gels showing more than two bands in all cases. The most likely explanation is that the restriction enzyme finds a restriction site within the insert and makes additional cuts. The extra recognition site is present within the *CP* region according to the sequence of the previously known TBRV sequence present in the GenBank of NCBI. Because of this, more than two bands are present as expected.

In the first restriction enzyme analysis for clones of cDNA sample 3 (Figure 6), a band for the vector could be seen at 3 kb. By summarizing the sizes for the other bands the total fragment size should be 2.3 kb, but this digestion gave a fragment of 1.8 kb plus a weak fragment of 0.3 kb. This is in total 2.1 kb which is smaller than the expected size for the *CP* fragment amplified with the first primer pair. After sequencing it appeared that the fragment was of plant origin. Since the sequence identity was quite high it is not likely that there has been any contamination with plant material. Therefore, the sequence match is probably a corresponding gene of raspberry, which is not present in the GenBank database.

In the restriction enzyme analysis for clones of cDNA sample 16 (Figure 8), it was also hard to explain all the bands. The vector band at 3 kb is present and the bands just above 1.5 kb

and at 0.5 kb can possibly be the insert of 2.3 kb from TBRV. Later, the sequencing did show a high sequence identity to a gene of *R. communis*, once again with high query coverage and low e-value. In addition, these sequences resulted in high sequence identity to chromosome 2 of *S. lycopersicum*. As stated above, those hits are probably the corresponding genes of raspberry.

For the second restriction enzyme analysis for clones of cDNA sample 3 (Figure 10), the insert should be approximately 1.7 kb. Depending on how the band sizes are estimated, the band just above 1 kb and the band between 0.5 and 0.6 kb can sum up to the insert size of 1.7 kb. As in all other cases the vector band at 3 kb is present. The sequencing results did not reveal any significant sequence identity with TBRV either. The three clones showed highest sequence identity with the oligopeptide transporter gene of *P. trichocarpa*, again possibly the corresponding gene of raspberry.

## **5. CONCLUSIONS**

The final conclusions of this bachelor degree project is that none of the sequencing results can confirm the previous ELISA results of TBRV presence in Belarusian raspberry plants.

The reason why the amplification failed is probably because the Belarusian TBRV isolates differ significantly in sequence from previously sequenced TBRV isolates. It may be due to differences in geographical origin. Another explanation may be that previously characterized isolates came from another host (*Robinia pseudoaccacia*, Accession number AY157994.1). Since the TBRV sequence can differ a lot between strains, the difference between the raspberry isolate in this project and the *R. pseudoaccacia* isolate is probably too large. Therefore, the primers designed based on the *R. pseudoaccacia* isolate might have problems with annealing.

Another thing one has to take into account is that the ELISA results were false. Maybe, the plants were not infected with TBRV, but with other viruses giving positive ELISA results.

To analyze this different isolate one can use another sequencing method, e.g. 454 sequencing, which is not relying on amplification and cloning. Another thing to try is to use degenerate primers suitable for the entire nepovirus subgroup b.

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