BACHELOR THESIS

Study of the early phase of viral infection in Chinese cabbage

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I. Annotation:

In this work, the replication behavior of CaMV and TuMV were monitored in the early stage of infection on Chinese cabbage.

II. Declaration

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III. Acknowledgments

First of all, I want to thank my supervisor Dr. Petrzik, who kindly gave me the possibility of doing my bachelor thesis in his laboratory and gave me the opportunity of getting to work closer with plants and viruses. I want to thank him also for the great patience he kept towards me, even if I was not doing all things right all the times. Furthermore, my thanks belong to Olga Kubešová for her great help and practical hints in the lab work and also for teaching me the Czech laboratory vocabulary. I further want to thank Tania Sarkisova, who also readily helped me all the times and during the writing of my thesis. Finally, my thanks go to my friends and family for their great support while I was writing my thesis.

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ABSTRACT

Viral infections in useful plants are economically very important factors. A severe infection can cause the loss of the whole harvest. In this way, Chinese cabbage is susceptible to two distinct viruses *Cauliflower Mosaic virus* (CaMV) and *Turnip Mosaic virus* (TuMV), making the product unmerchantable. We were interested in the viruses' replication behavior in the early phase of infection. We inoculated the leaves of the young plants with viral sap, and took a sample every day for CaMV and every second day for TuMV. With real-time PCR we could determine the relative amounts of present virus on every day of sampling. For TuMV, which is an RNA virus, we used reverse-transcription real-time PCR. This is an additional step in the procedure, where the viral RNA is reverse-transcribed into cDNA. By real-time PCR any present DNA can be specifically amplified with the corresponding primer, and at the same time the reaction can be monitored. We used SYBR green fluorescent dye, which preferentially binds to dsDNA and fluoresces when forming a complex. DNA segments specific for the plant's housekeeping genes and viral DNA segments are amplified in different vials at the same time. The software records the signal, and displays the cycle at which the fluorescence signal of the corresponding sample crosses a predefined threshold value. The relative amounts of virus present on each of the days were then calculated based on a mathematical model for PCR. The results show that we successfully infected Chinese cabbage, and that both viruses amplified after some days post-inoculation. The replicational behavior of both pathogens was obtained without influence of stress on the plant-virus interaction. These results can be used as a comparison in further analysis of plant-virus interactions under different experimental conditions.

1. Aim

The aim of this work was to study the behavior of two viruses differing in type of genome nucleic acid and genome content in early phase of infection on the same host. For this purpose the real-time PCR method was used for amplification, and determination of relative virus content in a time period of 0-8 and 0-16 days after mechanical inoculation.

2. Shortcut Definitions:

Shortcut	**Meaning**
CaMV	*Cauliflower Mosaic virus*
TuMV	*Turnip Mosaic virus*
ssRNA	single-stranded RiboNucleic Acid
(c)dsDNA	(circular) double-stranded DesoxyriboNucleic Acid
RdRp	RNA-dependent RNA-polymerase
NCR	Non-Coding Region
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
RT-rt-PCR	Reverse-Transcription real-time Polymerase Chain Reaction
kbp	Thousands of base pairs
cv.	cultivar

3. Introduction

3.1. Cauliflower Mosaic Virus – CaMV

3.1.1 Characteristics:

The CaMV is a small spherical particle of about 50 nm in diameter with an icosahedral structure. It belongs to the *Caulimoviridae* family, genus *Caulimovirus*. The virus contains a single circular double-stranded DNA molecule, and as such, is part of the pararetrovirus supergroup. The chromosome is replicated by reverse transcriptase but not integrated into the host's chromosome. CaMV appears in all temperate zones on earth, and there exist several strains, which in nature usually occur as a mixture. This virus is often found in mixed infections with Turnip mosaic virus.

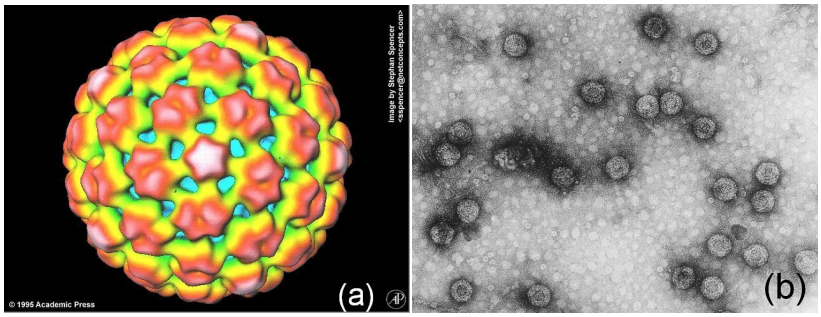

Figure 1: (a) Structure of a general icosahedral virus particle, view along the 5-fold axis (Spencer.S, 1995); (b) EM photograph of CaMV (ICTVdB - The Universal Virus Database)

3.1.2 Genome:

The DNA has about 8 kbp and is non-supercoiled due to 3 gaps, from which two are on the positive strand and one on the α (= minus) strand (Stavolone et al, 2005). The genome has six major ORFs and possibly two minor ORFs (Matthews, 1991; Astier et al, 2001; Mandahar 1999) which are listed in Table 1.

Table 1: CaMV proteins and functions

ORF	Protein MW (estimated in vitro)	Protein function
I	41 K	Cell-to-cell movement
II	19 K	Aphid transmission factor (ATF)
III	15K	Non-sequence-specific DNA binding → structural protein inside virion; ATF cofactor
IV	52 K	Capsid precursor
V	79 K	Reverse transcriptase, RNaseH, protease
VI	58 K	Disease induction, symptom expression; translational transactivator; inclusion body protein; virus assembly
VII + VIII	11 K + 12 K	unknown

Concerning ORF VII and VIII the opinions differentiate. Astier et al. (2001) and Mandahar (1999) state that gene VII does exit, but its function is unknown. Matthews cites the work of Givord et al.(1988), stating that both genes could be expressed *in vitro* and were found in low amounts *in vivo;* however no function could be assigned yet.

Furthermore, CaMV is known for a very powerful promoter: the 35S-promotor. It is responsible for the transcription of the biggest part of CaMV's genome. The name comes from the sedimentation coefficient of the transcript – 35S. The promoter is a very strong and constitutive promoter and is used in most genetically modified dicot plants, where it causes high levels of gene expression.

However, it seems not to work well in monocots, which is probably due to different quality and quantity of regulatory factors (Anonymous 1).

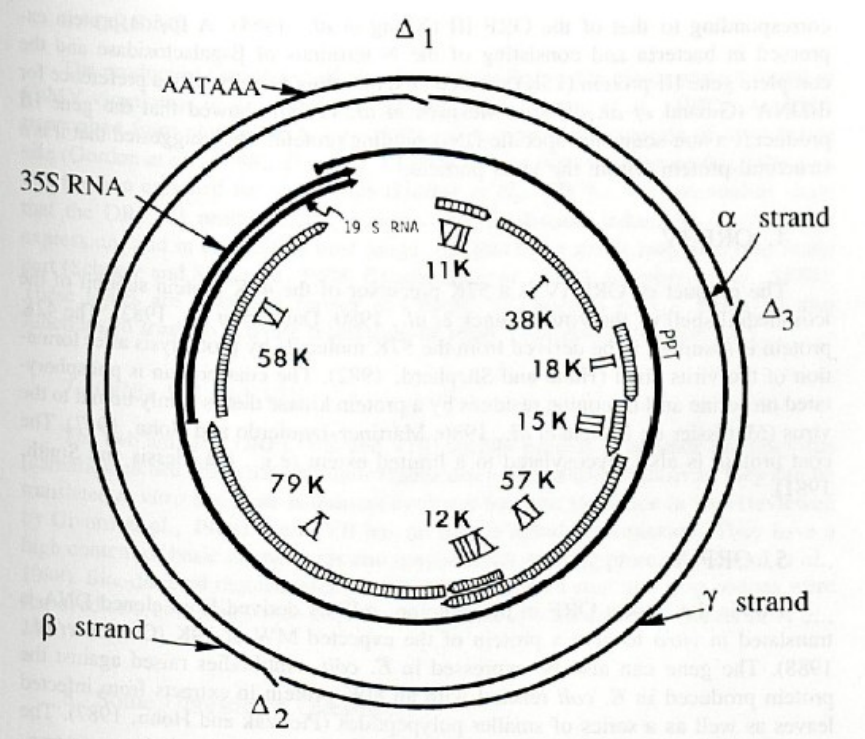

Figure 2: Map of CaMV's genome. On the α (coding) strand there is one interruption (Δ1). On the non codings strand (β and γ) there are two (Δ2 and Δ3). The eight potential ORFs with their corresponding MWs are depicted. Also the 35S and the 19S RNAs are indicated. (Matthews, 1991)

3.1.3 Replication:

After transmission to the host, the genome has to be transported to the nucleus. It was found that the coat protein contains a nuclear localization signal enabling it to reach the nucleus, but nothing is known about the decapsidation process (Leclerc et al. 1999). In the nucleus the gaps of the plasmid are repaired by removal of the overlapping nucleotides, and the DNA is supercoiled with host-histones to form a minichromosome. Then the host's DNA-dependent RNA-polymerase II transcribes it into two RNA subunits – 35S and 19S, both containing highly active promoters in the upstream region. After polyadenylation, the two mRNAs travel to the cytoplasm. The 19S RNA is translated in high amounts into proteins forming the electron-dense viroplasms and also transactivating translation of the 35S pregenomic RNA. The newly formed proteins enter the viroplasm, also called inclusionbody, inside of which the 35S mRNA is back-translated by reverse transcriptase into DNA, and the virions are assembled (Shababi et al, 2006). Some of this DNA goes back into the nucleus to start a new cycle. Finally, the virions leave the cell, probably through the plasmodesmata, transported by the inclusion bodies (Anonymous 2, Anonymous 3 , Astier et al, 2007).

3.1.4 Cell-to-cell movement

Two main theories exist about how CaMV spreads systemically. Citovsky, Knorr and Zambryski (1991) found that the protein generated from ORF I (Movement Protein – MP) binds do ssRNA of the 35S subunit in a cooperative way. They suppose that transport through the plasmodesmata (PD) occurs via a protein-RNA complex. Similarly, the 19S subunit might be transported. This is supported by findings of other authors, who state that indeed viral RNA is transported through the PD in RNA-protein complexes (Hohn et al, 2007). Citovsky et al. (1991) do not exclude a combination of this mechanism with another later occurring mechanism, which would imply the modification of the PD in a way that the whole virion can be transported. Other investigations (Ritzenthaler, Hofmann, 2007) showed that the MP forms tubules that stretch from the entry of the PD of one cell into the cytoplasm of the neighboring cell, and virions have also been found in the sieve elements of the phoelem. Apparently, the progeny virion is transported through the tubuli by interaction of the coat protein with the C-terminus of the MP projecting into the lumen of the tubules.

Recently, it was found (Harries et al., 2009) that protein 6 (P6) form ORF VI might, apart from many other roles, have a major role in cell-to-cell movement. This protein forms electron-dense inclusion bodies (EdIB) inside of which reverse transcription and formation of the progenies occurs. P6 interacts with the microfilaments inside the cell and enables intracellular movement, therefore, there is a great chance of this having also a role in intercellular movement, as P6 might transport the virions to the tubules that were built at the entrance of the PD.

3.1.5 Transmission:

CaMV is readily transmitted by aphids , from which at least 27 species are known to be vectors and act as such at any instance (Stepherd,1981). They are well suited because they do not destroy the plant they feed on. Transmission of the virus to the aphid is non-circulatory, this means that the virus attaches somewhere in the exterior mouth parts with some specific transmission bodies. These must be formed, so that the virus is able to adsorb to the vector. This seems to happen with a helper protein, which connects the receptors inside the vector with the virus capsid.

For infection of the host the aphid will make a puncture to check if the plant is suited for consumption, and only then it will insert its stylet for feeding – already the checking-puncture gives the virus enough time to infect the plant-host (Martinière et al, 2009).

Inside the host, transmission occurs via the vascular adhesion protein and the movement protein being attached to the inclusion bodies and transported through the plasmodesmata. There are no records about infections through seeds.

A host can be infected experimentally by rubbing the sap onto a leaf's surface and short rinsing with water for better penetration.

3.1.6 Host:

Potential hosts are most plant species from the *Cruciferae* family, including cauliflowers, Chinese cabbage, tendergreen mustard and others. The only exceptions are *Nicotiana clevelandii* and *Datura stramonium*, which both belong to the Solanaceae (Nightshades) family and are also susceptible to CaMV. According to the International Commitee on Taxonomy of Viruses dataBase (ICTV dB) also members of the *Resedaceae* family can be prone to infection with CaMV.

3.1.7 Symptoms:

CaMV can cause different symptoms, depending on the strain and the host. Some systemic infections are asymptomatic, others cause the mosaic and mottle disease. Symptoms are mottling, necrosis, leaf vein clearing (initially in young leaves), chlorosis, dark green vein banding, stunting of the whole plant. In some brassica species only the older leaves are affected. In some cases even death of the host can occur (Shepherd, 1981). In field the symptoms on leaves are hardly observable in early infection, but the yield is affected, as the plants give smaller heads of poor quality (Saunders, 1990; Shepherd, 1981). Furthermore, it was observed, that CaMV causes premature flowering of Chinese cabbage and turnip, and that at higher temperatures the symptoms of chronically infected plants are masked (Zitter et al, 1984).

3.2. Turnip Mosaic Virus – TuMV

3.2.1 Characteristics:

TuMV is a rod-shaped virus with a flexuous capsid and a length of 720 nm. The capsid protein is composed of 288 amino acids and is encoded by a cistron of 864 nucleotides (Tremblay et al, 1990). As a positive-sense ssRNA virus it is part of the *Potyviridae* family, genus *Potyviridae,* and is distributed all over the world (Tomlinson, 1970).

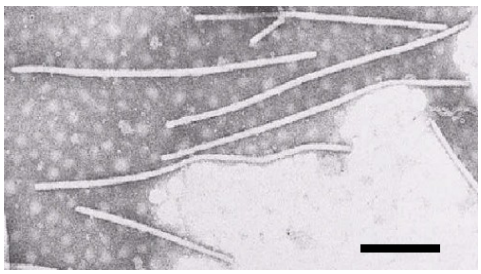

Figure 3: EM pricture of a purified preparation of TuMV.
The scale is 200 nm. (Tomlinson,1970)

3.2.2 Genome:

The genome is about 10 kb long, has a viral genome-linked protein (VPg) covalently linked to its 5' end, serving as primer for polymerase. The 3' end is polyadenylated. Furthermore, it encodes one large polyprotein with a 129 nucleotide long non-coding region before the start codon, and a NCR of 209 nucleotides after the termination codon, not counting the poly(A) tail.
Table 2 describes the genes from 5' to 3' end of the genome:

Table2: Protein functions of TuMV (Nicolas et al, 1992 ; Anonymous 4)

Protein	Other name	Function
P1	N-terminal protein	Protease
HC-Pro	Helper-component proteinase	Protease, aphid transmission, suppression of post-transcriptional gene silencing
P3	none assigned	unknown
p6K1	6 kDa protein 1	Involved in replication, but never detected in vitro/vivo
CI	Cytoplasmic inclusion protein	RNA-dependent RNA helicase
p6K2	6 kDa protein 2	Involved in replication, but never detected in vitro/vivo
VPg	Viral genome-linked protein	Activation of RdRp; overcoming host-resistance
NIa	Nuclear inclusion protein A	Protease, RNA-binding
NIb	Nuclear inclusion protein B	Core replicase (kind of RdRp)
CP	Capsid Protein	Encapsidation of viral RNA, regulation of viral RNA amplification, aphid transmission, systemic and cell-to-cell movement

3.2.3 Replication:

After penetration of the cell, the virus must be decapsidated. It seems the uncoating is a bi-directional process, which efficiently disassembles the capsid within 20 minutes and starts replication (Hull, 2002). This has been found with *Tobacco Mosaic Virus* (TMV), which is also a (+)-sense RNA virus with a rod shaped capsid. The process of uncoating seems to start on several sites along the capsid (rod) and is not dependent on pre-existing or induced enzymes (Shaw, 1973). The underlying mechanism for uncoating of the capsid was named co-translational disassembly (Wilson, 1984a). The 5' ends lack G residues and therefore interact more weakly with the coat protein. This enables the ribosome to attach to the RNA and to synthesize the RNA-dependent RNA-polymerase (RdRp) which starts disassembly at the 3' end in 5' direction, at the same time synthesizing the negative-sense strand. The (-)-sense strand will serve as a template for positive-sense RNA synthesis (Mandahar, 2006). The newly formed +RNA is translated into a polyprotein of about 358 kDa, whereby several constrains have to be overcome due to the leakage of an initial cap. The polyprotein encodes three viral proteases, which will cleave it into at least 10, fully functional proteins, which were described above (Leonard et al, 2004; Grangeon et al, 2010; Cotton et al, 2009). In TMV the mRNA of coat protein and genomic RNA are early products, implying the synthesis of RdRp and reproduction enhancers. Later the coat protein is synthesized as well as the MPs. This probably holds also for TuMV. According to Hull (2002) the whole process from uncoating to reassembly of TMV takes about 45 min, and it can be assumed that a similar schedule is valid for TuMV.

3.2.4 Cell-to-cell movement

For the family of *Potyviridae* it was found that their movement protein (MP) interact with proteins from the host translation apparatus. The elF4E is an important protein in eukaryotic translation, binding to the cap of mRNA – depending on the complex it forms the translation is up or down regulated. The potyviral VPg protein is located on a distinct place at the end of the virion and binds to the RNA 5' end. It specifically binds to an isoform of elF4E that leads to the transport of the virion through the plasodesmata. Arriving in the new cell the translational problem of cap leakage is easily overcome by the now present elF4E. Also the long-distance movement through sieve elements and phloem is probably directed by VPg (Cooper et al, 2006).

3.2.5 Transmission:

TuMV is transmitted by 40-50 aphid species. These are able to spread it in a non-persistent manner, characterized by an acquisition occurring within seconds to minutes, retention time lying within minutes, fast infection, happening within seconds to minutes and the virus being often easily transferred mechanically. Usually infection affects 95-100% of the harvest (Shukla et al, 1994).
Furthermore, the virus can be transmitted mechanically, by streaking the sap onto the leaves.

3.2.6 Host:

This virus infects a large variety of species from several families, including "*Amaranthaceae, Caryophyllaceae, Chenopodiaceae, Compositae, Cruciferae, Cucurbitaceae, Leguminosae-Caesalpinioideae, Leguminosae-Papilionoideae, Onagraceae, Orchidaceae, Papaveraceae, Phytolaccaceae, Solanaceae, Tetragoniaceae*" and other dicotyledonous families (ICTVdb, version 4). Some examples are: the various brassica species, head lettuce, watercress, radish, spinach, turnip, rhubarbe, mustard, various tobacco species, as well as bedding plants like zinnia and petunia are susceptible (Zitter et al, 1984).

3.2.7 Symptoms:

Common symptoms in cauliflower, cabbage and Brussels sprouts are black necrotic spots, mottling and ringspots. Other species show symptoms like mosaic, chlorotic ringspots in young leaves, distortion, stunting of the whole plant and color breaking of flowers. In cabbage necrotic spots are larger than those caused by CaMV, and even though not visible during the harvest, they can appear 2 to 5 months later during storage. These spots might already have been present earlier in deeper layers of the head. They appear on midribs, side veins and in the interveinal areas, being able to coalesce and, therefore, blight the whole leaf. Also the seeds can be affected, as the virus causes them to be empty or to have lesions, although they cannot transmit it.
According to Pound and Walker (1945) symptoms are most pronounced at temperatures between 22°C-30°C, and are completely masked at 16°C (Ferreira et al, 1991; Zitter et al, 1984; Anonymous 5).

3.3. Chinese Cabbage – Brassica rapa subsp. pekinensis cv. Nozaki

3.3.1 Synonyms:

Brassica pekinensis, Chinese cabbage, brassica campestris ssp. Pekinensis, napa cabbage (Uniprot.org)

3.3.2 Characteristics:

Chinese Cabbage is a dicot plant from the family of *Brassicacae* and as such counts to the cruciferous crop plants.

It probably evolved in China from the natural crossing of Pak Choi (*Brassica rapa* ssp. Chinensis – non-headed Chinese cabbage) and turnip (*Brassica rapa* var. rapa).
It is an biennial plant, but cultivated annually. During its vegetative stage its leaves form a more or less compact, conical rosette. The outer non-heading leaves are dark green, and the inner heading leaves rather whitish-green. At this stage they can reaches a size of 20 – 50 cm. In the reproductive stage (flowering state) Chinese cabbage forms a flowering stem, which develops leaves that are much smaller than the heading leaves in the vegetative state. The flowers are yellow and after pollination siliquae from. They are about 4 - 5 mm wide and 4 – 10 cm long, each containing 10 – 30 seeds (Dixon, 2007).

3.3.3 Mode of infection

The main cause of infection is transmission by insects. In the case of TuMV and CaMV several aphid species can be the vector. Helper proteins on the capsid bind to receptors in the mouth-part of the insect. When the animal feeds on the plant, it damages cuticle and cell wall of the plant, and the virus is readily infiltrated.
As to infection by manual inoculation, several mechanisms are possible for RNA viruses, but non of them has been proven yet. The virus might enter directly through a wound in the tissue, or the virus particle might attach to the cell surface and pass entirely or just its RNA. Other possibilities would be entry of the whole virus via the ectodesmata, invagination by the membrane, followed by formation of an endocytotic vesicle. (Shaw, 1999). Similar mechanisms are probable for DNA viruses.

3.3.4 Plant Defense and viral counter-attack

Apart from structural barriers like the cell walls and cuticles around it, plants developed many different internal mechanisms for defense against an invading pathogen. At first the basal defense is activated, which can be seen as an immune response. Thereby, the plant recognizes certain proteins of the pathogen as foreign and tries to destroy them (Freeman, Beattie 2008). Possible reactions are changing of the pH, cell wall fortification, papilla formation (polysaccharide polymers forming between an infected and a healthy cell), or production of active oxygen forms, inhibiting the replication and spread of the invader (Zsiros and co-workers). But viruses also evolved and found ways to overcome this first response of the plant by interfering with response inducing mechanisms. This phase is usually asymptomatic.
Another type of response is the hypersensitive response (HR). It is a much more drastic way of defense and ofter more pathogen-specific. The HR causes the plant to induce death of the infected cells, and can therefore be observed on the plant. As a consequence, the plant can acquire a systemic resistance (SAR), which is an enhanced disposition of plant resources for defense like salicylic acid and other chemicals.
A very sophisticated defense mechanism is gene silencing. The process is induced by dsDNA (transcriptional gene silencing - TGS) or dsRNA (post-transcriptional gene silencing - PTGS), which might appear as intermediates during replication of the virus. TGS has various techniques like methylation, enhancing or decreasing the transcription. By overproduction of a gene product the virus can be disturbed as much as by underproduction. For PTGS it is similar. Alien dsRNA is recongnized and host-enzymes degrade it to siRNAs (small interfering RNA) that assemble to big complexes, which will destroy homologous transcripts. siRNA probably also plays a major role in the aforementioned SAP, as several studies revealed high amounts of siRNA in the phloem of planst after infection. Likewise viruses developed mechanisms how to block the siRNA and other plant-strategies.

4. Materials and Methods

4.1. Preparation for infection, inoculation and sample taking:

The inocula were prepared from an asymptomatic leaf of infected *B. pekinensis* cv. Nozaki. One infected with CaMV and one with TuMV. With a mortar each of the leaves was smashed into a slurry with addition of 1 ml of 0.1 M phosphate buffer.
In total six Chinese cabbages had been grown before, and on the day of inoculation, in march, were still in the young state. Three were infected with one virus respectively. Wearing a glove, I dipped my finger into the slurry and rubbed it onto one leaf of each plant. The leaves were rinsed with distilled water, and the fist sample taken immediately.
For CaMV a sample was taken every day during 8 days – giving a total number of 9 samples.
Initially we had the same setup for TuMV, but during analysis we realized that the differences in relative concentration were not pronounced enough, and so, started over the experiment, this time taking a sample every second day. This gave a results over a time span of 16 days – giving a total number of 9 samples.
I took care of taking the samples at approximately the same time every (second) day.
Before analyzing the samples, another separate sample of each infected plant was taken to check if infection was successful.

4.2. CaMV procedure

4.2.1 DNA isolation

Nucleospin Plant II kit (Macherey-Nagel, Germany) was used for isolation of viral DNA from the leaf-samples.
With 400 µl lysis buffer the samples were homogenized and transferred to a vial. After addition of 10 µl RNAase A, the vials were placed into a heating block for 10 min at 65 °C. Then they were transferred into filter-vials and centrifuged at 11000 x g for 2 minutes. The supernatants were transferred into binding-filter vials, and 450 µl binding buffer were added, followed by 1 min centrifugation at 4,500 x g. Afterwards, the filters were subsequently washed with 400 µl wash buffer I, 700 µl wash buffer II, another 200 µl of wash buffer II and centrifuged (4,500 x g) for 2x 1 min between and 1x 2 min after the washing steps. The filters were put into a new collecting tube, 30 µl elution buffer were added and heated for 5 min. After another minute of centrifugation the vials were rinsed again with some solution buffer. Finally the DNA was transferred into a storage vials and put into the freezer.

4.2.2 Checking the separate CaMV sample:

The separate sample was analyzed for virus presence. A PCR was performed using 2 µl DNA, 0.5 µl of each of two virus-specific primers (132 R0 and 132 R1), 10 µl PPP-Master Mix (Top-Bio) and 7 µl DNAase free H_2O. The program was set as follows: 40 x (94/20" + 54/20" + 72/45").
PAGE was performed using 1 % agar in TBE-buffer (45 mM Tris-borat + 1 mM EDTA), and injecting 4 µl sample and middle range marker into the gel. The sample was run at 130 V for 15 min.

4.2.3 Sequencing

4 µl of sample 8 were run on 2 % agar with TBE-buffer at 125 V for 15 min. The band at 346 bp was cut out of the gel, and the DNA isolated using Nucleospin Extract II (Macherey-Nagel, Germany). 200 µl NT binding buffer were added to the sample and put into the heat block for 5 min. The suspension was transferred to a filter tube and centrifuged at 11 000 g for 1 min. The supernatant was discarded and the leftovers washed with 0.5 ml washing buffer. After another minute of centrifugation (11 000 g), buffer NT3 in ethanol was added, followed by centrifugation. Again the supernatant was discarded, and the filter dried by 2 min centrifugation. 25 µl elution buffer were added, and the membrane washed two times with the supernatant. To check, if extraction from the gel was successful, another PAGE was performed for 5 min using 2 µl extract.
A sequencing mixture was prepared from the extracted DNA using 4 µl DNA, 0.5 µl of each of the two primers (as above) and 3 µl DNAase free H_2O. This was then sequenced.

4.2.4 rt-PCR

For the real time-PCR two reaction mixtures were prepared. 180 µl Master mix (premix of SYBR green, Taq DNA Polymerase and 10x Taq buffer – Top-Bio s.r.o.) and 144 µl DNAase free H_2O were transferred to both vials. Then 9 µl of each of the control primers (359 A1 and 359 A2) were added to one of the mixtures, and another 9 µl of each of the two virus-specific primers (132 R0 and 132 R1) to the other vial. Into each of 9 tubes of the PCR plate, 2 µl of DNA sample were added and 38 µl of one of the reaction mixtures. This was mixed and half of it transferred to a second tube. The same was done for the second reaction mixture. Then the rt-PCR was started using following program:
1 x (95/1') + 40 x (95/20" + 50/20" + 72/40") + 1 x (72/5') in an iCycler apparatus (BIO-RAD).

4.3. TuMV procedure:

4.3.1 Isolation of RNA

Using the NucleoSpin RNA Plant kit (Macherey-Nagel, Germany) the RNA was isolated as follows.
The pellets were ground with a mortar, suspended in 350 µl lysis buffer and transferred to a NucleoSpin filtration tube. After centrifugation at 11 000 rpm for 1 min, 350 µl 70% Ethanol were added to the filtrate, and the solution transferred to an RNA-binding filter, followed by centrifugation for 30 sec (11 000 rpm). To the filter 350 µl MDB (Membrane Desalting Buffer) were added, and the tube centrifuged for 1 min. 95 µl rDNAase reaction buffer and 5 µl rDNAase were added to the filter, and incubation at room temperature for 15 min followed. Then the filter was washed with 200 µl of wash buffer I (centrifugation 30 sec.), 600 µl wash buffer II (centrifugation 30 sec.) and another 200 µl wash buffer II (centrifugation 2 min). The filter was put into a new collection tube, 30 µl elution buffer (SIGMA-ALDRICH life science) were added, and the extract centrifuged for 1 min.

4.3.2 Preparation of cDNA – reverse transcription

The iScipt cDNA Synthesis kit (BIO-RAD) was used to prepare the cDNA. The reaction mixtures were prepared by separately mixing 2 µl iScript reaction mix, 0.5 µl MMVL Rnase H+ reverse transcriptase and 7.5 µl of each RNA sample. The incubation times in the cycler were: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C.

4.3.3 PCR and PAGE

The separate sample was analyzed with PCR and PAGE. For this purpose, 2 µl cDNA, 7 µl nuclease free water, 10 µl PPPmix (Top-Bio s.r.o.) and 0.5 µl of each of two virus-specific primers were mixed. The iCycler (BIO-RAD) was programmed to 35x (94/20" + 50/30" + 72/1') + 72/5'. The amplification products were run on 1.5 % agarose gel at 130 V for 15 min in TBE buffer.
Also the rt-PCR products were verified for the presence of the virus by running them on polyacrylamide gel.

4.3.4 rt-PCR

Two mixed solutions were prepared, each filled with 150 µl Master mix (premix of SYBR green, Taq DNA Polymerase and 10x Taq buffer – Top-Bio s.r.o.) and 104 µl nuclease free water.
To one of them 8 µl of 2 primers for the housekeeping genes (control – 359 A1 and 359 A2) and to the other 2 virus-specific primers (TuE1 Pol F and Tu ex re2) were added. Finally, the solution was distributed over the wells on the PCR plate, each containing 3 µl of sample. The cycler was programmed to 35x (94/30" + 60/20" + 72/30").

4.4. Data processing

In order to explain the procession of the data, some therms have to be clarified. In real-time PCR the fluorescence of each sample in the holes of the PCR-plate is monitored. The baseline is defined as the noise of the record. For this purpose, the software calculates the mean fluorescence of the first 3 to 15 cycles and sets this as the baseline. The threshold is defined as the level of signal strength at which there is a statistically significant increase of signal relative to the calculated baseline signal. The threshold can be defined by the user or computed by the software. The software usually sets it at 10 times the standard deviation of the fluorescence signal of the baseline. The threshold cycle (Ct) is the cycle at which the fluorescent signal of the reaction crosses the threshold value. Assuming 100 % efficiency, estimation of the starting amount is possible as the Ct is inversely proportional to the latter. This means that we can assume that at each cycle twice as much DNA is present than before. Therefore, the earlier a reaction crosses the threshold, the more starting material was present.

This allows the computation of the relative amounts of nucleic acid present. The changes of the steady-state level of a gene of interest relative to the level of an invariant control gene are calculated (ΔCt). The control gene (e.g., housekeeping genes) can be seen as the internal standard and must not be influenced by the experimental conditions. The relative amount of nucleic acid (ΔC_T) is then assessed by: $\Delta C_T = 2^{-\Delta Ct}$ (1)

The threshold cycle (Ct) values of the virus-specific primers and those of the control primers were averaged and subtracted (ΔCt). Then ΔC_T was computed for each reaction and plotted against the corresponding day of sampling.

5. Results and Discussion

5.1. CaMV

5.1.1 Sequencing

As mentioned in the method section the product amplified by conventional PCR was sequenced. The sequencing result confirmed that the infection was successful, and that our product was indeed present.

5.1.2 rt-PCR

Figure 5 shows the real-time amplification signal of reference gene (a) and CaMV-specific amplification (b). The housekeeping genes are more abundant as they cross the threshold value earlier than the genes amplified by CaMV-specific primers.

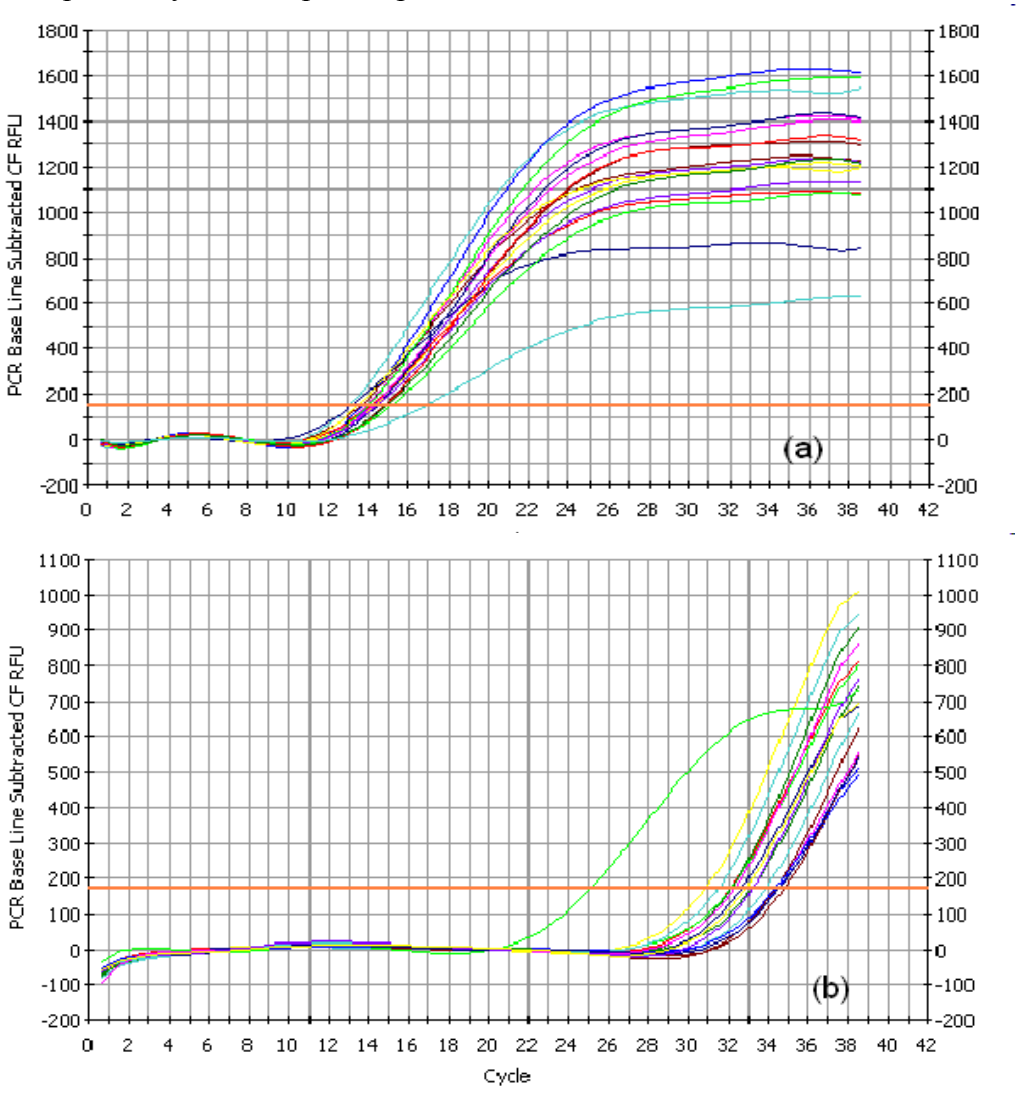

Figure 5: Amplification signal of housekeeping genes (a) and virus-specific genes (b) from the rt-PCR.

The data have been plotted in Figure 6 and show the relative amounts of viral DNA on each day of sampling. Interestingly there is a steep decrease of DNA during the first days, but in the course of the week the virus finally starts replicating again.

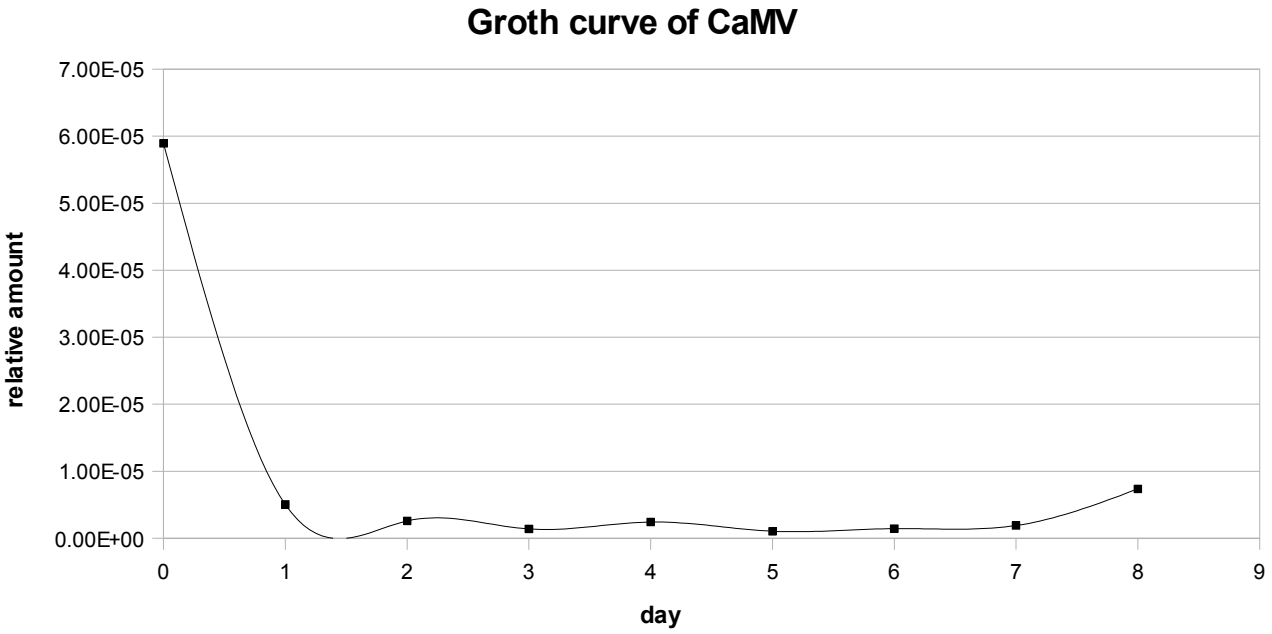

Figure 6: Relative amounts of CaMV DNA on each of 8 days. A strong decrease is observable at the beginning, but from the 7th day on an increase can be observed.

The sharp decrease that occurs the first day, can be explained by a very effective activation of the host's defense mechanism. Transcriptional silencing might be the cause, or a basal defense mechanism to which CaMV is specially susceptible. It cannot be an HR because no physiological symptoms could be observed.

The increase on the 2nd day might be due to a counter-attack of the virus, blocking the defense mechanism of Chinese cabbage. Translocation of genetic material to vicinal cells may also have occurred before a SAR could be launched. On the 3rd day post-inoculation again a decrease comes about. The plant might have found another way of blocking the virus replication, or simply a basal defense was started in newly infected cells. This phenomenon repeats on the 4th and 5th day. Only from the 7th day on the viral DNA is again more abundant and keeps increasing more steeply until the 8th day and probably further. I suppose that CaMV spread deeper into the tissue, and even if a SAR could be established by Chinese cabbage, a lack of resources could have weakened it, and defense was no more possible. Of course the virus might just have overcome the barriers created by the host. Moreover, the infection was probably just starting to get more serious after the 8th day. No symptoms on the infected leafs could be observed during the whole time of the experiment, but the rt-PCR showed that the virus was present and replicating. In their work, Monsion et al. (2008) mention that non-inoculated leafs showed symptoms 7-9 post-inoculation upon systemic infection with a mixed inoculum of allelic variants of CaMV.

5.2. TuMV:

5.2.1 PAGE

On Figure 7 we can see the product of the PCR with TuMV-specific primers separated by gel electrophoresis in TBE buffer. The band at 176 bp proofs the presence of reversely transcribed DNA of TuMV after rt-PCR. The next more intense band shows the presence of unused primer, primer-dimers or unspecific products synthesized after mishybridization of primers to the template. The product that is seen on the gel was from the samples taken 16 days post-inoculation.

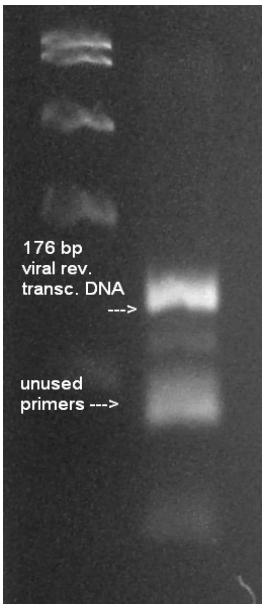

Figure 7: PAGE of rt-PCR product of reversely transcrived TuMV. The band at 176 bp shows the presence of an important amount of reversely transcribed DNA.

5.2.2 RT-rt-PCR

Picture 8 shows real-time amplification signal of reference gene (a) and virus-specific amplification (b). The curves passing the threshold at the left side are the housekeeping genes, the ones to the right show the viral component. The housekeeping genes are more abundant in the sample, and about eight cycles later the first viral gene is amplified above the threshold.

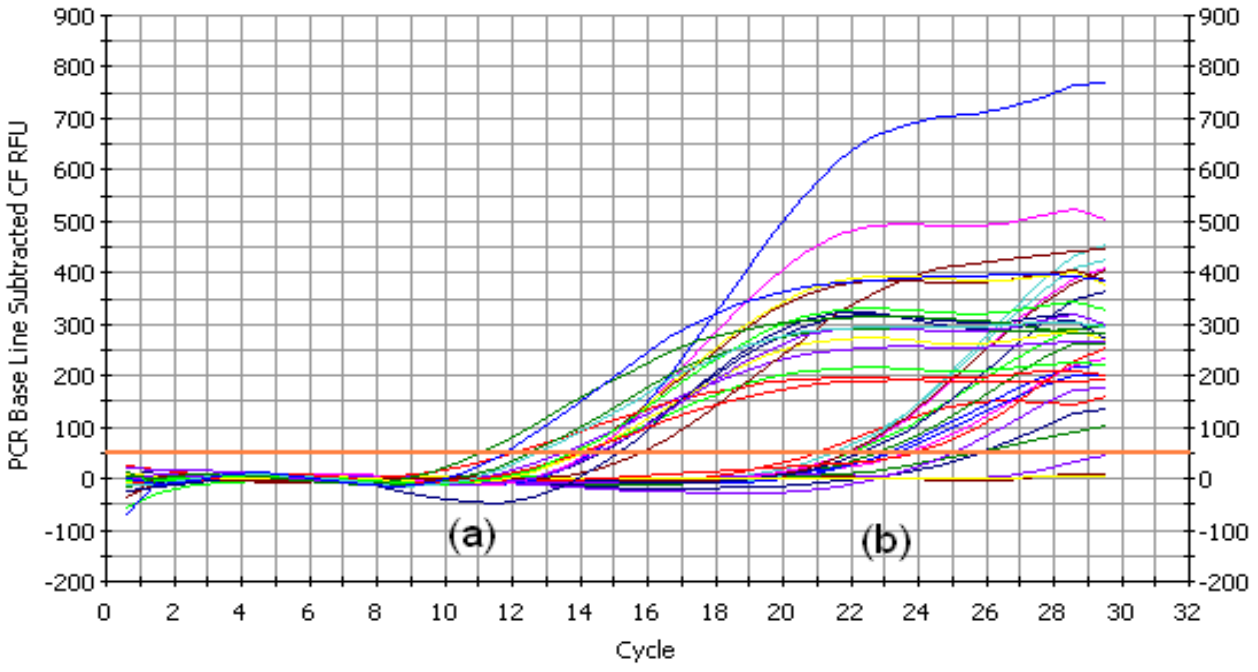

Figure 8: Amplification of (a) housekeeping genes and (b) TuMV-specific genes.

The following diagram (Figure 9) shows the obtained replication behavior of Turnip Mosaic Virus. The concentration is higher on the day of infection and follows a positive trend over the course of the days. The only exception is the sample on day six, showing a lower concentration than on any other day.

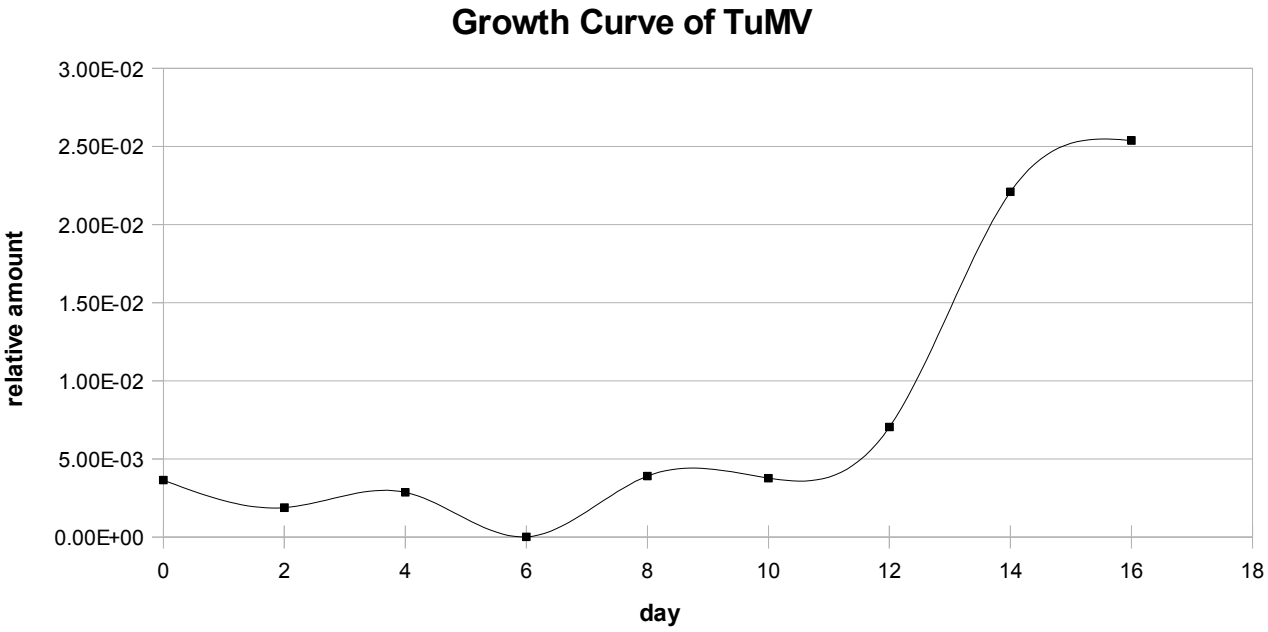

Figure 9: Relative amounts of TuMV nucleic acid over a time-span of 16 days. From the 11th day on a rapid increase can be observed.

The relatively high concentration of RNA right after infection (day 0) can be explained by the high abundance of virus particles on the surface. It represents the part of viruses that could immediately attach to the surface, since the rest was swapped off with distilled water. During the two following days, the amount of present viral nucleic acid decreases, probably because of the basal defense reaction of the plant. Between the 2nd and the 4th day, an increase can be observed again, the virus must have blocked the basal defense pathway. On the 6th day, a sharp decrease of viral RNA is observable. One theory about it is the activation of RNA silencing by Chinese Cabbage in response to the invading virus. After the 6th day, the amounts of nucleic acid increase again, therefore I suppose that TuMV successfully overcame the RNA silencing and was effectively replicating. I suppose that no HR occurred because no physiological symptoms could be observed on the infected plants. Reports about TuMV infecting *Brassica oleracea* state that the first symptoms were observed 14 days post-inoculation (Tomlinson, 1970), whereas we detected replication much earlier.

5.3. Method

The rt-PCR method is a simple, but efficient way to monitor the replication behavior of both TuMV and CaMV and certainly also of other viruses. Always the whole leaf area was inoculated, so that we can assume that infection occurred evenly. In the time period of sampling no physiological changes could be observed in the infected leaves of either plant, although via the rt-PCR we were able to detected quite some action during these apparently healthy days. This shows how sensitive the method is. The specialty lies in the specific amplification of a template within a mixture of DNAs and attachment of the SYBR green dye to every newly amplified dsDNA segment, allowing us to monitor

the reaction. For reverse transcription the MMVL Rnase H+ reverse transcriptase was used, to ensure that no RNA was left in the cDNA sample for the real-time PCR analysis.
This is important for the quantification as SYBR green doesn't only bind to dsDNA, but could weakly bind to RNA as well and thereby adulterate the results.
Our results show the relative amount of virus present on each (second) day after inoculation, without subjecting the plant to any form of stress. Therefore, the replicational behavior of the two distinct viruses we have found shows the development of the plant-virus interaction under optimal conditions.
Both of the viruses had a quite long lag-phase where they were fighting against the plant defense system. But once all barriers were overcome, CaMV and TuMV could replicate more efficiently.
It should be noted, that CaMV, despite of the fact that the inoculum was much less concentrated than TuMV sap, did not recover from the host plant's attack within eight days. In contrast, TuMV had already overcome its initial amount on the 8th day post-inoculation. But this is not the concern of the present work, and hence I will not go deeper into it.
rt-PCR and RT-rt-PCR are suitable methods for similar studies, for example for the observation of stress effects on the plant-virus interaction, monitoring of mixed infections or influence of genetic modification of either virus or Chinese cabbage. It is an easy and sensitive method, requiring little time and low expense.

6. Conclusion

We were able to infect Chinese Cabbage with CaMV and TuMV, to extract their NA and to perform a rt-PCR and RT-rt-PCR respectively. As the method was successful, we could map the replication behavior of the viruses during the given time periods on a common host. Not only the viruses differ in their structure and replication mode, but differences can also be observed in their replicative rhythms.
This study will serve as a comparison in future works about the influence of stress or genetic modification in either host or pathogen on the reaction of Chinese cabbage in response to an infection with CaMV and TuMV.

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