Molecular characterization of clover and dill infecting cryptic viruses - detection, protein interactions and evolutionary relationships

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ABSTRACT

Plant viruses are well known as disease causing agents in a large number of wild and crop plant species. However, there are also large communities of putatively benign viruses which co-exist with the host over generations without causing obvious symptoms and diseases: so called “persistent viruses”. The recent study sheds light on the biodiversity of persistent viruses of plants, which includes viruses from the families *Partitiviridae*, *Endornaviridae* and the proposed new family *Amalgaviridae*. All of these dsRNA viruses are widespread in plants. They cause generally no significant effects on their hosts and are only transmitted by cell division and through gametes at very high rates.

The largest family *Partitiviridae* includes plant (*Alphacryptovirus* and *Betacryptovirus*), fungal (*Partitivirus*) and protozoan (*Cryspovirus*) infecting viruses with bisegmented dsRNA genomes (CP, RdRp) and isometric virions. Cryptic viruses commonly occur in different plant species often in mixed infections without causing any symptoms. So far, numerous sequences have been determined for viruses of the genus *Alphacryptovirus*, but no sequence was available for any assigned member of the genus *Betacryptovirus*. Following extraction, cloning and sequence analysis of double-stranded RNA in this study, we report the molecular properties of three members of the genus *Betacryptovirus*, *White Clover Cryptic Virus 2*, *Red Clover Cryptic Virus 2* and *Hop Trefoil Cryptic Virus 2*, and two new putative betacryptoviruses found in crimson clover and dill. The results close a gap in the actual taxonomy and provide evidence for a distinct evolutionary lineage of dsRNA viruses of plants and fungi.

Our knowledge on the biology of cryptic viruses such as the influence on the host and molecular characteristics of their replication is very limited. Aside from sequence and structural analyses, the investigation of protein interactions is another step towards virus characterization. Therefore, ORFs of two type members *White Clover Cryptic Virus 1* and *2*, as well as the related viruses from Red Clover and dill were introduced into a bimolecular fluorescence complementation assay. We showed different kinds of protein dimerizations and observe the localization *in planta* of *Nicotiana benthamiana* leaf epidermal cells. We showed CP-CP dimerization with different localizations for all tested viruses. CP-deletion mutants were created to determine internal interaction sites. Moreover, RdRp self-interaction was found for all viruses, whereas CP-RdRp interactions were only detectable for the alphacryptoviruses. An intra-genus test of CPs

was successful in various virus combinations, whereas an inter-genus interaction was absent. This is the first report of *in vivo* protein interactions of members in the family *Partitiviridae*, indicating distinct features of the alpha- and betacryptoviruses.

To illuminate the large diversity of the persistent viruses in plants, two screening techniques were combined: Nucleic acid enrichment of RNA containing viruses by dsRNA isolation and a deep sequencing approach. So far metagenomic studies of plant infecting viruses have focused on experimentally or naturally infected plant material. In this study the target was to access the biodiversity of persistent viruses of plants. Therefore we isolated dsRNA from seed grown White Clover, Red Clover, Hop Trefoil and dill plants, cultivated under controlled greenhouse conditions. Deep sequencing resulted in 42 determined contigs fitting to persistent viruses. These contigs represent 52% of all reads. 15 putative new virus species belonging to persistent viruses could be identified as tentative members of the families *Partitiviridae*, *Endornaviridae* and *Amalgaviridae.* For the first time the suitability of a combination of dsRNA-screening and deep sequences techniques for the determination of the virome of persistent viruses was shown.

Keywords:

plant cryptic virus, taxonomy, protein-interactions, deep sequencing

ZUSAMMENFASSUNG

Pflanzenviren sind in der Regel als krankheitsauslösende Faktoren in vielen Wild- und Kulturpflanzen bekannt. Dennoch gibt es eine Vielzahl an Viren die mit ihrem Wirt seit Generationen hinweg in einer Art Koexistenz zusammen leben und offenbar keine Symptome und Erkrankungen hervorrufen: sogenannte „persistierende Viren“. In dieser Arbeit wird die Biodiversität persistierender Viren in Weiß-, Rot- und Gelbklee sowie Dill untersucht. Die dabei beleuchteten Viren gehören zu den Familien *Partitiviridae*, *Endornaviridae* und in die neu vorgeschlagene Familie *Amalgaviridae*. Diese dsRNA enthaltenden Viren sind in den untersuchten Pflanzen weitverbreitet, verursachen im Allgemeinen aber keine signifikanten Effekte bezogen auf ihren Wirt. Sie werden nur durch Zellteilung und die Keimzellen in hoher Rate übertragen.

Die größte Familie der *Partitiviridae* schließt Pflanzen-infizierende (*Alphacryptovirus* und *Betacryptovirus*), Pilz-infizierende (*Partitivirus*) und Protozoen-infizierende (*Cryspovirus*) Viren mit zweiteiligem dsRNA Genom und isometrischen Partikeln ein. Kryptische Viren kommen in der Regel in verschiedenen Kulturpflanzen vor, oftmals in Mischinfektionen ohne Symptome zu verursachen. Bisher konnten zahlreiche Sequenzen für Viren des Genus *Alphacryptovirus* bestimmt werden, jedoch waren keine Sequenzen für die bislang ausgewiesenen Mitglieder des Genus *Betacryptovirus* verfügbar. Nach Reinigung der dsRNA, gefolgt von Klonierungen und der anschließenden Sequenzanalyse konnten die molekularen Charakteristika von 3 Mitgliedern des Genus *Betacryptovirus* aufgedeckt werden. Dabei handelt es sich um *White Clover Cryptic Virus 2*, *Red Clover Cryptic Virus 2* und *Hop Trefoil Cryptic Virus 2* und vermutliche weitere zwei Betakryptoviren in Inkarnat Klee und Dill. Die Ergebnisse schließen dabei eine Lücke in der aktuellen Virustaxonomie und geben Hinweise auf eine ausgeprägte Verbindung zwischen dsRNA Viren von Pflanzen und Pilzen.

Unser Wissen über die Biologie kryptischer Viren, wie der Einfluss auf den Wirtsorganismus und die molekulare Charakteristik ihrer Replikation, ist sehr begrenzt. Abgesehen von Sequenz- und Strukturanalysen stellt die Untersuchung von Proteininteraktionen einen weiteren Schritt bezüglich der Viruscharakterisierung dar. Folglich wurden die offenen Leseraster von zwei Referenzmitgliedern *White Clover Cryptic Virus 1* und *2*, genauso wie die ihrer verwandten Viren aus Rotklee und Dill in ein bimolekulares Fluoreszenz-Komplementations-System überführt. Hiermit konnten

verschiedene Arten der Proteindimerisation aufgezeigt und deren Lokalisation in Epidermiszellen von *Nicotiana benthamiana in planta* beobachtet werden. Wir konnten für alle getesteten Viren eine CP-CP Dimerisation mit verschiedener Lokalisation nachweisen. CP-Deletionsmutanten wurden erstellt um interne Interaktionsstellen zu bestimmen. Darüber hinaus konnte für alle Viren RdRp- Selbstinteraktionen nachgewiesen werden, wohingegen CP-RdRp Interaktionen nur für Alphacryptoviren feststellbar waren. Ein CP intra-genus Test war in einer Vielzahl von Viruskombinationen erfolgreich, während eine inter-genus Interaktion fehlte. Dies ist die erste Studie über *in vivo* Proteininteraktionen von Mitgliedern der Familie *Partitiviridae*, wobei unterschiedliche Interaktionen von Alpha- bzw. Betacryptoviren aufgezeigt werden.

Um die große Diversität persistierender Viren in Pflanzen zu beleuchten wurden zwei Screeningverfahren kombiniert: Nukleinsäuren von RNA-Viren wurden durch dsRNA-Isolation angereichert und anschließend einer Hochdurchsatzsequenzierung unterzogen. Bisher wurden metagenomische Studien nur an gesammelten, virusinfizierten Umweltproben bzw. absichtlich infizierten Pflanzen vorgenommen. Das Ziel dieser Studie war es einen Zugang zur Biodiversität persistierender Viren in Pflanzen zu erhalten. Dafür wurde dsRNA aus Weiß-, Rot- und Gelbklee sowie Dill Pflanzen, die unter kontrollierten Gewächshausbedingungen aus Samen kultiviert wurden, isoliert. 42 ermittelte Contigs, auf die 52% sämtlicher Reads entfielen, konnten persistierende Viren zugeordnet. Hieraus resultierten 15 neue persistierende Viren, die als vorläufige Mitglieder in die Familien *Partitiviridae*, *Endornaviridae* und *Amalgaviridae* eingeordnet werden können. Die Ergebnisse zeigen erstmals die Eignung der Kombination von dsRNA-Screening Verfahren und Techniken der Hochdurchsatzsequenzierung für die Bestimmung des Viroms persistierender Viren.

Schlagworte:

Kryptische Pflanzenviren, Taxonomie, Protein-Interaktionen, Hochdurchsatz-Sequenzierung

CONTENTS

ABBREVIATIONS

%	percentage
α	alpha
A	adenine
abs.	absolute
A. tumefaciens	*Agrobacterium tumefaciens*
β	beta
BiFC	Bimolecular fluorescence complementation
BLAST	Basic Local Alignment Search Tool
bp	base pairs
°C	centigrade
C	Cytosine
ca.	circa
cDNA	copy DNA
CLSM	Confocal Laser Scanning Microscopy
cm	centimeter
CP	coat protein
cv.	cultivar
Da	Dalton = 1.66×10^{24} g
DCV-1	Dill Cryptic Virus 1
DCV-2	Dill Cryptic Virus 2
DNA	Desoxyribonucleic acid
DNase	Desoxyribonulease
ds	double stranded
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
et al.	*et alii*
g	gram
G	Guanine
GOI	gene of interest
ICTV	international committee on taxonomy of viruses
l	liter
kDa	kilo-Dalton
M	molar
mg	milligram
Min.	minute
µg	microgram
µl	microliter
µm	micrometer
µM	micro molar
M	molar
MCS	multiple cloning site
miRNA	micro RNA
ml	milliliter
mm	millimeter
mM	millimolar
mRFP	monomeric Red Fluorescent Protein

mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
nt	nucleoid
ORF	open reading frame
PCR	Polymerase Chain Reaction
pH	Negative decade logarithm of hydrogen ion concentration
ori	origin of replication
RCCV-1	Red Clover Cryptic Virus 1
RCCV-2	*Red Clover Cryptic Virus 2*
RNA	Ribonucleic acid
RNase	Ribonuclease
RdRp	RNA-dependent RNA Polymerase
s	Seconds
siRNA	small interfering RNA
ss	single stranded
ssp.	sub species
T	Thymine
Taq	*Thermus aquaticus*
TE	Tris-EDTA
U	units
UTR	untranslated region
V	Volt
v/v	Volume to volume
w/v	Weight to volume
WCCV-1	*White Clover Cryptic Virus 1*
WCCV-2	*White Clover Cryptic Virus 2*

1 General introduction

1.1 Cryptic viruses – met with little response in plant virology

Cryptic viruses are members of the family *Partitiviridae* including the plant infecting genera *Alphacryptovirus* and *Betacryptovirus*. Each monocistronic segment of the dsRNA genome is encapsidated separately in an isometric particle of 30-38 nm in diameter (Boccardo, 1987). These viruses have several properties that distinguish them from classical pathogenic viruses. Cryptic viruses occur only in low concentrations in their hosts, without showing any visible symptoms and apparently have less or no economic impact. These viruses can only be transmitted through gametes, but not by grafting, mechanical or vector inoculation. Nevertheless, the transmission by seeds and pollen occurs in very high rates and was found in a broad range of monocotyledonous and dicotyledonous plant species, such as alfalfa, beans, carnation, carrot, clover species, fire trees, pepper, radish, ryegrass, spinach and sugar beet (Milne, 1999).

Because of being non-pathogenic and limited in their transmission, cryptic viruses do not fit into the classical definition of a virus. First descriptions characterized them as virus-like particles (VLPs) in apparently healthy plants from seven species of beet (Pullen, 1968). These particles were inoculated to other herbaceous plants but did not cause an infection. Moreover, the particles could not be removed by heat therapy from meristem cultures, a method which works with other plant viruses. Therefore the VLPs were assumed to be part of the plants until a few beet plants were found to be free of VLPs (Kassanis, 1977) leading to the name Beet cryptic virus. Kassanis suggested the name “cryptic” to distinguish these viruses from the latent viruses which also caused no symptoms but were transmissible to other plants. Independent work groups in Japan described also the same VLPs in other cultivars and chose the name temperate viruses due to their resistance to thermotherapy (Natsuaki, 1984). Nevertheless, currently the putative virus does not meet the requirements for a clear virus description according to the Koch’s Postulates for viruses (Rivers, 1937). To fulfill these postulates:

1. The virus must be isolated from a diseased host and
2. cultivated in an experimental host or host cells.
3. A prove of lack of larger pathogens must be given and

4. the virus must produce comparable disease symptoms in the original host species or in related ones.

Due to the lack of detectable diseases and transmission, the term VLPs was still used up to until (Boccardo, 1983). Therefore, the requirements for virus characterization were revised (Kassanis, 1984) and resulted in a more modern and general virus definition (Hull, 2013). According to this definition a virus is:

1. dependent on the hosts protein-synthesizing machinery,
2. organized from pools of the required materials rather than by binary fission,
3. located at sites that are not separated from the host cell contents by a continuous lipoprotein bilayer membrane and
4. continually giving rise to variants through various kinds of change in the viral nucleic acid.

However, the progress of new technologies to detect viral sequences makes further criteria necessary to identify new viruses. Especially, the findings of a large number of potential viruses by the use of metagenomic approaches are in conflict to the strictness of the Koch's Postulates. It will be a major challenge in virology to keep the established taxonomy, and to integrate the advantages of the upcoming "viromic" studies (Mokili, 2012).

1.2 Genome organization and replication of cryptic viruses

Plant-infecting members of the family *Partitiviridae* encode the genome in two monocistronic segments of dsRNA. Findings of viruses with three or more fragments mostly represent mixed infections, or should be classified as satellite elements, due to the absence of a larger open reading frames (Ghabrial, 2008). The molecular weight of the individual components is between 0.8 to 1.6×10^6 Da at a fragment length of1.4 to 2.3 kbp (Boccardo, 1987). While the smaller dsRNA encodes for a viral coat protein (CP), the larger one encodes for a viral RNA-dependent RNA polymerase (RdRp).

The genome organization and replication of cryptic viruses is especially interesting in the context of dsRNA, which is acting as the genetic information storage. Naturally, dsRNA is not found in plants. For the dsRNA processing of the virus it has to contain its own RdRp or it depends on the presence of a helper virus. Cryptic viruses code for a virus-specific RdRp which is probably associated with the envelope protein (Buck,

1973). Plants are using the RNA interference (RNAi) as protection against RNA viruses by recognizing dsRNA and degrading it into smaller fragments (Hannon, 2002). Other plant viruses are known to avoid this counterstrike by using suppressor proteins (Carrington, 2001; Chapman, 2004). However, the CP and RdRp proteins of the cryptic viruses do not affect the RNA interference and other proteins were not reported (Yaqoob, 2006). It is assumed that dsRNA viruses provide all necessary enzymatic components for replication and transcription within the particle to circumvent the action of RNA interference (Bamford, 2002; Ghabrial, 2008). Required pores to interchange materials for transcription/replication can be observed by structural analyzes of the particle of the Partitivirus (Ochoa, 2008). As outlined in Figure 1 the dsRNA only occurs in the virus particle itself and serves here as a template for the also encapsidated RdRp (Boccardo, 1987).

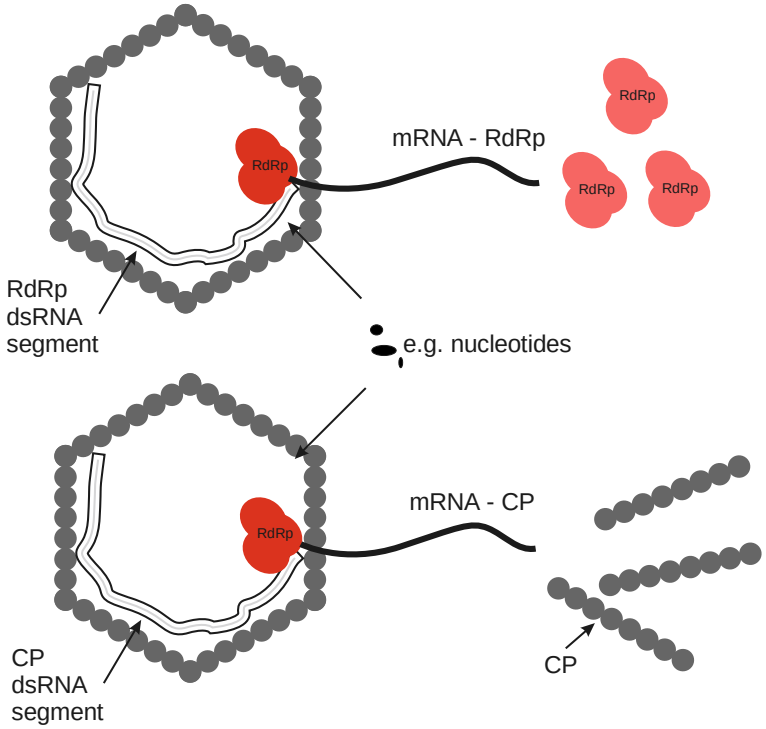


Figure 1: Replication strategy of cryptic viruses in the plant cell

The transcribed single-stranded RNAs pass from the particle into the cytoplasm by pores, where the CP and RdRp are translated. During the particle assembly, RNA and RdRp are packaged together by protein-protein and protein-RNA interaction within the CP. Only inside the assembled particle the RdRp switches to an active mode and starts to synthesize new dsRNA (Nibert, 2013) as illustrated in Figure 2.

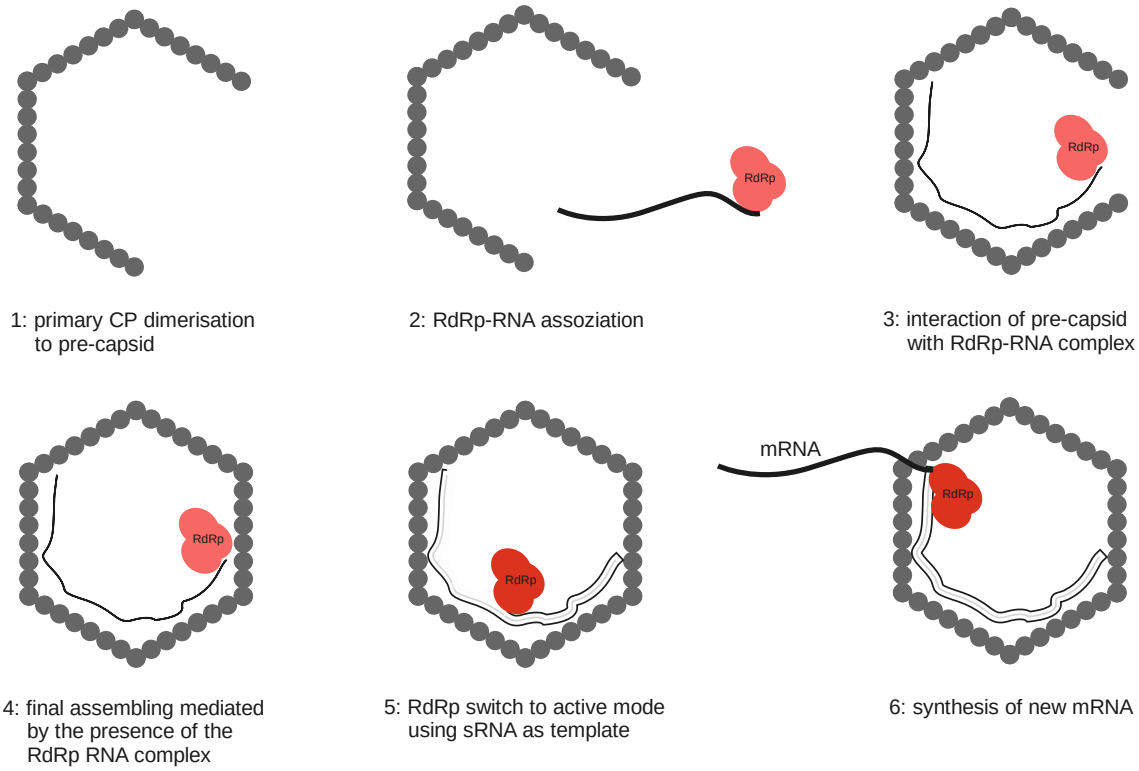


Figure 2: Proposed particle assembly of cryptic viruses in the plant cell

1.3 Particle morphology of cryptic viruses

Cryptic viruses have a relative simple particle morphology. The nucleic acids are enclosed by an isometric protein shell; additional lipid layers are not present. The outer diameter of the particles of the genus *Alphacryptovirus* is around 30 nm and of the genus *Betacryptovirus* around 38 nm. In electron microscope images the smaller particles appear featureless, whereas the greater particles of the genus *Betacryptovirus* show morphologically protruding subunits. From the results of the analysis via ultracentrifugation it is assumed that each one of the dsRNA bipartite genome is packaged separately into one particle. The molecular weight of the individual components is between 0.8 to 1.6 x 10^6 Da (Boccardo, 1987).

Recent X-ray diffraction studies focused on the structural analysis of the virus particles. A 3D-model was established for *Penicillium stoloniferum virus F* (PSV-F), a member of the genus *Partitivirus*, which is closely related to plant infecting alpha- and betacryptoviruses. Analyses showed a molded helix-rich envelope-protein with distinct surface curvatures consisting of 120 subunits, which is composed of 60 quasi symmetric coat protein dimers. Further possible pores could be modulated onto the particle surface. These could serve for the release of positive single-stranded RNA (ssRNA) after

transcription, which has been postulated for other dsRNA viruses (Prasad, 1996; Caston, 1997; Dryden, 1998; Zhang, 2003). However, the necessary RdRp protein could not be found in the viral particles in this structure analysis, maybe because of a weak link to the capsid protein (Ochoa, 2008).

Distinctive surface structures may be possible starting points for a host-parasite interaction, but experimental evidence is currently missing (Ochoa, 2008). Whether the particles allow an enzymatic activity as presented in other dsRNA viruses such as the Reovirus, has not be proven so far (Boccardo, 1987; Reinisch, 2002).

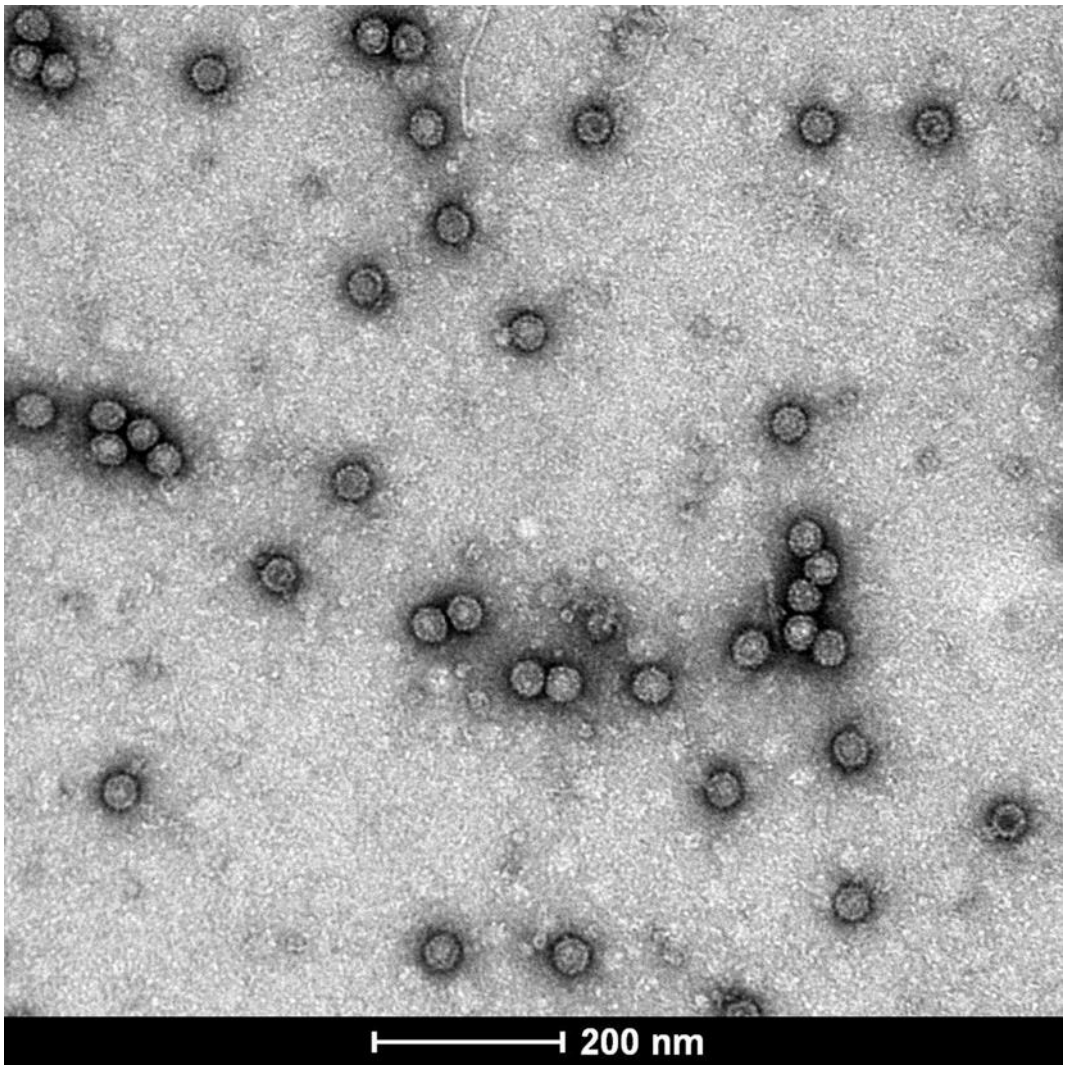


Figure 3: Immunosorbent electron microscopy of WCCV-2 particles trapped from a virus preparation from white clover (*Trifolium repens*), kindly provided by Dr. Frank Rabenstein, Julius Kuehn Institute, Quedlinburg

1.4 Interaction of *Partitiviridae* with their hosts

The presence of cryptic viruses generally does not result in pathological changes. Plants carrying cryptic viruses are usually symptomless. In previous works only a few cases have been described in which an infestation led to changes in the plant phenotype; these studies are summarized in table 1. However, some factors can cause to eventually misleading results:

1. Contamination of plants with fungi

Cryptic viruses occur in plants only in very low concentrations of 200 µg per kg in various tissues, mainly detectable in the mesophyll (Abou-Elnasr, 1985; Kuehne, 1989; Boccardo, 1989). In fungi, especially in older mycelium, significantly higher concentrations, such as 1 mg per g of tissue in the *Penicilliumstoloniferum virus S* are possible (Ghabrial, 2008). Therefore, overlooked infections with fungi, especially with endosymbiotic ones, can result in the misleading determination of the origin of the detected viruses even at low contamination levels (Kozlakidis, 2006).

2. Accumulation of cryptic viruses in the presence of pathogenic viruses

In mixed infections with other viruses present in the plant the concentration of cryptic viruses may increase in host plants. This might be a side effect of the down regulated defense mechanism of the plant by the viral suppressor proteins. An accumulation was shown for the *Beet cryptic virus* 1 and 2 in combination with *Beet mild yellowing virus* and *Beet yellows virus* infections (Kühne, 1989). Also in mixed infections of ryegrass cryptic virus with *Ryegrass mosaic virus* this effect was observed (Plumb, 1981).

In addition, interactions with other pathogens or environmental circumstances can result in misinterpretation of observed symptoms. Also, the infestation of cryptic viruses in fungi (*Partitivirus*) remains usually asymptomatic (Tavantzis, 2008). Studies on root sponge (*H. annosum*) showed, however, that the germination rate of basidiospores was reduced when the fruit body contained Partitivirus dsRNA (Ihrmark, 2004). Other tests, such as the infection of protoplasts by *Rosellinianecatrix* with purified particles of Partitivirus RnPV1 -W8 (Sasaki, 2006) showed no effect on the host. On the other hand, the lack of the availability of a cryptic virus free plant and the limited transmission of cryptic viruses can prevent further comparisons. Nevertheless, cryptic viruses interact with their host plant and can cause beneficial effects under special conditions. In future,

modern molecular techniques will shed light on the characteristics of cryptic viruses (Nakatsukasa, 2005).

Table 1: Studies with a reported influence of *Partitiviridae* to their host plants

Virus name	Host	Effect	Comments	References
Beet cryptic virus	Sugar Beet	mild chlorosis of leaves, reduced root fresh weight	could not be shown in other studies	Kassanis, 1978 White, 1978 Moir, 1983 Kühne, 1989
Radish yellow edge virus	Radish	yellow edging of leafs	weak, pathological change	Natsuaki, 1979
Beet cryptic virus	Sugar Beet	reduced yield	not detectable under drought conditions	Xie, 1994
White clover cryptic virus 1 (CP)	*Lotus japonicus*	root nodulation	led to an increased in the endogenous phytohormone abscisic acid and the suppression of root nodule formation	Suzuki, 2001 Nakatsukasa, 2005
Cherry chlorotic rusty spot / Amasya cherry disease associated partitivirus	Cherry	chlorotic rusty spots	disease presumed of fungal aetiology infecting with viruses	Coutts, 2004 Kozlakidis, 2006
Raphanus sativus cryptic virus 1	Radish	yellow edging of leafs	relationship to Radish yellow edge virus unclear	Chen, 2005
Curvularia thermal tolerance virus (unclassificated virus shows similarties to *Partitiviridae*)	Panic grass infected with Curvularia protuberata	three-way symbiosis result in thermal tolerance	association with a fungal endophyte	Márquez, 2007
Primula malacoides virus 1	Primula	yellowing-edge symptoms	authors described similar symptoms with Raphanus sativus cryptic virus 1	Chen, 2009
Pittosporum cryptic virus 1	*Pittosporum tobira*	chlorotic vein banding symptoms	interaction with the *Eggplant mottled dwarf virus*	Alabdullah, 2010
Citrullus lanatus cryptic virus	Watermelon	unclear, co-accumulation with Melon necrotic spot virus	not detectable in healthy watermelon plants	Sela, 2013

1.5 Origin and evolution of cryptic viruses

Because of their non-pathogenicity and limited transferability cryptic viruses, hold a special position in the field of virology. Kassanis (1984) formulated a new concept of viral definition contrary to classical virus definitions (see point 1.1). Molecular genetic analysis of cryptic viruses could contribute interesting aspects to the formation and evolution of viruses. Concerning the origin of viruses three possibilities are currently discussed (Forterre, 2006):

1.5.1 Origin of self-replicating molecules (coevolution)

According to the reduction theory, cryptic viruses could belong to a group of very old viruses descended from primitive pre-cellular life forms (RNA world). Later, they parasitized the earliest cells and reached co-evolution. These ancient viruses have lost their capability for infections, vector transmission and cell-to-cell transport in the course of time. Therefore, the virus participates in the binary fission of cells and reaches a high seed transferability (Kassanis, 1984). The non-pathogenicity of these viruses support this theory. Viruses increasingly adapt to their host during evolution to avoid destroying the host by the consequences of diseases so the outcome would be disadvantageous to themselves (see also Infect-and-Persist Strategy reviewed by Hilleman, 2004). However, this might be only applicable to some groups of cryptic viruses. Looking at sequence comparisons, some viruses are very similar to each other, although they were found in very distinct but related plant families. Moreover, phylogenetic studies rather anticipate a younger common ancestor for a related virus cluster as an origin from ancient viruses.

1.5.2 Virus emergence from host cell RNA or DNA molecules (escape hypothesis)

A different approach would be possible in the light of the escape hypothesis (Hendrix, 2000). It was suggested that viruses arise from some cell genes that escaped from the normal control mechanisms and become self-replicating entities (Hull, 2013). Cryptic viruses could represent a new group of viruses that evolutionarily have to be classified in front of the development of vector transmissibility (Kassanis, 1984). The starting point could be a self-replicating cellular mRNA (Koonin, 1993) which evolved through adoption of cellular proteins for packaging in a virus. A corresponding model for the Partitivirus was proposed by Ghabrial (1998). The *Totiviridae* which have a similar

genome structure to the cryptic viruses with only CP and RdRp, but with a non-segmented genome, are postulated as a possible intermediate. Another aspect supporting this theory are viral sequences which can be found in many plant genomes. Many of them are expressed as functional genes (Chiba, 2011; Liu, 2010). However, most plant genomes include only a part of the coat protein sequences, and RdRp sequences have not been reported so far. This fact together with phylogenetic analyses indicate that the integration of CP genes started from viruses to plants (Chiba, 2011). Moreover, no integrated sequences were found in plants which harbor the same cryptic viruses themselves (Roossinck, 2012).

1.5.3 Virus origin by degeneration of parasites

The reduction theory is based on the derivation of viruses from degenerated cells that eventually have parasitized normal cells. For the cryptic viruses in this model the presence of a pathogenic or endosymbiotic fungus could be helpful. A fungal or common origin for cryptic viruses is still an outstanding question (Kassanis, 1984; Ghabrial, 2008; Roossinck, 2010). Fungal infecting viruses have similar structural properties as cryptic plant viruses and also use mainly dsRNA genomes (Tavantzis, 2008). Similar approaches were discussed for the definition of fungal viruses and their evolutionary history (Buck, 1975; Frost, 1980). Fungal dsRNA viruses are widespread, mainly asymptomatic and dispersed in the mycelium over the contact to other hyphae and spores (Kassanis, 1984). Due to the lack of cell walls in fungal species, no viral movement proteins are needed. Phylogenetic analysis of cryptic plant viruses and the fungal cryptic viruses indicate a strong linkage (Ghabrial, 2008).This could erroneously lead to the belief that all findings of cryptic viruses in plant sources came from a contamination with an endosymbiotic fungus harboring the virus. However, this hypothesis could not be confirmed. Treatment of plants with systemic and superficial acting fungicides had no effect on the subsequent concentration of *White Clover Crypticvirus* 1 and 2 (WCCV) in *Trifolium repens*. Furthermore, no cross-reaction to the WCCV antiserum against mycoviruses of the group D and E was found (Boccardo, 1985). Nevertheless, it was possible to isolate cryptic viruses from protoplast cultures of *Vicia faba* (Abou-Elnasr, 1985) and sterile micro-plants of *Raphanus* L. (Natsuaki, 1985). Moreover, a fungal contamination was not detected in those plants in which dsRNA and particles were found (Boccardo, 1985). Also, in the betacryptovirus-harboring plants primula and hemp, no evidence for a fungal infection was found (Li,

2009; Ziegler, 2011). Recently, the observation of related viruses in distinctly related plants, does not support the notion that these plant species are infected or contaminated by a common specific endosymbiotic fungus (Boccardo, 1987).

1.6 Taxonomy of persistent infecting viruses

Viruses are well known as disease causing agents, but there is also a large community of benign viruses. These viruses have distinct features compared to the “classical” acute viruses summarized in table 2. This virus group includes the most studied viruses of plant infecting members of the family *Partitiviridae* which are called “cryptic viruses” due to the hidden lifestyle or “temperate viruses” due to their resistance to thermotherapy (Boccardo, 1987). Some other viruses show similar characteristics such as the Endornaviruses (Gibbs, 2005) and the newly proposed Amalgaviruses (Tzanetakis, 2013). These groups are now summarized according to their permanent infection as “persistent viruses” or due to their limited transmission ability as “uncultivable viruses” (Roossinck, 2011, 2013).

1.6.1 Definition of persistent viruses

Viruses are well known as disease causing agents, but there is also a large community of benign viruses. These viruses have distinct features compared to the “classical” acute viruses summarized in table 2. This virus group includes the most studied viruses of plant infecting members of the family *Partitiviridae* which are called “cryptic viruses” due to the hidden lifestyle or “temperate viruses” due to their resistance to thermotherapy (Boccardo, 1987). Some other viruses show similar characteristics such as the Endornaviruses (Gibbs, 2005) and the newly proposed Amalgaviruses (Tzanetakis, 2013). These groups are now summarized according to their permanent infection as “persistent viruses” or due to their limited transmission ability as “uncultivable viruses” (Roossinck, 2011, 2013).

Table 2: Properties of acute and persistent infecting viruses (adopted to Roossinck, 2011)

characteristic	acute lifestyle	persistent lifestyle
impact on host	symptomatic, may lethal or latent in different hosts	no obvious symptoms
infection periods	may be resolved by recovery	maintains for host lifetime and ongoing by their gametes
in plant distribution	use virus-encoded protein to moves systemically to host	passive via cell division, no active transport
transmission	horizontal via vectors, rarely by invasion of gametes	vertical nearly 100% via seed and pollen
virus titer	may establish very high concentration	generally low titer, may controlled by the host

1.6.2 Plant cryptic viruses

Cryptic viruses of plants are widespread in mono- and dicotyledonous plant species and are currently classified in the genera *Alphacryptovirus* and *Betacryptovirus* of the family *Partitiviridae* by the International Committee on Taxonomy of Viruses (ICTV). In addition, the family contains the genera *Partitivirus* and *Cryspovirus* which include viruses that infect fungi and protozoan, respectively (Ghabrial, 2012).

Phylogenetic analyses divided viruses of the genera *Alphacryptovirus* and *Partitivirus* into two clearly distinct clusters (Ghabrial, 2008; see Figure 4). Further characteristics such as dsRNA size, presence of interrupted poly(A) stretches and particle sizes support the clustering (see Table 3). Therefore, a revision of the taxonomic structure of the family *Partitiviridae* is needed. An actual ICTV proposal (Nibert, 2013) recommends an assignment of the clusters Alphacryptovirus II and Partitivirus II into two new genera (*Gammapartitivirus* and *Deltapartitivirus*) within the family *Partitiviridae*. Furthermore, the recent genera *Betacryptovirus* is proposed to be combined with the Partitivirus I cluster to the new genus *Betapartitivirus*. Also the cluster Alphacryptovirus I will be assigned to the genus *Alphapartitivirus*.

1.6.3 Amalgavirus

A new group of plant viruses have been identified in the last decade. Their genome comprises of one dsRNA fragment containing two overlapping ORFs with putative CP function and RNA-dependent RNA polymerase motifs (Martin, 2006; 2011; Liu, 2009;

Sabanadzovic, 2009; 2010), see also Table 3 for details. These viruses share several properties with plant infecting cryptic viruses:

- associated with symptomless infections in their respective hosts,
- transmission via grafting and mechanical inoculation attempts failed, and they show
- very high rates of seed transmission.

The OFR2 is likely expressed via +1 translational frameshift or a stoprestart mechanism resulting in a fusion protein, similar to viruses of the fungal family *Totiviridae*. However, phylogenetic analysis shows a distant relatedness to members of the family *Partitiviridae* (Martin, 2011).

1.6.4 Endornavirus

The genomes of endornaviruses consist of a linear dsRNA of 10–18 kbp in length with only one open reading frame (ORF) (Roossinck, 2010). These ORFs likely encode a single polypeptide that is thought to be processed by a proteinase, and at least one conserved motif of an RdRp can be found (Gibbs, 2000). Endornaviruses seem not to form true virions and are usually present at low numbers of copies (Horiuchi, 2004). Besides in plant host, these viruses have been found in fungi and protests (Roossinck, 2011). Also endornaviruses share several properties with plant infecting cryptic viruses (symptomless, only transmitted thought their gametes).

Table 3: Genomic features of genera with persistent viruses (* new genera in ICTV review)

Genus	Particle size (nm)	genome size (kbp) RdRp + CP	poly-a-stretch	RdRp size		CP size	
				AS	kDa	AS	kDa
*Alphapartitivirus**	30	2.0 + 1.8	+	616	72	488	54
*Betapartitivirus**	38	2.3 + 2.3	+	715	83	666	74
*Gammapartitivirus**	30	1.8 + 1.6	-	537	62	433	47
*Deltapartitivirus**	30	1.7 + 1.5	-	483	55	366	41
Cryspovirus	31	1.7 +1.4	-	524	62	319	37
*Amalgavirus**	?	3.5	-	798	91	377	41
Endornavirus	?	13	-	?	?	?	?

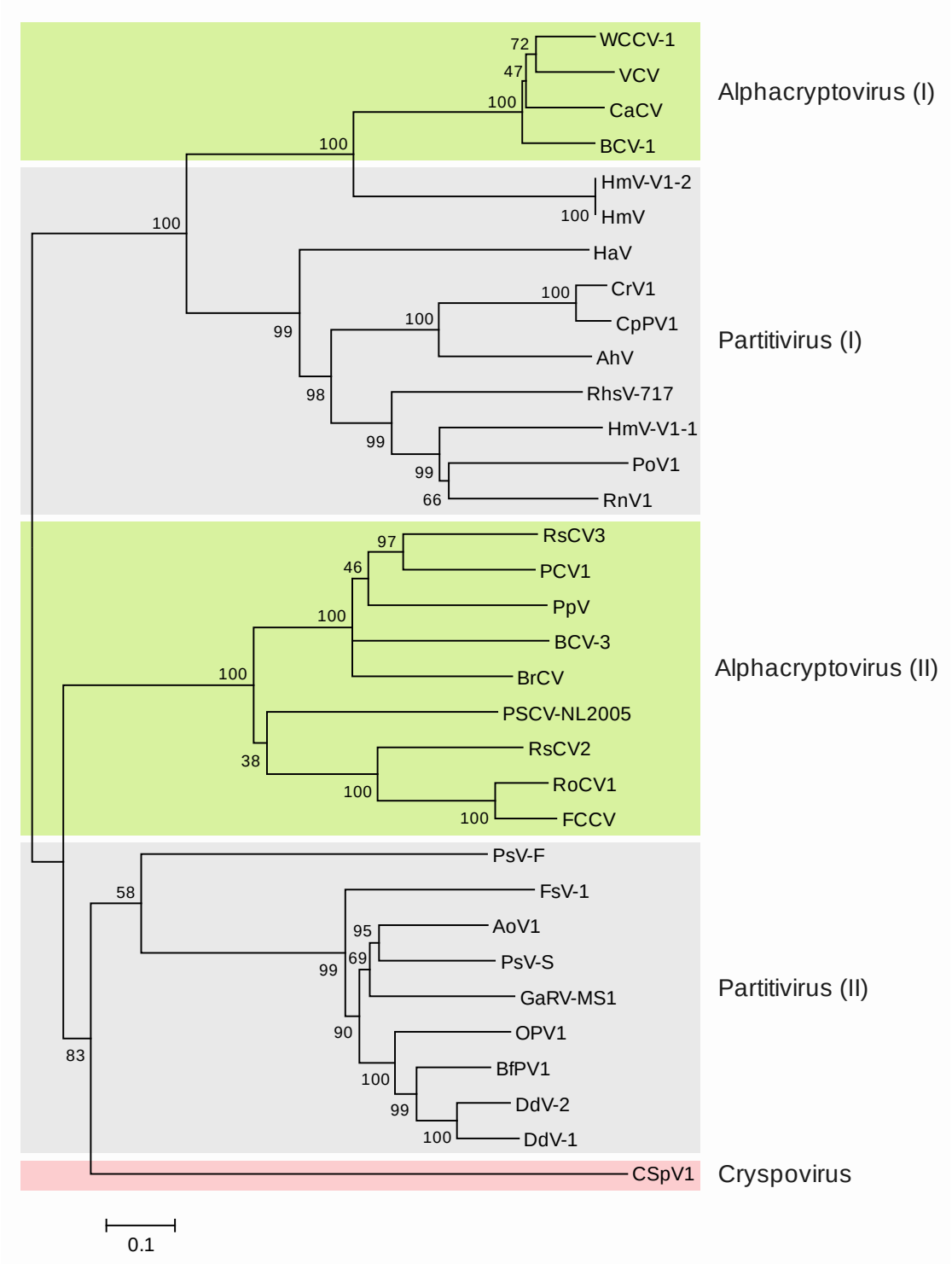


Figure 4: Neighbor-joining phylogenetic tree based on the complete amino acid sequences of RdRps of members and probable members of the family *Partitiviridae*. The amino acid sequences were aligned using the program CLUSTAL W. For virus names and abbreviations, see Table 4. The phylogenetic tree was generated using the MEGA4 phylogenetic package. Bootstrap percentages out of 1000 replicates are indicated at the nodes.

Table 4: Viruses of the family *Partitiviridae* with their actual and proposed future genus assignment

Virus name	Acronym	GenBank accession	Proposed new genus
Genus *Partitiviruses*			
Aspergillus ochraceous virus 1	AoV1	EU118277, EU118278	*Gammapartitivirus*
Atkinsonella hypxylon virus	AhV	L39125, L39126, L39127	*Betapartitivirus*
Ceratocystis resinifera virus 1	CrV1	AY603051, AY603052	*Betapartitivirus*
Discula destructiva virus 1	DdV1	AF316992, AF316993, AF316994, AF316995	*Gammapartitivirus*
Discula destructiva virus 2	DdV2	AY033436, AY033437	*Gammapartitivirus*
Fusarium poae virus 1	FpV1	AF047013, AF015924	*Betapartitivirus*
Fusarium solani virus 1	FsV1	D55668, D55668	*Gammapartitivirus*
Gremmeniella abietina RNA virus MS1	GaRV-MS1	AY089993, AY089994, AY089995	*Gammapartitivirus*
Helicobasidium mompa virus	HmV	AB025903	*Betapartitivirus*
Heterobasidion annosum virus	HaV	AF473549	*Betapartitivirus*
Ophiostoma partitivirus 1	OPV1	AM087202, AM087203	*Gammapartitivirus*
Penicillium stoloniferum virus F	PsV-F	AY738336, AY738337	*Gammapartitivirus*
Penicillium stoloniferum virus S	PsV-S	AY156521, AY156522	*Gammapartitivirus*
Pleurotus ostreatus virus 1	PoV1	AY533036, AY533038	*Betapartitivirus*
Rhizoctonia solani virus 717	RhsV-717	AF133290, AF133291	*Betapartitivirus*
Rosellinia necatrix virus 1 – W8	RnV1	AB113347, AB113348	*Betapartitivirus*
Tentative *Partitiviruses*			
Botryotinia fuckeliana partitivirus 1	BfPV1	AM491609, AM491610, AM491611	*Gammapartitivirus*
Ceratocystis polonica partitivirus 1	CpPV1	AY247204, AY247205	*Betapartitivirus*
Helicobasidium mompa partitivirus V1-1	HmV-V1	AB110979	*Betapartitivirus*
Helicobasidium mompa partitivirus V1-2	HmV-V1-2	AB110980	*Betapartitivirus*
Genus *Alphacryptovirus*			
Beet cryptic virus 1	BCV-1	EU489061, EU489062	*Alphapartitivirus*
Beet cryptic virus 3	BCV3	S63913	
Vicia cryptic virus	VCV	AY751737, AY75138	*Alphapartitivirus*
White clover cryptic virus 1	WCCV1	AY705784, AY705785	*Alphapartitivirus*
Tentative *Alphacryptovirus*			
Black raspberry cryptic virus	BrCV	EU082132	*Deltapartitivirus*
Carrot cryptic virus	CaCV	FJ550604, FJ550605	*Alphapartitivirus*
Fragaria chiloensis cryptic virus	FCCV	DQ093961, DQ355440, DQ355439	*Deltapartitivirus*
Pepper cryptic virus 1	PCV1	DQ361008	*Deltapartitivirus*
Pinus sylvestris cryptovirus	PSCV	AY973825	*Deltapartitivirus*
Pyrus pyrifolia cryptic virus	PpV	AB012616	*Deltapartitivirus*
Raphanus sativus cryptic virus 2	RsCV2	DQ218036, DQ218037, DQ218038	*Deltapartitivirus*
Raphanus sativus cryptic virus 3	RsCV3	FJ461349, FJ461350	*Deltapartitivirus*
Rose cryptic virus 1	RoCV1	EU413666, EU413667, EU413668	*Deltapartitivirus*
Genus *Cryspovirus*			
Cryptosporidium parvum virus 1	CSpV1	U95995, U95995	*Cryspovirus*

1.7 Methods to classify cryptic viruses on a molecular level

In this thesis a broad range of techniques was used to characterize cryptic plant viruses. Therefore, the three main procedures will be shortly presented here.

1.7.1 DsRNA-Screening as a universal tool for unspecific virus diagnosis

In the history of plant virology a wide spectrum of diagnostic tools has been developed. These tests of virus infection can be subdivided into four categories: First, attempts based on the symptomatology on host or indicator plants. Next, methods depending on physical properties of the virus particle, which can be determined by microscopy techniques. And last, other methods using properties of viral proteins for serological procedures. Most modern approaches involve properties of the viral nucleic acid for specific amplification and detection of viruses (Hull, 2013).

However, most of these techniques need already some knowledge for the detection of the virus infection. Viruses lack any similar properties, like ribosomal RNA in pro- or eukaryotes, which could be used for the development of a universal detection method. Therefore, different approaches have to be utilized to address this problem in the discovery of novel viruses (Roossnick, 2011) see table 5:

Table 5: Methods for discovery of viruses and their ability to detect new viruses (adapted from Roossnick, 2011)

Method	Specificity	Sensitivity	Labor	Cost	Comment
Indicator plants	moderate	moderate	slow	low	need of virus transmission problem of latent infection
Electron microscopy	low	moderate	slow	high	virus particles needed purification required
Serology	moderate	moderate	fast	moderate	detection of related virus with polyclonal antibodies
(RT)PCR	high	high	fast	high	use of degenerate primer for conserved virus motif
Hybridization	none	moderate	fast	high	need of comparative sample e.g. healthy plant
Deep sequencing	none	high	fast	high	use of total RNA or virus enriched nucleic acids

After looking at the available techniques it is obvious that the deep sequencing technique offers the greatest potential to detect unknown viruses. Despite the development of new technologies in this field, it is not efficient to start the sequencing of the whole transcriptome of a plant and search for RNA molecules obtained from viral infections. Therefore, different methods can be used for virus enrichment in a sample:

- subtractive hybridization (Adams, 2009)
- purification of particles by ultracentrifugation (Hugenholtz, 2008)
- isolation of siRNAs (Kreutze, 2009)
- isolation of dsRNA (Roossnick, 2010)

For the characterization of cryptic viruses and other persistent viruses the isolation of dsRNA is the method of choice: There is no virus-free host plant available, particles are not reported for all viruses and cryptic viruses use dsRNA as their genome. Due to the "cryptic lifestyle" of these viruses appropriate concentrations of siRNAs cannot be expected. Moreover using dsRNA for sequence determination will have additional advantages:

- The genome or intermediates of a broad range of plant viruses can be tackled.
- DsRNA has a high stability and
- Covers the entire genome sequence of a virus.
- DsRNA is less prone to errors than a single transcripts of the virus (due to the double stranded structure)

1.7.2 Application of deep sequencing for virus determination

Metagenomic studies assess the diversity of life forms by sequencing approaches. The invention of deep sequencing methods allows studying microbial populations, even of uncultivable ones, by the use of universal genes like ribosomal RNA or ITS regions. This enables the assessment of the biodiversity of higher organisms by amplicon sequencing in a cost and time effective way.

However, there is a lack of such universal targets in virus sequences. Therefore additional virus enrichment steps are needed to obtain viruses in environmental samples. Recent studies use filtration and centrifugation approaches to isolate viruses from different sources (Roossinck, 2012). Many viral related sequences were found in different water sources (Djigeng, 2009; Rosario, 2009; Tamaki, 2012), human body parts (Zhang, 2006; Nakamura, 2009; Reyes, 2010) and animal excrements (Blinkava, 2010; Li, 2010). However, the greatest restriction regarding virus metagenomics is the missing determination of the original host of the identified viral sequences.

Further studies put their focus on plant virology (summarized in Table 6). Most approaches use selected plant sources to analyze an individual plant virome. Such "ecogenomic" studies allow a more detailed view on biodiversity of viruses spread over

different cultivars. However, none of these approaches put their focus on persistent infections.

Table 6: Plant ecogenomic deep sequencing studies:

Culture	Enrichment	Study aim / output	Reference
Tomato (infected), *Liatris spicata*	subtractive hybridization	proof of principle; determination of new *Cucumovirus*	Adams, 2009
Sweetpotato (infected)	siRNA	proof of principle; obtain unexpected viruses	Kreuze, 2009
Cucumis melo, N. benthamiana, A. thaliana, S. lycopersium (infected)	siRNA	proof of principle; detection of 9 different viruses	Donaire, 2009
diverse cultivars form field	dsRNA	massive parallel sample sequencing; several thousand new plant viruses	Roossinck, 2009
Grapevine (naturaly infected)	dsRNA	full genome assembly of gapevine viruses, determination of new viruses	Coetzee, 2010
Grapevine	dsRNA	reveals a virome dominated by mycoviruses	Rwahnih, 2011
Nicotiana tabacum Xanthi (infected with *Cucumber Mosaic virus*)	CP amplicons	deep sequencing of a recombinant virus populations in transgenic and nontransgenic plants	Morroni, 2013
Sweet orange	siRNA	determination of a new *Enamovirus*	Vives, 2013

The techniques of sequencing methods are constantly evolving. The choice of a technology depends on different factors such as the number of samples, sample throughput and the available capital (Mardis, 2013). However, the following aspects are important for the user:

- depths of sequencing for sensitivity
- sufficient read lengths for a good assembling
- accuracy of reads for correct sequences
- cost efficacy for multiplexing samples
- time for sample preparation

From the perspective of virologists it is rather crucial to gain useful information from the data. The first objective is the identification of viruses in the pool of sequences, ideally from the determination of the full genome lengths. Moreover, other questions can be answered like the relative concentration or sequence variation of the viruses within the sample. The biggest challenge is to eliminate the contamination of other sources, mainly sequences from microorganisms and the host. There is a wide range of bioinformatics applications, which can be combined for the evaluation of deep

sequencing data. Therefore an own bioinformatics pipeline was established, summarized in Figure 5:

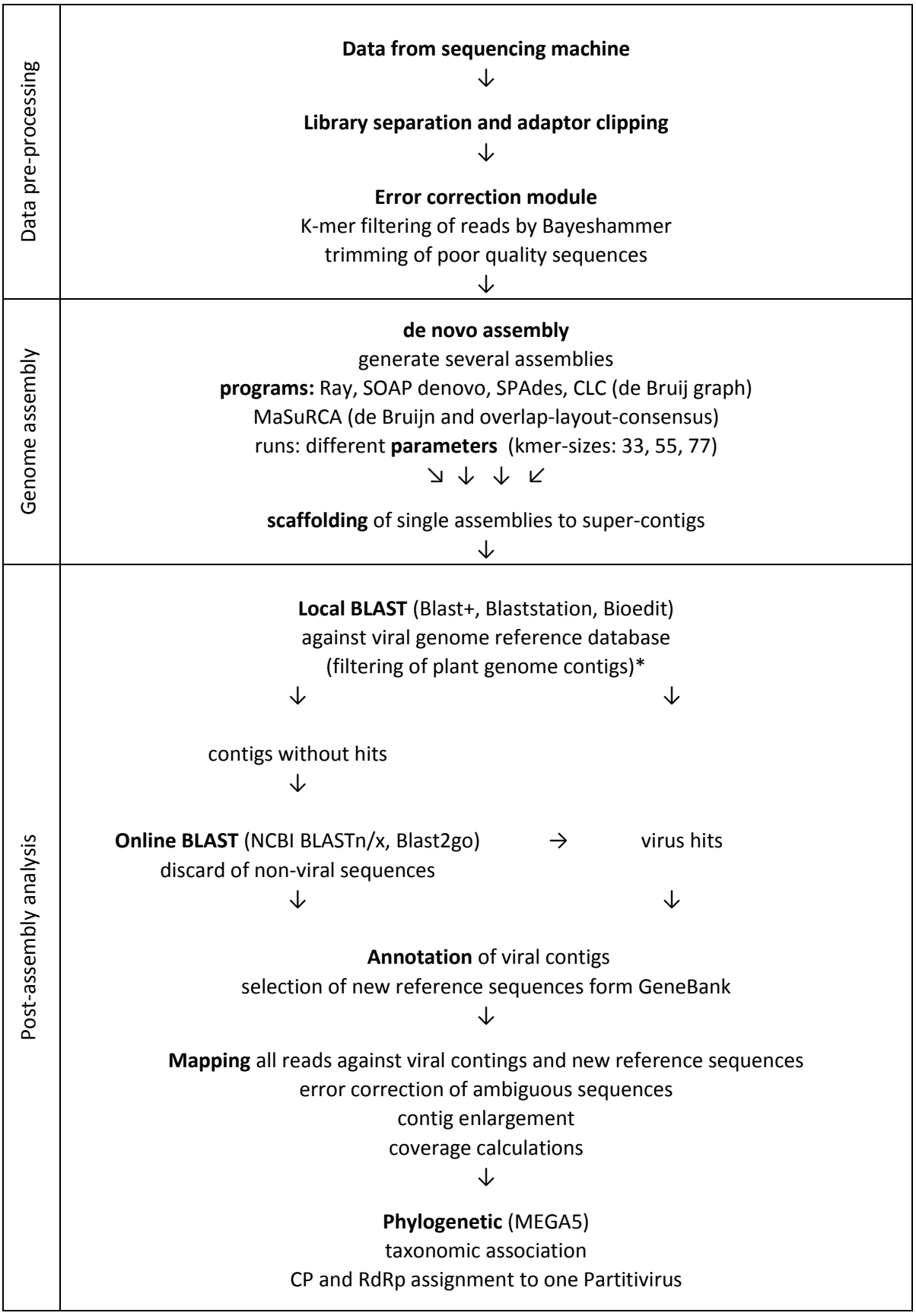


Figure 5: Bioinformatics pipeline (*optimal, not used in this study)

One major advantage of the pipeline is the use of different programs and parameters for the main *de novo* assembly step, which resulted in different pools of contigs. The further combination of the single contigs results in a new super assembly, including improvements to lengths and numbers of contigs as shown in Figure 6. Once a genome has been assembled, a number of further analyses are possible, like quality control, comparison to reference sequences, variant detection and annotation.

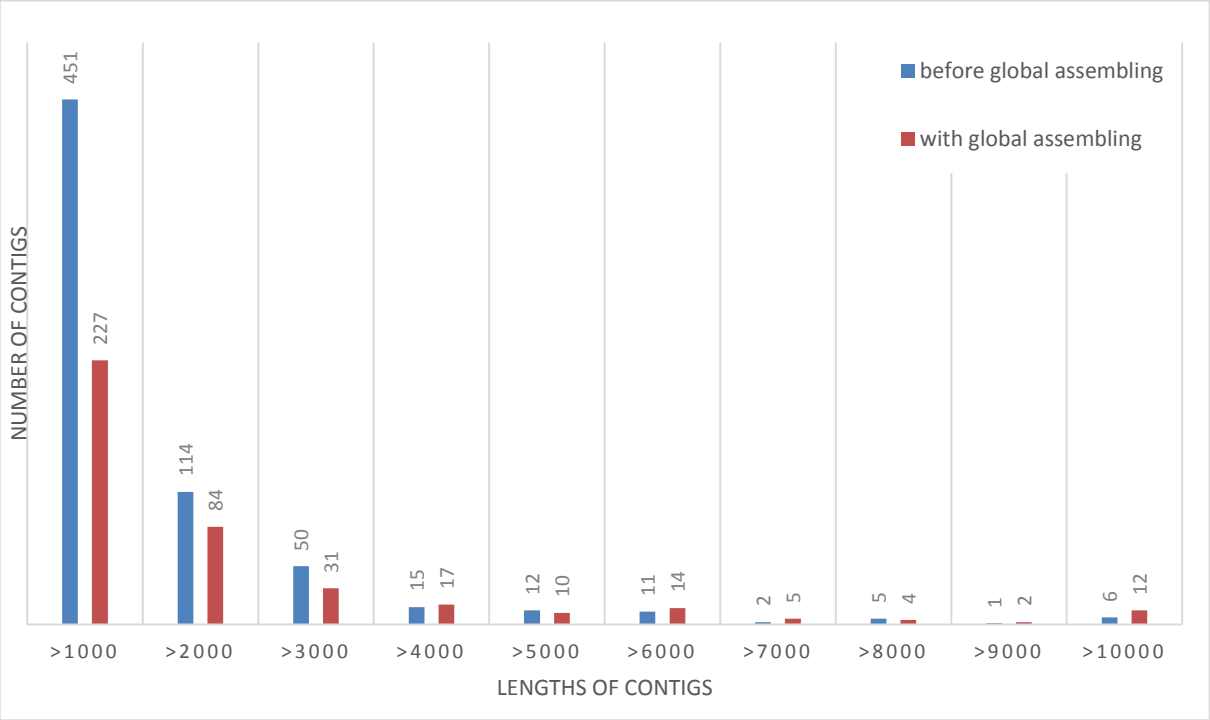


Figure 6: Contig lengths comparison of single assemblies with the combination assembly

1.7.3 Bimolecular fluorescence complementation to determinate protein interaction of cryptic viruses

Our knowledge about the biology of cryptic viruses like the influence on the host and molecular characteristics of their replication is very limited. Most recent studies have put their focus on sequence determinations and the evolutionary relationship of fungal Partitiviruses (Ghabrial, 2008; Lesker 2013a). Aside from structural analyses, the investigation of protein interactions is a next step towards virus characterization. Proteins are involved in almost all biological processes in living cells and their interactions play also a key role in viral life cycles.

Several methods were established to identify and characterize protein-protein interactions. Besides different *in vitro* methods (Phizicky, 1995), the popular *in vivo* yeast two-hybrid (YTH) system (Fillds, 1989) the bimolecular fluorescence complementation (BiFC) analysis became a powerful alternative for studying protein-protein interactions (Hu, 2002; Walter, 2004). Major advantages of this system are the high specificity and great stability of the reconstituted chromophore complex and its intrinsic fluorescence under natural conditions. Furthermore, it is possible to localize the protein interactions in the cell.

The two proteins of interest (POI) are fused to the non-fluorescent N-terminal or C-terminal fragment of a fluorescent protein. If the POI interact with one another, both parts of the reporter become reconstituted and fluorescence can be detected (Figure 7).

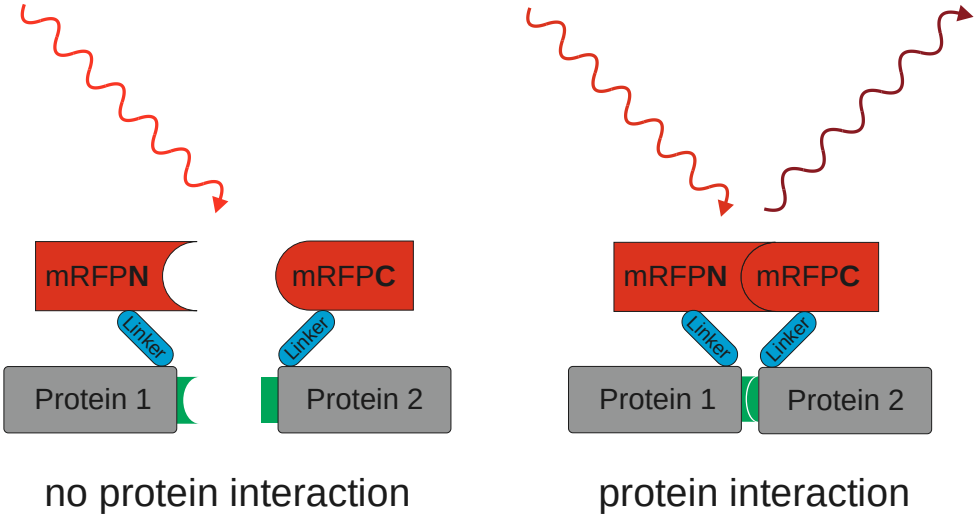


Figure 7: Reconstituted chromophore complex by a protein interaction

It has been suggested that each target protein is fused to both the N- and C-terminal fragments of the fluorescent reporter protein in turn, and that the fragments are fused at each of the N- and C-terminal ends of the target proteins. This allows for a total of four different permutations, when protein self-interactions is being tested (Figure 8):

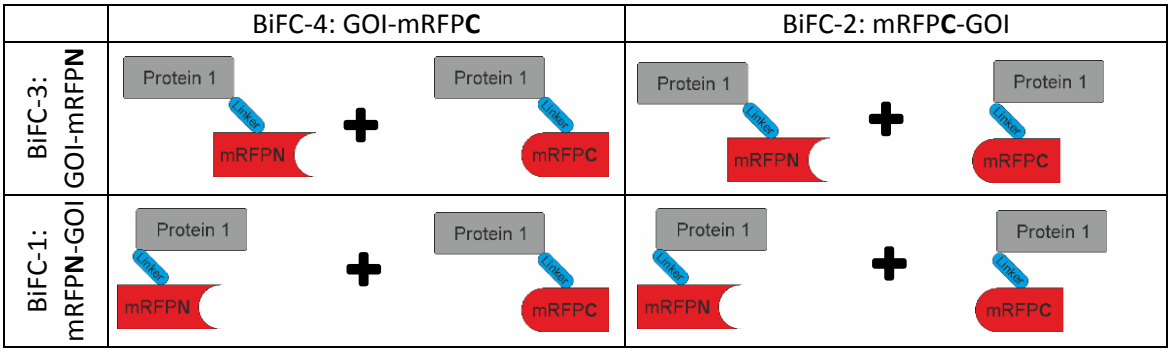


Figure 8: Possible permutations of a protein self-interaction

This difference can be used as internal control for the assay. Depending on the different conformation of fusion protein, each combination has to be tested, to exclude false negative results. Cryptic viruses consist of two proteins, this result in 16 permutations to explore all possible RdRp/CP combination and CP/RdRp self-interaction for each virus as shown in Figure 9:

mRFPN \ mRFPC	CP-mRFPC	mRFPC-CP	RdRp-mRFPC	mRFPC-RdRp
CP-mRFPN	CP vs CP		CP vs RdRp	
mRFPN-CP				
RdRp-mRFPN	RdRp vs CP		RdRp vs RdRp	
mRFPN-RdRp				

Figure 9: Interaction matrix to investigate CP-RdRp protein interactions

To test all possible combination will reduce false-negative results trigger by potential blocking of interactions-sites due to fusion of the reporter parts (Takashi, 1999). All observed interactions were localized in epidermal leaf cells of *Nicotiana benthamiana* after agroinoculation.

1.8 Objectives

Cryptic viruses are members of the family *Partitiviridae* which are widespread in mono- and dicotyledonous plant species. Although they do not cause economic losses, they can be responsible for misleading results in diagnostic approaches (Boccardo, 1987). Partitiviruses infecting plants have no known natural vectors nor can them be transmitted by mechanical means or grafting. However, they are transmissible at very high rates through seed and pollen. Therefore, classical virological methods, such as transmission experiments or reverse genetic systems are difficult to perform. So the information on the biology of cryptic viruses is very limited.

The first objective in the study of cryptic viruses was the verification of the status of the genus *Betacryptovirus* in the taxonomy. The last study of viruses of this genus was published about 28 years ago (Boccardo, 1985) and so far no sequence information is available. Other phylogenetic studies reported a distant relationship between members of *Partitiviridae* and recommended a reconsideration of the taxonomy (Ghabrial, 2008). However, this was not appropriate, due to the lack of sequences of the previously assigned genus. Therefore, dsRNAs of descriped host plants (White Clover, Red Clover, and Hop Trefoil) were isolated and were used for the sequence determination of Betacryptoviruses.

The second part of this work covers a protein interaction approach to shed light on the biological characteristics of cryptic viruses. The dsRNA nature and their limited transferability reduce the opportunities to analyze the virus group. Aside from performed sequence and structural analyses (Ochoa, 2008) the investigation of protein interactions is another step towards the characterization of this virus group. The aim was to verify expected and hypothesized protein interactions of CP and RdRp proteins. For the reproducibility and clarification of functional relationships among the cryptic viruses, three alphacryptoviruses and three betacryptoviruses from White Clover, Red Clover and Dill were submitted to a Bimolecular Fluorescence Complementation Analysis.

The last part of this thesis focuses on the biodiversity of persistent virus infections of plants. Therefore a metagenomic approach was performed by using the next generation sequencing technique to access unknown viral infections in plants. Most plant infecting viruses use single-stranded RNA as their genome and generate double-stranded RNA during replication. A specific isolation of dsRNA offers an easy way for RNA-virus

screening in plants without previous knowledge about the virus. However, additional and laborious work steps are necessary for a determination of the viral sequences. High throughput sequencing technologies can omit time-consuming manual sequencing analyses. In addition, they enable the detection of lowest target amounts even in mixed infections. Therefore, dsRNA purification in combination with deep sequencing analysis was used to analyze known cryptic viruses of plants described above and determine the full virome to evaluate the phylogenetic relationship of the family *Partitiviridae*.

Together these studies will help to understand the relevance of the diversity of these persistent infecting viruses, regarding their interaction with the host and the linkage to fungal viruses.

2 Molecular characterization of five betacryptoviruses infecting four clover species and dill

2.1 Abstract

The family *Partitiviridae* includes plant (*Alphacryptovirus* and *Betacryptovirus*), fungal (*Partitivirus*) and protozoan (*Cryspovirus*) viruses with bisegmented dsRNA genomes and isometric virions. Cryptic viruses commonly occur in different plant species without causing any symptoms. So far numerous sequences are determined and assigned to the genus *Alphacryptovirus*, but no sequence is available for the described members of the genus *Betacryptovirus*. In this study, we report the molecular properties of the betacryptoviruses *White clover cryptic virus 2*, *Red clover cryptic virus 2* and *Hop trefoil cryptic virus 2* and two new putative members found in crimson clover and dill by extraction, cloning and sequence analyses of double-stranded RNA. Members of *Betacryptovirus* show common sequence motifs with members of the *Partitivirus*. In phylogenetic analyses members of the genus *Betacryptovirus* forms a new sub-cluster within the clusters of the genus *Partitivirus.* The results support a distinct evolutionary lineage of dsRNA viruses of plants and fungi.

2.2 Introduction

Cryptic viruses in plants are widespread in mono- and dicotyledonous plant species and are currently classified in the genera *Alphacryptovirus* and *Betacryptovirus* of the family *Partitiviridae* (Brunt, 1996; Ghabrial, 2012). In addition, the family contains the genera *Partitivirus* with the fungi infecting viruses, and *Cryspovirus* with the protozoan infecting viruses (Boccardo, 1983; Ghabrial, 2012). In general the genome of cryptic viruses is composed of two monocistronic dsRNA segments of approximately 1.5 to 2.5 kbp in size. The larger segment encodes a putative RNA-dependent RNA polymerase (RdRp) and the smaller one the coat protein (CP). Both dsRNA molecules are individually encapsidated in non-enveloped isometric particles measuring 30 - 40 nm in diameter (Boccardo, 1987; Ghabrial, 2008; Ghabrial, 2012).

Plant infecting cryptic viruses have no known natural vectors and are also not transmitted by mechanical means or grafting. Nevertheless, they are transmitted at very high rates, nearly 100%, through the gametes if both parents are infected (Ghabrial 1998). Because they do not encode proteins which have homologies to movement proteins of other viruses, the transmission occur in a passive way by cell division, thereby also infecting seed and pollen (Ghabrial, 2008). Cryptic viruses seem to be well adapted to their hosts, they even persisting for years in tissue cultures and surviving thermotherapy (Szego, 2010). A very low concentration of cryptic viruses in their host plants does not lead to any visible symptoms and has apparently no drastic impact on quality and yield in crop plants. Although they do not cause economic losses in their host plants, they can be responsible for misleading results in diagnostic approaches based on the detection of RNA (Boccardo, 1987; Suzuki, 2001). Plant cryptic viruses of the family *Partitiviridae* are widely spread in various species, often in mixed infections with different cryptic viruses and other kinds of dsRNA viruses, like endornaviruses (Fukuhara, 2008) and viruses similar to southern tomato virus (Mel'nichuk , 2005; Szego, 2010).

Based on a restricted transmissibility and the nature of dsRNA of cryptic viruses interesting aspects regarding their evolution, genetics and interaction with their hosts arise. The reduced set of proteins, consisting only of coat protein (CP) and RNA-dependent RNA polymerase (RdRp), and the absence of any further proteins for virus movement might point to the origin of these viruses. The nature of dsRNA is rather common for viruses occurring in various fungi. Here, no movement proteins are necessary because of the absence of cell walls separating the cells in fungi (Ghabrial, 2008). Furthermore the relationship between e.g. cryptic viruses of plants and fungal viruses, as determined by phylogenetic analysis, is evident. Sequence analyses of viruses in the genera *Alphacryptovirus* and *Partitivirus* show a high degree of diversity of cryptic viruses (Ghabrial, 2008). It is remarkable that some plant infecting cryptic viruses reveal more similarity to the fungal viruses of the genus *Partitivirus* than to the plant viruses belonging to the genus *Alphacryptovirus.* Both genera can be split into two major clusters (Ghabrial, 2012). Therefore, a reconditioning of the current taxonomy should be considered. However, the classification of the genus *Betacryptovirus* is still unsolved, because of missing sequence information. To elucidate the relationship of the above mentioned genera we determined and analyzed additional sequences of alphacryptoviruses and putative betacryptoviruses.

Viruses of the genus *Betacryptovirus* were described in white clover, red clover, hop trefoil and carrots (Ghabrial, 2012). Comparing to other members of the *Partitiviridae* a betacryptovirus genome is consisting of two dsRNA segments of 2.1 to 2.4 kbp, which is slightly bigger than that of viruses grouped into the genus *Alphacryptovirus*. Furthermore the particles, measuring 38 nm, are larger than those of the genus *Alphacryptovirus* that are only 30 nm in diameter. Additionally, a serological discrimination of viruses of the different genera *Alphacryptovirus* and *Betacryptovirus* is possible (Boccardo, 1987).

The last time the genus *Betacryptovirus* has been described is about twenty years ago (Boccardo, 1985). In the present study described host plants were analysed by dsRNA extraction concerning conceivable infections by viruses of the genus *Betacryptovirus*. It was possible to isolate dsRNA with the mentioned size out of white clover (*Trifolium repens*), red clover (*Trifolium pratense*), hop trefoil (*Medicago lupulina*) and dill (*Anethum graveolens*), and getting the first sequence information demonstrating a linkage to fungal viruses of the genus *Partitivirus*. In addition a comparative immune-capture RT-PCR using a betacryptovirus specific antibody confirmed the results.

Moreover the increased use of deep sequencing screening in plant diagnostics discovers more and more latent and cryptic virus infections in plants. A well classified sequence pool of wide spread cryptic viruses could be helpful to avoid incorrect assignments of viral sequences (Suzuki, 2001; Liu, 2012).

2.3 Materials and methods

2.3.1 Plant material

Seedlings of white clover (*Trifolium repens* cv. Lirepa; Bruno Nebelung GmbH, Münster, Germany), red clover (*Trifolium pretense* cv. Nemaro; Bruno Nebelung GmbH, Münster, Germany), hop trefoil (*Medicago lupulina*, Samenkiste Karlsruhe, Germany), crimson clover (*Trifolium incarnatum*, Samenkiste Karlsruhe, Germany), persian clover (*Trifolium resupinatum* Bruno Nebelung GmbH, Münster, Germany), dill (*Anethum graveolens var hortorum*; Borena Köln, Germany) and six cultivars of carrot (*Daucus carota*; Lange rote stumpfe ohne Herz 2, Rossmann GmbH, Burgwedel, Germany; Nantaise 2; Pariser Markt 5, Quedlinburger Saatgut GmbH, Quedlinburg, Germany; Rothild, REWE GmbH, Köln, Germany; Rote Riesen 2; Sugarnax 54,

Gartenland GmbH, Aschersleben, Germany) were grown under standard greenhouse conditions. A total weight of 20 g of fresh leaves was used for each dsRNA extraction.

2.3.2 Extraction of dsRNA

Leaves were homogenized in liquid nitrogen and stored at -80°C until use. Extraction of dsRNAs was carried out as described by Morris and Dodds (Morris, 1979) with some modifications. To enrich dsRNAs and eliminate most of contaminating ssDNA, dsDNA and ssRNA, a phenol/chloroform extraction and chromatography through a CF-11 cellulose column was done in the presence of 16.5% ethanol followed the elution by digestion with RNase-free DNase I (Roche) and with RNase T1 (Roche). After further purification by another round of CF-11 column chromatography and ethanol precipitation the dsRNA pellet was dissolved in 50 µl TE (10/0.1). The purity and concentration of dsRNAs was estimated by agarose gel electrophoresis. Gel separated dsRNAs were excised from the gel and purified by extraction with NucleoSpin (NucleoSpin MACHEREY-NAGEL GmbH, Düren, Germany) and subsequently used for cloning.

2.3.3 Amplification, cloning and sequence determination

Complementary DNA (cDNA) was synthesized using purified dsRNA as a template. The cDNA clones were obtained by RT-PCR with tagged random primers (Grothues, Cantor et al. 1993) using RevertAid Reverse Transcriptase (Fermentas) and Phusion Flash PCR Master Mix (Finnzymes) according to the manufacturer's instructions. The amplified PCR-fragments were ligated into the CloneJET Vector (Fermentas) and transformed into *Escherichia coli* NM522 cells (Hanahan, 1983). Plasmids were isolated according to Birnboim and Doly (Birnboim , 1979) and sequenced by SEQLAB (Göttingen, Germany). Initial sequence information formed the basis for designing sequence specific oligonucleotides for further gene walking steps. Complete sequences of the ends were determined by a modified RACE procedure (Roche, Applied Science) based on oligo(dT) and oligo(dG) primed cDNA by using a terminal deoxynucleotidyl transferase (Thermo Scientific). Similar sequences of cloned cDNAs and their corresponding putative proteins were identified by BLAST searches in the GenBank database. Multiple alignments of nucleotide and amino acid (aa) sequences were done by ClustalW (Thompson, 1994). Phylogenetic analysis and construction of neighbor joining phylogenetic trees were done with MEGA version 5 (Tamura, 2011).

2.3.4 Immunocapture RT-PCR and immunoelectron microscopy (IEM)

Plant extracts were prepared form 1 g leaves homogenized in 500 µl extraction buffer (20 mM Tris-HCl, 138 mM NaCl, 1 mM PVP, 0.05% Tween-20, 3 mM KCl; pH 7.4) and centrifuged for 5 min at 12.100 x g. The resulting supernatant was used for sample coating.

Sterile 200 µl PCR-Tubes (Biozym) were coated with 50 µl of 1:200 diluted antibody (provided by Dr. Piero Caciagli from the Institute of Plant Virology, Turin, Italy) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6) overnight at 4°C. After three washes with PBS-T buffer (138 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl, 0.05% Tween-20; pH 7.4) 50 µl of plant extracts were added and incubated again overnight at 4°C and washed three times. For cDNA synthesis, all components were purchased from Thermo Scientific, 19 µl water, 6 µl 5xRT-Buffer, 1 µl Random Hexamer Primer, 3 µl 10 mM dNTP mix were added directly in the sample coated tubes. After an incubation for 5 min at 95°C 100 Units RevertAid Reverse Transcriptase and 10 Units RiboLock RNase Inhibitor were added to the tubes and incubate for 60 min at 42°C. 3 µl of cDNA was added to 10 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific), 5 µl water, 1 µl 10mM Primer BetaUniCPs (5'-CCGCTTAGAAATTAAGAATTCACATTCGTCTA-3') and 1 µl 10mM Primer BetaUniCPas (5'-ATCATTGAGCAAGAAGTAGCCATA-3'). The tubes were heated at 98°C for 10 s, followed by 30 reaction cycles (1 s at 98°C, 15 s at 56°C and 15 s at 56°C) and a final step of 15 s at 72°C. The PCR products obtained were analyzed on 1% agarose gels containing 0.5 mg/l ethidium bromide.

For electron microscopy, 50 g of leaf material from white clover was subjected to a virion purification method as described by Boccardo (1987). The preparation, which proved to contain only a few spherical virus particles after negative staining with 2 % aqueous uranyl acetate, was tested by immunosorbent electron microscopy (ISEM) and decorated as described by Milne (1984). For the coating of grids (ISEM) and decoration, the WCCV-2 antiserum was diluted 1:1000 and 1:50, respectively.

2.4 Results

2.4.1 DsRNA analysis

The aim of this study was to verify the presence of the formerly described betacryptoviruses and putative betacryptoviruses by including additional clover species and herbs. For sequence analyses of putative betacryptoviruses from white clover, red clover, hop trefoil, dill, persian clover and seven carrot cultivars a dsRNA extraction was performed. In about 80 % of the samples different dsRNA patterns were detected with 1 to 8 segments ranging in size from approx. 0.5 to 14 kbp. However, most of the bands were located in the range of approx. 1.5 kbp to 3.5 kbp. DsRNAs of putative betacrypticviruses were detectable in the size range from 2.2 to 2.4 kbp for white clover, red clover, hop trefoil, crimson clover and dill (Fig. 1). No dsRNA in the predicted range of 2.2 to 2.4 kbp for betacryptoviruses could be found in any of the seven varieties in the described host plant carrot.

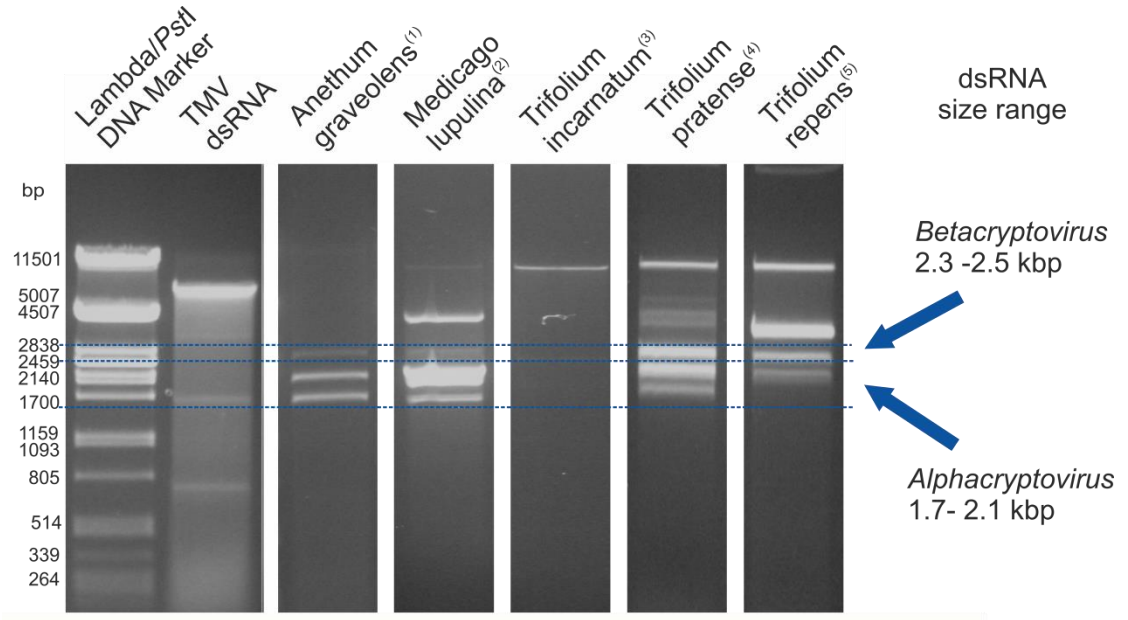


Figure 1: Electrophoretic analysis of dsRNA in 1% agarose gels with TAE buffer. The gels were stained with ethidium bromide. Lanes: Lambda - *Pst*I DNA-Marker; TMV (dsRNA-Marker); purified dsRNA from 1: dill (*Anethum graveolens*), 2: hop trefoil (*Medicago lupulina*), 3: crimson clover (*Trifolium incarnatum*), 4: red clover (*Trifolium pratense*), 5: white clover (*Trifolium repens*)

2.4.2 Nucleotide sequence analysis

DsRNA bands in the size range of the putative betacryptoviruses were gel purified and used for RT-PCR. Two dsRNAs deriving from white clover, red clover, hop trefoil, incarnate clover and dill were further analysed. The larger segment, representing RNA 1 was approx. 2.4 kbp in size, while the smaller one (RNA 2) was about 77-90 bp shorter (Table 1). Each segment includes a single open reading frame (ORF) with untranslated regions (UTR) at the 5'- and 3'- ends. RNA 1 encodes an RNA-dependent RNA polymerase (RdRp) and RNA 2 the respective coat protein (CP). The 5-UTR consists of 103-110 bp in case of RNA1 and 91-96 bp in case of RNA 2. Aligning of the 5'UTRs revealed a high degree of sequence similarities: all newly determined dsRNAs start with the sequence AGAUU followed by short stretches of U and C. In addition, a strictly conserved sequence motif of 18 nucleotides AGAAUUCACAUUCGUCUA was identified at position 39 to 57 (Fig. 2).

```
Virus RNA       ---------->     <------------    --->    <---
CanCV  CP    -GAUUUUUCUAAAGC-GCCCCGC-CUU-UAGUUAUUAAGAAUUUACAUUCGUCUAUAUUA
CrCV2  CP    AGAUUUAUUUAAAGCCGCCCCAAGCUC-UAAAUAUUAAGAAUUCACAUUCGUCUAAGUUA
DCV2   CP    AGAUUUUAUUAAAGC-GCCCCGAACUU-UAGUAAUUAAGAACUCACAUUCGUCUAAAUUA
HTCV2  CP    AGAUUUUUCUAAAGC-GGCCC---CGC-UAGAAAUUAAGAAUUCACAUUCGUCUACAUUA
PrMCV  CP    -GAUUUUUUUAAACCGGCCCCCCGCU--UAAAAAUUUAGAAUUUACAUUCGUCUACAAAA
RCCV2  CP    AGAUUUUUCUUAAGC-GCCCCGC-CUC-AAGAAAUUAAGAAUUCACAUUCGUCUAAAUUA
WCCV2  CP    AGAUUAUUCUUAAUC-GGCCC-C-CGA-AAGAAUUUAAGAAUUCACAUUCGUCUAUAUUA
CanCV  RdRp  -GAUUUUUUAUAAGCGCCCCCCGCC-UUAUUUAACUAAGAAUUAACAUUCGUCUAAGUUA
CrCV2  RdRp  AGAUUUAUUUAAAGCCGCCCCGUGC-UCUGAAUAUUAAGAAUUCACAUUCGUCUAACCUC
DCV2   RdRp  AGAUUUUAUUAAAGCGCCCACC----UUUAAUAUUUGAGAAUUCACAUUCGUCUAAAUUA
HTCV2  RdRp  AGAUUUUUUAUAAGCGGCCCCCGCC--UAUAAUAUUUAGAAUUCACAUUCGUCUAAUUUU
PrMCV  RdRp  -GAUUUUUUUAAACCGGCCCCCCGA-CUUAAAAAUUUAGAAUUCACAUUCGUCUACGAUA
RCCV2  RdRp  AGAUUUAUUUAAAGCGGCCCCCGCC---CUAAUAUUUAGAAUUCACAUUCGUCUACUAAU
WCCV2  RdRp  AGAUUUAUUUAAGACGGCCCCCCCCGUUCUAAUAUUUAGAAUUCACAUUCGUCUAUCCAU
              ****:   * *: *   *:*             :* ****:* ***********
```

Figure 2: Consensus sequences of the 5-UTR-regions of the genomic segments of putative betacryptoviruses. Arrows above indicate the ability to form potential stem-loop-structures (mfold). "*" indicates identical positions and ":" conserved positions with only one substitution.

Secondary structure analyses of the 5' UTRs revealed two conserved stem-loop structures at position 4-36 and 40-52 (see Fig. 2). These structures were found in all RNAs except for WCCV2-RNA1. Figure 3 shows the putative secondary structure of RNA1 of the putative betacryptoviruses from RCCV-2-RNA1 and WCCV2-RNA2. These stem-loop structures are likely to have an important role in dsRNA replication and virus assembly (Li, 2009).

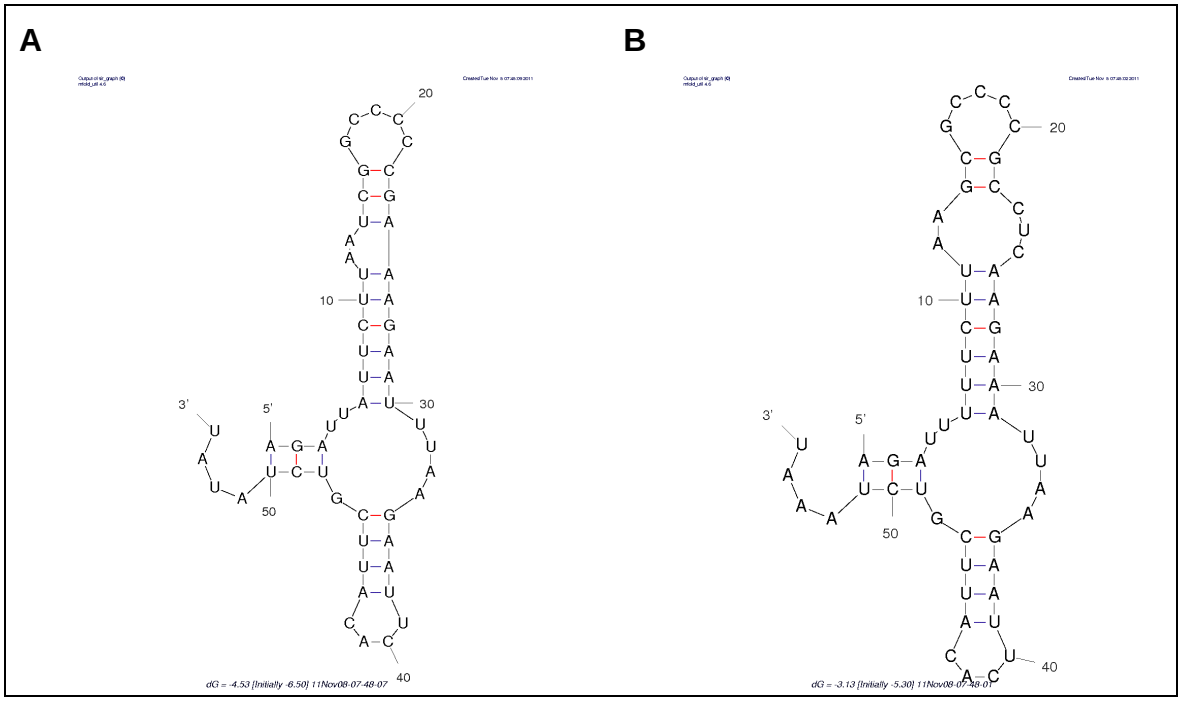


Figure 3: Potential conserved stem-loop-structures in 5-UTRs. Structure A: WCCV-2 CP coding strand (RNA2) structure B: RCCV-2 RdRp coding strand (RNA1) predicted by mfold

Open reading frames of 2235-2238 bp in RNA1 and 2019-2021 bp in RNA2 were identified. The ORFs were followed by 3‘-UTRs and a conserved C-rich region at the very 3’-terminal end of the RNAs consisting of a 3-5 nucleotide poly C-stretch. The 3-UTR of RNA1 is about 88 bp larger than the corresponding sequence of RNA2, which has a length of about 236 bp. The same applies to the length of interrupted poly A-stretches. In RNA2 these regions are about 78 bp and therefore more as twice as long as in RNA1 where they reached 37 bp. RNA1 codes for a protein with 745-746 aa with a calculated molecular mass of 87 kDa. The protein displays typical domains on aa-position 390-600, described for viral RdRps (Marchler-Bauer, 2009). RNA2 codes for a protein with 673-674 aa and a calculated molecular mass of 75-76 kDa. Further analyses did not reveal any conserved regions.

2.4.3 Immunocapture RT-PCR

The IC-RT-PCR was successfully performed leading to an estimated band size of 413 bp for the detection of WCCV-2 and RCCV-2 (Fig. 5). However, an omission of the

antibody coating step revealed also bands of the expected sizes. To prevent binding of virus particles to uncoated surfaces, we used an additional blocking step (incubation with 5% milk in PBS-T overnight a 4°C after coating with the antibody) to ensure the specific trapping of virus particles by the antibody. RT-PCR with primers specific to WCCV1 after immunocapture failed to detect this virus, indicating the specific detection of WCCV-2 only.

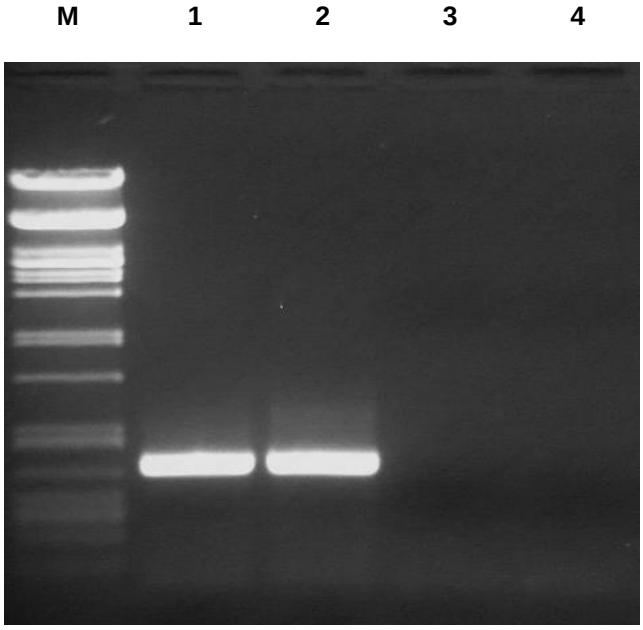


Figure 4: Electrophoretic analysis of Immunocapture RT-PCR fragments. Lanes: M: Lambda - *Pst*I DNA-Marker; 1: white clover (*Trifolium repens*); 2: red clover (*Trifolium pratense*); 3: negative control, *Nicotiana benthamiana*; 4: white clover (*Trifolium repens*) without antibody coating step

When adsorption preparations were used in electron microscopy, the preparation from white clover plants contained very few isometric particles after negative staining with uranyl acetate. In ISEM, however, numerous isometric particles measuring 38 nm in diameter were visualized on grids coated with the WCCV-2 antiserum, resulting in an approximately 100-fold increase in the number of virions on the grids (Fig. 5). Moreover, these particles were densely decorated after incubation with the WCCV-2 antiserum, further supporting the assignment of this virus to the genus *Betacryptovirus*.

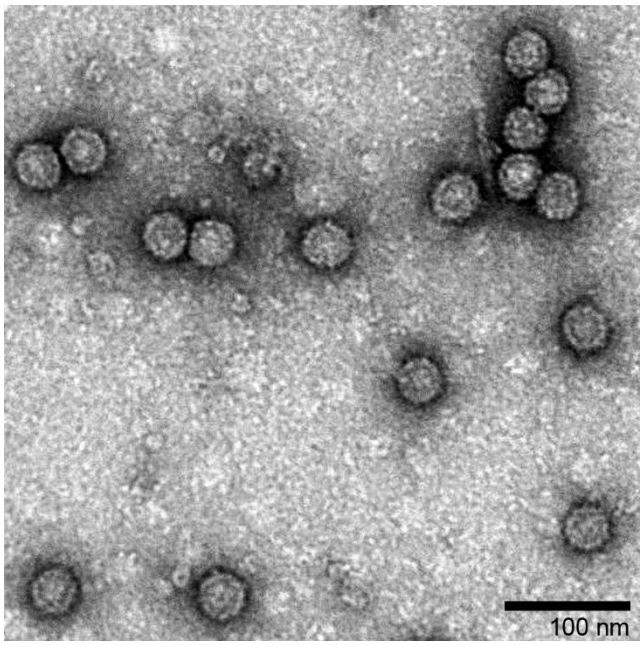


Fig. 5 Immunosorbent electron microscopy of particles trapped from a preparation from white clover (*Trifolium repens*) on a grid coated with WCCV-2 antiserum. Virions obtained after isopycnic centrifugation in Cs2SO4 gradients were negatively stained with 2 % uranyl acetate. The bar represents 100 nm

2.4.4 Phylogenetic analysis

A BLASTP search with RNA1 (RdRp) as well as RNA2 (CP) showed a strong sequence similarity to Primula malacoides virus (Li, 2009) and Cannabis cryptic virus (Ziegler, Matousek et al. 2011). Furthermore, additional homologies to viruses of the genus *Partitivirus* that infect the plant pathogenic fungus *Rhizoctonia solani* (Rhizoctonia solani virus 717 (RhsV-717) (Strauss,2000) and *Rosellinia necatrix* (Rosellinia necatrix partitivirus 1-W8 (RnV1) (Sasaki, 2005) were found.

Phylogenetic trees based on RdRp as well as on CP sequences showed a wide diversity of viruses in the family *Partitiviridae* whereas plant and fungal viruses of the genera *Alphacryptovirus* and *Partitivirus*, respectively, were each divided into two clusters (I + II) as already described (Ghabrial, 2012) (Fig. 4). Distinctive features are homologies of RdRp sequences which are closer between the clusters Alphacryptovirus I and Partitivirus I and Alphacryptovirus II and Partitivirus II than between the clusters within the individual genera.

This is also shown for other sequence parameters like larger RNAs and protein sizes and the presence of poly A-stretches (not described for members of clusters II) in 3-

UTRs of viruses grouped in cluster I comparing to those belonging to cluster II (Table 1).

Table 1: Overview of the molecular characteristics of selected members within the family of *Partitiviruses*, new sequences in bold letters

		RdRp coding RNA				CP coding RNA			
Genus (Clusters) / Virusnames	**Genbank (RdRp; CP)**	bp	ORF	AS	kDa	bp	ORF	AS	kDa
Betacyptovirus									
Cannabis Cryptic Virus (CaCV)	JN196536; JN196537	2420	109-2343	745	87.2	2290	92-2107	672	74.9
Crimson Clover Cryptic Virus 2 (CCCV-2)	JX971982; JX971983	2444	109-2347	746	87.2	2354	96-2118	674	75.4
Dill Cyptic Virus 2 (DCV-2)	JX971984; JX971985	2430	103-2338	745	87.1	2354	96-2115	673	75.3
Hop Trefoil Cyptic Virus 2 (HTCV-2)	JX971980; JX971981	2431	110-2348	746	87.2	2349	91-2110	673	75.4
Primula Malacoides Virus 1 (PmV1)	NC_013109; NC_013110	2390	110-2282	724	84.2	2344	94-2113	673	74.8
Red Clover Cryptic Virus 2 (RCCV-2)	JX971984; JX971985	2430	107-2342	745	86.7	2353	94-2113	673	75.7
White Clover Cryptic Virus 2 (WCCV-2)	JX971976; JX971977	2435	109-2347	746	87.1	2348	92-2111	673	75.6
Partitivirus I									
Atkinsonella hypoxylon partitivirus (AhV)	NC_003470; NC_003471	2180	40-2035	665	77.6	2135	72-2028	652	73.7
Ceratocystis polonica partitivirus (CpPV1)	NC_010705; NC_010706	2315	53-2042	663	76.9	2252	92-2075	661	73.2
Ceratocystis resinifera partitivirus (CrV1)	NC_010755; NC_010754	2207	53-2042	663	76.8	2305	93-2076	661	73.3
Fusarium poae virus 1 (FUPO-1)	NC_003884; NC_003883	2203	54-2073	673	78.3	2185	112-2023	637	70.5
Pleurotus ostreatus virus 1 (PoV1)	NC_006961; NC_006960	2296	79-2197	706	82.2	2223	115-2023	636	71.1
Rhizoctonia solani virus 717 (RhsV-717)	NC_003801; NC_003802	2363	86-2276	730	85.8	2206	79-2128	683	76.4
Rosellinia necatrix partitivirus 1-W8 (RnV1)	NC_007537; NC_007538	2299	76-2203	709	83.8	2279	80-2138	686	76.6
Alphacryptovirus I									
Beet cryptic virus 1 (BCV-1)	NC_011556; NC_011557	2008	94-1942	616	72.5	1783	123-1590	489	53.4
Carrot cryptic virus (CaCV)	FJ550604; FJ550605	1971	95-1943	616	72.7	1776	119-1589	490	54.4
Vicia cryptic virus (VCV)	NC_007241; NC_007242	2012	93-1941	616	72.9	1779	119-1580	487	53.9
White clover cryptic virus 1 (WCCV-1)	NC_006275; NC_006276	1955	75-1923	616	72.9	1708	105-1566	487	54.2
Partitivirus II									
Aspergillus ochraceous virus (AoV1)	EU118277; EU118278	1754	67-1684	539	62.3	1555	101-1400	433	47.0
Botryotinia fuckeliana partitivirus 1 (BfPV1)	NC_010349; NC_010350	1793	62-1682	540	62.6	1566	102-1410	436	46.8
Discula destructiva virus 1 (DdV-1)	NC_002797; NC_002800	1787	65-1682	539	62.4	1585	99-1407	436	47.6
Discula destructiva virus 2 (DdV-2)	NC_003710; NC_003711	1781	62-1679	539	62.4	1611	103-1429	442	47.9
Fusarium solani virus 1 (FsV-1)	NC_003885; NC_003886	1645	16-1573	519	59.7	1445	40-1279	413	44.2
Gremmeniella abietina RNA virus (GaRV-MS1)	NC_004018; NC_004019	1782	63-1680	539	62.1	1586	100-1429	443	47.1
Ophiostoma partitivirus 1 (OPV1)	AM087202; AM087203	1744	70-1687	539	62.7	1567	109-1399	430	46.3
Penicillium stoloniferum virus S (PsV-S)	NC_005976; NC_005977	1754	66-1683	539	62.3	1582	113-1415	434	46.8
Alphacryptovirus II									
Fragaria chiloensis cryptic virus (FCCV)	NC_009519; NC_009521	1734	183-1620	479	55.8	1479	191-1235	348	38.8
Pepper cryptic virus 1 Jal-01 (PCV1)	JN117276; JN117277	1563	93-1530	479	54.5	1512	96-1332	412	47.6
Raphanus sativus cryptic virus 2 (RsCV2)	NC_010343; NC_010344	1717	179-1610	477	55.3	1521	209-1247	346	38.2
Raphanus sativus cryptic virus 3 (RsCV3)	NC_011705; NC_011706	1609	101-1544	481	54.9	1581	94-1216	374	42.7
Rose cryptic virus 1 (RoCV1)	NC_010346; NC_010347	1749	183-1674	497	55.9	1485	190-1234	348	38.8

Members of the genus *Betacryptovirus* form a sub-cluster within cluster *Partitivirus* I. A pair-wise sequence identity among representative members of the different clusters shows high similarities up to 50% identity in the RdRp Proteins (see Tab. 2). They also show similar sequence parameters, e.g. similar RNA-sizes, presence of interrupted poly A-stretches, and similar deduced sizes of the capsid proteins. Also, the estimated particle sizes of 34-35 nm (Li, 2009; Ziegler, 2011) clearly differ from that estimated for alphacryptoviruses (30 nm), which are lying in a size range of the cluster Partitivirus I.

Table 2: Summary of pair-wise amino acid sequence identities in percent of selected members within the family *Partitiviridae*

RdRp / CP	CaCV	CCCV-2	DCV-2	HTCV-2	PmV1	RCCV-2	WCCV-2	AhV	CpPV1	CrV1	FUPO-1	PoV1	RhsV-717	RnV1	VCV	WCCV-1	DdV-1	PsV-S	PCV1	RsCV2
CaCV	-	73	76	73	74	74	73	32	32	33	46	46	43	50	19	19	17	17	13	14
CCCV-2	57	-	76	76	75	77	75	33	33	33	46	46	44	50	21	21	16	17	16	15
DCV-2	69	59	-	79	77	78	76	35	35	35	48	49	45	51	21	21	17	17	15	15
HTCV-2	61	61	64	-	76	84	82	34	35	35	47	48	45	50	20	21	16	17	15	15
PmV1	60	58	61	61	-	77	76	34	33	34	46	46	44	48	20	20	16	17	14	13
RCCV-2	59	59	60	73	56	-	90	34	35	35	46	48	44	50	20	21	17	18	15	14
WCCV-2	59	57	58	68	56	77	-	34	34	35	47	47	44	49	20	21	17	18	15	15
AhV	16	15	17	16	15	15	15	-	56	55	37	37	38	35	20	22	16	15	14	16
CpPV1	17	16	16	17	17	16	16	41	-	96	34	35	36	35	20	20	16	17	16	15
CrV1	16	15	15	16	16	15	15	40	88	-	34	36	37	35	20	20	17	18	16	16
FUPO-1	20	18	20	20	21	19	20	18	21	20	-	60	47	50	18	19	16	16	12	13
PoV1	19	19	20	19	20	20	19	18	22	21	58	-	45	54	20	20	16	16	13	16
RhsV-717	34	32	34	34	33	33	32	16	16	15	20	20	-	45	23	22	18	18	15	15
RnV1	31	30	29	31	31	30	29	14	14	14	18	18	33	-	20	20	16	16	14	14
VCV	8	8	8	8	9	8	7	6	10	9	7	6	6	7	-	84	14	15	14	13
WCCV-1	11	10	10	8	10	10	9	8	9	9	9	8	8	7	56	-	15	16	13	14
DdV-1	8	8	6	7	8	8	9	7	7	7	6	6	6	8	9	9	-	70	17	17
PsV-S	8	8	8	7	9	8	8	9	7	6	8	7	6	8	9	9	54	-	17	18
PCV1	9	11	9	10	10	10	10	5	8	7	6	7	9	7	11	8	9	8	-	34
RsCV2	8	8	9	8	10	9	8	6	9	10	10	10	10	8	8	8	8	10	4	-

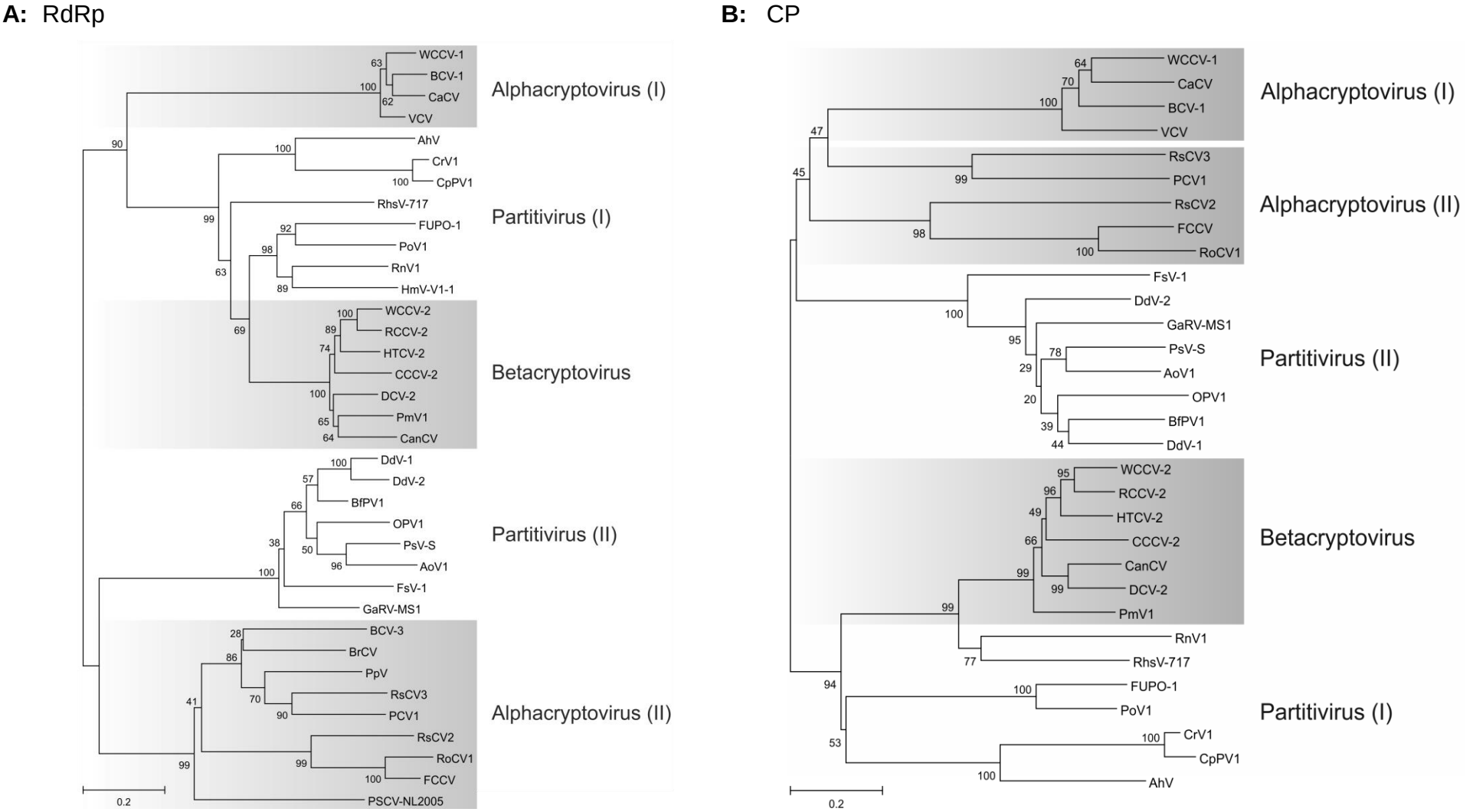


Figure 4: Evolutionary relationship of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bar represent in the units of base substitutions per site. All ambiguous positions were removed for each sequence pair. Abbreviations see table 1 except of BCV-3: Beet Crytic Virus 3 [AAB27624], BrCV: Black raspberry cryptic virus [EU082132], Pyrus pyrifolia cryptic virus [AB012616], PSCV-NL2005: Pinus sylvestris partitivirus [AY973825], HmV-V1 Helicobasidium mompa partitivirus [AB110979]

2.5 Discussion

In this study the complete sequences of 5 viruses were determined and classified into the genus *Betacryptovirus*. Three dsRNAs pairs from well-known hosts such as white clover (Boccardo, 1985), red clover (Boccardo, 1983) and hop trefoil (Luisoni, 1987) with sizes of about 2.4 kbp described for a typical betacryptovirus (Boccardo, 1985) were isolated, cloned and their sequences were determined leading to white clover cryptic 2, red clover cryptic 2 and Hop trefoil virus 2. In addition two new unexpected putative viruses of the genus *Betacryptovirus* were identified in dill (*Anethum graveolens)* tentatively named dill cryptic virus 2 (DCV2) and crimson clover (*Trifolium incarnatum)* tentatively named crimson clover cryptic virus 2 (CrCV2). Furthermore two potential members of the genus *Betacryptovirus* were identified from Primula and Canabis by searching the GenBank using comparative sequence analyses. For these viruses, particle sizes with a diameter of 35 nm were described, equivalent to the size of well-known betacryptoviruses (Li, 2009; Ziegler, 2011; Ghabrial, 2012). The separation of different cryptic viruses out of mixedly-infected plants can be difficult (Boccardo, 1985, 1987) and a classification by particle size should be used only if plants containing a single virus infection. DsRNAs from white clover at 2 kbp could be assigned to WCCV-1. Moreover, additional dsRNAs in dill were classified as belonging to the *Alphacryptovirus* cluster I (~2 kbp) and *Alphacryptovirus* cluster II (~1.6 kbp; results not shown). A clear demarcation of viruses belonging to the genus *Betacryptovirus* to those of the genera *Alphacryptovirus* and *Partitivirus* can be made by sequence similarities and differences. Already described putative betacryptoviruses have essentially larger dsRNAs and protein sizes (Table 1). Approximate similarities to viruses of the genus *Partitivirus* can be found. Sequences of the viruses RnV1 and RhsV-717 that infect plant-pathogenic fungi show high similarities to the sequences of the genus *Betacryptovirus*.

Phylogenetic analyses have shown the division of viruses in the genera *Alphacryptovirus* and *Partitivirus* into two clusters (Ghabrial, 2008, 2012). Viruses of the genus *Betacryptovirus* generate an own sub-cluster, which relates to the *Partitivirus* cluster I. The current taxonomic classification does not reflect those findings in an adequate way. So the type member of the genera *Betacryptovirus* (WCCV2) and *Partitivirus* (AhV) are closer to each other, than viruses of different clusters of the

genus *Alphacryptovirus* and *Partitivirus*. Further characteristics such as dsRNA size, presence of interrupted poly A-stretches and particle size support a reorganization of the taxonomic units. Therefore the clusters *Alphacryptovirus* II and *Partitivirus* II should be assigned to two new genera within the family *Partitiviridae*. The sequence information collected now for betacryptoviruses should eliminate any uncertainties in classification of newly identified cryptic viruses (Li, 2009; Ziegler, 2011), especially those, where data are generated by further use of deep sequencing methods.

The strong similarity of plant cryptic viruses to mycoviruses could lead to the possibility that the described potentially betacryptoviruses were associated with endosymbiotic fungi which have been found in several plants. This opportunity was already discussed in previous publications (Boccardo, 1985). A fungal contamination was not detected in those plants in which dsRNA and particles have been found (Boccardo, 1985). Even in the betacryptovirus-harboring host plants primula and hemp, no evidence for fungi was found (Li, 2009; Ziegler, 2011). Beside different kinds of clover mentioned above betacryptoviruses were also found in unrelated plants like dill, Primula and hemp. This argument is contradictory to a contamination by a common specific endosymbiotic fungus (Boccardo, 1987). Furthermore viruses of the genus *Alphacryptovirus*, obtained from protoplast cultivation show high sequence similarities to fungal partitiviruses (Abou-Elnasr, 1985).

The close genetic relationship of fungal and plant viruses within the family *Partitiviridae* might be an evidence of horizontal virus transmission (Ghabrial, 1998; Roossinck, 2010), particularly because closely genetically related partitiviruses were found in pathogenic fungi. Additionally, the high sequence similarity of individual viruses that were found in different plant families could be explained by a vector mediated transmission. Phytopathogenic fungi can act as vectors for a couple of different plant viruses (Rochon, 2004; Lubicz, 2007). An uptake and/or delivery of viruses by fungi may also apply for viruses of the family *Partitiviridae* provided that the virus is somehow succeeding to enter the gametes to establish a permanent infection. However, experimental approaches supporting such a transmission of cryptic viruses (Mel'nichuk, 2005) were unable to provide sufficient evidence for a horizontal transmission (Szego, 2010). Nevertheless, the high sequence identity of cryptic viruses within different plant families supports the idea of vector transmission (Ghabrial, 1998; Roossinck, 2010). A drawback to verify this theory is probably the low virus titer within the plant, which could be influenced by the host plant as shown for endornaviruses

(Urayama, 2010). An additional aspect is the horizontal gene transfer from parts of cryptic viruses to eukaryotic nuclear genomes (Liu, 2010; Chiba, 2011). This gene transfer was found for the partitivirus (cluster I) member RnV1, which is phylogenetic closely related to the described betacryptovirus cluster, to the genome of *Acyrthosiphon pisum*. The integration of sequence parts most likely occurred when the viruses were first introduced into noninfected species, probably as a result of an antiviral immunity response (Liu, 2010, 2012). Concerning that no integration of betacryptovius-like sequences could be found in genomes of plants or fungus, suggests an ancient origin for these viruses rather than an out coming from their hosts. However, based on the close genetic relationship of the genera *Betacryptovirus* and *Partitivirus* further investigations concerning plant-fungus interaction might provide evidence for the potential of horizontal interkingdom transmission of cryptic viruses.

3 In planta protein interactions of three Alphacryptoviruses and three Betacryptoviruses from White Clover, Red Clover and Dill by Bimolecular Fluorescence Complementation Analysis

3.1 Abstract

Plant-infecting viruses of the genera *Alpha-* and *Betacryptovirus* within the family *Partitiviridae* cause no visible effects on their hosts and are only transmitted by cell division and through gametes. The bipartite dsRNA genome is encoding an RNA-dependent RNA polymerase (RdRp) and a coat protein (CP). Aside from sequence and structural analyses, the investigation of protein interactions is another step towards virus characterization. Therefore, ORFs of two type members *White Clover Cryptic Virus 1* and *2* (WCCV-1 and WCCV-2), as well as the related viruses from Red Clover and Dill were introduced into a bimolecular fluorescence complementation assay. We showed CP-CP dimerization for all tested viruses with localization for alphacryptoviruses at the nuclear membrane and for betacryptoviruses close to cell walls within the cytoplasm. For CPs of WCCV-1 and WCCV-2 deletion mutants were created to determine internal interaction sites. Moreover, RdRp self-interaction was found for all viruses, whereas CP-RdRp interactions were only detectable for the alphacryptoviruses. An intra-genus test of CPs was successful in various virus combinations, whereas an inter-genus interaction of WCCV-1CP and WCCV-2CP was absent. This is the first report of in vivo protein interactions of members in the family *Partitiviridae,* indicating distinct features of the alpha- and betacryptoviruses.

3.2 Introduction

Cryptic viruses, widespread in mono- and dicotyledonous plant species, are currently classified in the genera *Alpha- and Betacryptovirus* of the family *Partitiviridae* (Brunt, 1996; Ghabrial, 2012). Additionally, the family contains the genera *Partitivirus* and *Cryspovirus*, which include viruses infecting fungi and protozoa, respectively

(Boccardo, 1983; Ghabrial, 2012). The genome of cryptic viruses is composed of two monocistronic dsRNA segments of approximately 1.5 to 2.5 kbp in size. While the larger segment encodes a putative RNA-dependent RNA polymerase (RdRp), the smaller one encodes the coat protein (CP). Both dsRNA molecules are individually encapsidated in non-enveloped isometric particles measuring 30-40 nm in diameter (Boccardo, 1987; Ghabrial, 2008; Ghabrial, 2012). There are no known natural vectors of plant-infecting cryptic viruses, and they are not transmitted by mechanical means or grafting. Nevertheless, a very high rate of transmission by the gametes is found, nearly 100 %, if both parents are infected (Ghabrial, 1998). Cryptic viruses do not encode proteins with homology to so far known movement proteins of other viruses. Hence their transmission occurs in a passive way by cell division, thereby also infecting seed and pollen (Ghabrial, 2008). There seems to be a good adaption of cryptic viruses to their hosts, reaching only a low virus titer, persisting for years in tissue culture and withstanding thermotherapy (Boccardo, 1987). No visible symptoms are caused by cryptic viruses, and apparently they do not lead to drastic impact on quality and yield in crop plants. Although economic losses in their host plants are not obvious, they can be responsible for misleading results in diagnostic approaches based on RNA detection (Boccardo, 1987; Suzuki, 2001). Plant viruses of the family *Partitiviridae* frequently occur in various species, often in mixed infections with different cryptic viruses and other kinds of dsRNA viruses, such as *Endornaviruses* (Fukuhara, 2008) and viruses similar to Southern tomato virus (Sabanadzovic, 2009; Martin, 2011).

First studies dealing with cryptic viruses were done in the early 1980´s, followed by the first description of their genome structure and particle sizes (Boccardo, 1987). Various attempts of virus transmission were made but only an exclusive transmission by seeds and pollen was found. The relationship to mycoviruses was proven by several serological investigations; based on these findings together with particle and genome sizes the classification into the genera *Alphacryptovirus* and *Betacryptovirus* was established. RdRp polymerase activity linked with virus particles was confirmed by enzyme assays (Marzachi, 1988). The first viral sequence became available for *Beet cryptic virus 3* (Xie, 1993). The first complete sequence of an alphacryptovirus, namely *White clover cryptic virus 1* (WCCV-1) was published by Boccardo in 2005 (Boccardo, 2005), the first betacryptovirus *White clover cryptic virus 2* (WCCV-2) was determined in 2013 (Lesker, 2013a). Phylogenetic analyses revealed further subdivision of the genus *Alphacryptovirus* in two clusters and a relationship between herbal and fungal

viruses in the family *Partitiviridae* was shown (Ghabrial, 2008; Lesker, 2013). Several studies suggest a viral influence on its host. For example, dsRNA patterns were linked to yellow edge symptoms in radish (Chen, 2006). In addition, an artificial expression of the WCCV-1CP gene in Lotus japonicus influenced the growth of the roots (Nakatsukasa-Akune, 2005). However other studies in crop plants were not able to demonstrate any symptoms despite a virus infection or significant impact on yield (Ghabrial, 2008). In some cases an increase of dsRNA concentration has been observed when an additional plant virus was present together with a cryptic virus (Boccardo, 1987).

A cryptic virus with a dsRNA genome, but also any other RNA containing virus using dsRNA as a replication intermediate, faces a problem during its replication cycle. Plants natural defense mechanisms generally recognize dsRNA, which is subsequently degraded. RNA viruses have evolved special proteins - suppressors of silencing - to protect themselves in various ways from RNA degradation (Waterhouse, 2001). Cryptic viruses do not have such kinds of proteins, so they have to hide their dsRNA from the plants natural defense. It is assumed that the dsRNA only occurs in the virus particle itself and here serves as a template for the also encapsidated RdRp (Boccardo, 1987; Ghabrial, 2008). The transcribed single-stranded RNA passes from the particle through pores into the cytoplasm, where CP and RdRp are translated (Nibert, 2013). During particle assembly RNA and RdRp are packaged by protein-protein and protein-RNA interaction together with the CP. Only inside the assembled particle the RdRp switches to an active mode and starts to synthesize new dsRNA (Nibert, 2013).

Recent X-ray diffraction studies focused on the structural analyses of virus particles. A 3D model was established for *Penicillium stoloniferum virus F* (PSV-F) a member of the genus *Partitivirus*, which is closely related to plant infecting alpha- and betacryptoviruses. The particle composition follows a T=1 symmetry consisting of 120 subunits (Ochoa, 2008). Furthermore, pores were found suitable for mRNA transfer; however RdRp was not localized in particles (Pan, 2009). A biological characterization of cryptic viruses is difficult because of their features, like a limited transmission. This also applies to the establishment of reverse genetic systems due to the dsRNA nature of these viruses.

After genetic studies concerning plant cryptic viruses (Ghabrial, 1998; Lesker, 2013) identification and investigation of protein–protein interactions present a further step in

understanding the virus biology of the alpha- and betacryptoviruses. Several methods were established to identify and characterize protein–protein interactions. Besides different *in vitro* methods (Phizicky, 1995), the yeast two-hybrid (YTH) system (Fields, 1989) is the most popular *in vivo* method to detect protein interactions. However, this system relies on the yeast nucleus under artificial conditions. Protein interactions requiring biologically relevant modifications or a specific subcellular localization are not detectable (Stolpe, 2005). Therefore, bimolecular fluorescence complementation (BiFC) analysis was developed and became a powerful alternative for studying protein-protein interactions (Hu, 2002; Walter, 2004). The two proteins of interest (POI) are fused to the non-fluorescent N-terminal or C-terminal fragment of a fluorescent protein. If the POI interacts with each other, both parts of the reporter become reconstituted and fluorescence can be detected. Significant advantages of this system are the high specificity and great stability of the reconstituted chromophore complex and its intrinsic fluorescence under natural conditions. Furthermore, it is possible to localize the protein interactions in the cell.

In this study, an optimized BiFC-system (Zilian, 2011) was used to investigate for the first-time protein interactions of viruses belonging to the family *Partitiviridae* in planta. The aim was to verify expected and hypothesized protein interactions. Firstly, we focused on the CP dimerization, which is the starting point of virus assembly. Sixty of these dimers are building the particle structure of *Partitiviridae* with a T=1 symmetry, whereas no additional viral components are needed for this domain swapping. Furthermore, we hypothesized an interaction of CP and RdRp. This interaction is proposed for the last steps of the virus assembly to introduce the RdRp in the particle and to activate the transcription (Ghabrial, 2008). Additionally, self-interaction of the RdRp was tested. For clarification of functional relationships among the cryptic viruses and to establish negative controls for the BiFC-system the CP and RdRp of one virus were tested versus proteins of two other virus members of the same genus (interspecies interactions). Moreover, an intergenus interaction with the CPs of WCCV-1 and WCCV-2 was performed. Additionally, we used deletion mutants to narrow down the part involved in the CP-CP interaction of the two type members of alphacryptovirus, WCCV-1 and betacryptovirus, WCCV-2, respectively.

3.3 Results and Discussion

Due to the formerly described infection cycle of the cryptic viruses in plants, different protein-protein and protein-RNA interaction could be expected. The primary domain shaping of CP proteins to dimers forms the basis of the final capsid structure. Multiple interaction sites were found by structural analyses (Pan, 2009; Tang, 2010), so a CP self-interaction could be expected in different permutations, as well as in distinct deletions mutants. Moreover, due to the fundamental similarities, interactions between CPs from viruses found in related host plants (intra genus) are most likely. The only other encoded protein, the RdRp, has to be packaged into the particle, where it is assumed to recognize higher CP- or RNA-structures to start transcription and the synthesis of the dsRNA genome (Ghabrial, 2008). The viral genome within the particle is hidden from the plant defense mechanisms centered on the recognition of dsRNA.

Another important step in the virus life cycle is to ensure the passive transport of cryptic viruses during cell division, especially to the gametes. Due to the lack of movement proteins for active transport via plasmodesmata, the cryptic viruses had to develop mechanisms to establish in meristem cells, which enable them to withstand thermotherapy (Boccardo, 1983). An interaction and in planta localization approach could be the first step to provide more hints to understand the "cryptic strategy".

An optimized BiFC system was used to elucidate protein interactions of six different cryptic viruses from the genera *Alphacryptovirus* and *Betacryptovirus*. For this purpose, the type members WCCV-1 and WCCV-2 from *Trifolium repens* (Boccardo, 1985) and two closely related cryptic viruses from *Trifolium pratense*, namely Red clover cryptic virus 1 and *Red clover cryptic virus 2* (Luisoni, 1987) were used. In addition, the more distantly related Dill cryptic virus 1 and Dill cryptic virus 2 (Lesker, 2013a) from *Anethum graveolens* of the family *Apiaceae* are also included in the study.

3.3.1 Establishment of internal controls

Initially, the Plum pox virus coat protein and deletion mutants thereof served as positive and negative controls, i.e. to verify protein-protein interactions detected by the BiFC system. The development of controls with proteins of cryptic viruses is limited, because these viruses encode only two proteins, which largely reduces the number of possible interaction partners. To circumvent this drawback, proteins of closely and distantly related cryptic viruses from two different genera were used in this study to broaden up

the spectrum of potential interaction partners. To ensure the association of the monomeric red fluorescent protein (mRFP) fragments each CP and RdRp protein was fused to the N- as well as the C-terminal fragment. This allowed a screening of multiple combinations of fusion proteins for fluorescence complementation in all permutations. (Figure 1 and Figure 2). A total of four different BiFC binary vectors (BiFC 1-4) resulted, which carry the RdRp and CP genes, respectively, of distinctive cryptic viruses. Finally, for each CP and RdRp self-interaction four constructs and for the RdRp-CP interaction eight-constructs were available to test the interactions.

In at least one combination of each construct (Virus - CP/ RdRp – BiFC-Vector 1-4) an interaction was found (Figure 1 and Figure 2). This indicates a correct translation of fusion proteins, because in case of binary vectors BiFC3 and BiFC4 the GOI was fused upstream to the reporter gene. In the BiFC1 and BiFC2 vectors identical GOI-fragments from BiFC3 and BiFC4 were used, and the final constructs were verified by restriction enzyme digest and sequencing. Therefore, the different permutation and cross species tests of each construct served also as either additional positive or negative control. In case of BiFC2 mRFPC-RCCV-1CP only positive interactions with all test partners were identified, whereas with all other constructs, at least one negative interaction was determined. Thereby, additional control measurements with BiFC2 mRFPC-RCCV1CP were performed to exclude false-positive results, e.g. testing without any interacting partner, which reveals no fluorescence (data not shown). In addition, different localizations with BiFC2 mRFPC-RCCV1CP were found in several interactions, indicating the correct and specific determination of interactions and no general and unspecific interaction of the test partners.

Interestingly, in several cases interactions were not found in all kinds of permutations. Especially RdRp self-interactions were only found in combinations when the RdRp was fused N-terminal as well as C-terminal to the mRFP with the BiFC2/3 vectors or *vice versa* with the BiFC1/4 vectors (Figure 1 and 2). This indicates that testing of all permutations might be beneficial in case of all BiFC systems. If only one permutation is tested with a negative result, all other permutations should also be tested to avoid the oversight of possible interacting partners. This applies to studies on the localization of interactions, too. In case of self-interactions of RCCV-1CP and DCV-1CP an association with the nuclear membrane was evident (Figure 1) with all BiFC combinations. However, the same expected localization of WCCV-1CP was found only in one permutation (BiFC2/3).

Figure 1. Interactions of the *RNA-dependent RNA polymerase (*RdRp) and *coat protein (*CP) of alphacryptoviruses WCCV-1, RCCV-1 and DCV-1. Grey shaded areas indicate self-interactions of CP and/or RdRp. Symbols: "–": no fluorescence; "n.t.": not tested; "+++"/"++"/"+": for strong/medium/low fluorescence signals; "###"/"##"/"#": almost all/mean number of/only a few cells detected with fluorescence; capital letters indicate localization of fluorescence in the cell: "C": cytoplasm, "I": inclusions in the cytoplasm, "N": nucleus, "NM": nuclear membrane. Bimolecular fluorescence complementation (BiFC) constructs are represented in the vertical line with BiFC3 (CP-mRFPN): "CP-●", BiFC1 (mRFPN-CP): "●-CP"; or BiFC3 (RdRp-mRFPN): "RdRp-●", BiFC1 (mRFPN-RdRp)": ●-RdRp" and in the horizontal line with BiFC4 (CP-mRFPC): "CP-●", BiFC2 (mRFPC-CP): "●-CP" or BiFC4 (RdRp-mRFPC): "RdRp-●", BiFC2(mRFPC-RdRp): "●-RdRp".

		WCCV-1 with mRFP**C** fusion				**RCCV-1** with mRFP**C** fusion				**DCV-1** with mRFP**C** fusion			
		CP-●	●-CP	RdRp-●	●-RdRp	CP-●	●-CP	RdRp-●	●-RdRp	CP-●	●-CP	RdRp-●	●-RdRp
WCCV-1 with mRFP**N** fusion	CP-●	-	+++ ## NM	-	-	-	++ # I	n.t.	n.t.	-	-	n.t.	n.t.
	●-CP	++ ## C+N	-	-	-	++ # I	++ ## I	n.t.	n.t.	-	-	n.t.	n.t.
	RdRp-●	-	-	-	++ ## C+N+I	n.t.	n.t.	-	-	n.t.	n.t.	-	-
	●-RdRp	-	+ # C+N+I	++ ## C+N	-	n.t.	n.t.	-	-	n.t.	n.t.	+ # C+N	-
RCCV-1 with mRFP**N** fusion	CP-●	-	+++ ### NM	n.t.	n.t.	+++ ### NM	+++ ### NM	-	-	+++ ### N	+++ ### N	n.t.	n.t.
	●-CP	-		n.t.	n.t.	+++ ### NM	+++ ### NM	-	-	+++ ### N	+++ ### N	n.t.	n.t.
	RdRp-●	n.t.	n.t.	-	-	-	+ # C+N	-	+ # C+N	n.t.	n.t.	-	-
	●-RdRp	n.t.	n.t.	-	-	+ # I	++ ## I	+ # C+N	-	n.t.	n.t.	++ # C+N	-
DCV-1 with mRFP**N** fusion	CP-●	-	+++ ### NM	n.t.	n.t.	+++ ### N+I	+++ ### N	n.t.	n.t.	+++ ### NM	+++ ### NM	++ ## I	-
	●-CP	-	+++ ### NM	n.t.	n.t.	+++ ### I	+++ ### N	n.t.	n.t.	+++ ### NM	+++ ### NM	+++ ## NM+I	-
	RdRp-●	n.t.	n.t.	-	-	n.t.	n.t.	-	-	-	-	++ # I	-
	●-RdRp	n.t.	n.t.	++ ## C+N	-	n.t.	n.t.	++ ## I	-	++ ## C+N	+++ ### N+I	+++ ## C+I	+++ ## I

Figure 2. Interactions of RdRp and CP of betacryptoviruses WCCV-2, RCCV-2 and DCV-2. Grey shaded areas indicate self-interactions of CP and/or RdRp. Symbols: "–": no fluorescence; "n.t.": not tested; "+++"/"++"/"+": for strong/medium/low fluorescence signals; "###"/"##"/"#": almost all/mean number of/only a few cells detected with fluorescence; capital letters indicate localization of fluorescence in the cell: "C": cytoplasm, "I": inclusions in the cytoplasm, "N": nucleus, "NM": nuclear membrane. BiFC constructs are represented in the vertical line with BiFC3 (CP-mRFPN): "CP-●", BiFC1 (mRFPN-CP): "●-CP"; or BiFC3 (RdRp-mRFPN): "RdRp-●", BiFC1 (mRFPN-RdRp): "●-RdRp" and in the horizontal line with BiFC4 (CP-mRFPC): "CP-●", BiFC2 (mRFPC-CP): "●-CP" or BiFC4 (RdRp-mRFPC): "RdRp-●", BiFC2(mRFPC-RdRp): "●-RdRp".

		WCCV-2 with mRFP**C** fusion				**RCCV-2** with mRFP**C** fusion				**DCV-2** with mRFP**C** fusion			
		CP-●	●-CP	RdRp-●	●-RdRp	CP-●	●-CP	RdRp-●	●-RdRp	CP-●	●-CP	RdRp-●	●-RdRp
WCCV-2 with mRFP**N** fusion	CP-●	+++ ### C+I	+++ ### C+I	-	-	+++ ### C+I	+++ ### C+I	n.t.	n.t.	+++ ### I	++ ## I	n.t.	n.t.
	●-CP	+++ ### C+N	++ ## I	-	-	-	+++ ## I	n.t.	n.t.	-	-	n.t.	n.t.
	RdRp-●	-	-	-	++ ## C+N	n.t.	n.t.	-	+ # C+N	n.t.	n.t.	-	-
	●-RdRp	-	-	++ ## C+N	-	n.t.	n.t.	+ # C+N	-	n.t.	n.t.	++ ## C+N	-
RCCV-2 with mRFP**N** fusion	CP-●	+++ ### C+I	+++ ## C+I	n.t.	n.t.	+++ # C+I	+++ ## I	-	-	+++ ## C+N+I	++ # I	n.t.	n.t.
	●-CP	+++ ### C+I	+++ ## I	n.t.	n.t.	+++ ## C+I	+++ ### I	-	-	+++ ### C+I	++ ## C+I	n.t.	n.t.
	RdRp-●	n.t.	n.t.	-	+ # C+N	-	-	-	+ # C+N	n.t.	n.t.	-	-
	●-RdRp	n.t.	n.t.	+ # C+N	-	-	-	+ ## C+N	-	n.t.	n.t.	+ # C+N	-
DCV-2 with mRFP**N** fusion	CP-●	+ # I	+ # I	n.t.	n.t.	+++ ## I	-	n.t.	n.t.	+++ ### I	+++ ### I	-	-
	●-CP	-	-	n.t.	n.t.	-	-	n.t.	n.t.	++ ## C+N	++ ## C+N	-	-
	RdRp-●	n.t.	n.t.	-	-	n.t.	n.t.	-	-	-	-	-	+ # C+N
	●-RdRp	n.t.	n.t.	+ # C+N	-	n.t.	n.t.	+ # C+N	-	-	-	+ # C+N	-

3.3.2 CP dimer formation

The particles of the *Partitiviridae* are composed of 120 CP subunits forming 60 dimers, which corresponds to a T=1 symmetry (Ghabrial, 2012). For virus assembly of cryptic viruses, interactions between CP subunits, the RdRp and RNA are necessary. A certain degree of self-assembly without any other viral element occurs for the CP subunits of viruses. Furthermore, even entire particles without encapsidated RNA were found in case of isometric viruses (Buck, 1973, 1974). CP dimers act as starting points for the assembly process (Pan, 2009).

An interaction of the CP was detected for all alpha- and betacryptoviruses (Table 1 and Table 2). Detection of WCCV-1 CP-CP interaction depended on the localization of the fused protein in relation to the mRFP-fragment as described above. Furthermore, differences in the number of cells showing fluorescence and also in the intensity of the fluorescence were observed. A strong fluorescence signal was found in the majority of epidermal cells within the analyzed leaf regions (Figure 1).

The CP interaction of viruses of the same genus in plant cells was localized in as similar manner, but differs in the alpha- and betacryptoviruses. Concerning the alphacryptoviruses all three tested viruses showed CP homo-dimer formation. A localization of CP-CP homo-dimers at the membrane surrounding the nucleus were visualized (Figure 1; Figure 3A,B), in regard to RCCV-1 and DCV-1 even in all four permutations. Prominent deposits could be found associated with the outer membrane without fluorescence inside the nucleus. In addition, CP-CP hetero-dimers were detected between WCCV-1, RCCV-1 and DCV-1 (Figure 3E), respectively, but again not in all permutations.

In a similar way, all intragenus permutations of the CP of the betacryptoviruses were tested. A distinct localization for CP interactions of viruses from the genus *Betacryptovirus* was absent (Figure 2). In contrast to the alphacryptoviruses protein-protein interactions were mainly detected in marginalized deposits in the cytoplasm close to the cell wall (Fig.4A). These inclusion bodies in the cytoplasm can consist of biologically inactive proteins. However, CP-CP interactions were also detected by fluorescence in the cytoplasm and the nucleus for WCCV-2 and DCV-2. Moreover, CP-CP interspecies interactions were as well detected between WCCV-2, RCCV-2 and DCV-2 (Figure 4C), respectively, but similar to alphacryptoviruses not in all permutations.

Lastly, no intergenus interaction between the CP of alphacryptovirus WCCV-1 and the betacryptovirus WCCV-2 was detected independent of BiFC permutations (results not shown).

However, except for WCCV-1 a CP interaction was demonstrated in all permutations of the tested viruses, which indicates interacting domains or areas independent of free N- and C-termini of the CPs. The 40 N-terminal amino acids of Partitivirus CPs were not involved in the structure of the particle resolved by 3D structure analyses (Pan, 2009; Tang, 2010). Probably, they are located at the inside of the virus particle and ensure the arrangement of the dsRNA within the particle (Nibert, 2013) or they are located at the surface of the virion.

A more precise localization in cell compartments could be reached with other techniques like immune labeling electron microscopy in the host plants or *in situ* hybridization. However, the distinctive location of primary virus assembly sites showed in this study may indicate that the viruses of the genera *Alphacryptovirus* and *Betacryptovirus* use different compartments to co-exist in one cell.

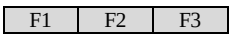
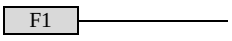
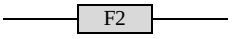
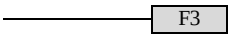
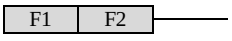
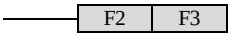
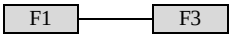
3.3.3 Localization of protein interaction sites in the CPs

The putative interaction domain within the WCCV-1CP and WCCV-2CP was approximated by dividing the coding frame into three parts. The fragments vary from 150 to 273 amino acids, so that protein structures should be formed. However, possible secondary structures were not taken into consideration for the choice of the selected regions. Moreover, each potential interaction of different fragments was tested with only two fusion permutations, resulting in a limited degree of freedom for protein adjustments. The particle structure, as outlined above, implied multiple interactions within a single CP for dimer formation. In addition, protein regions are known that are probably not at the surface of viral particles (Pan, 2009; Tang, 2010) and more likely bind RNA inside the particle (Nibert, 2013).

To narrow down the interacting domains in the CP of WCCV-1 and WCCV-2, six different deletion mutants were created (Tab. 1). We obtained only a few interactions for the alphacryptovirus WCCV-1CP mutants, similar to the findings for the full-length CP permutation tests. In the used BiFC2/3 permutation (mRFPC-F/F-mRFPN) only seven interactions out of 48 possible combinations tested positively. The full-length CP in the BiFC3 vector was interacted with all other BiFC2 (mRFPC-CP) fragments except for BiFC2-F2. Additionally, we also detected interactions for BiFC2-F1/2 with BiFC3-

F2 and BiFC3-F2/3. Furthermore, the localization of the observed fluorescence in the BiFC2-F1 and BiFC2-F1/2 combination changed from the nuclear membrane to the cytoplasm and nucleus compared to the interaction of the full-CP used as a positive control (Table 1).

Table 1. Schematic overview of the tested alphacryptovirus WCCV-1CP deletion mutants; "–" no interaction visible; "+++"/"++"/"+": for strong/medium/low fluorescence signal; "###"/"##"/"#": almost all /mean number of /only few cells detected with fluorescence; capital letters for localization in the cell: "C": cytoplasm, "N": nucleus, "NM": nuclear membrane.

BiFC3: mRFPN-F \ BiFC2: F-mRFPC	**full**	**F1**	**F2**	**F3**	**F1/2**	**F2/3**	**F1/3**
full 	+++ ### NM	+ # C+N	-	+++ ### NM	+ # C+N	++ ## NM	+++ ### NM
F1 	-	-	-	-	-	-	-
F2 	-	-	-	-	++ ## C+N	-	-
F3 	-	-	-	-	-	-	-
F1/2 	-	-	-	-	-	-	-
F2/3 	-	-	-	-	++ ## C+N	-	-
F1/3 	-	-	-	-	-	-	-

Tab 2: Schematic overview of the tested *Betacryptovirus* WCCV-2CP deletion mutants; "–"no interaction visible; "+++"/"++"/"+": for strong/medium/low fluorescence signal; "###"/"##"/"#": almost all /mean number of /only few cells detected with fluorescence; capital letters for localization in the cell: "C": cytoplasm, "I": Inclusion in cytoplasm

BiFC4: mRFPC-F / BiFC3: mRFPN-F	**full**	**F1**	**F2**	**F3**	**F1/2**	**F2/3**	**F1/3**
full F1 F2 F3	+++ ### C+I	-	+++ ### I	+ # I	++ ## I	+ # I	-
F1 F1	-	-	-	-	-	-	-
F2 F2	-	-	+++ ### I	-	++ # I	++ ## I	-
F3 F3	-	-	++ ## I	-	-	-	-
F1/2 F1 F2	++ ## C+I	-	++ # I	-	++ # I	++ ## I	-
F2/3 F2 F3	-	-	++ ## I	-	-	-	-
F1/3 F1 F3	-	-	-	-	-	-	-

The orientation of the fusion in respect to the reporter part seems to be critical for the dimerization. The association with the C-termini of the full-length CP resulted in five detected interactions with CP fragments, whereas the opposite direction did not. It is particularly interesting to note that only if both partners include the F3-part the fluorescence was located on the nuclear membrane. This might be an indication that the C-terminus is involved in the protein localization perhaps it provides its own signal peptide sequence for the protein targeting.

A similar approach was performed for the betacryptovirus WCCV-2 (BiFC3/4 permutation; F-mRFPN-F/F-mRFPC; Table 2). Overall thirteen interactions out of 48 possible combinations were identified. Fluorescence was mainly located in inclusions within the cytoplasm of the plant cells. Most interactions were detected for mutants still including the F2 part. In contrast, no interactions were observed in any combination with F1 and F1/3 fragments. Furthermore, BiFC3-F3 mutant interacts with BiFC4-F2

and BiFC4-F3 with the BiFC3 full length CP, but F3 in BiFC3 and BiFC4 did not interact with itself (Figure 4E). Conversely, almost all positive combinations of interaction required the F2 part in both partners, and additionally, for the F2 fragment an interaction with the F3-mRFPN fusion was shown. This furthermore indicates that the middle part - F2 - of the WCCV-2 protein is particularly important for primary dimerization and probably also for the forming of inclusions within the cytoplasm.

3.3.4 RdRp dimerization

The RdRp of *Partitiviridae* is located within the virus particle and produces transcripts of the dsRNA genome. The transcripts are delivered through pores of the particle into the cytoplasm (Ghabrial, 2012). The tested viruses have only one dsRNA per particle and accordingly just one RdRp molecule will be packaged (Buck, 1973, 1974). So RdRp self-interaction seems not necessary. However, in all alpha- and betacryptoviruses a potential RdRp interaction was found, almost always in the BiFC1/4 permutation (mRFPN-RdRp with RdRp-mRFPC) and BiFC2/3 (mRFPC-RdRp with RdRp-mRFPN) combination, in which the RdRp was fused N- and C-terminal to the mRFP.

The fluorescence was predominantly observed in the cytoplasm and the nucleus as shown for WCCV-1 (Figure 3C) and WCCV-2 (Figure 4B). For DCV-1, an RdRp interaction was detected with all permutations except BiFC1/4 (mRFPN-RdRp with RdRp-mRFPC). The fluorescence was observed equally distributed throughout the cytoplasm but also in inclusions within the cytoplasm. Additionally, an RdRp interaction of RCCV-2 and DCV-2 was shown resembling the homologous interaction (Figure 4D).

However, RdRp interactions were less frequent than CP interactions. Overall, also the fluorescence intensity and frequency of cells showing fluorescence was lower compared to the CP interactions (Figure 1 and Figure 2), indicating for a weak and fragile self-interaction. Furthermore, a close proximity of overexpression, aggregation and mis-localization of RdRp proteins may contribute to the interaction determined by the BiFC-system. Dimer formation of RdRps was also described for other virus families (Qin, 2002). However, these clearly differ in their replication cycle from *Partitiviridae*. Former publications gave no evidence of a RdRp self-interaction. Therefore, further analyses like yeast two hybrid analyses concerning the weak but clearly detectable RdRp self-interaction might confirm the results.

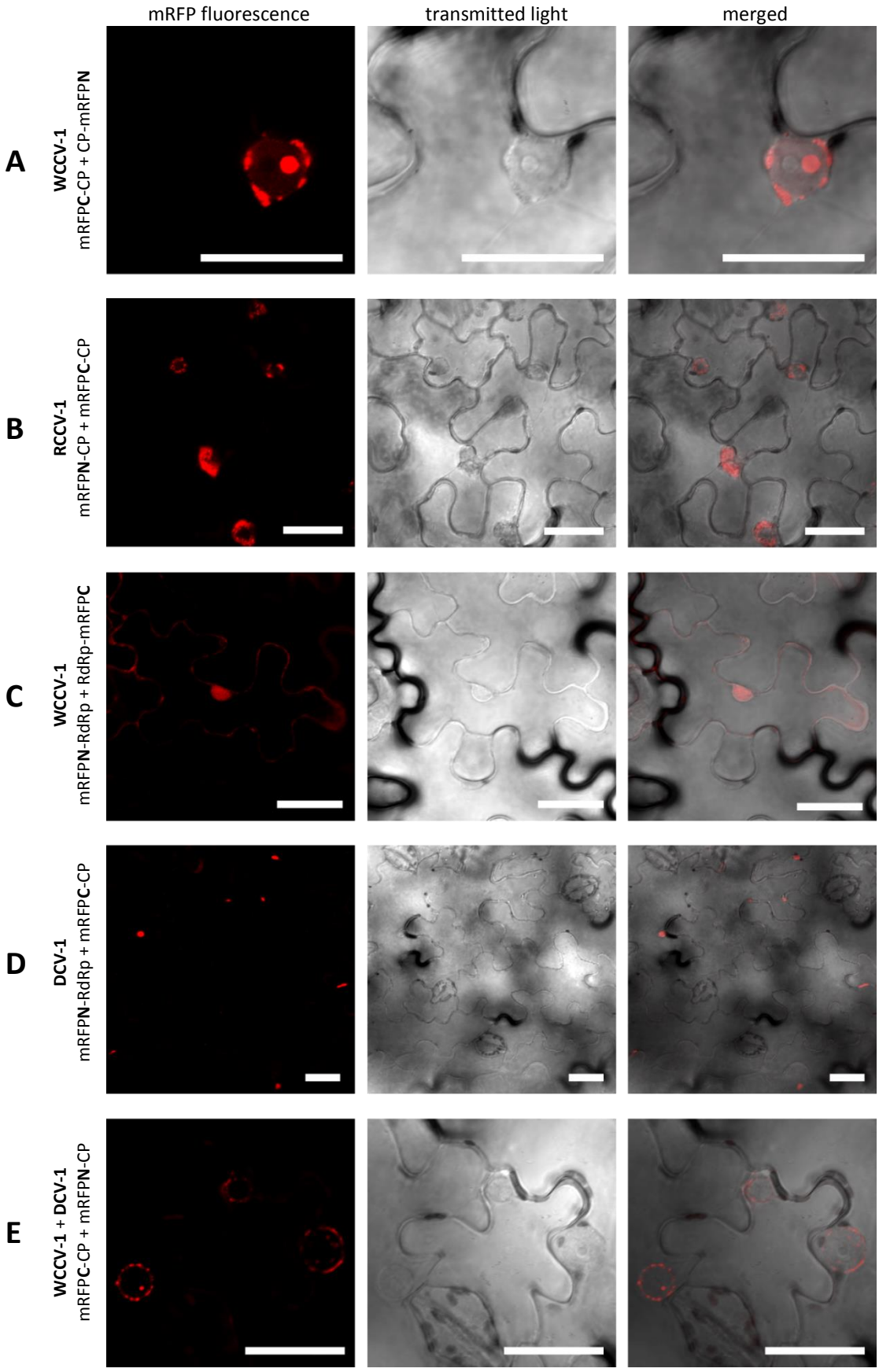


Fig 3: Selected interactions of proteins of alphacryptoviruses. BiFC of mRFP in *N. benthamiana* epidermal cells at 3 days p.i. CLSM images for the mRFP fluorescence, the transmitted light mode of chlorophyll and merged pictures with the transmitted light mode of cells. Bars, 30 µm.

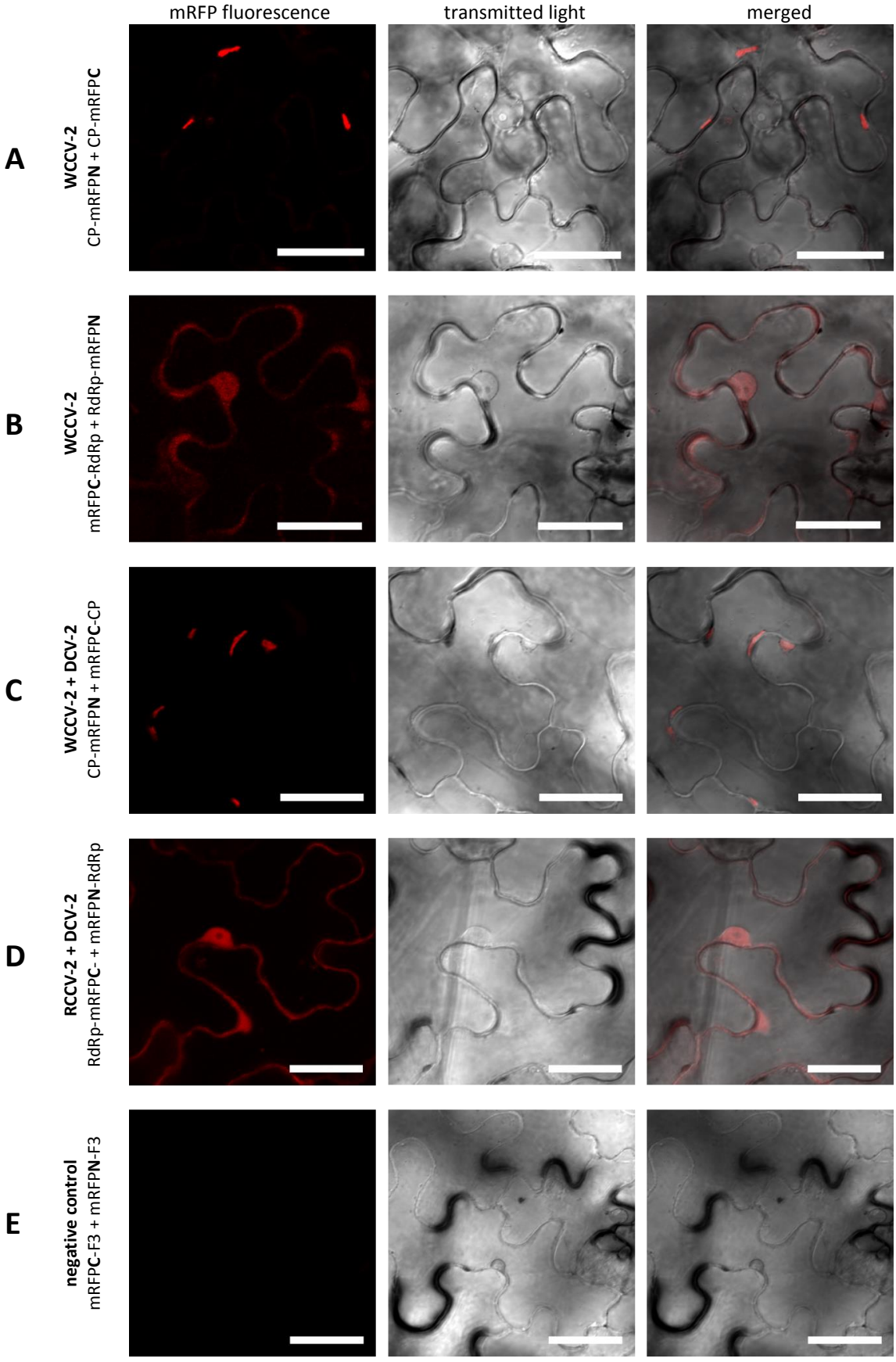


Fig 4: Selected interactions of proteins of betacryptoviruses. BiFC of mRFP in *N. benthamiana* epidermal cells at 3 days p.i. CLSM images for the mRFP fluorescence, the transmitted light mode of chlorophyll and merged pictures with the transmitted light mode of cells. Bars, 30 µm.

3.3.5 CP-RdRp interactions

During particle assembly of cryptic viruses, RNA and RdRp have to be assembled with the CP (Ghabrial, 2012). 3D structural analyses have shown pores within the particle that might support the transfer of newly synthesized RNA by RdRp from the particle (Pan, 2009; Tang, 2010). These pores are small but flexible, so that an interaction between RNA and / or RdRp resulting in a transfer of RNA can be supposed (Pan, 2009; Nibert, 2013). RdRp could not be shown in structural analyses (Pan, 2009). It is postulated that RdRp is not covalently attached to the inside of the particle (Ghabrial, 2012). Nevertheless, the RdRp should be localized within the particle to transcribe and to convert ssRNA into dsRNA (Ghabrial, 2008).

An interaction of CP and RdRp has been observed in at least one permutation of all alphacryptoviruses. Several permutations showed a medium fluorescence in a few cells. In regard to WCCV-1 only the BiFC1/2 combination (mRFPN-RdRp with mRFPC-CP) delivered a positive signal (Tab.1). Three interactions were found in fusions of RdRp with mRFPN and CP with mRFPC in the closely related RCCV-1 but these interactions were not detectable in the opposite orientation of the tested proteins. In contrast, we observed plenty of intensely fluorescent cells in case of DCV-1, in all permutations of the C-terminal RdRp fusions (Figure 1). The localization of the proteins within the cell was not homogeneous; positive fluorescence signals were mainly found in inclusions in the cytoplasm (Figure 1; Figure 3D), in some permutations in the cytoplasm itself and in the nucleus and nuclear membrane.

So the localization, in case of WCCV-1 and RCCV-1, clearly differs from RdRp and CP self-interactions, where greater deposits in the cells were missing. A localization of CP-RdRp interaction at or near the outer nuclear membrane as described for the primary dimer fusion was found in one permutation only. This might be an indication for a later CP-RdRp interaction step within the framework of virus assembly in the cytoplasm.

In contrast to alphacryptoviruses no interaction between CP and RdRp was found in the three viruses of the genus *Betacryptovirus* (see Figure 2). One reason could be that the CP-RdRp interaction is weaker in manifestation and therefore, harder to verify with the used BiFC-system. The particles differ from those of the alphacryptovirus in particle size - 38 vs. 30 nm - and the presence of prominent subunits on the particle surface (Ghabrial, 2008). Other factors like higher structures of CPs or the presence of full-length RNA may be a prerequisite and essential for CP-RdRp interaction. Concerning

these points further analyses like trimolecular fluorescence complementation analysis (Rackham, 2004) might be helpful.

3.3.6 Cross species interaction of CP and RdRp

Cryptic viruses are interesting regarding their evolutionary relationship to one another, because a horizontal transmission of those viruses via vectors is not known (Ghabrial, 2012). However, there is a high sequence homology in these viruses, even though they occur in different plant families (Ghabrial, 2008). From the phylogenetic point of view, a horizontal transmission with a vector is more likely than a coevolution between the virus and the host before primeval times (Lesker, 2013a). Besides using interspecies tests as internal controls it was interesting to find out if protein interactions can also be established among the viruses within one genus.

We tested the CP and RdRp hetero dimerization of related viruses within one genus from white clover and red clover, furthermore, of more distantly related viruses from dill. CP dimers were detected between all viruses within one genus (Figure 1 and Figure 2). Concerning the alphacryptoviruses a strong fluorescence was found in almost all cells, localized in analogy to the already described CP dimers in the membrane of the nucleus (Figure 3E). Nevertheless, also a different localization in the nucleus for the dimers RCCV-1 CP and DCV-1 CP was visible. Moreover, eight permutations with WCCV-1 revealed no interaction and in another three permutations only a few cells were detected with low fluorescence signals from inclusions located in the cytoplasm.

In regard to the betacryptoviruses a similar localization for CP hetero-dimers were observed and overall fewer combinations showed a positive signal with a lower number of cells and fewer intensities of fluorescence, especially in combination with the more distantly related DCV-2. Interactions occurred in all tested virus combinations. In particular, it was noticed that interactions were not found with all permutations, compared to self-interactions, the fluorescence was weaker and the localization changed. This was also the case for the RdRp hetero dimerization detected within the genus *Alphacryptovirus* for DCV-1 with WCCV-1 and RCCV-1, respectively, in the BiFC1/4 (mRFPN-RdRp with RdRp-mRFPC) combination as already described above, but not between WCCV-1 and RCCV-1. In addition, a dimerization for all RdRps of the betacryptoviruses was observed.

However, in case of WCCV-1 only a few permutations were found to react positive, which could indicate that RCCV-1 and DCV-1 with much more interactions could be

better analysed in our BiFC system in *N. benthamiana*. Furthermore, an imperfect assembly of virus CPs might cause a malfunction in the further localization. In case of heterologous tests CP subunits of the same virus might preferentially interact, thereby not leading to a fluorescence signal, because of missing one reporter part. Heterologous protein interactions can also induce different localizations like the occurrence of deposits within the cytoplasm in case of the alphacryptovirus. Concerning this, further data are needed for the precise localization and description of steps involved in the virus replication cycle.

3.4 Experimental Section

3.4.1 Construction of the expression plasmids for BiFC

The pCB:GOI-mRFPN (BiFC 1), GOI-mRFPC (BiFC 2), pCB:mRFPN-GOI (BiFC 3) and pCB:mRFPC-GOI (BiFC 4) expression plasmids were generated as described by Zilian and Maiss (2011).

3.4.2 Construction of the plasmids for full-length protein interaction

The coding sequence of the CP or RdRp, respectively of WCCV-1, WCCV-2, RCCV-1, RCCV-2, DCV-1, DCV-2 were RT-PCR-amplified using dsRNA preparations from White Clover, Red Clover and Dill with RevertAid Premium Reverse Transcriptase and Phusion Flash Master Mix (Thermo Scientific) as described previously (Lesker, 2013). New Sequences are stored in Genbank under accession numbers: RCCV-1RdRp: KF484724, RCCV-1CP: KF484725, DCV-1RdRp: KF484726 and DCV-1CP: KF484727. Fragments were generated by using primers, which include specific restriction endonuclease sites (*Bam*HI or *Bgl*II and *Sal*I or *Xho*I) for cloning into the BiFC vectors (Supplementary Table S1). Fragments were first cloned into pJET1.2 (Thermo Scientific) according to the manufacturer's protocol, digested with the appropriate restriction enzyme and ligated into the binary BiFC-plasmids, which were digested with *Bam*HI/*Sal*I or were cloned by a Gibson Assembly (New England Biolabs) approach (Gibson, 2011). The sequences were verified by sequencing and restriction enzyme digests.

3.4.3 Construction of the plasmids for deletion mutants of WCCV-1CP and WCCV-2CP

The open reading frame of each CP was divided into six fragments by PCR mutagenesis using Phusion Flash DNA polymerase (Thermo Scientific). The F1, F3, F1/2, F2/3 and F1/3 fragments of WCCV-1CP, encoding aa 1–150, 151–338 and 339–487, respectively were generated using the BiFC2:mRFPC-WCCV-1CP and BiFC3:WCCV-1CP-mRFPN vectors as templates. The same fragments of WCCV-2CP, using aa 1-200, 201-473 and 474-673 were created from the BiFC3: WCCV-2CP-mRFPN and BiFC4: WCCV-2CP-mRFPNC vectors.

3.4.4 Transient protein expression in N. benthamiana leaf epidermal cells and CLSM

The BiFC plasmids and pCB:p35TBSVp19, encoding the TBSV p19 protein as a suppressor of gene silencing, were used for the electroporation into *A. tumefaciens* strain GV2260 (Hoekema, 1983). Agrobacteria cultures harbouring the plasmids were prepared for infiltration according to Zilian & Maiss (2011) (Zilian, 2011). The infiltration of young leaves of *N. benthamiana* plants 4 to 5 weeks old was performed by using *A. tumefaciens* mixtures containing the BiFC1-4 plasmids and pCB:p35TBSVp19 binary plasmid. All infiltrated plants were incubated at room temperature for 3 days. Discs of infiltrated *N. benthamiana* leaves were investigated with a Leica TCS SP2 confocal laser scanning microscope. The excitation at 543 nm of the mRFP domain was performed by using the green neon laser. The emitted light was captured at 600–610 nm, thus creating consistent-recording conditions. Visualization of the chlorophyll autofluorescence was made by excitation at 488 nm with the argon/crypton laser and subsequent fluorescence detection at 690–740 nm. Digital capture and processing of the images were performed by using the Leica confocal software.

3.5 Conclusions

Our results revealed various differences in protein interactions between alpha- and betacryptoviruses which are not only caused by different protein and particle sizes. As already described, betacryptoviruses differ from alphacryptovirus in terms of the presence of prominent arches on the virus particle surface (Boccardo, Lisa et al. 1987). For the betacryptoviruses a related Partitivirus the *Fusarium poae virus* 1 was analyzed

by X-ray crystallography (Pan, 2009; Tang, 2010). As long as no 3D structure for the alphacryptoviruses is described, it will be difficult to compare these structures of the two genera in a meaningfull way. However, it is assumed that they share distinctive features, including a quasi-symmetric CP protein dimerization and formation of a T=1 capsid structure by 60 dimers by domain swapping (Nibert, 2013). Nevertheless, in this protein interaction study, we are able to find differences between the viruses of two plant infecting genera of the family *Partitiviridae.* We obtained expected CP – RdRp interactions only for the members of the genus *Alphacryptovirus*. The localization of the CP dimers were observed for WCCV-1, RCCV-1 and DCV-1 in the nuclear membrane, whereas the fluorescence signals for the WCCV-2, RCCV-2 and DCV-2 was located in inclusions within the cytoplasm of epidermis cells. CP mutants of WCCV-1 and WCCV-2 showed a different localization of interaction sites in the CP.

From the perspective of the evolutionary relationship, it is interesting to verify protein interactions between viruses in one genus infecting distant host plants and to find no interaction between the type members of genus *Alphacryptovirus* and *Betacryptovirus* in the same host. Together with the different localization of the CP -CP interactions and findings of the CP - RdRp interactions only in the alphacryptoviruses primary indications are given for striking differences in the molecular life cycle of these two virus genera. However, this study is the first protein interaction approach *in planta* for viruses of the family *Partitiviridae* so far and merely one further step to understand the biology of the viruses of the family *Partitiviridae.*

Appendix

Table A1. Oligonucleotides used for amplification of plant cryptic virus ORFs. Virus-specific sequences are shown in lower case at the 3'end; Restriction enzyme recognition sequences/Gibson Assembly sites in upper-case characters.

Virus/Vector	Primer name	Primer sequence
WCCV1	BiFC_WCCV1_CP_s	cgGGATCCatgaatcaagacactcctctcgcc
	BiFC_WCCV1_CP_as	acgcGTCGACttcagcacggttggcagcttg
	BiFC_WCCV1_R_s	gaAGATCTatggattacctaatcactgcatttaaccg
	BiFC_WCCV1_R_as	acgcGTCGACctcgcctggagcattgataaacaa
RCCV1	BiFC_RCCV1_Rs	gaAGATCTatggattacttcatatccgcatttaac
	BiFC_RCCV1_Ras	ccgCTCGAGctcgccaggtgcattgatg
	BiFC_RCCV1_Cs	cgGGATCCatgaatcacaacactcctcctgc
	BiFC_RCCV1_Cas	acgcGTCGACttcagcacggttggcagc
DCV1	BiFC_DCV1CPs	cgGGATCCatggaccccaacgtccctattgc
	BiFC_DCV1CPas	acgcGTCGACttcggcgcggttcgcggcct
	GA12_GA_DCV1Rs	GGATCTGGTGGAGGTGGATCCatggattacctcacaac cgcattc
	GA12_GA_DCV1Ras	GAGGATCGATCCTTAGTCGACctcagcaggatccttaa gaaataag
	GA34_GA_DCV1Rs	GAAGGAGATATAACAATGGGATCCatggattacctc acaaccgcattc
	GA34_GA_DCV1Ras	CCAGATCCACCTCCGTCGACctcagcaggatccttaag aaataag
WCCV2	BiFC_WCCV2_R_s	cgGGATCCatgcctcacaactccactcgc
	BiFC_WCCV2_R_as	acgcGTCGACcgggaaatttcttgtggcaggca
	GA12_WCCV2CP_s	GGATCTGGTGGAGGTGGATCCatgtctcctgatgagaa ccccac
	GA12_WCCV2CP_as	GAGGATCGATCCTTAGTCGACgacagcggggtagga ttcatag
	GA34_WCCV2CP_s	GAAGGAGATATAACAATGGGATCCatgtctcctgat gagaaccccac
	GA34_WCCV2CP_as	CCAGATCCACCTCCGTCGACgacagcggggtaggattc atag
RCCV2	BiFC_RCCV2_R_s	gaAGATCTatgccgttcaactctgctcg
	BiFC_RCCV2_R_as	ACGCgtcgacCGGGAAATTTCTTGTGGCGGG
	BiFC_RCCV2_CP_s	cgGGATCCatgtctactgaagagacccttcct
	BiFC_RCCV2_CP_as	ACGCgtcgacAACAGCGGGGAAGGACTCATA

4 Deep Sequencing of double-stranded RNA as a tool to assess the presence of unknown RNA viruses in plants

4.1 Abstract

So far metagenomic studies of plant infecting viruses have used experimentally or naturally infected plant material. In this study we put the focus on the biodiversity of persistent viruses of plants, which include viruses from the families *Partitiviridae*, *Endornaviridae* and the proposed new family *Amalgaviridae*. All of these dsRNA containing viruses are widespread in plants. Generally, they cause no significant effects on their hosts and are only transmitted by cell division and through gametes at very high rates.

Therefore we isolated dsRNA obtained from seed grown White Clover, Red Clover, Hop Trefoil and dill plants, cultivated under controlled greenhouse conditions. The dsRNA was pooled and introduced to a cost effective benchtop sequencing technology. The resulting 15 million read-pairs were assembled and compared to GenBank via BLAST searches. 42 contigs fit to persistent viruses which cover 52% of all reads. Viruses were assigned to the four individual plant species using specific primers generated from the contigs for RT-PCRs of dsRNA and total RNA preparations. Phylogenetic analyses suggest at least five clusters of different plant infecting cryptic viruses in the family *Partitiviridae*. A total of eight putative new virus members were determined. Furthermore, three new putative *Endornaviruses* and four putative members of the new genus *Amalgavirus* were discovered. Almost all contigs cover at least 97% of the corresponding reference sequences, missing only about 20 bp on the 5'- and 3'- end, respectively.

These results show for the first time the suitability of the combination of dsRNA-screening and deep sequences techniques for the determination of persistent viruses from seed-grown plants.

4.2 Introduction

For a period of nearly 100 years studies concerning plant viruses focused mainly on crop plant infecting viruses. However, from a metagenomic point of view this is just the tip of an iceberg of viromes of plants. As with non-cultivatable microbes in biodiversity, persistent viruses in plants are the vast unknown in viral ecology (Rosario, 2011). Most of the viruses make use of a dsRNA genome and belong to cryptic viruses of the family *Partitiviridae*, *Endornaviridae* and the proposed family *Amalgaviridae* (Martin, 2011).

Currently there are no known natural vectors of persistent viruses, and they are not transmitted by mechanical means or grafting (Brunt, 1996). Nevertheless, persistent viruses have a very high rate of transmission which occurs passively by cell division, thereby also infecting seed and pollen (Boccardo, 1987). It appears that persistent viruses are very well adapted to their hosts, reaching only a low virus titer, persisting in tissue culture for years and withstanding thermotherapy (Boccardo, 1985). No visible symptoms are caused by cryptic viruses, and apparently they do not have a drastic impact on the quality and the yield of crop plants.

However, recent studies indicate an influence of persistent viruses on their hosts under special environmental conditions and/or an involvement in plant defense reactions leading to hypervirulence (Nakatsukasa-Akune, 2005; Marquez, 2007; Chiba, 2013). Moreover, in many plant species an integration of viral coat protein sequences into the host genome was detected (Liu, 2010; Chiba, 2011). This, together with the knowledge about strong virus–fungi relationships, a complex interaction between plants, fungi and residing persistent viruses has been suggested (Roossinck, 2010, 2013).

Although the ICTV recognizes currently over 900 plant virus species, many of the persistent viruses are not included in the current taxonomy. This is caused by the fact that for proper taxonomic classification of these viruses rules like Koch's postulates, determination of host range and elucidation of transmission roads are difficult to fulfill. New developments in the field of molecular biology, especially in high throughput sequencing, allow us a close look inside the plant sequence pool today (Roossinck, 2011).

There is a broad spectrum of techniques which can be applied to detect viruses in plants, such as electron microscopy, ELISA and PCR. However, with the exception of electron microscopy the other diagnostic tools require previous knowledge of the expected virus, for instance, the availability of specific antibodies or sequence information for the design of the detection oligonucleotides. To gain access to an unknown virus, classical

approaches such as indicator plants and electron microscopy can give first clues to a specific group. But not all viruses are detectable via a host range study or can be classified by their particle structures. Moreover, these methods are laborious and time consuming. Other techniques focus on the enrichment of viral agents in a sample. Virus purification by ultracentrifugation (Prata, 2012) does not cover all types of viruses and depends on the presence, stability and the type of virus particles. Techniques focusing on determination of the viral sequence, such as subtractive hybridization, can reduce host sequences from the gene pool. Nevertheless, in the past it was very expensive and time consuming to find viral sequences by random sequence determinations in those pools (Hull, 2013).

The invention of deep sequencing methods allows us to study microbial populations, in a cost and time effective way. Universal genes, such as ribosomal RNA or ITS regions, permit the assessment of the biodiversity of higher organisms by amplicon sequencing and identification by barcoding. The lack of such universal targets in viral sequences hampers this kind of approach. A possible way to find a potential virus infection in plants is the isolation of the total RNA. Considering not all viruses have a poly-A tail the number of further purification steps can be reduced. Therefore, it is uneconomical to always sequence a full transcriptome of a plant for virus detection. In recent metagenomic studies of viruses three approaches were applied to reduce the complexity of a sample:

1) The subtractive hybridization of cDNAs from an infected versus a healthy plant can remove the majority of expressed host genes. The approach depends on the availability of a respective peer plant with the identical genotype, which was grown under the same environmental conditions. Furthermore, the infection with a virus has an influence on the transcription of host genes, which reduces the efficacy of the elimination of host nucleic acids. However, in a recent study the approach was used to identify known and unknown viruses in *Liatris spicata*. Approximately 20-40 % of the reads were related to viral sequences, covering 97 % of the genome of the previous infected virus (Adams, 2009).

2) Viruses are targets and inducers of RNA silencing (RNAi) that constitutes the basic antiviral defense mechanism in plants. The resulting small interfering RNAs (siRNAs) can be used as a universal target to detect virus infections in the plant (Kreuze, 2009). However, one limitation of the method is the small size of the resulting sequences of around 24 base pairs (bp), which limits its specificity and the possibility to generate full

coverage contigs of the virus genome. Moreover, plants use their own small micro RNAs (miRNAs) derived from its own genome for the regulation of transcription and translation. In a study focusing on viruses in sweet potatoes, 15-40 % of the obtained siRNA sequences delivered hits to the infecting viruses. The largest contig covers 3115 bp of a virus genome with the total size of 10996 bp (Kreuze, 2009).

3) The viral infection of a plant can also be assessed with the extraction of double-stranded RNA (dsRNA). Most plant infecting viruses use RNA as genetic material, and dsRNA as an intermediate form for their replication (Weber, 2006). Even some DNA viruses use RNA intermediates for replication and RNA transcripts for their gene expression (Gu, 2006). However, because large dsRNAs (>30 bp) are not common in plant cells, a specific isolation of dsRNA will allow a broad screening for viral RNAs in a given plant. Different studies used deep sequencing of dsRNA to reveal viral infections of grapevine plants. Most reads (70 %) fit to viral sequences, whereas contigs cover at least 90 % of the compared reference genomes. Additionally, different virus strains can be determined (Coetzee, 2010), such as the 26 putative fungus infecting viruses that were identified by Rwahnih (2011) using this method. These results identify dsRNA-sequencing as a suitable method to assess viral infections, covering even cryptic and latent viruses of plants.

The aim of our study was to determine the spectrum of mixed cryptic infections of known host plants, utilizing dsRNA extraction and a deep sequencing approach with the Ilumina MiSeq platform performed by GATC Biotech. We used clover species in which mixed cryptic infections had been described before (Boccardo, 1987; Lesker, 2013a): White clover (*Trifolium repens*) is infected by the type members *White Clover Cryptic virus 1* and *2* from the genus *Alphacryptovirus* and *Betacryptovirus*, and at least one other cryptic virus (Boccardo, 1985). The full sequences of both viruses had been determined before (Boccardo, 2005; Lesker, 2013a) and were used as an internal control to verify sequencing results. For a further analysis of the cryptic virus diversity in different plants, we included more cultivars: The closely related red clover (*Trifolium pratense*), the distantly related clover species hop trefoil (*Medicago lupulina*) and as an out-group species from a far related plant family dill (*Anethum graveolens*). To turn limited financial resources into high revenues we pooled the individually extracted dsRNA for the use in one cDNA library preparation. This library was then mixed together with another library 1:1 and sequenced in a 2x150 bp MiSeq run. We expected

that this should result in a depth of 1.8 mio reads (500 megabases) for each individual cultivar to assess the virome from seed-grown cultivars.

4.3 Materials and methods

4.3.1 Plant material

Seeds of white clover (*Trifolium repens* cv. Lirepa), red clover (*Trifolium pretense* cv. Nemaro), hop trefoil (*Medicago lupulina*) and dill (*Anethum graveolens* var. *hortorum*), from different breeders, were germinated and grown under controlled greenhouse conditions.

4.3.2 RNA extraction

Double stranded RNA was isolated from 20 g plant material of each plant species using a cellulose extraction protocol, as described previously in Lesker (2013a). The total RNA was extracted from 0.1 g fresh weight leaf material with the peqGOLD Plant RNA kit (Peqlab, Erlangen, Germany) and TRIzol Reagent (Life Technologies, Carlsbad, USA) following the instruction manuals.

4.3.3 Sequencing

Sequencing was performed by GATC Biotech (Konstanz, Germany) using the Illumina Miseq platform. The concentrations of the dsRNA from the four plant species were determined by photometric measurements (Nanodrop, Thermo) and by agarose gel analysis. Samples were pooled by mixing equal amounts of dsRNAs from the different species. Of the mixed dsRNA material, 1µg was used for the library preparation (Ilumina TruSeq Stranded Total RNA Sample Preparation kit) by GATC using standard conditions and including a heat step for 8 min at 95° C leading to the denaturation and fragmentation of the dsRNA. Together with another library sample, the resulting DNA template was quantified, diluted and hybridized to the flow cell. Clusters were generated using the MiSeq v2 2x150bp sequencing kit.

4.3.4 Bioinformatics

Generated paired-end sequence data were analyzed using an own pipeline: Adaptor cleaned sequences that passed quality filtering were subjected to an error correction by

BayesHammer. Corrected reads were assembled using different assemblers (Ray, SOAPdenove, SPAdes and MaSuRCA). Combined scaffolds were used to search the NCBI database for viral sequence similarities with BLASTn, BLASTx and the Blast2go software (Conesa, 2005). Selected sequences of putative cryptic viruses were further analysed by MEGA5 (Tamura, 2011) to obtain evolutionary relationships. Mapping was performed to enlarge and correct contigs, and to determine the shares of the reads that belong to virus and host genes using Geneious R7 (Biomatters, Auckland, New Zealand).

4.3.5 RT-PCR detection of viral sequences

Selected contigs were used to design specific oligonucleotides to detect each putative virus fragment from the original dsRNA extractions as well as from independent total RNA purifications. Different cDNA libraries from each plant species were prepared with RevertAid Premium Reverse Transcriptase components (Thermo Scientific, Waltham, USA): 5 µl RNA, 10 µl 5X Reaction Buffer, 5 µl 10 mM dNTP Mix, 1 µl Reverse Transcriptase, 1 µl RiboLock RNase Inhibitor, 1 µl Random Hexamer Primer, and 27 µl nuclease-free water. For dsRNAs a 5 min 95°C denaturation step including the Hexamer Primer preceded cDNA synthesis was also included. After 60 min incubation at 50°C, 1 µl of cDNA was added to 5 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific), 3 µl water, 2 µl 10 mM specific oligonucleotide mix. The tubes were heated at 98°C for 10 s, followed by 30 reaction cycles (1 s at 98°C, 15 s at 58°C and 15 s at 72°C) followed by a final elongation step of 15 s at 72°C. The PCR products were analyzed on 1 % agarose gels containing 0.5 mg/l ethidium bromide.

4.4 Results

4.4.1 Sequencing, de novo assembly and annotation

We received preformatted fastaq data form GATC Biotech. The 2x150 bp paired-end data yielded in 15,220,100 reads with 4,566,030,000 sequenced bases. Processing with the bioinformatics pipeline resulted in an assembly of 1567 contigs with a size distribution between 214 and 23,666 bp. Contigs were annotated via BlastX and grouped in host genomic, microorganisms and viruses of the family *Partitiviridae* (subdivided in the proposed genera *Alphapartitivirus*, *Betapartitivirus*,

Deltapartitivirus), the family *Endornaviridae*, the proposed family *Amalgaviridae* and other viruses. Annotated sequences are summarized in Tables 1 to 3.

4.4.2 Mapping of total reads

Grouped contigs were used as reference sequences for a re-mapping of the 30.4 million unpaired MiSeq reads. Despite the use of a specific dsRNA-isolation method most reads map to plant genes (11,653,920) and fewer reads (320,920) identify contigs of microorganism (see also Figure 1). A share of 7 % of reads (2,053,634) could not be mapped to the reference sequences. The largest cluster of viral reads fit to genus *Alphapartitivirus*: 6,666,771 reads map to 16 contigs. Further shares of the reads were assigned to the genus *Deltapartitivirus* (11 contigs with 4,594,566 reads) and *Betapartitivirus* (8 contigs with 3,199,731 reads) resulting in 48 % of the total reads being mapped into the family of *Partitiviridae*. Three contigs of putative *Endornavirus*es could be determined with a total of 3 % of the reads (1,042,179), and four contigs grouped to the genus *Amalgavirus* with 77,904 reads (0.3 %). Additionally, contigs of other viruses were found (results not shown) which fit about 1 % of the total reads. The individual coverage of the contigs varied from 9x (contig Nr. 167) to 250,000x for the contig Nr. 457 (for details see Tables 1 to 3).

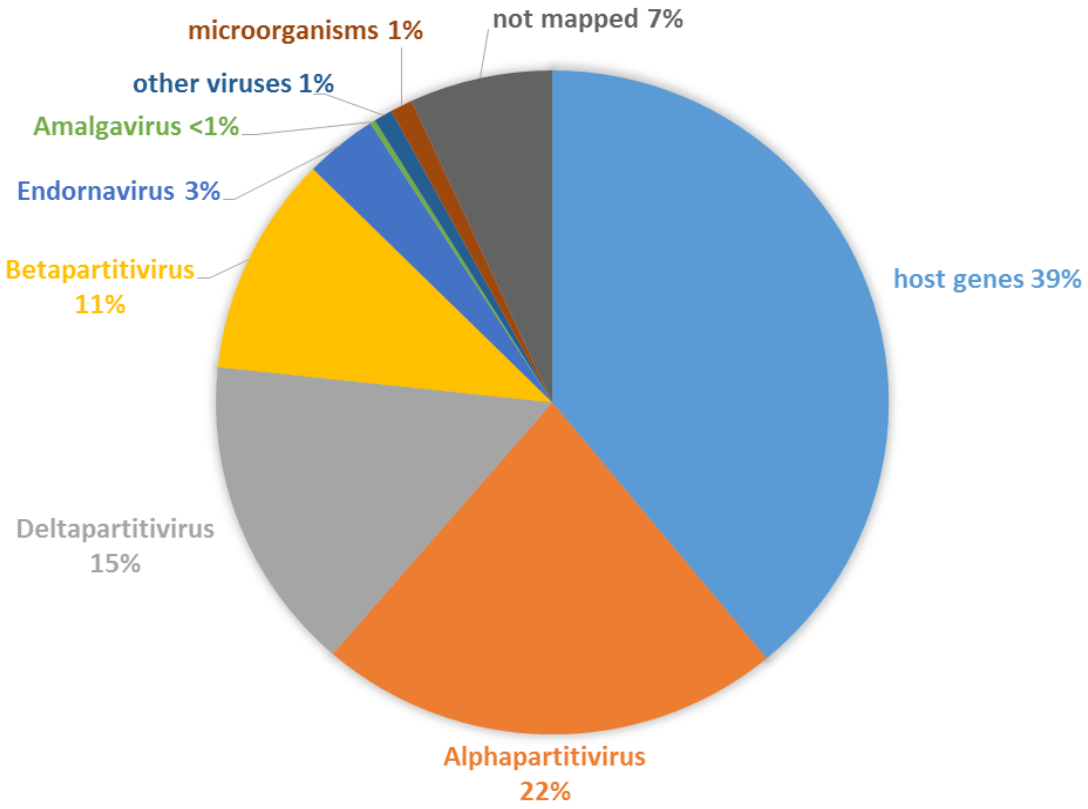


Figure 1: Share of contigs mapping to plants, *Partitiviridae*, *Endornavirus* and *Amalgavirus*

4.4.3 Sequence analysis and phylogenetic classification

For the analyzed cultivars several cryptic viruses have been already described. Sequences of two cryptic viruses, one of the genus *Alphapartitivirus* and one of the genus *Betapartitivirus*, are available for White Clover, Red Clover and Dill. A further *Betapartitivirus* was isolated from Hop Trefoil (Boccardo, 2005; Lesker, 2013a, 2013b). All related 14 genomic fragments were found in the present study with nearly 100 % of sequence identity and genome coverage. Furthermore, an additional 21 contigs with similarities to viruses belonging to the family *Partitiviridae* were identified. After generating of specific primer pairs for each of the contigs, each dsRNA and total RNA preparation of the 4 cultivars were tested by RT-PCR allocating each new putative virus of the family *Partitiviridae* to the different cultivars: 7 viruses were found in Dill and Hop Trefoil, respectively, 6 viruses were identified in Red Clover and an additional one in White Clover (see also Table 1). Together with the already assigned viruses we identified in Dill at least 6, in Hop Trefoil 5, in Red Clover 6 and White Clover 3 putative cryptic viruses by the presence of CP and RdRp sequences.

Obtained contigs fitting to the family *Partitiviridae* were used together with selected GeneBank genomes for a separate global alignment of RdRp (Figure 2) and CP sequences (Figure 3) using the muscle aligner in MEGA5.2. The resulting phylogenetic trees show a diverse relationship of viruses, even of viruses detected in the same host: 4 main clusters of the proposed genera *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus* and *Deltapartitivirus*. No contigs fit to the fungal *Gammapartitivirus* cluster, even if contig-1 from White Clover cannot be clearly assigned to a cluster. All expected 8 *Betapartitivirus* sequences were found in one cluster. The branch of the genus *Alphapartitivirus* is very diverse, whereby at least 3 subclusters can be recognized. Viruses in the first subcluster (A1) are closely related to plant infecting alphacryptoviruses with homology to the type member White Clover Cryptic Virus 1. The second subcluster (A2) consists of more distantly related viruses of plant as well as fungal hosts. The third subcluster (A3) includes the plant pathogenic fungus *Rosellinia necatrix partitivirus* 2 and clearly separates from A1 and A2. Because this subcluster also contained viral sequences which were found within the genome of different host plants, we included a genomic sequence from *Arabidopsis thaliana* (GeneBank gi: 4757414) in the CP phylogenetic analyses (Figure 3). Most of the newly determined sequences were assigned to the subcluster A3. Also the main cluster of the genus *Deltapartitivirus* can now be split into 2 subclusters (D1, D2). This becomes

particularly evident from the RdRp tree (Figure 2). However, in case of D1 and D2 more variability is recognized within the CP tree. We were also able to assign different virus sequences from one host plant to the different subclusters, e.g. contig 65 and contig 457 from Dill.

The assessment of contigs with complete open reading frames from one host plant into the various RdRp and CP subclusters enabled us to establish new potential bipartite viral genomes resulting in 8 new putative cryptic viruses tentatively named Dill Cryptic Virus 3 (CP: contig 18, RdRp: contig 457), Dill Cryptic Virus 4 (CP: contig 175, RdRp: contig 65), Hop Trefoil Cryptic Virus 1 (CP: contig 1, RdRp: contig 84), Hop Trefoil Cryptic Virus 3 (CP: contig 673, RdRp: contig 749), Hop Trefoil Cryptic Virus 4 (CP: contig 25 and 27, RdRp: contig 2), Red Clover Cryptic Virus 3 (CP: contig 52, RdRp: contig 15), Red Clover Cryptic Virus 4 (CP: contig 760, RdRp: contig 688) and Red Clover Cryptic Virus 5 (CP: contig 19, RdRp: contig 755).

Table 1: Contigs match to the family *Partitiviridae*

Genus	Host plant	Contig Nr.	length (bp)	Protein	Best BlastX hit – Virus name (GenBank Accession)	Ident	coverage	Nr. of Reads
Alphapartitivirus	Dill	506	1859	CP	Dill Cryptic Virus 1 (NC022615)	100%	99%	644
		626	1991	RdRp	Dill Cryptic Virus 1 (NC022614)	100%	98%	26.796
		680	1798	CP	Rosellinia necatrix partitivirus 2 (NC020235)	38%	98%	27.924
		766	1999	CP	Rosellinia necatrix partitivirus 2 (NC020235)	30%	99%	1.013
	Hop Trefoil	1	1827	CP	Vicia cryptic virus (EF173390)	72%	99%	13.492
		84	1939	RdRp	Vicia cryptic virus (EF173392)	91%	99%	133.116
		673	1967	CP	Rosellinia necatrix partitivirus 2 (NC020235)	35%	99%	43.930
		749	1907	RdRp	Vicia faba partitivirus 1 (DQ910762)	75%	99%	561.751
	Red Clover	757	2238	CP	Red Clover Cryptic Virus 1 (NC022617)	100%	99%	49.270
		747	2030	RdRp	Red Clover Cryptic Virus 1 (NC022616)	100%	99%	737.985
		52	1909	CP	Diuris pendunculata cryptic virus (JX891460)	32%	99%	3.902
		15	1043	RdRp	Chondrostereum purpureum cryptic virus 1 (AM999771)	39%	54%	15.510
		760	2098	CP	Rosellinia necatrix partitivirus 2 (NC020235)	37%	99%	80.540
		688	1965	RdRp	Vicia faba partitivirus 1 (DQ910762)	74%	99%	143.842
	White Clover	744	1849	CP	White Clover Cryptic Virus 1 (NC006276)	99%	99%	3.947
		634	1960	RdRp	White Clover Cryptic Virus 1 (NC006275)	99%	97%	81.716
Betapartitivirus	Dill	649	2375	CP	Dill Cryptic Virus 2 (NC021148)	100%	99%	19.628
		8	2579	RdRp	Dill Cryptic Virus 2 (NC021147)	100%	99%	625.948
	Hop Trefoil	48	1246	CP	Hop Trefoil Cryptic Virus 2 (NC021099)	100%	53%	9.476
		597	1967	RdRp	Hop Trefoil Cryptic Virus 2 (NC021098)	100%	76%	46.064
	Red Clover	585	2290	CP	Red Clover Cryptic Virus 2 (NC021097)	100%	97%	98.306
		694	2445	RdRp	Red Clover Cryptic Virus 2 (NC021096)	100%	99%	991.009
	White Clover	637	2285	CP	White Clover Cryptic Virus 2 (NC021095)	100%	97%	78.130
		492	2576	RdRp	White Clover Cryptic Virus 2 (NC021094)	100%	99%	1.330.826
Deltapartitivirus	Dill	18	1556	CP	Pepper cryptic virus 1 (JN117277)	46%	99%	108.294
		457	1649	RdRp	Pepper cryptic virus 1 (JN117276)	71%	99%	2.725.723
		175	1053	CP	Raphanus sativus cryptic virus 2 (NC010345)	38%	71%	502
		65	1729	RdRp	Raphanus sativus cryptic virus 2 (NC010343)	68%	99%	4.155
		13	1224	RdRp	Persimmon cryptic virus (NC017989)	70%	78%	3376.70
	Hop Trefoil	25	1560	CP	Beet cryptic virus 2 (HM560702)	44%	98%	82.450
		2	1580	RdRp	Beet cryptic virus 2 (HM560703)	78%	99%	482.616
		27	1635	CP	Beet cryptic virus 2 (HM560704)	40%	99%	12.321
	Red Clover	19	1855	CP	Pepper cryptic virus 1 (JN117277)	35%	99%	146.448
		755	1728	RdRp	Pepper cryptic virus 1 (JN117276)	56%	99%	692.789
	White Clover	118	1884	RdRp	Penicillium stoloniferum virus F (NC007221)	27%	99%	1.451

Table 2 Contigs match to the family *Amalgaviridae*

Genus	Host plant	Contig Nr.	leghts (bp)	Protein	Best BlastX hit – Virusname (GenBank Accession)	Ident	coverage	Nr. of Reads
Amalgavirus	Hop Trefoil	590	3488	Fusion	Southern tomato virus (NC011591)	52%	99%	8,617
	Red Clover	858	1467	Fusion	Southern tomato virus (NC011591)	59%	43%	369
	White Clover	167	3471	Fusion	Southern tomato virus (NC011591)	51%	99%	204
	White Clover	32	3296	Fusion	Southern tomato virus (NC011591)	46%	96%	31,159

Table 3 Contigs match to the family *Endornaviridae*

Genus	Host plant	Contig Nr.	leghts (bp)	Protein	Best BlastX hit – Virusname (GenBank Accession)	Ident	coverage	Nr. of Reads
Endornavirus	Red Clover	177	7495	Poly	Grapevine endophyte Endornavirus (NC019493)	79%	61%	1,088
	Red Clover	759	12862	Poly	Astrovirus MLB1 (FJ402983)	24%	99%	428,563
	White Clover	7	15110	Poly	Bell pepper Endornavirus (AB597230)	45%	99%	661,195

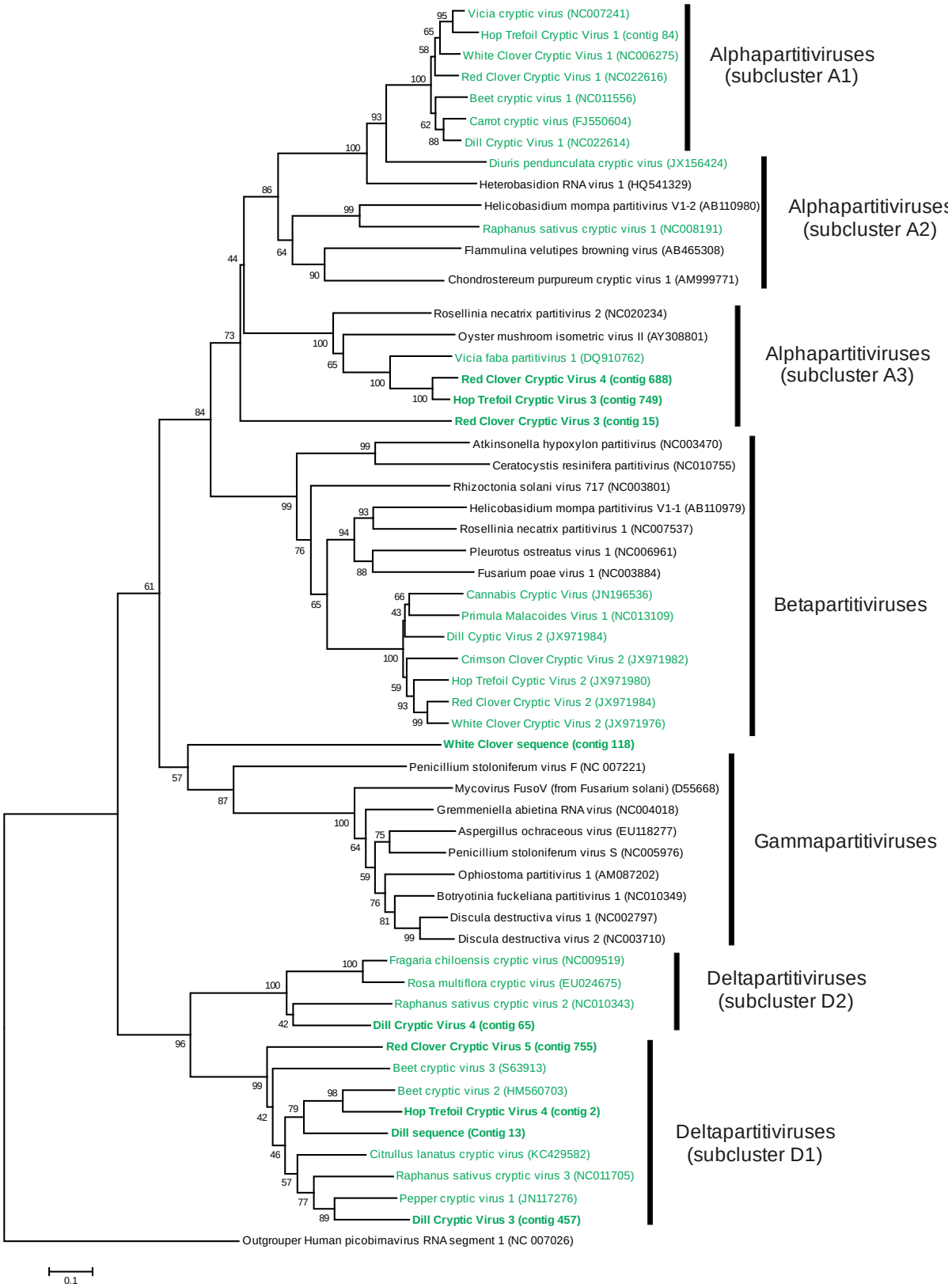


Figure 2: Evolutionary relationship of taxa using RdRp sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bar represents base substitutions per site. All ambiguous positions were removed for each sequence pair. Putative plant infecting viruses are in green color. New sequences are in bold letters.

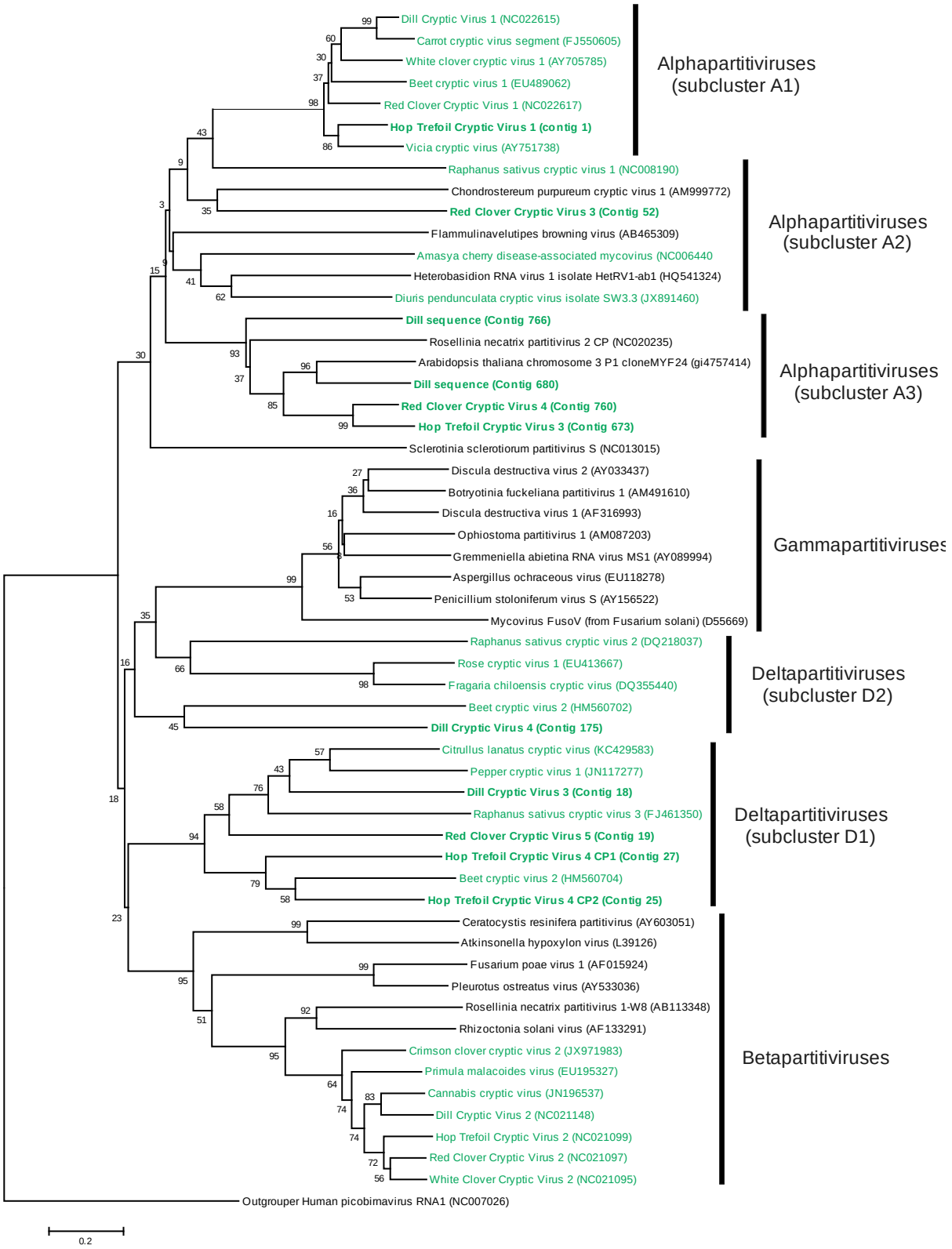


Figure 3: Evolutionary relationship of taxa using CP sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bar represents base substitutions per site. All ambiguous positions were removed for each sequence pair. Putative plant infecting viruses are in green color. New sequences are in bold letters.

4.4.4 Discussion

We successfully performed deep sequencing using from dsRNA as starting material to assess the cryptic virome of 3 clover species and dill. This was the first metagonomic approach focused on persistent plant viruses from the family *Partitiviridae*, the genus *Endornavirus* and the proposed genus *Amalgavirus*. We used non infected plant material grown from seeds under controlled greenhouse conditions, to prevent infection with natural occurring common viruses.

Suitability of sequence purity for virus determination

The Illumina MiSeq platform provided by GATC delivered over 15 million paired-end reads. To limit costs, four samples were mixed to create one library, where theoretically each virome consists of 3.8 million read pairs. Despite the use of a specific dsRNA isolation method, 39 % of all reads aligned to plant genes, mostly to chloroplast derived nucleic acids. This was also the case in other studies, dealing with dsRNA sequencing. After a deep sequencing of dsRNA isolated form grapevine, 14 % of the reads mapped to the host genome (Coetzee, 2010). Our percentage is at least twice as high although we did not use infected plant material from fields, where dsRNA may occur in larger amounts. However, at least 0.7 to 1.9 million read pairs fit to viruses found in each cultivar. In total, 53 % of all reads match to viral sequences. This share of viral sequences is higher as reported in other studies using siRNA or virus enriched total RNA, with a maximum of 40 % in case of infected plant material (Kreuze, 2009; Adams, 2009). Persistent viruses are in general quite lower in titer (Roossinck, 2011, Lifestyle) underlining the suitability of deep sequencing approaches for these entities. Besides Partitiviridae and Endornaviruses no other viruses were found which could be clearly assigned to mycoviruses. This likely indicates a plant origin of the found viruses.

The genome coverage of obtained contigs concerning comparison with their reference sequences is better when using dsRNA as a template than with contigs from total or siRNA. Moreover, dsRNA represents the full genome or replicative intermediate forms of a virus, leading to an increase of correctness and completeness of the determined sequences. In our study only 7 contigs out of 42 missed substantial genome parts, the vast majority (83 %) of the genome fragments seem to be nearly complete with a method-inherent absence of approx. 20 bp from the 5’- and 3’-end, respectively. Therefore, dsRNA sequencing is most suitable for the detection of unknown plant viruses.

Large diversity of cryptic infection

We were able to detect at least 8 putative new members of the family *Partitiviridae*, 4 putative new viruses of the proposed genus *Amalgavirus* and 3 putative new members of the genus *Endornavirus*. Four additional sequences, which were assigned to the genera *Alphapartitivirus* and *Deltapartitivirus* were also found, indicating that additional cryptic viruses may be present in the virome of these cultivars. A large diversity of persistent viruses was identified in the tested cultivars: Dill contains at least 6 persistent viruses of the family *Partitiviridae*, Hop Trefoil harbors 5 viruses (4 from genera of the *Partitiviridae* and 1 from the genus *Amalgavirus*), Red Clover contains 8 persistent viruses (5 belonging to the *Partitiviridae*, 1 belonging to *Amalgavirus* and 2 belonging to *Endornavirus*) and White Clover contains 6 viruses (3 from the *Partitiviridae*, 2 fitting to the *Amalgavirus* and 1 to *Endornavirus*). Mixed infections with up to 5 plant cryptic viruses in one host have been reported before, for example in clover and carrot (Boccardo, 1987). However, reporting the present study we report for the first time sequences of more than 3 viruses of the family *Partitiviridae* in one host plant. For fungal hosts, multiple sequences of up to 9 Partitiviruses have also been reported before (Vainio, 2013; Yaegashi, 2013). This data imply a more in-depth picture of the large diversity of persistent viruses in their hosts, which might be extended by further studies.

Putative horizontal transmission events

The significance of such diversity can be discussed from different points of view. The basis of the theory is an interaction triangle consisting of the viruses, their hosts: plants, pathogenic or symbiotic fungi and the environmental conditions. One, so far unresolved point is the putative source or origin of these plant persistent viruses. The high sequence similarity of individual viruses found in different plant families strengthens the hypothesis of a vector mediated transmission. This could be evidence of horizontal virus transmission from a plant pathogenic or symbiotic fungus, especially when the close genetic linkage to the fungal viruses within the family *Partitiviridae* (Ghabrial, 1998; Roossinck, 2010, Lifestyle) is considered. This applies in particular for the subcluster A1 in genus *Alphapartitivirus* and the group of plant borne viruses within the fungal *Betapartitivirus*es (see Picture 3 -4). Phytopathogenic fungi can act as vectors for a number of different plant viruses (Lubicz, 2007; Rochon, 2004). However, to establish a permanent infection a virus has to enter the germ line cells, e.g. by exchange during plant-fungus interaction (Kankanala, 2009). This event would be rarer than a

transmission from a plant to a fungus. Because once the virus transmitted from a plant to fungal cells, the virus could go through the cell wand free mycel into cells which produce spores to become persistent (Roossinck, 2012).

Horizontal gene transfer of viral sequences into plant genomes

Another aspect is the insertion of viral sequences into the plant host genome. Many of these genome integrations contain parts similar to the coat protein open reading frames of the fungal *Rosellinia necatrix pratitivirus* 2 (Liu, 2010). We determined 5 contigs (see Figure 3, subcluster A3) that show a strong relationship to genomic sequences, e.g. of the chromosome 3 from *Arabidopsis thaliana*. These newly characterized sequences could be helpful to detect more of such integrations. However, phylogenetic data indicate a transfer of sequences to the plant genomes (Chiba, 2011). Most genomic integrations encode only parts of viral references. Moreover no integration of viral sequences could be found in the genome of plants, which harbor the virus themselves (Rossinck, 2012). This suggests an ancient origin for these viruses rather than a release from their hosts.

Host influence of cryptic viruses

Nevertheless, many of the viral integrations in genomes seem to be expressed like genes, indicating a potential function for the plant. The role of plant viruses in plant ecology, including potential beneficial effects was proven by other studies (Xu, 2008; Roossinck, 2011, Nat Rev). An interaction can be advantageous for plants in special environmental conditions. A transcription of the coat protein of the White Clover Cryptic Virus 1 can regulate root nodulation in the presence of adequate nitrogen (Nakatsukasa-Akune, 2005). Moreover, in plants the additional presence of an endophytic fungus resulted in an increased thermal tolerance after viral infection (Marquez, 2007). Furthermore, persistent viruses are involved in the plant defense mechanism. Infections by cryptic viruses can result in hypervirulent effects through the interaction of the pathogenic fungus with a host plant. The same studies speculate about a potential use of persistent viruses to control fungal diseases (Ghabrial, 1998). Although this metagenomic study reveals a more detailed picture of the plant cryptic virus diversity, further investigations are needed to understand the biology of these and other persistent viruses.

5 General discussion

5.1 Revision of the taxonomy of the family *Partitiviridae*

The first part of this thesis had its focus on the sequence determination of members of the genus *Betacryptovirus* in the family *Partitiviridae*. Numerous sequences have been determined for viruses of the genus *Alphacryptovirus* so far, but no sequence was available for any assigned member of the genus *Betacryptovirus* (Ghabrial, 2008). In this study the complete sequences of five viruses were determined and classified into the genus *Betacryptovirus*. Three dsRNA pairs with typical sizes of Betacryptoviruses (Boccardo, 1985) were isolated from already known hosts: white clover, red clover and hop trefoil (Boccardo, 1983, 1985; Luisoni , 1987). Furthermore, two new putative viruses of the genus *Betacryptovirus* were unexpectedly identified from dill (*Anethum graveolens)* and crimson clover (*Trifolium incarnatum).* Additionally, comparative sequence analyses indicated that viruses identified from Primula and Cannabis (Li, 2009; Ziegler, 2011) are potential members of the genus *Betacryptovirus*.

Previous phylogenetic analyses of the family *Partitiviridae* divided the genera *Alphacryptovirus* as well as the genus *Partitivirus* into two clearly distinct clusters (Ghabrial, 2008, 2012). Additionally, the findings of this thesis show that the genus *Betacryptovirus* forms an own sub-cluster, which is closely related to the Partitivirus I cluster. Therefore, the type members of the genera *Betacryptovirus* (WCCV2) and *Partitivirus* (AhV) are closer related to one another than viruses of different clusters of the genera *Alphacryptovirus* and *Partitivirus*. The current taxonomic classification does not satisfactorily reflect these findings. Further characteristics such as dsRNA size, presence of interrupted poly(A) stretches and particle sizes support the need for a revision of the taxonomic structure of the family *Partitiviridae*.

The results gathered in this study fill a gap in the virus taxonomy of *Partitiviridae* revealing a relationship of plant cryptic viruses to fungal viruses. In collaboration with US-American colleagues a proposal was submitted to the ICTV which suggests an adaption of the taxonomy of these viruses based on the findings of this thesis (Nibert, 2013).

However, further phylogenetic analyses of sequences stored in GenBank indicate more putative members of the family *Partitiviridae*. In most cases sufficient data about the host, particle size and further relationships are missing. Therefore, these sequences cannot be assigned officially to viruses within the family *Partitiviridae* by the ICTV. This problem is especially acute for putative viral sequences obtained from deep sequencing approaches (Brister, 2010). Nevertheless, metagenomic data of viral populations provide important information for taxonomic approaches.

Cryptic viruses are widespread in mono- and dicotyledonous plant species, in some cultivars up to five viruses were detected. Because of the missing transferability of cryptic viruses, each virus that can be found in one host represents a new species in the family. If we push our thinking to the increasing upcoming sequence data of viruses, this will result in an inflation of newly described viruses. To circumvent this problem, it might be helpful to define a quasi-species for each cluster in a virus family. All new determined cryptic-like sequences in the future could be assigned directly to synthetic template species if relevant thresholds are reached (Fauquet, 2005; MacDiamid, 2013).

5.2 Biodiversity of cryptic viruses

The deep sequencing results obtained from seed grown cultivars under controlled conditions show a large diversity of viruses belonging to the family *Partitiviridae* within each host plant. Up to five sequence pairs in a single host plant represent five individual viruses, which is in accordance with other observations in cultivars such as carrots (Natsuaki, 1990). Each virus can be assigned to a specific sub cluster in phylogenetic analyses. Interestingly some clusters contain plant host as well as fungal host infecting viruses (*Alphapartitivirus*, *Betapartitivirus*), whereas other clusters include only plant or fungal host infecting viruses (*Gammapartitivirus*, *Deltapartitivirus*). Moreover the viruses of each sub cluster are sometimes very similar to one another even if the harboring host plants belong to different plant families (sub cluster A1, cluster of betacryptoviruses, see 4.4.3 Figure 1), whereas other clusters contain very distinct members (sub cluster A2). High sequence similarities can indicate a common origin, especially when looking at isolated sub clusters. However, the information on the sequence stability of cryptic viruses is very limited. Therefore, we should exercise a degree of caution when evaluating phylogenetic approaches like molecular clocks. Sequence determinations of *Blueberry latent viruses*, a member of the

putative cryptic like genus *Amalgavirus*, show an extremely stable population structure. The diversity does not exceed 0.5% among all isolates obtained from all over the USA and Japan (Martin, 2012). However, a sub cluster of plant infecting cryptic viruses within a main cluster which includes hosts of numerous plant pathogenic fungi suggests the possibility of a potential horizontal transmission event.

Another point is the diversity in the composition of types of cryptic viruses which were found in different cultivars. Some plant species e.g. carrot (Natsuaki, 1990) contain almost all members of the different sub cluster, whereas in other host plants e.g. spinach (Natsuaki, 1983) only one cryptic virus could be determined (Boccardo, 1987). Furthermore, a change in the occurrence of cryptic viruses is also observed. While in earlier studies *Beet Cryptic Virus*1 (BCV-1) occurred frequently in sugar beet cultivars (Lukacs, 1994), it is now very rarely found in actual cultivars (Szego, 2010). This drift in a viral population may be explained by human influences with changing varieties and/or potential interaction with altered fungal species or pathotypes. The disappearance of BCV-1 might be connected with the parallel introduction of rhizomania-resistant beet cultivars using the Rz1 gene (Szego, 2010). However, it is purely speculative to draw a connection between these two phenomena. Nevertheless, in the context of results from other studies (see Introduction point 1.4), it can be hypothesized that the diversity of cryptic viruses is influenced by environmental conditions and not only by their capability of seed transmission.

5.3 Transmission routes of cryptic viruses – throughout the plant, to a fungus and into the plant genome

5.3.1 Vertical transmission in the host plant - the hitchhiker strategy

The cryptic lifestyle of plant infecting *Partitiviridae* is one key feature of their wide distribution throughout the plant kingdom (see introduction point 1.6.1). The deprivation of plant defenses via in-particle transcription ensures the virus propagation in the cell, but also prohibits any active transport mechanism for the distribution within the plant (Roossinck, 2010). This feature abolishes the necessity of any further protein, and only the RdRp for genome replication and transcription and CP as the protecting capsid are needed to build up a full virus. However, cryptic viruses have to become hitchhikers during cell propagation, even in the meristem up to the gametes to ensure

their “survival”. Meristems are normally devoid of systemic pathogens, probably due to the absence of differentiated conducting tissues (Alam, 2013). However, cryptic viruses even overcome thermotherapy. Thermal treatment that produces a greater viral degradation when repeated over time can lead to elimination of “classical” viruses (Kassanis, 1957; Boccardo, 1987). The underlying mechanisms, which could stabilize cryptic viruses have not yet been resolved. Structural analysis of the virus particle confirms hidden transcription and replication. The results of the protein-interaction study (Part 3) verified the necessary CP self-interactions for the assembly of the virus. However, the propagation during cell division is still unclear. In recent studies cryptic particles were localized in the cytoplasm (Natsuaki, 1985) or seem to be associated with the nucleus (Boccardo, 1987). The localization of protein-interactions in our BiFC approach confirms these findings. The association with cell components may indicate a passive distribution during cell division. However, cryptic viruses seem to be well adapted to their hosts and fit best to the infect-and-persist strategy (Hillemann, 2004).

5.3.2 Horizontal transmission between plant and fungi – a chicken-and-egg problem

A linkage of plant infecting cryptic viruses to fungal viruses of the genus *Partitivirus* has been demonstrated by numerous phylogenetic studies. Also the sequence determination of the genus *Betacryptovirus* (Part 2) and the additional deep sequencing approach (Part 4) indicate a common origin of *Partitiviridae* from plant and fungal hosts. However, experimental approaches to analyze such a transmission of cryptic viruses (Mel'nichuk, 2005) were unable to provide sufficient evidence for a horizontal transmission (Szego, 2010). On the other hand, phytopathogenic fungi can act as vectors of a diverse range of different plant viruses (Rochon, 2004). Therefore, an uptake or delivery of viruses by fungi may also apply for viruses of the family *Partitiviridae* provided that the virus is capable of entering the gametes to establish a permanent infection. Nevertheless, such an event seems very rare or only has taken place in ancient times. Overall, there is no evidence for a direct transmission of cryptic viruses from plants to fungi, no virus was found in both kingdoms via sequencing approaches so far. Despite of large amounts of determined sequences of cryptic viruses from fungal origin, most studies put their focus on pathogenic mushrooms. However, it is more likely that an endosymbiotic species acts as a vector. However, the endosymbionts could be very different and host-specific. Findings of similar viruses in different distinct families do

not support the notion that these plant species are infected or contaminated by a common specific endosymbiotic fungus (Boccardo, 1987). Furthermore, viruses of the genus *Alphacryptovirus* obtained from plant protoplasts show high sequence similarities to fungal partitiviruses (Abou-Elnasr, 1985).

Even if horizontal transmission occurs, the question about the origin still remains. Is the plant the original host or the vector of cryptic viruses? DsRNA viruses are commonly found in fungi without the need of any movement protein (see Introduction point 1.2). However, such a virus can only become persistent by an interaction of the harboring fungus with the plant gametes. The opposite case appears more likely: An infected plant will release the virus through an interaction with a pathogenic or endosymbiotic fungus. Once the viruses or viral transcripts enter a fungal cell, it can be easily speared through the mycelium to the spore producing cells and therefore become persistent (Roossnick, 2012).

Despite of the fungal linkage of plant cryptic viruses, other possible vectors should be kept in mind. Plant feeding insects can or other viruses can serve as a vector for plant cryptic viruses. In a study of the Melon necrotic spot virus (MNSV), which is naturally transmitted by the fungus vector (*Olpidiumradicale*), a new cryptic virus was found only in mechanical with MNSV inoculated melons but not in MNSV free plants (Sela, 2013).

Nevertheless, an experimental evidence for all putative transmission routes is still missing. Upcoming studies with the use of reverse genetic approaches, like infection assays (Potgieter, 2013) and further localizing studies *in planta,* e.g. with the applied BiFC system, will shed light on these transmission events.

5.3.3 Insertions of sequences of cryptic viruses in plant genomes – the transmission terminus

An additional aspect of cryptic viruses is the horizontal gene transfer (HGT) to eukaryotic nuclear genomes. Coat protein-like sequences of cryptic viruses were found in different plants, such as *Arabidopsis, Brassica* ssp. and *Nicotiana* ssp. (Chiba, 2011; Liu, 2010). It is generally assumed that the direction of the HGT is from the virus into the host, and not by the virus capturing genes: Most insertions represent pseudo genes that include deletions, internal stops and frame shifts. The patchy phylogenic distribution indicates a recently occurred introduction, rather than an escaped gene.

Cryptic RdRps belong to the picorna-like superfamily, and have similar topologies as the Totiviruses, which suggest an ancient origin (Liu, 2010). Moreover, no integrated sequences were found in plants which harbor cryptic viruses themselves (Roossinck, 2012).

The integration of genome parts most likely occurred when the viruses were first introduced into non-infected species, probably as a result of an antiviral immune response. The mechanism is quite unclear, because the integration of viral RNA sequences into the host genome has to involve reverse transcription and recombination events (Liu, 2010). Nevertheless, cryptic viruses intimately associated with their host cell over a long evolution time (Ghabrial, 1998). This increases the likelihood of an integration event, for example triggered by a helper virus, which uses revers transcription or during cell division mechanisms (Liu, 2010). Observations of virus particles and protein-interactions associated with the nucleolus may indicate the subcellular site for such events (Boccardo, 1987; Part 3).

However, based on the close genetic relationship between viruses from genera of the family *Partitiviridae* that infect plants and fungi, further investigations concerning plant-fungus interactions will provide evidence for the potential of horizontal inter-kingdom transmission and integration of cryptic viruses. The findings of this study delivered new viruses that show similarities to potential plant genome localized sequences. This applies in particular for subcluster A3 (see point 4.4.3, Figure 2), where, for the first time, four new CP sequences were identified from plant hosts which show similarities to sequences in the genome of *Arabidopsis*.

5.4 Significance of diversity of persistent viruses in plants

In the past, viruses were commonly described as pathogens. Their negative impact is mainly caused by the effect on plant defense suppression and interference with plant metabolic processes during replication and transmission. The “persistent strategy” avoids such significant influences on their hosts, because no active transport or suppressor activities are needed. Cryptic viruses seem to be almost perfectly adapted to their harboring host plants. However, our knowledge concerning the origin and importance of the large diversity of persistent viruses in plants is incomplete. Recent studies have led to another view on viruses other than a cause of disease associated patterns. Viruses may interact with their hosts like epigenetic elements in nature,

providing characteristic features to them which are beneficial under specific conditions (Márquez, 2007; Roossinck, 2010). Therefore, also an integration of viral sequences into the host genome can be justified. Many of the viral integrations in genomes seem to be expressed like regular genes, indicating a potential function for the plant (Liu, 2010).

The role of plant viruses in plant ecology was elegantly shown by other studies (Xu, 2008; Roossinck, 2011). At first an interaction with viruses can be helpful for plants in case of special environmental conditions. An expression of the coat protein of the *White Clover Cryptic Virus 1* in lotus can regulate root nodulation in the presence of adequate nitrogen supply through the plant hormone abscisic acid. In the opposite direction the level of the virus titer was reduced during nodulation in white clover (Nakatsukasa-Akune, 2005). Beet plants infected with cryptic viruses show reduced yield under normal conditions, but no effect could be found if the plant were exposed to drought stress (Xie, 1994). Moreover, the additional presence of an endophytic fungus resulted in a thermal tolerance of plants after viral infection (Marquez, 2007).

Also other persistent viruses which belong to the family *Endornaviridae* have an impact on their hosts. The presence of an *Endornavirus* is correlated with male sterility in *Vicia faba* by an unknown mechanism (Pfeiffer, 1998).

Another issue concerns the involvement of persistent viruses in the plant defense mechanism. In the presence of other pathogenic viruses, the amount of dsRNA is increased (Boccardo, 1987) which makes it even possible for the cryptic viruses to infect the plant (Sela, 2013).Infections by cryptic viruses can result in hypervirulence effects as demonstrated by the interaction of a pathogenic fungus with a host plant. This may have the potential for controlling fungal diseases (Ghabrial, 1998).Therefore, the diversity of cryptic viruses in specific hosts might be an adaptation to the presence of a fungal pathogen like the disappearance of the *Beet cryptic virus 1* by the introduction of rhizomania-resistant cultivars (Szego, 2010).

Even if metagenomic studies reveal a more detailed picture of the plant virus diversity, further investigations are needed to understand the biology of persistent viruses. Interaction and *in planta* studies e.g. with the BiFC system will shed more light on this interesting virus group. These viruses could have the potential to play an important role in interactions between plants, endophytes, pathogens under specific environmental conditions.

6 Outlook

Although persistent viruses are very common, the knowledge about the biology is very limited. Future research will go undoubtedly in the direction of their potential role as mutualists rather than agents of diseases (Roossinck, 2010). Even if more and more metagenomic data will become available the main pitfall of studies concerning plant cryptic viruses is the lack of a revers genetic system. Firstly isogenic virus free lines of a model plant, which naturally harbors cryptic viruses is needed. Due to the lack of a response to the usual virus elimination methods like thermotherapy, new techniques e.g. the use of artificial micro RNAs in combination with callus regeneration may help to achieve a breakthrough (Ossowski, 2008). Moreover, an efficient infection system has to be established for testing the effect on the hosts, like it was performed for the related fungal viruses (Sasaki, 2006). Due to the fact of a limited transmission of persistent viruses further ecogenomic studies may be able to provide an evidence for a putative vector transmission event. Deep sequencing approaches may be able to detect proposed endosymbioic of pathogenic fungi, which can be tested for the presence of viral sequences that were previously determined from their host plant. This step will help to evaluate the biological potential of persistent viruses, which may lead to a probable use as cross-protection agents against plant-pathogenic fungi (Ghabrial, 2008) or as inducers of stress-tolerance to environmental conditions (Marquez, 2007; Roossinick, 2012). Therefore further approaches are needed to establish viromics as an interface to optimize plant cultivation systems.

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PUBLICATIONS

Monograph:

Ghabrial S.A., Nibert M.L., Maiss E., **Lesker T.**, Baker T.S., Tao Y.J. (2012): Family Partitiviridae. In: *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses (ICTV)*, ed. by King A.M.Q., Adams M.J., Carstens E.B., Lefkowitz E.J. (Elsevier, Amsterdam, 2012), pp. 523–534

Further publications:

A) Publications with peer review process

1. **Lesker, T.**, Maiss E. (2013): In planta protein interactions of three Alphacryptoviruses and three Betacryptoviruses from White Clover, Red Clover and Dill by Bimolecular Fluorescence Complementation Analysis. In: Viruses, Volume 5, Issue 10 Page 2512-2530

2. **Lesker, T.**, Rabenstein F., Maiss E. (2013): Molecular characterization of five betacryptoviruses infecting four clover species and dill. In: Archives of Virology, Volume 158, Issue 9, Page 1943-1952

B) Publications without peer review process

1. **Lesker, T.**, Maiss E. (2013): Deep Sequencing of double-stranded RNA as a tool to assess the presence of unknown RNA viruses in plants. In: Proceedings of the 45th Annual Meeting of the DPG - working group "Virus Diseases of Plants" 14.-15.10.2013, Julius Kühn - Institut Braunschweig

2. **Lesker, T.**, Maiss E. (2013): Protein interactions of alphacryptoviruses and betacryptoviruses from white clover, red clover and dill by bimolecular fluorescence complementation analysis. In: Proceedings of the 45th Annual Meeting of the DPG - working group "Virus Diseases of Plants" 14.-15.10.2013, Julius Kühn - Institut Braunschweig

3. Blockus S., **Lesker, T.**, Maiss E. (2013): Determination of full-length nucleotide sequence of Asparagus virus 1. In: Proceedings of the 45th Annual Meeting of the DPG - working group "Virus Diseases of Plants" 14.-15.10.2013, Julius Kühn - Institut Braunschweig

4. Rentz P., Herbst R., Hohe A., Haage U., **Lesker, T.**, Maiss E. (2013): Screening of Epiphyllum sp. by dsRNA isolation of virus infections. In: Proceedings of the 45th Annual Meeting of the DPG - working group "Virus Diseases of Plants" 14.-15.10.2013, Julius Kühn - Institut Braunschweig

5. **Lesker, T.**, Maiss E. (2012): Molecular characterization of potential dsRNA virus from Trifolium sp. - Cryptic virus M is an amalgam of Totiviridae and Partitiviridae. In: Proceedings of the 44th Annual Meeting of the DPG - working group "Virus Diseases of Plants" 08.-09.03.2012, Julius Kühn - Institut Quedlinburg

6. **Lesker, T.**, Maiss E. (2011): Molecular analysis of the genus Betacryptovirus of the family Partitiviridae. In: Proceedings of the 43rd Annual Meeting of the DPG - working group "Virus Diseases of Plants" 31.03.-01.04. 2011, Georg-August-University of Göttingen; DPG working group reports (2011) 140-141

7. **Lesker, T.**, Blockus S., Maiss E. (2011): DsRNA - Screening of Asparagus officinalis. In: Proceedings of the 43rd Annual Meeting of the DPG - working group "Virus Diseases of Plants" 31.03.-01.04. 2011, Georg-August-University of Göttingen; DPG working group reports (2011) 141

8. **Lesker, T.**, Göing J., Rose H., Schneider C., Korte J., Maiss E. (2010): DsRNA screening - Isolation, molecular characterization and phylogenetic analysis of possible dsRNA viruses in vegetables, herbs and ornamentals. In: Proceedings of the 57th German Plant Protection Conference 06.-09.09.2010, Humboldt University of Berlin; Julius Kühn-Archiv No. 428 (2010) 404-405

9. **Lesker, T.**, Myrach T., Menzel W., Willenborg J., Vetten H.-J., E. Maiss (2009): Cloning and phylogenetic analyses of putative plant cryptic viruses. In: Proceedings of the 5th Joint meeting of the DPG working group “Virus Diseases of Plants“and the „Dutch Association for Plant Virology, 08.-09.04.2009, Hamburg; DPG working group reports (2009) 76-77

C) Bachelor and Master thesis

1. **Lesker, T.** (2008): Continuative characterization of the vicia cryptic virus and molecular analysis of putative cryptic viruses form Apium graveoens, Cucumis sativus, Petroselinum crispum and Spinacia oleracea. In: Master thesis No 13 of Institute of Plant Diseases and Plant Protection, Gottfried Wilhelm Leibniz Universität, Hannover, Germany

2. **Lesker, T.** (2006): Determination of the host range of Potato Virus Y and distribution of Agrobacterium tumefaciens in Nicotiana tabacum "Samsun nn" after agroinfection. In: Bachelor thesis No 15 of Institute of Plant Diseases and Plant Protection, Gottfried Wilhelm Leibniz Universität, Hannover, Germany

The following above mentioned publications have evolved from my doctoral dissertation:
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