Universidade de Évora

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Carla Marisa Reis Varanda

Thesis presented to obtain the PhD degree in
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Supervisor: Professor Maria Ivone Esteves da Clara
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Thesis publications

The present work is based on the following manuscripts:

- **Varanda, C.M.R**.; Félix, M.R.F.; Clara, M.I.E. (2008). DsRNA analysis and RT-PCR assays to evaluate viral infections: the case of necroviruses in olive. Journal of Plant Pathology, 90 (2): 385.

- **Varanda C.M.R**., Cardoso, J.M.S.; Félix, M.R.F.; Oliveira, S.; Clara, M.I.E. (2010). Multiplex RT-PCR for detection and identification of three necroviruses that infect olive trees. European Journal of Plant Pathology, 127: 161-164.

- **Varanda, C.M.R**.; Silva, M.S.M.R; Félix, M.R; Clara, M.I.E.(2011). Evidence of *Olive mild mosaic virus* transmission by *Olpidium brassicae*. European Journal of Plant Pathology, 130: 165-172.

- **Varanda, C.M.R**.; Félix, M.R.; Soares, C.M.; Oliveira, S.; Clara, M.I.E. Specific amino acids of *Olive mild mosaic virus* coat protein are involved on transmission by *Olpidium brassicae*. Journal of General Virology (major revisions).

Other publications

- Félix, M.R., **Varanda, C**., Cardoso, J.M.S., Clara, M.I.E. (2004). Soil transmission of an olive isolate of Olive latent virus 1. "Plant Protection towards the 21st century", Proceedings of the 15th International Plant Protection Congress p 447.

- Félix, M.R.; Cardoso, J.M.S.; **Varanda, C.M.R**.; Oliveira, S.; and Clara, M.I.E (2005). Complete nucleotide sequence of an Olive latent virus 1 isolate from olive trees. Archives of Virology, 150 (11): 2403-2406.

- Félix, M.R.F.; **Varanda, C**.; Cardoso, J.M.S.; Clara, M.I.E. (2006). Plant root uptake of *Olive latent virus* 1 and *Olive mild mosaic virus* in single and mixed infections. Proceedings of XII MPU Congress, Rhodes, Greece, 516-517.

Abstract

OMMV is an olive infecting necrovirus, likely a recombinant between TNV-D, with which it shares a high identity in its coat protein (CP), and OLV-1. Design of specific primers to use in RT-PCR, allowed for the first time molecular discrimination of OMMV from TNV-D. RT-PCR application to an olive orchard survey revealed OMMV predominance. Its transmission by *Olpidium brassicae* was demonstrated here for the first time and further examined. OMMV wild-type, a natural non-transmissible OMMVL11 mutant containing two mutations, a construct OMMVWT/OMMVL11 and single mutants obtained by site-directed mutagenesis were found similarly infectious. Sequencing, homology modelling and transmissibility assays showed that the single mutation of Asn residue to Tyr in position 189 of the CP amino acid sequence was located internally in the particle and abolished virus-zoospore adsorption and transmissibility. This indicates that mutation altered CP conformation on zoospore recognition sites. These findings may have important epidemiological implications in the olive crop.

Caracterização molecular da transmissão de *Necrovirus* (*Tombusviridae*) de *Olea europaea* L. por fungos vectores

OMMV é um necrovirus que infecta a oliveira e que terá surgido a partir de recombinação entre TNV-D, com o qual compartilha uma elevada identidade na cápside proteica (CP), e OLV-1. O desenho de 'primers' específicos para utilização em RT-PCR permitiu, pela primeira vez, a discriminação entre OMMV e TNV-D. A aplicação de RT-PCR a um olival revelou a predominância de OMMV. A transmissão por *Olpidium brassicae* foi aqui pela primeira vez demonstrada e examinada. OMMV 'wild type', um mutante natural OMMVL11 não transmissível contendo duas mutações e mutantes obtidos por mutagénese dirigida revelaram-se igualmente infecciosos. Sequenciação, modelagem por homologia e ensaios de transmissão mostraram que a mutação do resíduo Asn para Tyr na posição 189 da sequência de aminoácidos da CP se localiza no interior da partícula e inibe a adsorção virus-zoosporo e a transmissão. Estes resultados indicam que a mutação alterou a conformação da CP em locais de reconhecimento do zoosporo. Os resultados aqui obtidos podem ter importantes implicações na cultura da oliveira.

Abbreviations

≈	approximately
aa	amino acid
bp	base pair
BSA	bovine serum albumin
^{o}C	degree Centigrade
ca.	*circa*
cDNA	complementary DNA
CP	coat protein
cv.	cultivar
DAS-ELISA	double antibody sandwich-Enzyme linked immunosorbent assay
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxyribonucleiotide triphosphate
dsRNA	double-stranted RNA
DTT	dithiothreitol
E	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
e.g.	example given
EU	European Union
IgG	immunoglobulin G
g	gram
g	gravity acceleration
h	hour
IPTG	isopropyl-[beta]-D-thiogalactopyranoside
ITS	internal transcribed spacer
kb	kilobase
kDa	kiloDalton
M	molar
min	minutes
mg	miligram
mL	mililiter
mM	milimolar
M-MLV	Moloney Murineleukemia Virus
MW	molecular weight
ng	nanogram
nm	nanometer
No.	number
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PVP	polyvinyl pyrrolidone
rDNA	ribosomal DNA
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid

Rnase	ribonuclease
rpm	rotations per minute
RT	readthrough
RT-PCR	reverse transcription-polymerase chain reaction
s	seconds
SDS	sodium dodecyl sulphate
sl	sensu lato
sp.	species
SSC	sodium shloride/sodium citrate
STE	sodium/Tris/EDTA buffer
T	Triangulation
TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA buffer
TNA	total nucleic acid
Tris	tris hydroximethyl aminomethane
U	unit
UV	ultra violet
V	Volt
Vis	visible
v/v	volume/volume
WT	wild type
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside
µg	microgram
µl	microliter
µm	micrometer

Viruses

ArMV	*Arabis mosaic virus*
CLRV	*Cherry leaf roll virus*
CLSV	*Cucumber leaf spot virus*
CMV	*Cucumber mosaic virus*
CNV	*Cucumber necrosis virus*
LBVaV	*Lettuce big-vein associated virus*
MLBVV	*Mirafiori lettuce big-vein virus*
MNSV	*Melon necrotic spot virus*
OLRSV	*Olive latent ringspot virus*
OLV-1	*Olive latent virus 1*
OLV-2	*Olive latent virus 2*
OLV-3	Olive latent virus 3
OLYaV	Olive leaf yellowing associated virus
OMMV	*Olive mild mosaic virus*
OSLV	Olive semi-latent virus
OVYaV	Olive vein yellowing associated virus
OYMDaV	Olive yellow mottling and decline-associated virus

PepMV	*Pepino mosaic virus*
SLRSV	*Strawberry latent ringspot virus*
TMV	*Tobacco mosaic virus*
TNV	Tobacco necrosis virus
TNV-A	*Tobacco necrosis virus A*
TNV-D	*Tobacco necrosis virus D*

Nucleotide bases

A	Adenine
T	Thymine
G	Guanine
C	Cytosine

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
O	Pyl	Pyrrolysine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
U	Sec	Selenocysteine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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Chapter 1. Introduction

Olive (*Olea europaea* L.) is a traditional Mediterranean crop susceptible to several pests and diseases, among which are 15 different viruses (Cardoso *et al*., 2005; Alabdullah *et al.*, 2010). Symptoms associated to their presence in olive trees range from general decline, defoliation, deformation of leaves and fruits, yellowing, necrosis and low rooting capacity of cuttings of some cultivars as compared to that of healthy ones. Moreover, several olive viruses are not limited to olive and can infect other crops where they can cause serious diseases. In Portuguese olive orchards, levels of viral infection have reached 100% of tested samples, in several sites (Félix *et al*., 2002; Varanda, 2005; Varanda *et al*., 2006), with the necroviruses *Olive latent virus* 1 (OLV-1), *Olive mild mosaic virus* (OMMV) and *Tobacco necrosis virus D* (TNV-D) infections accounting for 21% in one orchard (Varanda *et al*., 2006).

The major olive importing markets, such as Argentina, Australia, China and India demand certified virus-free material (Grieco *et al*., 2000). In addition, the European Union Directive 93/48 of 23-06-1993, *Conformitas Agraria Communitatis* (CAC), states that all olive propagative material produced and commercialized within the EU must be free from all viruses.

These observations make essential the development of sensitive and reliable techniques that allow not only detection but also virus identification. This will allow to increase knowledge on virus epidemiology, namely the mechanisms of transmission (how, when and to which hosts they are transmitted), which are indispensable tools for the development of preventive measures to minimize viral dissemination and contribute for the successful control of the diseases they are associated with.

The main goal of this study was to determine if *Olpidium brassicae*, a known vector of several important plant viruses, namely TNV isolates, had a role on the high dissemination of necroviruses, namely OMMV and if so, elucidate the molecular basis of OMMV-*O. brassicae* interaction that enables specific adsorption to fungal zoospores and subsequent transmission.

To reach this goal specific objectives were pursued:

1) identify the predominant Olpidium species present in a highly necrovirus infected olive orchard;

2) develop a multiplex molecular identification method for OMMV, TNV-D and OLV-1 to allow their discrimination and the determination of each necrovirus level of infection, in olive orchards;

3) determine OMMV levels of soil transmission to plants by the fungus *O. brassicae*;

4) compare the ability of *O. brassicae* zoospores to capture and transmit OMMV wild type (WT) with that of OMMV mutants;

5) evaluate the role of OMMV coat protein (CP) in viral adsorption to zoospores and subsequent transmission;

6) identify specific regions of the OMMV WT genome involved in fungus zoospore acquisition and subsequent viral transmission by comparison with that of OMMV natural mutants deficient in fungal transmission.

Chapter 2. Literature Review

2.1. Viruses in *Olea europaea* L.

The possible existence of a virus disease in olive (*Olea europaea* L.) was first suggested by Pesante, in 1938, after observing a symptomatology consisting of foliar abnormalities, phloem necrosis and progressive decline of the tree (Martelli, 1999). At the time no graft transmission assays were attempted to demonstrate viral presence but tests performed in 1953, by Fogliani, using olive trees showing symptomatology similar to that described by Pesante, were virus-negative. Subsequent studies revealed that these symptoms, named leptonecrosis, were in fact due to a physiological disorder caused by boron deficiency (Martelli, 1999). In the 1950s there were several references to diseases with a possible viral cause (Table 2.1), partially supported by successful graft-transmission tests. However, virus particles were never observed (Martelli, 1998; Martelli, 1999).

Table 2.1: Graft transmissible diseases of olive

Disease	Country	First record
Partial paralysis	Argentina	Nicolini and Traversi, 1950
Sickle leaf	USA, Israel, Chile, Portugal	Thomas, 1958
Infectious yellows	Italy	Ribaldi, 1959
Foliar deformation	Italy	Corte *et al.*, 1961
Spherosis	Israel	Lavee and Tanne, 1984

The first reference to the presence of virus particles in olive tissues dates from 1977, when Pacini and Cresti detected virus-containing tubules in thin-sectioned pollen grains of a symptomless tree of cv. 'Corregiolo', in Italy. Later, in 1979, Savino and co-workers isolated *Strawberry latent ringspot virus* (SLRSV) from that tree providing the first evidence of a true virus infection in olive (Martelli, 1999). Since then, 15 virus species belonging to 8 different genera have been isolated and identified in olive (Martelli, 1999; Cardoso *et al*., 2005; Alabdullah *et al*., 2010). These are geographically distributed practically wherever the olive crop is grown (Table 2.2).

SLRSV and *Arabis mosaic virus* (ArMV) were the first viruses to be identified in olive (Savino *et al*., 1979). The role of SLRSV in causing 'bumpy fruits' disease in cv. 'Ascolana tenera' was confirmed (Marte *et al*., 1986). The same symptoms were observed in cv. 'Negrinha de Freixo', in Portugal and also found to be associated to SLRSV presence (Henriques *et al*., 1992). This virus has also been detected in symptomless olive trees from other cultivars in Italy

(Savino *et al*., 1979), Portugal (Henriques, 1994; Félix *et al*., 2002) and Spain (Bertollini *et al*., 1998).

Table 2.2: Viruses detected in olive trees and their geographical distribution

Virus Species	Virus genus	Virus family	First record in olive	Geographical distribution
Strawberry latent ringspot virus (SLRSV)	*Sadwavirus*	*Secoviridae*	Savino *et al*. (1979)	Italy,Portugal,Spain,Egypt, USA,Lebanon, Syria
Arabis mosaic virus (ArMV)	*Nepovirus*	*Comoviridae*	Savino *et al*. (1979)	Italy,Portugal,Egypt,USA, Lebanon, Syria
Cherry leaf roll virus (CLRV)	*Nepovirus*	*Comoviridae*	Savino and Gallitelli (1981)	Italy,Portugal,Spain,Egypt, USA,Lebanon, Syria
Cucumber mosaic virus (CMV)	*Cucumovirus*	*Bromoviridae*	Savino and Gallitelli (1983)	Italy,Portugal,Syria
Olive latent ringspot virus (OLRSV)	*Nepovirus*	*Comoviridae*	Savino *et al*. (1983)	Italy,Portugal,Spain,Syria
Olive latent virus 1 (OLV-1)	*Necrovirus*	*Tombusviridae*	Gallitelli and Savino (1985)	Italy,Jordan,Turkey,Japan, Portugal, Lebanon, Syria
Olive latent virus 2 (OLV-2)	*Oleavirus*	*Bromoviridae*	Castellano *et al*. (1987)	Italy,Lebanon, Syria
Olive vein yellowing-associated virus (OVYaV)	*Potexvirus*	*Alphaflexiviridae*	Faggioli and Barba (1994)	Italy
Olive yellow mottling and decline-associated virus (OYMDaV)	*Unassigned*	*Unassigned*	Savino *et al*. (1996)	Italy
Tobacco mosaic virus (TMV)	*Tobamovirus*	*Virgaviridae*	Triolo *et al*. (1996)	Italy
Olive semi-latent virus (OSLV)	*Unassigned*	*Unassigned*	Materazzi *et al*. (1996)	Italy
Olive leaf yellowing-associated virus (OLYaV)	*Unassigned*	*Closteroviridae*	Sabanadzovic *et al.* (1999)	Italy,Lebanon,Israel,Egypt, USA,Syria
Olive mild mosaic virus (OMMV)	*Necrovirus*	*Tombusviridae*	Cardoso *et al.* (2004)	Portugal, Italy
Tobacco necrosis virus D (TNV-D)	*Necrovirus*	*Tombusviridae*	Cardoso *et al*. (2009)	Portugal
Olive latent virus 3 (OLV-3)	*Marafivirus*	*Tymoviridae*	Alabdullah *et al.* (2009)	Portugal,Turkey,Syria,Malta, Lebanon, Greece,Tunisia,Italy

Adapted from Félix and Clara, 2008

Other viruses isolated from symptomless olive trees are *Cherry leaf roll virus* (CLRV) (Savino and Gallitelli, 1981), *Cucumber mosaic virus* (CMV) (Savino and Gallitelli, 1983), *Olive latent virus 1* (Gallitelli and Savino, 1985; Martelli *et al*., 1995; Félix and Clara, 1998) and *Olive latent virus 2* (OLV-2) (Castellano *et al*., 1987). OLV-1 has also been isolated from olive trees with yellow leaves (Savino *et al*., 1996). ArMV (Savino *et al*., 1979), *Olive latent ringspot virus* (OLRSV) (Di Franco *et al*., 1993) and Tobacco necrosis virus *sensu lato* (TNV sl) (Félix and Clara, 2002) (see section 2.2.) are frequently associated with weakened or defoliated olive trees. Olive yellow mottling and declining-associated virus (OYMDaV), Olive vein yellowing-associated virus (OVYaV) and Olive leaf yellowing-associated virus (OLYaV) were found associated with 'yellow mottling and declining', 'vein yellowing' and 'leaf yellowing' diseases, respectively (Martelli, 1999). Olive semi-latent virus (OSLV) was detected in olives showing 'vein clearing' disease and *Tobacco mosaic virus* (TMV) was found associated with 'vein banding' symptoms (Triolo *et al*., 1996). *Olive mild mosaic virus* (OMMV) was isolated from a tree of cv. 'Galega vulgar', in Portugal, showing a mild mosaic in leaves (Cardoso *et al*., 2005) (see section 2.2.). More recently, the same authors have complete sequenced a TNV isolate

from an asymptomatic tree of cv. 'Verdeal Alentejana', identifying it as a TNV-D species (TNV-D^P) (Cardoso *et al*., 2009). Olive latent virus 3 (OLV-3) is the newest described virus known to infect olive and was found in an apparently healthy tree in Italy (Alabdullah *et al*., 2009).

Many of these viruses are not limited to olive, which may function as a virus reservoir, and can be transmitted from this to other crops where they can cause serious diseases. SLRSV, ArMV, CLRV, SLRSV, TNV, TMV and CMV are ubiquitous, polyphagous and of high economic relevance in other crops. OLV-1 has been also isolated from citrus (Martelli *et al*., 1996), tulip (Kanematsu *et al*., 2001) and tomato (Hasiów-Jaroszewska *et al*., 2011); OLV-2 was isolated from castor bean in Greece (Grieco *et al*., 2002) and OMMV was isolated from tulips in The Netherlands (Pham *et al*., unpublished). The other viruses have only been detected in olive.

The use of viral detection and identification methods is essential in sanitary selection programs to aid in producing virus-free plants. Demands such as the European Union Directive 93/48 concerning the *Conformitas Agraria Communitatis*, determining that all olive nursery material to be traded within the EU must be free from several harmful organisms including all viruses, rendered diagnostic compulsory. Viral identification is needed to understand virus epidemiology in nature, contributing to a more successful control of the diseases they are associated with. The fact that virus infections are frequently symptomless, together with the low concentration and irregular distribution in woody plants, are some constraints that have contributed to the development of highly sensitive and reliable techniques.

Biological assays such as mechanical inoculation has allowed to recover several viral isolates from field growing olive trees. It is an easy and cheap test to perform, however, some viruses are not mechanically transmissible and results are erratic mostly due to inactivators present in olive tissues (Félix and Clara, 2008). Serological assays such as the double antibody sandwich – enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) are relatively sensitive but their reliability using olive tissues is often low. The virus low concentration, its irregular distribution in the plant, and the presence of inhibitor substances capable of interfering with the test, sometimes lead to erratic results (Martelli, 1999).

Double-stranded RNA (dsRNA) analysis is a technique based on the analysis of dsRNA that are synthesized by RNA viruses during replication in the host cell. DsRNA are viral specific and correspond to the full length virus genome and to subgenomic RNA species that accumulate in the infected cells. DsRNA are readily extracted from infected olive tissues by phenol treatment and chromatography on cellulose columns, followed by gel electrophoresis, using the technique described by Morris and Dodds (1979) with some changes (Rei, 1995; Grieco *et al*., 2000; Saponari *et al*., 2001). The size and pattern of extracted dsRNA provide an

indication of the virus that is present in plant tissues by comparison with those described for other known viruses (Martelli, 1999). Interpretation of the electrophoretic pattern is sometimes difficult, in case of multiple infections or contamination of plant samples with arthropods (Gunasinhe and German, 1988) or fungi (Tooley *et al*., 1989) infected with their own RNA viruses. DsRNA analysis allows to detect viruses that are not mechanically transmitted or that occur in low concentration in their host, as happens with olive viruses. Another advantage of this technique is that it is not affected by phenolic compounds found in olive tissues (De Nino *et al*., 1997) that frequently interfere with other viral diagnostic methods. This technique was frequently used to survey olive trees (Martelli *et al*., 1995; Savino *et al*., 1996; Saponari *et al*., 2002a), often showing high levels of infection. DsRNA have also been used as templates for molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and molecular hybridization (Martelli, 1999; Varanda *et al*., 2006; Félix and Clara, 2008). This has allowed to show that necrovirus in olive are not detected by dsRNA analysis. No dsRNA gel electrophoretic patterns expected for necrovirus replication were observed when 161 olive trees were surveyed, yet the use of these dsRNA as templates in RT-PCR assays revealed that 35 of those trees were necrovirus-infected (Varanda *et al*., 2006). This is most probably due to the viral low titre in the tissues and the low sensitivity of dsRNA analysis as a method *per se* to detect olive necroviruses.

Molecular techniques based on genome amplification have been used to identify olive viruses, whose genome is fully or partly sequenced, through the design of appropriate primers. All viruses so far known to infect olive have an RNA genome but only a few have been sequenced. Viral or total RNA extracted from infected tissues is reverse transcribed (RT) through a transcriptase enzyme that converts RNA to complementary DNA (cDNA) prior to polymerase amplification. A region of cDNA can then be amplified in a reaction in presence of a DNA polymerase and specific primers complementary to regions of the transcribed genome. Several RT-PCR assay formats have been optimized for the diagnosis of olive viruses. Monospecific RT-PCR and multiplex RT-PCR, if one virus or several are to be detected; separate RT and PCR reactions (RT-PCR) or as one (single step RT-PCR) and nested PCR, consisting on a second amplification of an amplified product resulting from a previous PCR. Monospecific tests have been used to identify SLRSV, ArMV, CLRV, OLRSV, OLYaV, CMV, OLV-1, OLV-2, TNV-D and/or OMMV (Grieco *et al*., 2000; Cardoso *et al*., 2004; Varanda, 2005). Primers initially used to identify TNV-D in RT-PCR assays, also detect OMMV, as they are complementary to sequences within the CP gene in which they have high identity (Cardoso *et al*., 2005) (see section 2.2.). Hence the need to design specific primers to discriminate between the 2 viruses, which was done in the present work. Single step RT-PCR has been optimized for

the detection of SLRSV, ArMV, CLRV, CMV, OLYaV, OLRSV, OLV-1 and OLV-2 (Faggioli *et al*., 2002; 2005). Bertollini *et al.* (2001) have developed a single-step multiplex RT-PCR for the simultaneous detection of six of the olive infecting viruses. Nested RT-PCR tests have been successfully used in the detection of OLV-1, OLV-2, OLRSV, ArMV, SLRSV and CLRV from total nucleic acid (TNA) extracted from infected olive tissues (Pantaleo *et al*., 1999). In 2003, Bertolini *et al.* developed a multiplex nested RT-PCR, to detect CMV, CLRV, SLRSV and ArMV and the bacteria *Pseudomonas savastanoi* pv. *savastanoi* in olive tissues. This test showed to be 100 fold more sensitive than single step multiplex RT-PCR. Different olive tissue extracts have been used as template for RT-PCR. Grieco *et al.* (2000) showed that OLV-1 and OLYaV were the only viruses to be detected directly in plant sap by RT-PCR. As for the other viruses, RT-PCR products were only obtained using total nucleic acids or dsRNA preparations as template. The quality of viral RNA template is an important limitation in RT-PCR assays. The use of dsRNA extracted from olive tissues as template allows to eliminate much of the contaminants that may interfere with the viral genome amplification. The use of larger amounts of sample that can be collected from different parts of the tree when dsRNA fraction is used, minimizes the constraint of the low viral concentration and uneven distribution within the plant (Nolasco *et al*., 2000; Bertolini *et al*., 2003).

The epidemiological research on viruses occurring in olive trees represents an essential contribution to improve the quality of this crop, whose demands have been continually raising.

The high frequency of viral infections found in Portugal, as in other countries where surveys have been made, such as Italy, suggests the efficient virus disseminating means in nature (Félix *et al*., 2002; Saponari *et al*., 2002a).

Olive viruses are believed to spread in nature mainly through the use of rooted cuttings and of seedlings originated from infected mother plants. Not much is known about the vectors and their role in viral dissemination in the olive crop. The only references to tests of insect transmission to olive were done with OLYaV and OLV-3 (Sabanadzovic *et al*., 1999; Alabdullah *et al*., 2009). A specific OLYaV RNA sequence was detected in *Euphyllura olivina* and in *Pseudococcus* sp. present in plants infected with this virus but no definitive conclusions were drawn (Sabadadzovic *et al*., 1999). Alabdullah *et al.* (2009) tested *E. olivina* and *Saissetia oleae* for OLV-3 transmission without success although the virus was presumably acquired by *E. olivina*.

SLRSV was suggested to be seed transmitted when it was found to be present in pollen, however this possibility was never proved. CLRV and OLV-1 have been detected in flowers and pollen and OLV-1 was shown to be seed-transmitted (Lobão *et al*., 2002; Saponari

et al., 2002b). The transmission of OLV-1 through soil in the absence of biological vectors has been demonstrated in herbaceous plants (Martelli *et al*., 1996; Félix *et al.*, 2006), however, in such cases, the virus infected plant roots but did not disseminate to other parts of the plant.

The fungus *Olpidium brassicae* (Wor.) Dang., known to be a vector of TNV sl (see section 2.2.) among other viruses (Teackle, 1962; Adams, 1991), has been consistently found in close association to the root surrounding soils of olive TNV sl - infected plants (Félix and Clara, 2001). These authors have used lettuce plants to bait *O. brassicae* from soils where TNV sl – infected olive trees were growing. This resulted in the observation of fungal zoosporangia and resistance spores and in the infection of lettuce plants, suggesting that this virus may also be transmitted by *O. brassicae* in the olive crop (Félix and Clara, 2001).

Preliminary transmission studies with OMMV have shown that OMMV is naturally transmitted to roots of herbaceous hosts only when high amounts of viruses are used to inoculate the roots (Félix *et al*., 2006). When healthy plants were placed in pots where infected plants had grown, no transmission was observed. However when healthy plants were immersed in a suspension of 160 µg of OMMV/10 mL, they became infected. The same authors showed that when healthy plants were placed in pots where double OMMV and OLV-1 infected plants had grown, these became infected with both viruses suggesting a possible synergistic effect between these two viruses (Félix *et al*., 2006).

As to other olive infecting viruses, no transmission tests were performed using olive plants but their modes of transmission are well studied in other hosts, as are the cases of the nematode-transmitted SLRSV, CLRV and ArMV and the insect-transmitted CMV. Seed or pollen transmission has been reported for SLRSV, CLRV, ArMV, CMV and TMV in other plants (Murant, 1981; Eppler, 1983; Yang *et al*., 1997; Okada *et al*., 2000).

2.2. *Olive mild mosaic virus*

OMMV belongs to genus *Necrovirus*, family *Tombusviridae.* It was originally isolated from olive trees and initially identified as TNV based on serologic techniques using a broad range TNV antiserum (TNV broad range, Loewe Phytodiagnostic, Germany) (Louro, 2004). This antiserum consists in an artificial mixture of antisera for: TNV-A (Kassanis), PV 180; TNV-A type (tomato), TNV (apple); TNV (bell peeper), PV 197; TNV (cherry); TNV Grogan, PV 198; TNV-D (Kassanis), PV 192; TNV-D (soil, Germany), PV 219; TNV-E (Aschersleben); TNV (Paul), PV 218 and TNV (tulip), PV 44. In this literature review, previous studies where TNV was identified serologically will be referred as TNV *sensu lato* (sl). In 2004, OMMV was identified as a TNV-D isolate based on the sequencing of the coat protein (CP) gene (Cardoso *et al*., 2004) but in 2005, after complete sequencing it was concluded that the virus was a distinct species and was named *Olive mild mosaic virus* (Cardoso *et al*., 2005).

Based on the deduced CP amino acid (aa) sequence, OMMV showed 86.2% identity with that of TNV-D and 40.7% identity with that of OLV-1 (Cardoso *et al*., 2004). Its RdRp showed a high identity, 91.2%, with that of the necrovirus OLV-1 and very low with that of TNV-D, 33.6%. p6 and p8 of OMMV showed a high identity with those of OLV-1 (Cardoso *et al*., 2005). Due to the high identities of OMMV with the other two necroviruses, these authors proposed that the origin of OMMV may have resulted from a recombination event occurred during simultaneous replication of TNV-D and OLV-1 in a host cell. An earlier existence of OMMV has been recently revealed based on new sequence data (Genbank accession numbers EF 201608, EF 201607, EF 201606 and EF 201605), of the virus associated to the Augusta disease, first observed in tulips in 1928, in The Netherlands, which was previously ascribed to TNV sl by Kassanis, in 1949.

OMMV genomic RNA is similar to the other members of the Family *Tombusviridae* (Figure 2.1.), it has 3683 nts and 5 open reading frames (ORFs). The 5'-proximal ORF (ORF1) (nt 60 - 668) encodes a polypeptide of 202 aa and a molecular mass of 23 kDa (p23), and is predicted to be involved in RNA replication. ORF2 results from the readthrough of the amber stop codon (nt 666 – 668) of ORF1 to the termination codon at nt 2232 – 2234, encoding a polypeptide with a molecular mass of 82 kDa (p82) and was identified as the RdRp. The ORF3 (nt 2218 – 2439) overlapps ORF2 by 17 nts and encodes a 73 aa polypeptide with a molecular mass of 8 kDa (p8). ORF 4 (nt 2443 – 2613) encodes a 56 aa polypeptide with a molecular mass of 6 kDa (p6) (Cardoso *et al*., 2005). p8 and p6 are predicted to be involved in virus movement. The 3'-proximal ORF (ORF5) (nt 2636 – 3445) encodes a polypeptide of 269 aa with 29 kDa (p29) and was identified as the CP (Cardoso *et al*., 2004).

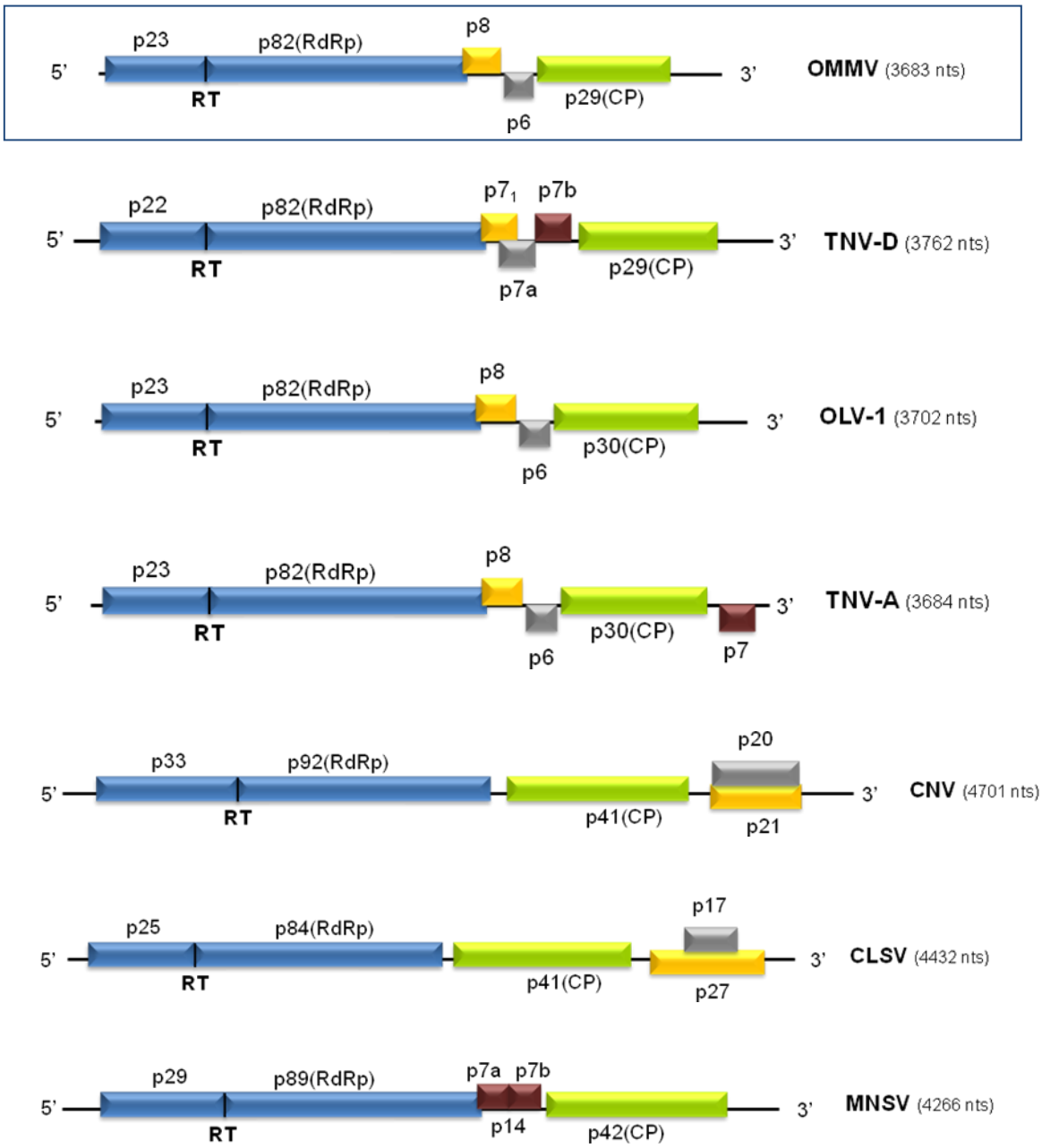


Figure 2.1: Genome structures of several viruses belonging to family *Tombusviridae*. Viruses belonging to genus *Necrovirus* (OMMV – *Olive mild mosaic virus*; TNV-D – *Tobacco necrosis virus D*; *OLV-1 – Olive latent virus 1*; TNV-A – *Tobacco necrosis virus A*) and some of the viruses transmitted by *Olpidium* species *in vitro* (CNV – *Cucumber necrosis virus (Genus Tombusvirus*); CLSV – *Cucumber leaf spot virus* (Genus *Carmovirus*), MNSV – *Melon necrotic spot virus* (Genus *Carmovirus*)). ORFs are shown as boxes with predicted sizes indicated above or below. RT: readthrough; RdRp: RNA polymerase RNA dependent; CP: Coat protein.

Necroviruses are sized *ca*. 28 nm in diameter, exhibit a T=3 icosahedral symmetry and are composed of 180 copies of three conformationally distinct subunits: A, B and C (Figure 2.2.). The virions belonging to genus *Necrovirus* are composed of capsid proteins that lack the protruding domain, and consequently their surfaces have a smoother appearance (Van Regenmortel *et al*., 2000). All of the viruses in the *Tombusviridae* lack the helicase motif.

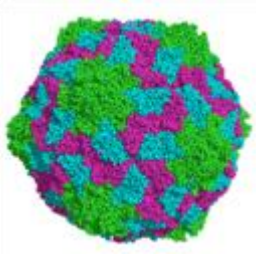


Figure 2.2.: Three dimensional representation of the whole virus assembly of the necrovirus Tobacco necrosis virus coat, highlighting the three conformationally distinct subunits, coloured in different colours. Figure prepared with Pymol by Cláudio Soares (ITQB).

2.3. Plant virus transmission

Transmission is an important step in the biological cycle of plant viruses as it ensures their maintenance and survival. For transmission to be successful, plant viruses must rely on vectors to penetrate the otherwise impermeable cuticle or cell wall, for transport among hosts and in some cases, for long term protection in the absence of suitable hosts (Hull, 2002; Andret-Link and Fuchs, 2005; Raccah and Fereres, 2009). Plant viruses are vectored predominantly by arthropods, nematodes, fungi and plasmodiophorids; they can also be transmitted through seed, pollen and vegetative plant material (Gray and Banerjee, 1999; Rochon, 2004; Andret-Link and Fuchs, 2005; Ng and Falk, 2006; Hogenhout et al., 2008; Raccah and Fereres, 2009)

The transmission process requires several successive steps: acquisition of virus from an infected source; stable retention of viruses at specific sites through binding of virions to receptors; release of the virus from its vector and delivery of virions to a site of infection in a susceptible host cell (Andret-Link and Fuchs, 2005; Rochon, 2007). Among these basic criteria, different modes of vector-mediated virus transmission have been characterized (Gray and Banerjee, 1999; Hull, 2002; Rochon, 2007). In the 1930's, Watson and Roberts proposed the terms 'nonpersistent' and 'persistent' (Watson and Roberts, 1939) currently applied to virus short retention in the vector (minutes to hours) or to extended retention (hours to indefinitely), respectively. For viruses showing an intermediate retention in their vector, the term 'semipersistent' is applied. Nonpersistent and semipersistent viruses are referred to as 'noncirculative' because they are not internalized by vectors. In other words, they do not enter the haemocoel (vector body cavity) or cross any vector cell membrane (Gray and Banerjee, 1999), as opposite to persistent viruses that are referred to as 'circulative'. These can be further divided into 'propagative' (viruses that replicate in the vector) and 'nonpropagative' (viruses that do not replicate in the vector) (Gray and Banerjee, 1999). Although these general modes of transmission were derived from studies on aphids, thrips and leafhoppers, they apply to transmission by most arthropod vectors with piercing and sucking mouthparts but to a lesser extent by nematodes, fungi and plasmodiophorid vectors (Rochon, 2007).

The transmission of a virus by a vector is often characterized by some degree of specificity in that a plant virus can be transmitted by one or a few vector species but not by others (Andret-Link and Fuchs, 2005). The specificity of transmission is explained by several characteristics including a recognition event between the virion, or a viral protein motif, and a site of retention in the vector (Brown and Weislher, 1998). With few exceptions, members of a specific virus genus are transmitted by similar vectors (e.g. *Potyvirus* are transmitted by

aphids) (Ng *et al*., 2004). This is most likely related to the fact that a major specificity determinant for vector transmission is the viral coat protein (Hull, 2002), which sequence and structure is a major criterion for virus classification (Van Regenmortel *et al*., 2000). Transmission is also facilitated by other virus-encoded determinants referred to as 'helper components' (Rochon, 2007).

2.3.1. Virus transmission by fungal vectors

There are currently three species of *Olpidium* sp. known to vector plant viruses. These are within the true fungi in the phylum *Chytridiomycota* and consist of *Olpidium bornovanus*, *O. brassicae* and *O. virulentus*, the latter was previously identified as *Olpidium brassicae* species (Sasaya and Koganezawa, 2006; Rochon, 2007).

Studies based on host specificity, ability to transmit plant viruses and molecular analysis of the complete rDNA-ITS regions of *O. brassicae* have confirmed that fungal strains that infect non-crucifer plants (such as lettuce and tomato) are different of those infecting crucifer plants (such as cabbage and mustard) and therefore the two groups should be classified as two distinct species. The crucifer strains require zoospore mating to develop resting spores upon plant infection contrary to the noncrucifer strains (Sahtiyanci, 1962; Koganezawa *et al*., 2005; Sasaya and Koganezawa, 2006). Based on the differences between crucifer and noncrucifer strains, Sahtiyanci (1962) divided *Pelotrachelus* (=*Olpidium*) *brassicae* into two distinct species, *P. brassicae* and *P. virulentus*, respectively, but his proposal was not adopted. In 2005, Koganezawa proposed the nomenclature *Olpidium virulentus* (Sahtiyanci) Karling for the non crucifer strain and *O. brassicae* for the crucifer strain (Koganezawa *et al*., 2005). The rDNA-ITS regions of *O. virulentus*, *O. brassicae* and *O. bornovanus* were analysed by Sasaya and Koganezawa (2006) by sequencing the amplified product obtained in PCR assays, using primers ITS1 and ITS4 (White *et al*., 1990), sized 632 bp for *O. virulentus*, 600 bp for *O. brassicae* and 1137 bp for *O. bornovanus*. However, the use of those primers in PCR assays does not allow the identification of *O. brassicae* and *O. virulentus* by simple visualization of amplified PCR products in gel due to the close size of the amplicon, *ca*. 600 bp. More recently, a multiplex PCR assay to discriminate the three species of *Olpidium* was developed by Herrera-Vásquez *et al*. (2009). This method is rapid and does not require sequencing of the amplified product, as they have very different sizes being easily differentiated after gel electrophoresis, 579 bp for *O. virulentus*, 204 bp for *O. brassicae* and 977 bp for *O. bornovanus*. Since then, molecular identification allowed to identify *O. virulentus* as a fungal vector of *Pepino mosaic*

virus (PepMV) (Alfaro-Fernandez *et al*.,, 2010); of a dianthovirus causing the necrotic streak of fique (Gonzalez *et al*., 2010) and of *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) (Maccarone *et al.*, 2010). In this review, *O. brassicae sensu lato* (sl) will be used to denote both *O. brassicae* and *O. virulentus* when citing reports prior to 2009.

Olpidium spp. produce motile zoospores as a means of dispersal, as well as resting spores that enable long-term survival (Figure 2.3).

The zoospores of *O. bornovanus* and of *O. brassicae* sl have a single, posterior whiplash flagellum and display a characteristic 'jerky' swimming pattern. The body of the zoospore of *O. bornovanus* is ellipsoidal and has about 4.5 by 8 µm, whereas the zoospore of *O. brassicae* sl is spherical and has only approximately 3.3 by 5.6 µm. The zoospores of *O. bornovanus* have longer flagella, the zoosporangia are larger, and the resting spores are smooth rather than stellate (Barr, 1968). The zoospore body and flagellum are surrounded by a membrane sheath and an external matrix containing specific mannose and fucose-containing oligosaccharides and glycoproteins (Kakani *et al*., 2003).

Electronmicroscopy studies have shown two phases in the infection process by *O. brassicae* (Temmink and Campbell, 1969). The initial stage of zoospore encystment likely involves withdrawal of the flagellum, composed of axoneme plus axonemal sheath, into the zoospore body or, instead, the wrapping of flagellum around the developing cyst (Matthews, 1970; Gibbs and Harrison, 1976). However the first hypothesis is more likely as newly encysted zoospores often show a 'whorl of membranes' believed to result from the rupture of the axonemal sheath inside the zoospore body (Temmink, 1971; Stobbs *et al*., 1982). After encystment, the zoospore secretes a thin outer cyst wall (Matthews, 1970; Gibbs and Harrison, 1976). In the second phase, 2 hours later, each zoospore produces an infection canal, which can be seen penetrating the wall of the nearest root cell (Gibbs and Harrison, 1976), and the cyst cytoplasm enters the host cell (Figure 2.4) (Matthews, 1970). In response to fungal infection, the host cell produces a papillum. In addition, a hole appears in the cyst at the junction of the papillum, providing a means for the cyst protoplast to enter the host cell and initiate infection, leaving behind the original plasmalemma, the tonoplast and the cyst wall (Matthews, 1970; Gibbs and Harrison, 1976; Rochon *et al.*, 2004). The thallus (cyst cytoplasm surrounded by the plasmalemma) enlarges over the next one or two days, still separated from the root cell cytoplasm only by a thin membrane. Thalli develop a thick wall outside of the plasmalemma, become multinucleated, develop into zoosporangia and form individual zoospores. Zoospores are then released into the soil medium surrounding root cells via an exit

tube formed by the mature zoosporangium (Gibbs and Harrison, 1976). Thalli may also develop into resting spores but do not become multinucleated structures.

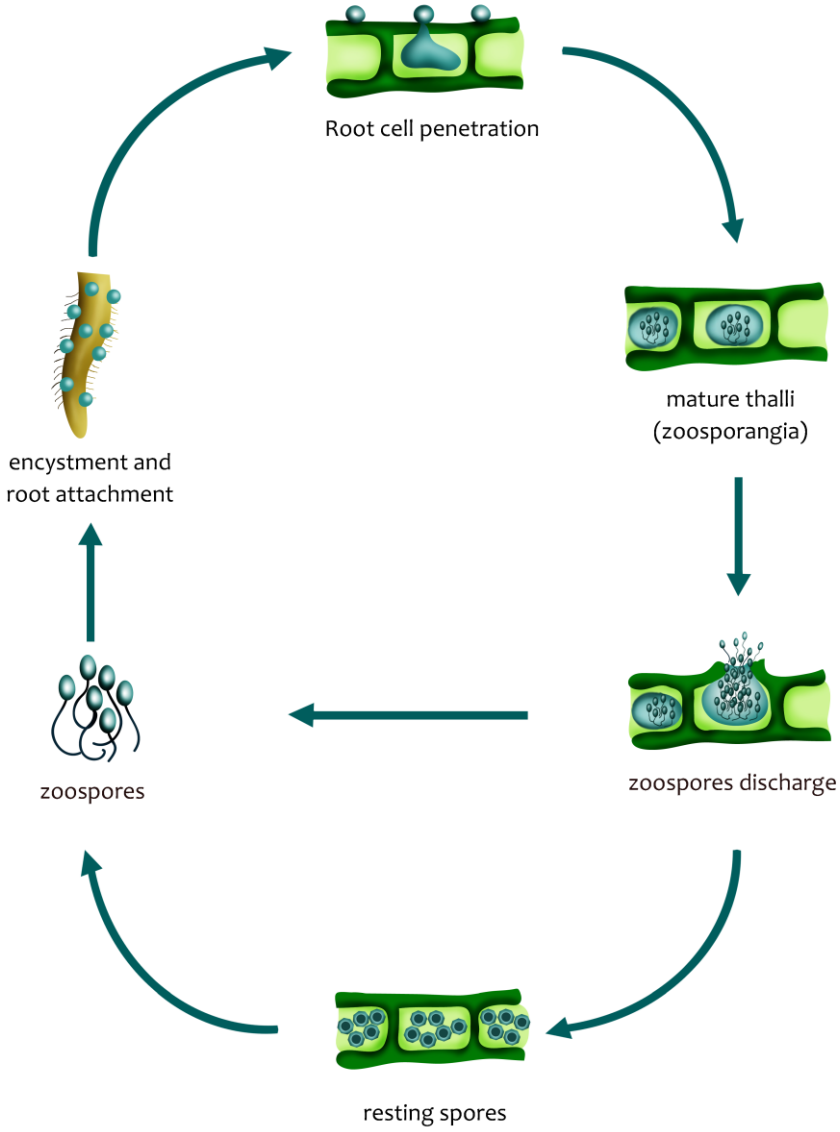


Figure 2.3: Schematic representation of *Olpidium brassicae* lyfe cycle

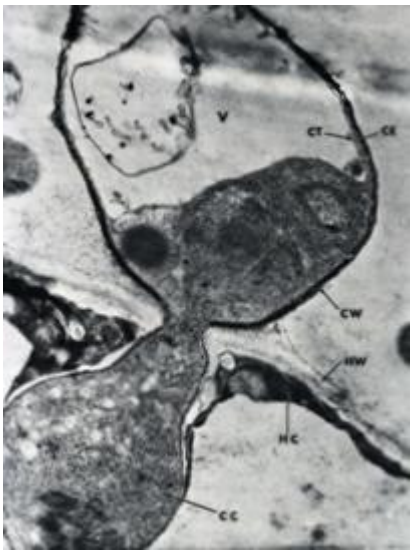


Figure 2.4: Infection of a root cell by *Olpidium* sp.. Electron micrograph showing contents of an encysted zoospore entering the host cell. CC – cyst cytoplasm; HC – host cytoplasm; HW – host wall; CW – cyst wall; CT – cyst tonoplast; CE – cyst ectoplast; V – vacuole (Matthews, 1970).

2.3.1.1. Virus-vector relationships

Two types of virus-vector relationships have been described: *in vitro* and *in vivo* (Campbell and Fry, 1966; Campbell, 1996). They are distinguished by the mode of virus acquisition by the vector and the location of the virus in relation to the vector resting spore.

Both *O. bornovanus* and *O. brassicae* sl transmit viruses in the *in vitro* manner. All of the viruses transmitted *in vitro* by *Olpidium* spp. belong to family *Tombusviridae* and are in the genera *Tombusvirus*, *Aureusvirus*, *Carmovirus*, *Dianthovirus* and *Necrovirus* (see Figure 2.1.).

In vitro transmission involves the independent release of zoospores and of viruses in the soil or aqueous medium outside of the plant root system followed by adsorption of the virus particle to the surface of zoospores (Campbell, 1988; Rochon *et al*., 2004).

Viruses transmitted in the *in vitro* manner require at least 2 distinct stages for successful transmission: a relative short (≈ 5 – 15 min) acquisition stage in which virus particles are specifically attached to both the plasmalemma of the zoospore body and the axonemal sheath of the flagellum (Temmink *et al*., 1970; Gibbs and Harrison, 1976) and a release stage in which virus gains access to the root cell cytoplasm following encystment of virus-bound zoospores on root cells (Rochon *et al*., 2004).

Many lines of evidence support the notion that the association between the virus and the zoospore is highly specific (Adams, 1991; Campbell, 1996) and reflects the biological associations observed in nature. For example, Temmink *et al*. (1970) showed that high levels of TNV sl particles are adsorbed to the zoospore of a highly transmitting isolate of *O. brassicae*, and no virus was observed under the electron microscope using a nonvector isolate. Similar results were obtained showing that *Cucumber necrosis virus* (CNV) specifically adsorbs to its vector, *O. bornovanus*, but not to *O. brassicae* (Stobbs *et al*., 1982; Kakani *et al*., 2001; Kakani *et al*., 2003).

The manner in which acquired virus is subsequently transmitted to root cells is not known. The protoplast of encysted zoospores is injected into the root cell cytoplasm, leaving behind the plasmalemma. This raises the question as to how virus, which is adsorbed to the zoospore plasmalemma, eventually enters the root cytoplasm. One hypothesis is that the virus bound to the axonemal sheath enters the zoospore protoplasm during the retraction of the flagellum (Temmink, 1971; Stobbs *et al*., 1982; Campbell, 1996). Indeed, Stobbs *et al*. (1982), in studies of CNV transmission by *O. bornovanus*, found that encysting zoospores contained large amounts of virus particles in between 'whorls of membranes' formed inside the zoospore following flagellar retraction (Figure 2.5.).

Another unknown aspect is the means by which the virus within the zoospore protoplasm, becomes associated with the root cell cytoplasm. It is assumed that either this event occurs prior to thallus cell delimiting membrane that separates it from the host cell cytoplasm (Temmink, 1971), or the viral nucleic acid is able to traverse this membrane to enter the root cell cytoplasm as suggested by Rochon *et al.* (2004).

Once virus become associated with the host cell cytoplasm, virus replicate and spread without the aid of the vector. Later, zoospores on one hand and free virions on other hand are released into the soil becoming available for other rounds of acquisition and entrance into cells of new host plants. It is not known why viruses transmitted in the *in vitro* fashion do not become incorporated into zoospores during coinfection of the plant. Viruses may not be present in the same root tissue where spores develop or they may not have the capability of crossing from the root cell cytoplasm into the developing thallus, or if they do enter resting spores they may not remain infectious there (Rochon *et al*., 2004).

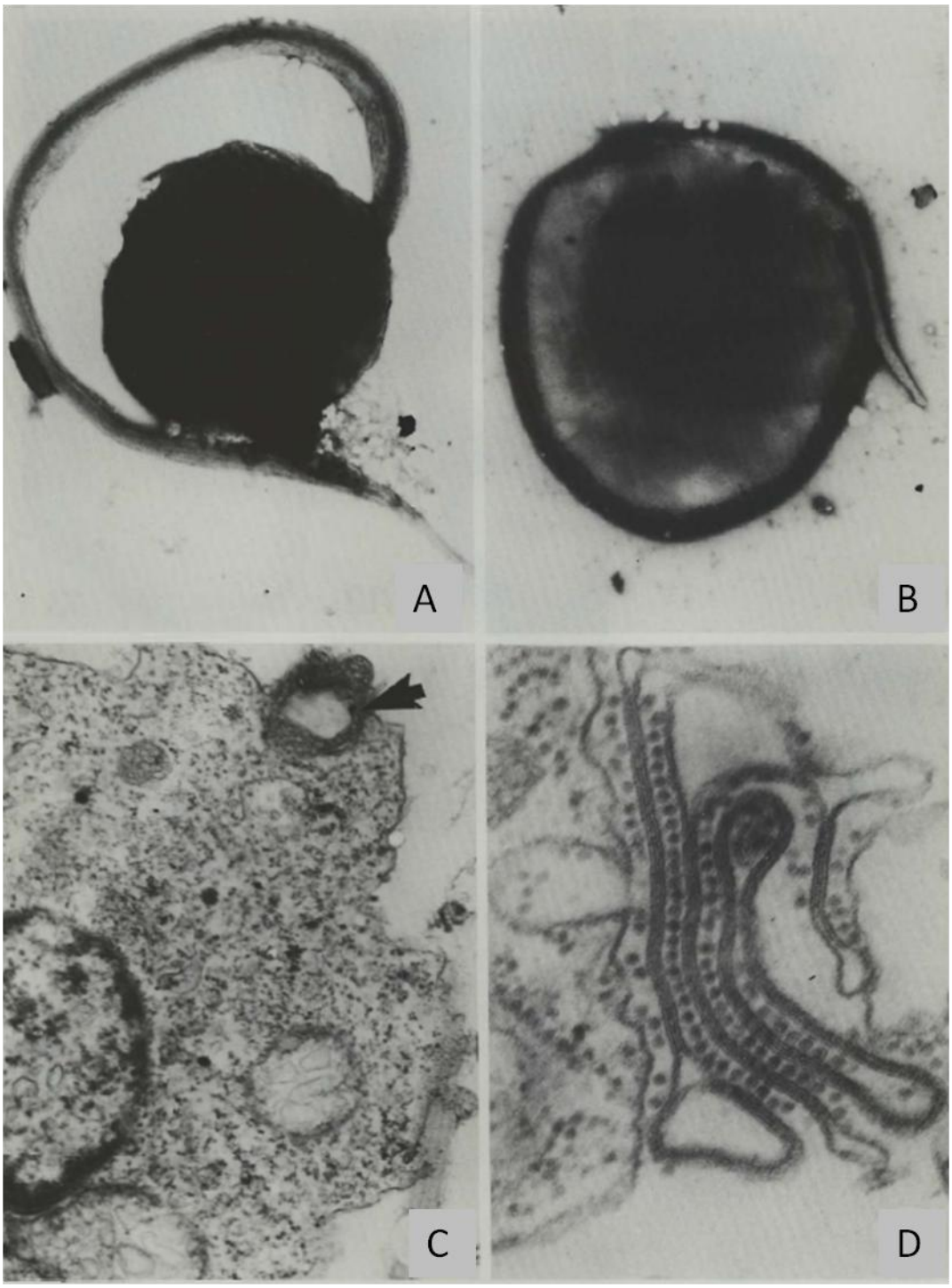


Figure 2.5.: Flagellar retraction of the zoospore, A – showing the 'loose' coiling of the flagellum (x8000); B – showing 'tight' coiling of the flagellum (x10000). Whorl of membranes (arrow) with associated CNV particles, C – x15000; D – x60000 (Stobbs *et al*., 1982)

The CP of plant viruses plays an important role in transmission. The reciprocal exchange of the CP gene of CNV and that of a nontransmissible tombusvirus, the cherry strain

of *Tomato bushy stunt virus* (TBSV-Ch), showed that particles containing the modified TBSV-Ch genome with the CNV CP were efficiently transmitted and those containing the original TBSV-Ch genome were not (McLean *et al*., 1994). Similar observations were obtained when Robbins *et al*. (1997) performed the reciprocal exchange of the CP gene of CNV with that of a non transmissible mutant (LL5).

Particular aa within the distinct domains of the CP have shown to be essential for transmission. A single Leu to Phe mutation at position 294 and a single Val to Ala change at position 295 in the CNV CP protruding domain, reduced viral attachment to *O. bornovanus* and therefore lowered transmission efficiency (Kakani *et al*., 2001). An Ile to Phe mutation at position 300 in the MNSV CP protruding domain, led to loss of fungal transmission by abolishing binding of particles to zoospores (Mochizuki *et al*., 2008). A single amino acid mutation (Glu to Lys) in the CNV CP shell domain resulted in decrease of transmission efficiency of CNV by *O. bornovanus*, mostly by decreasing binding efficiency, indicating that specific regions of the CP can mediate zoospore adsorption (Robbins *et al*., 1997). Studies on CNV CP mutants showed that specific amino acids located in the interior of the particle, located near the cavity on the particle pseudo-threefold axis (the trimer), also decrease the efficiency of zoospore binding and subsequent transmission (Kakani *et al*., 2001) likely by affecting subunit-subunit interactions and virion conformation.

The loss of transmissibility is most of the times in part due to inefficient attachment of virions to the zoospore surface. However, Kakani *et al.* (2004) have shown that a particular aa was responsible for the loss of CNV transmissibility even though no decrease in binding efficiency to zoospores of its fungal vector was observed. In that study, the authors showed that zoospore bound CNV particles are conformationally different from native CNV and similar to that of swollen CNV, a structural state that results from electrostatic repulsion of negatively charged residues accompanied by externalization of the normally inward facing CP RNA binding domain and arm (Figure 2.6.) (Kakani *et al*., 2004). A CNV mutant with a change Pro to Gly at position 73 in the CNV CP arm region was incapable of swelling and although its binding efficiency was similar to that of the wild type CNV, it was not fungal transmitted.

The involvement of glycoproteins in CNV attachment was suggested when zoospores after treatment with protease and periodate reduced binding of CNV. Binding of CNV to zoospores was competitively and specifically inhibited by mannose- and fucose-containing oligosaccharides, leading the authors to suggest that binding involves attachment to specific sugars that are prominent on the zoospore surface (Kakani *et al*., 2003).

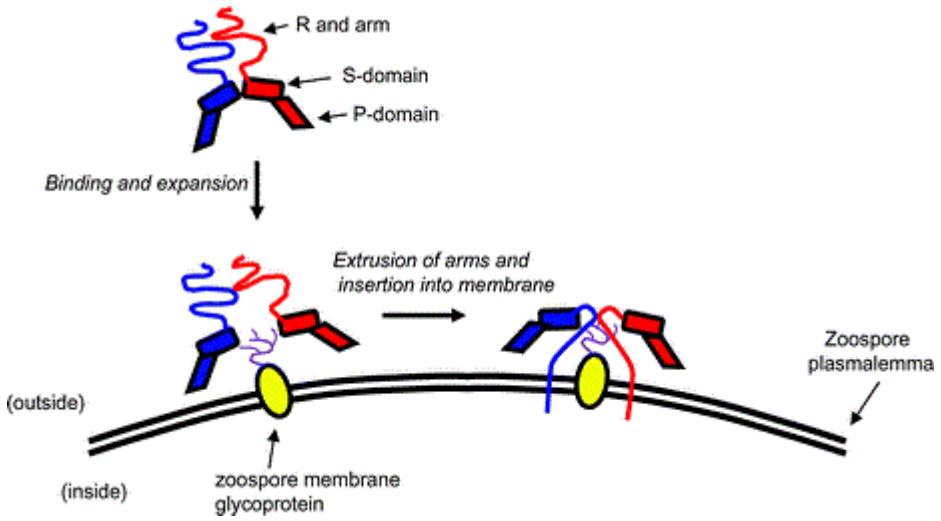


Figure 2.6.: Model for CNV binding to zoospores of *O. bornovanus* showing the steps in the binding of CNV capsid to the zoospore plasmalemma (Rochon *et al*., 2004).

The only species of *Olpidium* sp. known to transmit viruses in the *in vivo* manner is *Olpidium brassicae* sl. All the viruses transmitted *in vivo* by *O. brassicae* sl belong either to the Ophioviruses (*Mirafiori lettuce big-vein virus* (MLBVV), *Tulip mild mottle mosaic virus*) or to the Varicosaviruses (Freesia leaf necrosis virus, *Lettuce big-vein associated virus* (LBVaV), *Lettuce ring necrosis virus*, Tobacco stunt virus), both in unassigned families. As stated previously in this work, *O. virulentus* has recently been found to be the vector of the *in vivo* transmitted MLBVV and LBVaV (Maccarone *et al*., 2010).

In vivo transmission involves the acquisition of the virus by the fungus in coinfected root cells. In this mode of transmission, viruses are not acquired by zoospores in media outside the host (Gibbs and Harrison, 1976). As with *in vitro* acquisition, the means by which virus enters the root cell cytoplasm following injection of the protoplast is not known. Virus particles in a coinfected cell could cross the thin membrane of the sporangium and thereby be incorporated into zoospores during further development.

The virus is believed to be present within zoospore protoplasm and resting spores (Campbell, 1988; Rochon *et al*., 2004). However, attempts to detect virus in resting spores have been difficult, maybe due to the low number of particles per spore or the low proportion of spores that contain particles. So far the best evidence is that resting spores are viruliferous even following harsh chemical treatment and long-term storage (Campbell, 1996). Direct

evidence of the presence of viruses in the resting spores has been found in plasmodiophorid vectors (*Polymyxa graminis*, *P. betae* and *Spongospora subterranea*) which transmit virus in the in vivo manner. *Soil-borne wheat mosaic virus* movement proteins and viral RNA were detected in plasmodia and resting spores of *P. graminis* (Driskel *et al*., 2004), although virions were not found in resting spores. Also, Verchot-Lubicz *et al.* (2007) found *Beet necrotic yellow vein virus* encoded proteins in resting spores of its plasmodiophorid vector, *P. betae*; this suggests that the virus may replicate in its vector.

Chapter 3. Material and Methods

3.1. Viral strains

Olive fruits harvested in Autumn and 2-year stems collected in Spring from 54 olive trees belonging to a collection of ecotypes of the cv. 'Galega vulgar' growing in the south of Portugal were screened for necrovirus infection by multiplex RT-PCR assays. *Nicotiana benthamiana* plants infected with OLV-1 (GM6 isolate, Félix *et al*., 2005), *Chenopodium murale* plants infected with OMMV (wild type, Cardoso *et al*., 2005) and *C. murale* plants infected with TNV-D (V8i isolate, Cardoso *et al*., 2009) were used as positive controls in those assays. Plants were maintained in a chamber at 22-25 ºC with a 14 h photoperiod.

C*a*. 100 g of symptomatic plant material previously inoculated with either viral RNA, infected *N. benthamiana* or *C. murale* leaves, in the presence of 0.05 M sodium phosphate buffer (pH 7.0) and carborundum, were used for virus purification. Leaves were ground in cold 0.1 M sodium phosphate buffer (1:3 w/v) and 1 % sodium ascorbate, the homogenate filtered, clarified in the presence of a mixture of butanol and chloroform (1:1 v/v), concentrated by differential centrifugation (Zhang *et al*., 1993) and further purified by ultracentrifugation through sucrose density gradient columns (10 – 40 % in 0.02 M sodium phosphate buffer, pH 7.0) at 150000 *g* for 40 min. The single light scattering virus band was recovered and concentrated by ultracentrifugation at 150000 *g* for 4 h. Virus concentration was determined at 260 nm in a UV/Vis spectrophotometer DU 530 Life Sciences (Beckman) and the extinction coefficient used was $E^{1\%}_{260} = 5.0$.

A transcript of a full-length cDNA clone of OMMV (pUC18OMMV) was used (Cardoso *et al*., submitted) was used for inoculation of plants, after plasmid DNA extraction and transcription (see sections 3.9 and 3.10).

An OMMV variant, OMMV L11, was obtained following 15 serial mechanical inoculations of single local lesions induced in *C. murale* plants by the above OMMV transcript.

A mutant, designated as OMMV/OMMVL11 was obtained by substituting the OMMV CP gene by that of OMMVL11 (see section 3.11).

3.2. Fungal strains

For the recovery of *Olpidium* species from soil, 5-days old cabbage (*Brassica pekinensis*) seedlings were used as bait plants. Soil samples were collected from 8 different

sites of an olive orchard (Mirandela, northeast of Portugal) where necroviruses had been found in a high percentage in olive trees (Varanda *et al*., 2006).

Fungal isolates were maintained on Chinese cabbage plants (*Brassica pekinensis*) growing in a chamber at 16-22 ºC with a 14 h photoperiod.

For morphological identification of baited *Olpidium* species, seven weeks after sowing bait plants, these were carefully removed, the roots washed, immersed into an aqueous 10% KOH solution and autoclaved at 121ºC for 15 minutes. Roots were stained overnight in 1% trypan blue lactoglycerol (1 water: 1 glycerol: 1 lactic acid), destained in an aqueous 50% glycerol solution for 18 h, essentially as described by Philip and Hayman (1970) and observed under a light microscope for the presence of fungal resting spores with typical morphology.

Molecular identification of the Olpidium species was carried out by PCR assays (see section 3.5.3.3.), using DNA of *O. brassicae* and *O. virulentus* as positive controls in PCR assays that were kindly supplied by Herrera-Vásquez (Universidad Politécnica de Valencia, Spain).

To obtain a single sporangial culture, *Olpidium*-infected cabbage roots were homogenized in chilled 0.5 M glycine–NaOH (pH 7.6) in a blender for 30 s, the resulting extract was filtered and a drop of the filtrate was observed under a light microscope (Lin *et al*., 1970). A single mature sporangium was collected and serially transferred 5 times to small drops of cold sterile-water to eliminate possible contaminating zoospores of a different origin. The single isolated sporangium was placed in a 30 mL plastic pot containing a mixture of sterile sand and vermiculite where 5 day old cabbage seedlings were growing.

3.3. Nucleic acid extraction

Total RNA fraction was extracted from symptomatic host plants using the commercial RNeasy Plant Mini Kit (Qiagen). About 100 mg of infected tissue were macerated in presence of liquid nitrogen and manufacturer's instructions were followed. The RNA quantification was achieved using the Quant-it™RNA assay kit (Invitrogen) in the Qubit Fluorometer (Invitrogen).

Double stranded RNA were extracted from healthy and infected herbaceous hosts, *N. benthamiana* and *C. murale*, olive fruits and 2-year olive stem scrappings. About 20 g of sample tissue were ground in liquid nitrogen and mixed with 45 mL of cool 2x STE (1x STE: 0.1 M NaCl, 0.05 M Tris, 0.5 mM Na_2EDTA, pH 7), 17 mL of 10 % SDS, 1 mL of a bentonite suspension (45 mg bentonite/mL of 0.1 M KH_2PO_4), 1 mL of 2-mercaptoethanol, 50 mL of phenol-chloroform (1:1) (phenol water saturated, pH 7.5, containing 0.1 % 8-

hydroxyquinoline). This homogenate was shaken for 45 min and then centrifuged at 10000 *g* for 15 min. 17 % ethanol and 2 g of CF11 cellulose (Whatman) were added to the recovered supernatant. The mixture was shaken for 1 h and then passed through a disk of fabric material plugged in a disposable syringe. The cellulose column was then washed with 20 volumes of 17 % ethanol in 1x STE buffer at room temperature and dsRNA fraction was eluted with 4 volumes of 1x STE buffer with no ethanol (Morris and Dodds, 1979). Eluate was then digested in a one-tube reaction with RN*ase*-free DN*ase* (Fermentas) (0.6 mg/mL) in 10 mM $MgCl_2$ and DN*ase*-free RN*ase* (Fermentas) (0.5 µg/mL) in 2x SSC (1x SSC: 0.15 M NaCl, 0.015 sodium citrate, pH 7.0) and Proteinase K (Fermentas) (0.1 mg/mL) as in Saldarelli *et al*. (1994). Enzyme was removed from the treated dsRNA fraction by TE-saturated (pH 8.0) phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation (Sambrook *et al*., 1989). Final dsRNA pellet was ressuspended in 30 µL of ultrapure water.

Fungus infected cabbage plants were kept without watering for 3 days prior to placing the roots in distilled water for 30 min. The resulting spore suspension was filtered through Whatman No. 4 filter paper, the filtrate was centrifuged at 1000 *g* for 10 min, the pellet ressuspended in sterile distilled water and the zoospore yield determined with a Fuchs-Rosenthal counting chamber. *Ca*. 5 x 10^6 zoospores were mechanically lysed and total DNA was directly extracted using the commercial DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. The DNA quantification was achieved using the Quant-it™DNA assay kit (Invitrogen) in the Qubit Fluorometer (Invitrogen).

3.4. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA)

DAS-ELISA was done according to Loewe-Phytodiagnostica protocol, using the Tobacco necrosis virus broad range antiserum (Loewe-Phytodiagnostica) and Nunc Maxisorp microtiter plates.

Each sample consisted of *ca*. 0.5 g of cabbage roots that were previously washed carefully for 10 min with a 1% SDS aqueous solution and then with running tap water for 3 h. Plant tissues were macerated in the presence of sample buffer (1:10) (2% PVP MW 40000, 0.2% BSA in washing buffer (0.137 M NaCl, 8 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.05% Tween 20, pH 7.4) pH 7.4) and centrifuged at 8000 *g* for 8 min.

In the first step of incubation the microtiter plate wells were coated with 200 µL of a solution of IgG diluted 1:200 in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and

incubated for 4 h at 37 ºC. Then, the antiserum was removed, the plate washed 4 times with washing buffer for 3 min each wash. 190 µL of each sample to be tested was placed in the plate wells and incubated over night at 4 ºC. Plates were then subjected to 5 washes of 3 min each. 190 µL of antibody-alkaline phosphatase-conjugate, diluted 1:200 in sample buffer were added to the wells and incubated for 4 h at 37 ºC. Then, 5 washes of 3 min each were done as above. 190 µL of enzyme substrate (1 mg/mL of substrate 4-nitrophenyl phosphate di-sodium-salt) prepared in substrate buffer (9.7% diethalonamine, 1 mM $MgCl_2.6H_2O$, pH 9.8) were placed in each well of the plate and incubated for 1-2 h at room temperature.

The enzymatic reaction was monitored at 405 nm after 1 h in a microtiter reader Model 680 (BioRad). Samples were considered positive when absorbance values were greater than twofold the average of the negative control values.

Each sample was tested twice in each microtiter plate. Positive controls were supplied by Loewe Phytodiagnostica and negative controls consisted of healthy cabbage roots.

3.5. PCR based assays

3.5.1. Primers

Specific primers to be used in multiplex RT-PCR for the identification of olive necroviruses, were designed as to exhibit similar annealing temperatures and based on TNV-D and on OMMV genomic published sequences, NC_003487 and NC_006939, respectively, retrieved using the Nucleotide Sequence Search program located in the Entrez Browser (http://www.ncbi.nlm.nih.gov/Entrez), aligned using BioEdit (version 7.0.9.0) (Hall, 1999).

The pair of primers designed for OMMV identification hybridize in the RdRp (OMMVd5') and in the CP gene (OMMVd3') regions whereas those specific for TNV-D identification are complementary to regions within the RdRp gene (TNVDd5' and TNVDd3'), originating amplicons sized 934 bp and 278 bp, respectively in PCR based tests (Figure 3.1).

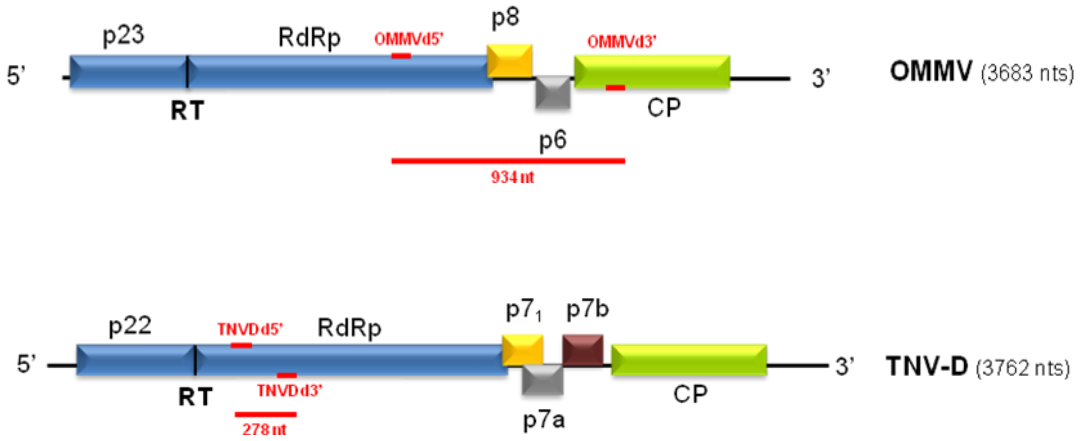


Figure 3.1: Schematic representation of OMMV (top) and TNV-D (bottom) genomes, indicating the location of the gene products and of the specific primers. RT: readthrough; RdRp: RNA polymerase RNA dependent; CP: coat protein.

These specific primers were used in multiplex RT-PCR assays together with primers specific for OLV-1 (Martelli *et al.*, 1996) (Table 3.1).

Table 3.1: Primers used for virus identification and for mutagenesis in PCR based assays. Mutations in mutagenic primers are underlined.

Virus	Primer	Position	Sequence 5' - 3'	Amplicon (bp)
OLV-1	PB	2720-2738	TTTCACCCCACCAAATGGC	747
	PA	3448-3466	CTCACCCATCGTTGTGTGG	
OMMV	OMMVd5'	1857-1875	CCGTGCCAAACACAATCTC	934
	OMMVd3'	2771-2791	CCTAGATCTTCTGGGCTAAGC	
TNV-D	TNVDd5'	303-322	GTAGGTGACAAGGACGCTGA	278
	TNVDd3'	560-581	GGATAGCGACTTTTTAGCCGCT	
OMMV	OMMVcoat5'	2613-2630	GACATTTACTATAACACC	877
	OMMVcoat3'	3471-3489	AAGGGTAGATATGTGGGCG	
OMMVA39T	sense	3185-3215	TCTGCGCTAAATAGCTACAGCTCTGGAGGGG	–
	antisense		CCCCTCCAGAGCTGTAGCTATTTAGCGCAGA	
OMMVG60A	sense	3265-3297	CAGCACAATAGGCAACACTGCCTTCACTGCTCT	–
	antisense		AGAGCAGTGAAGGCAGTGTTGCCTATTGTGCTG	

Specific primers (OMMVcoat5' and OMMVcoat3') encompassing the CP ORF of OMMV were designed based on the OMMV genomic sequence, NC_006939 (Table 3.1). The plus sense primer (OMMVcoat5') lies 6 nucleotides downstream of the OMMV CP ORF and the minus sense primer (OMMVcoat3') lies 25 nucleotides upstream of the OMMV CP ORF (Figure 3.2). The use of these primers in RT-PCR assays yields a fragment of *ca*. 877 nt.

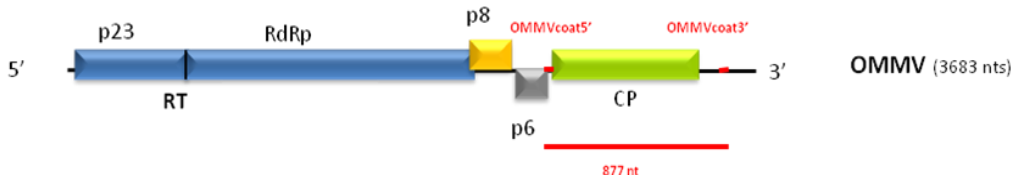


Figure 3.2: Schematic representation of OMMV genome, indicating the location of the gene products and of primers used for OMMV CP amplification in PCR reactions. RT: readthrough; RdRp: RNA polymerase RNA dependent; CP: coat protein.

Amplicons obtained following RT-PCR were observed in gel after electrophoresis to confirm the expected size, further purified by GFX PCR DNA and Gel band purification kit (GE Healthcare) and cloned into pGEM-T® Easy Vector (Promega, Madison, WI, USA) to confirm the corresponding genomic region sequence.

The mutagenic oligonucleotide primers used in *in vitro* site directed mutagenesis were designed within the OMMV CP ORF using Stratagene's web-based QuickChange® Primer Design Program, based on OMMV genomic sequence NC_006939 (see Table 3.1).

For *Olpidium* spp. identification, a primer specific for *O. virulentus* (OLPvirF) and a primer specific for *O. brassicae* (OLPbraF) were used together with one common reverse primer (OLPR) (Herrera-Vasquez *et al*., 2009) in a multiplex PCR assay (Table 3.2).

Table 3.2: Primers used for *Olpidium* sp. identification in PCR assays

Fungus	Primer	Position	Sequence 5' - 3'	Amplicon (bp)
O. virulentus	OLPvirF	53-76	AACCCAAGACCTGCCCCCAAAAG	579
	OLPR	609-632	TCCTCCGCTTATTGATATGCTTA	
O. brassicae	OLPbraF	396-416	AGCTATAGCTCACCCTCTTT	204
	OLPR	577-600	TCCTCCGCTTATTGATATGCTTA	

3.5.2. cDNA synthesis

For cDNA synthesis, 1 µg of denatured dsRNA fractions or 1 µg of total RNAs were used in a 20 µL reaction with 200 U of M-MLV reverse transcriptase (Invitrogen) in the presence of 150 ng of random hexamers (Promega), denatured for 10 min at 70 ºC and incubated on ice for 15 min. Reverse transcription was performed in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, for 1 h at 37 ºC, followed by 5 min at 70ºC for enzyme denaturation.

3.5.3. PCR

3.5.3.1. Optimization of multiplex RT-PCR assays

Each pair of primers was first tested individually in monospecific PCR assays. One µL of cDNA was used in a 50 µL reaction with 2.5 units of *Taq* DNA Polymerase (Fermentas) performed in 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)2SO_4$, 0.1% Triton X-100, 0.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer. Amplifications were carried out in a Thermal Cycler (BioRad) following 35 cycles at 94º C for 1 min, 54º C for 1 min and 72º C for 2 min, and a final extension step of 72º C for 10 min.

For multiplex PCR experiments, several parameters were tested to establish the optimal conditions for the amplification of targets: concentration of primers (0.1 to 0.5 µM) and magnesium chloride (0.75 mM to 2.5 mM), annealing temperature (50ºC to 60ºC) and time (30 s to 1.5 min). The final adopted conditions were similar to those used in monospecific PCR, except that 0.3 µM OMMV primer concentration and an annealing temperature of 56º C were used.

3.5.3.2. OMMV CP amplification

1 µL of cDNA was used in a 50 µL reaction with 2 U of FideliTaqDNA Polymerase (USB corporation) performed in 10 mM Tris HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer (OMMVcoat5' and OMMVcoat3'). Amplifications were carried out in a Thermal Cycler (BioRad) following an initial denature at 94 ºC for 1 min, 35 cycles at 94 ºC for 30 seconds, 53 ºC for 1 min and 68 ºC for 1 min and 30 seconds, and a final extension step of 68 ºC for 5 min. Amplicons of 877 bp were visualized in gel upon electrophoresis, as described in 3.5.4.

3.5.3.3. *Olpidium* spp. molecular identification

10 ng of DNA were used in a 50 µL reaction with 2.5 U of DreamTaq DNA Polymerase (Fermentas) performed in 1x DreamTaq buffer, 0.2 mM dNTP's, 0.2 µM of each primer. Amplifications were carried out in a Thermal Cycler (BioRad) programmed for a 5 min initial denaturation at 94 ^{o}C, followed by 35 cycles of denaturation at 94 ^{o}C for 45 s, annealing at 55 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min, and a final extension step at 72 ^{o}C for 10 min. Amplified products were visualized as in section 3.5.4.. Products of 579 bp reveal the presence of *O. virulentus* and products of 204 bp reveal the presence of *O. brassicae*.

3.5.4. Agarose gel electrophoresis

Fractions of dsRNA, plasmid DNA and RT-PCR products were visualized in 1 % agarose gel following electrophoresis in 0.5x Tris-borate-EDTA buffer (TBE) (1x TBE: 0.1 M Tris, 0.09 M boric acid, 1 mM EDTA, pH 8.5) at 80 V for 1 - 2 h. 1 Kb DNA Plus Ladder (Invitrogen) was used as molecular marker. Gels were stained with ethidium bromide (0.5 µg/mL) and photographed under UV light using a Kodak DC 120 camera (Sambrook *et al*., 1989).

3.5.5. Purification of RT-PCR products

RT-PCR products were purified from agarose gels following electrophoresis, using the Gel band purification kit (GE Healthcare).

3.6. DNA sequencing and sequence analysis

DNA sequencing reactions were performed by Macrogen (Seoul, Korea). Sequence analysis was carried out using BioEdit (version 7.0.9.0) (Hall, 1999).

3.7. Cloning vectors

The pUC18 vector (Invitrogen) is a high copy *E. coli* cloning vector. It is a double stranded circular DNA with *ca*. 2.7 Kb and includes a gene for antibiotic resistance to ampicillin (amp^{R}) and a gene for the enzyme beta-galactosidase (lacZ) (Figure 3.3). pUC18 has a region of

E. coli operon lac which contains a 5' – terminal part of the lacZ gene encoding the N – terminal fragment of beta-galactosidase and is capable of complementation with the C – terminal piece encoded by the bacterial chromossome. When the 2 fragments get together they form a fully functioning enzyme that degrades X-Gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside), into a blue product.

The expression of lacZ gene is induced by IPTG (isopropyl-[beta]-D-thiogalectopyranoside), an analog of lactose. The disruption of the lacZ gene by insertion of the desired DNA leads to white colonies due to the absence of enzyme activity.

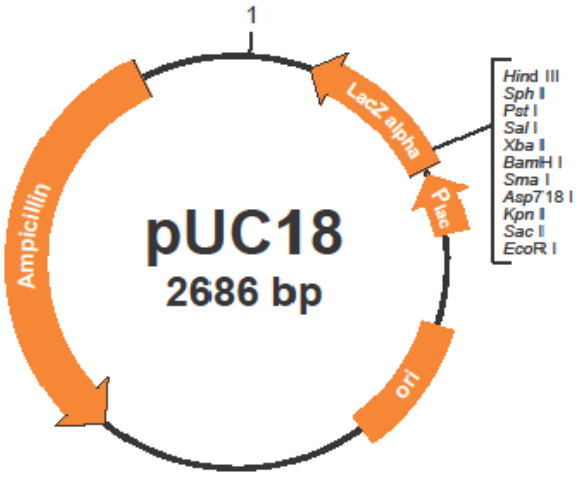


Figure 3.3: Schematic representation of pUC18 showing the multiple cloning site which allows the insertion of the desired DNA fragment and the disruption of the lacZ gene (Adapted from www.invitrogen.com).

The pGEM-T easy vector (Promega) is a high copy cloning vector. It is a double stranded linearized DNA with *ca*. 3.0 Kb and, as pUC18, includes a gene for antibiotic resistance to ampicillin and the lacZ gene containing the cloning site (Figure 3.4).

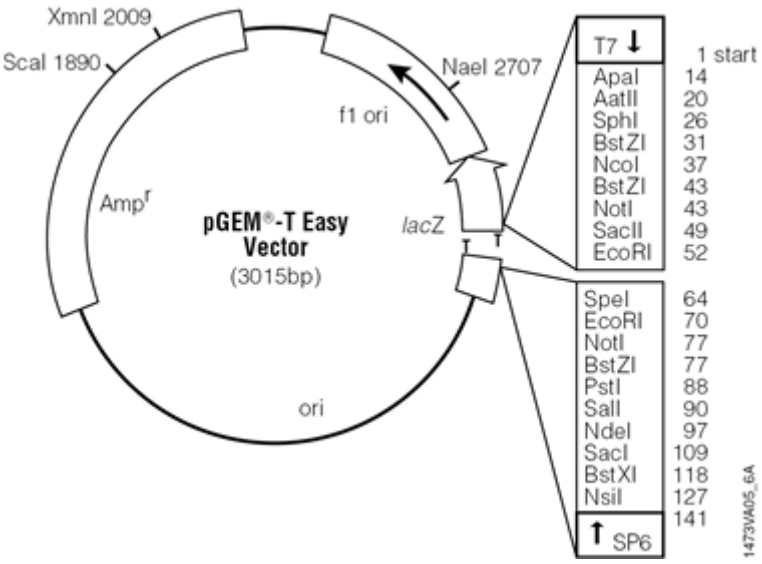


Figure 3.4: Schematic representation of pGEM®-T Easy Vector showing the multiple cloning site which allow the insertion of the desired DNA fragment and the disruption of the lacZ gene (Adapted from www.promega.com).

3.8. Competent cells and transformation conditions

E. coli JM109 competent cells ready to transform are components of the pGEM®-T Easy vector system II (Promega). Transformation was performed following the manufacturer's instructions using 5 µL of ligation reaction for each 50 µL of competent cells. This mixture was incubated on ice for 20 min, subjected to a heat shock at 42 ºC for 45 s and cooled on ice for 2 min. 950 µL of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) were added to the mixture and then shaked for 1.5 h at 37 ºC at 150 rpm. Cells were centrifuged at 1000 *g* for 10 min at room temperature and pellet was ressuspended in about 100 µL of the supernatant, plated on low salt LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5, 1.5% agar) supplemented with 100 µg/mL of ampicillin, 0.5 mM IPTG and 80 µg/mL X-Gal, for selection of recombinant clones, and incubated over night at 37 ºC.

3.9. Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli* cells using DNA-spin™ Plasmid DNA Purification Kit (Intron) following manufacturer's instructions, after growing cells in low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) supplemented with 100 µg/mL of ampicillin and grown over night at 37 ºC at 175 rpm.

3.10. *In vitro* transcription and inoculation of plants

The full length cDNA of OMMV (pUC18OMMV); the full length cDNA of OMMV/OMMVL11 (pUC18OMMV/OMMVL11) and the full length cDNA of the two site-directed OMMV mutants (pUC18OMMVN189Y and pUC18OMMVA216T), cloned in pUC 18 vectors, were used for *in vitro* transcription and inoculation of plants. To this effect, plasmid DNAs were linearized after digestion with *Sma*I restriction endonuclease which cuts at a unique site of pUC18 vector (see section 3.7.). They were further purified with QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions. *In vitro* transcription was achieved using RiboMax^{TM} Large Scale RNA Production System – T7 (Promega). About 2 µg of linear plasmid DNA were used in transcription reaction in the presence of T7 RNA Polymerase according to manufacturer's instructions. Following transcription, DNA templates were removed by digestion with DNase (1 U/µg of template DNA) and the transcripts were purified by extraction with phenol: chloroform (5:1) acid equilibrated (pH 4.7) (Sigma) and ethanol precipitated. About 2 µg of synthesized RNA were mechanically inoculated directly onto carborundum dusted leaves of a *Chenopodium murale* plant maintained in a growth chamber as described in 3.1.

3.11. Construction of OMMV/OMMVL11

The construct OMMVWT/OMMVL11 was obtained by substituting the OMMV CP gene by that of OMMVL11. To do this, RNA of OMMVL11 was extracted as in 3.3. and cDNA copies of the CP gene region were obtained by RT-PCR as described in 3.5.3.2. using primers flanking the CP ORF (see section 3.5.1.). The OMMV L11 CP RT-PCR product was ligated into pGEM easy vector (Promega) (see section 3.7.) (Figure 3.5.). 50 ng of pGEM-T vector (Invitrogen) were used in a 10 µL reaction with 1 U of T4 DNA ligase (Promega) in 1x ligation reaction buffer

(Promega) with 90 ng of OMMV L11 CP fragment. Ligation was done by incubation for 1 h at room temperature. Transformation conditions were as described in 3.8. and plasmid DNA was extracted as in 3.9.. DNA from this clone and from puc18OMMV (Figure 3.6.A and 3.6.B), was digested with *EcoN*I (*Xag*I)and *HPa*I (*KspA*I), which cut at unique sites flanking the CP ORF (nt 2643 and nt 3454 of OMMV genome, respectively).

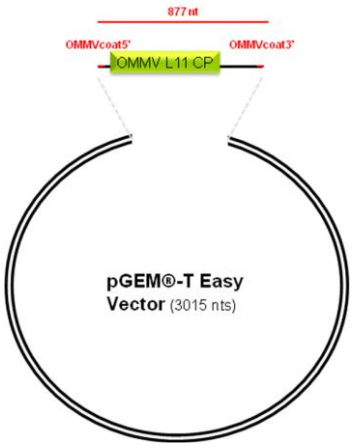
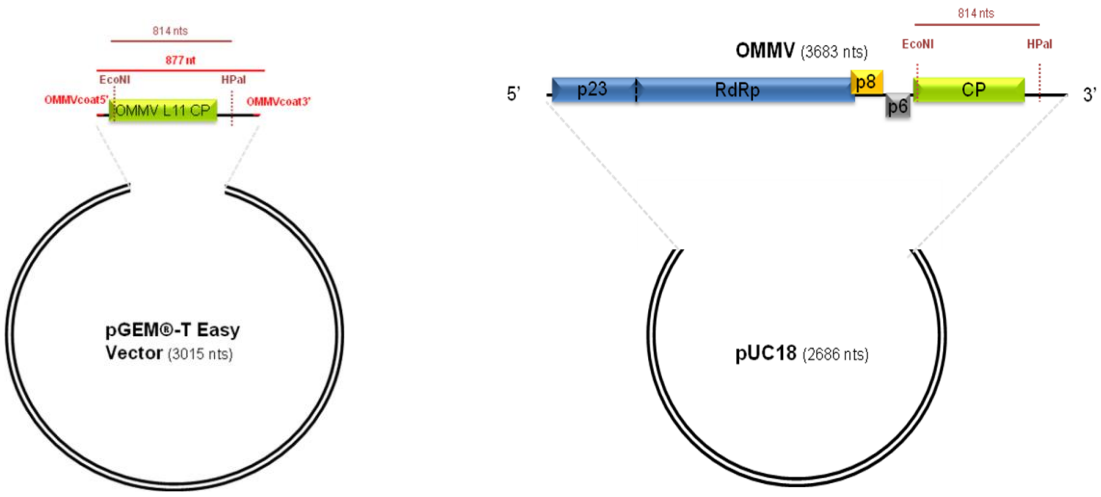


Figure 3.5: Schematic representation of pGEMOMMVL11CP.



A **B**

Figure 3.6.: Schematic representation of A: pGEMOMMVL11CP and B: pUC18OMMV, indicating the *EcoN*I and *HPa*I restriction sites flanking the CP ORF

The OMMV L11 generated amplicon in RT-PCR, sized *ca*. 811 nt, was ligated into *EcoN*I - *HPa*I digested 5558 nt pUC18OMMV fragment to produce pUC18OMMV/OMMVL11 (Figure 3.7.). 60 ng of digested puc18OMMV were used in a 20 µL reaction with 1 U of T4 DNA ligase (Promega) in 1x ligation reaction buffer (Promega) with 30 ng of digested OMMV L11 fragment. Ligation reaction was accomplished by incubating at 14 ºC for 20 h and 1 h at 24 ºC. Sequencing allowed to confirm the sequence of the entire transferred region of the OMMV L11 CP gene. *In vitro* transcription and inoculation of plants were done as described in 3.10. except that 3.5 µg of linear plasmid DNA were used. Symptomatic tissues of inoculated plants were used to inoculate healthy plants for further virus purification to be used in fungus transmission assays.

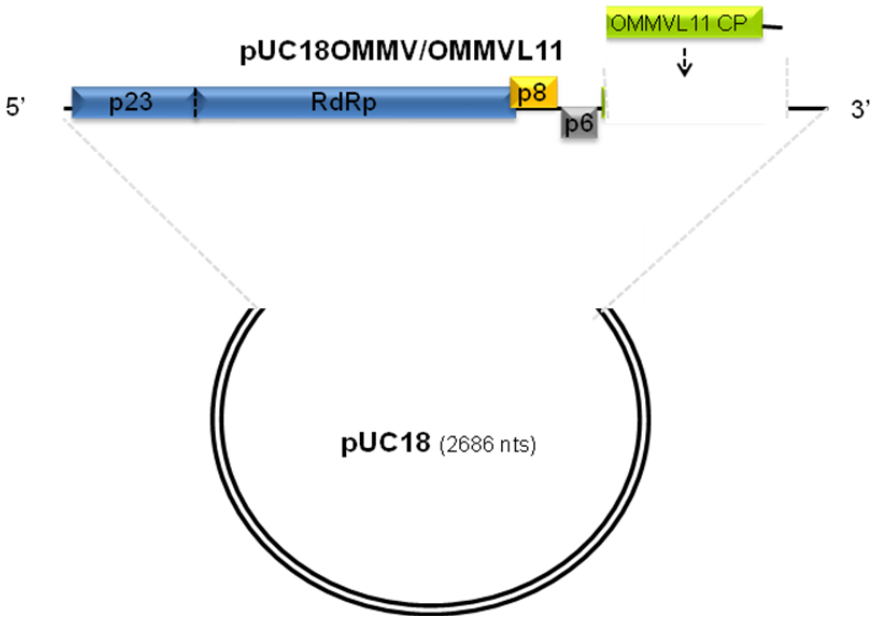


Figure 3.7.: Schematic representation of pUC18OMMV/OMMVL11

3.12. *In vitro* site-directed mutagenesis

In vitro mutagenesis was carried out to introduce mutations into OMMV WT cDNA to assess their role in the OMMV L11 transmissibility by *O. brassicae*. Plasmid DNA which contains the viral full-length cDNA of a clone of puc18OMMV was extracted as in 3.9. and used as template for site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. *In vitro* site-directed mutagenesis allowed to produce mutants OMMVN189Y and OMMVA216T, containing a single mutation each.

3.13. Assays to evaluate virus binding to fungus zoospores

100 µg of each purified virus OMMV, OMMV L11, OMMV/OMMVL11, OMMVN189Y and OMMVA216T were separately incubated with $1x10^6$ *O. brassicae* zoospores mL^{-1} in 10 mL 50 mM glycine-NaOH (pH7.6) for 20 min. Zoospores were then pelleted by centrifugation at 2800 *g* for 7 min. Supernatant containing unbound virus was further ultracentrifuged at 117000 *g* for 3 h, the pellet was resuspended in 30 µL of 0.02 M sodium phosphate buffer and the amount of virus in the pellet was estimated spectrophotometrically. Experimental controls were carried out as in section 3.14. except that no zoospores were included.

3.14. Evaluation of virus transmission by fungus

Five µg of each purified OMMV, OMMV L11, OMMV/OMMVL11, OMMVN189Y and OMMVA216T were added to 100 mL of 50 mM glycine-NaOH (pH 7.6) containing $1x10^6$ zoospores mL^{-1}, and as a control, to glycine solution alone. After a 20 min period to allow virus acquisition, 1 mL of the suspension was poured into 100 mL plastic cups containing 5 days old cabbage seedlings growing in sterile sand. Six days later, plants were taken and roots were carefully washed with a solution of SDS 1% to remove mechanically adsorbed virus to the root surface, then washed with abundant tap water and tested by DAS-ELISA as described in 2.7 to evaluate viral presence within the plant root cells. Similar experiments were conducted in the absence of fungal zoospores. A hundred pots, containing 10 plants each, were used in each experiment, which was repeated 5 times, involving a total of 500 pots for each virus.

3.15. Analysis of OMMV CP structure by homology modelling

Analysis of the structure of OMMV CP by comparative modelling approaches, was performed by Cláudio Soares from Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, using the MODELLER package (version 9v6) (Sali and Blundell, 1993), based on the TNV CP structure. The TNV CP structure was solved as a repetition (60 times) of a trimer unit, composed of the same protein, but presenting different observable lengths (meaning that a different number of residues can be identified in the X-ray structure) and some conformational differences (Oda *et al*., 2000). Due to the invisible parts in the crystal, OMMV protein sequence was only aligned with each one of these chains considering

the segments 79-269, 80-269 and 57-269, with chains A, B and C, respectively. Modelling the whole virus capsid, which contains 180 polypeptide chains, was outside of the reach of MODELLER. Therefore, a “minimum contact unit” was modelled, consisting in one central trimer surrounded by three other trimers, in a total of 12 chains. This arrangement has most of the multimer contacts of the central trimer unit, and is, therefore, adequate to analyse the effects of mutations.

The alignment made with Modeller align2d procedure was used to derive 40 models, and the one with the lowest value of the objective function was chosen. The Ramachandran plot of this model showed that 95.7% of the residues are found in most favoured regions, and 4.3% found in additional allowed regions. No residues were found in generously allowed or disallowed regions which evidences the good quality of the model.

To understand the effect of two mutations on the virus particle structure, namely OMMVN189Y and OMMVA216T, a double mutant containing them was built, using the same procedure used for the wild type.

Chapter 4. Molecular detection and identification of *Olpidium* spp. and of necroviruses in olive orchards

Olpidium spp. are known to be vectors of several viruses, namely *Olpidium brassicae* sl-transmitted TNV sl. Previous studies showing high levels (21%) of necrovirus infection in an olive orchard led to the search for *Olpidium* spp. in that soil and find out if it had any role in virus transmission. In this chapter, *Olpidium* spp. were morphologically and molecularly identified in PCR assays, using specific primers designed by Herrera-Vásquez *et al*. (2009). In addition, a multiplex RT-PCR assay was developed to discriminate the three necroviruses known to infect the olive crop, since there was no molecular sensitive and fast technique to differentiate OMMV from TNV-D. For that, two sets of specific primers for each OMMV and TNV-D were designed and their use in RT-PCR assays together with the primers specific for OLV-1 (Martelli *et al*., 1996) was optimized. Several parameters were tested to establish the optimal conditions for the multiplex PCR using, as template, dsRNA fraction extracted from herbaceous hosts and from olive trees. Application of this method allowed for the first time to determine levels of infection of OMMV and of TNV-D in olive trees.

4.1. Results

4.1.1. Detection and identification of *Olpidium* spp.

Roots of the cabbage bait plants growing in the 8 different samples of soil of a high necrovirus infected olive orchard were stained and observed under a light microscope. Zoospores showed a single posterior whiplash flagellum and a rapid and jerky movement, typical of most *Chytridiomycota* members. The presence of abundant stellate resting spores (Figure 4.1.) suggested that the fungus could be either *O. brassicae* or *O. virulentus* species. Reliable differentiation between these two species is only possible at molecular level.

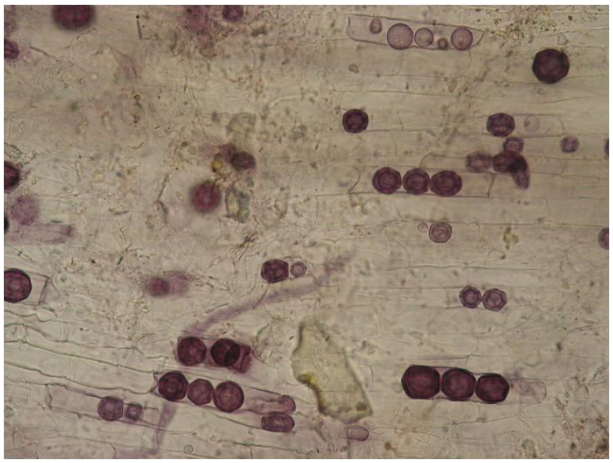


Figure 4.1: Root cells of cabbage bait plants revealing typical stellate spores of *Olpidium brassicae* sl.

The use of primers specific for *O. brassicae* and *O. virulentus* in multiplex PCR assays using fungal DNA extracted from roots of the bait plants, resulted in the amplification of a genomic sequence sized 579 bp and/or 204 bp in all the 8 soil samples tested. The product sized *ca*. 579 bp, revealed *O. virulentus* presence in 2 soil samples and the product sized *ca*. 204 bp, revealed *O. brassicae* in 7 (Figure 4.2).

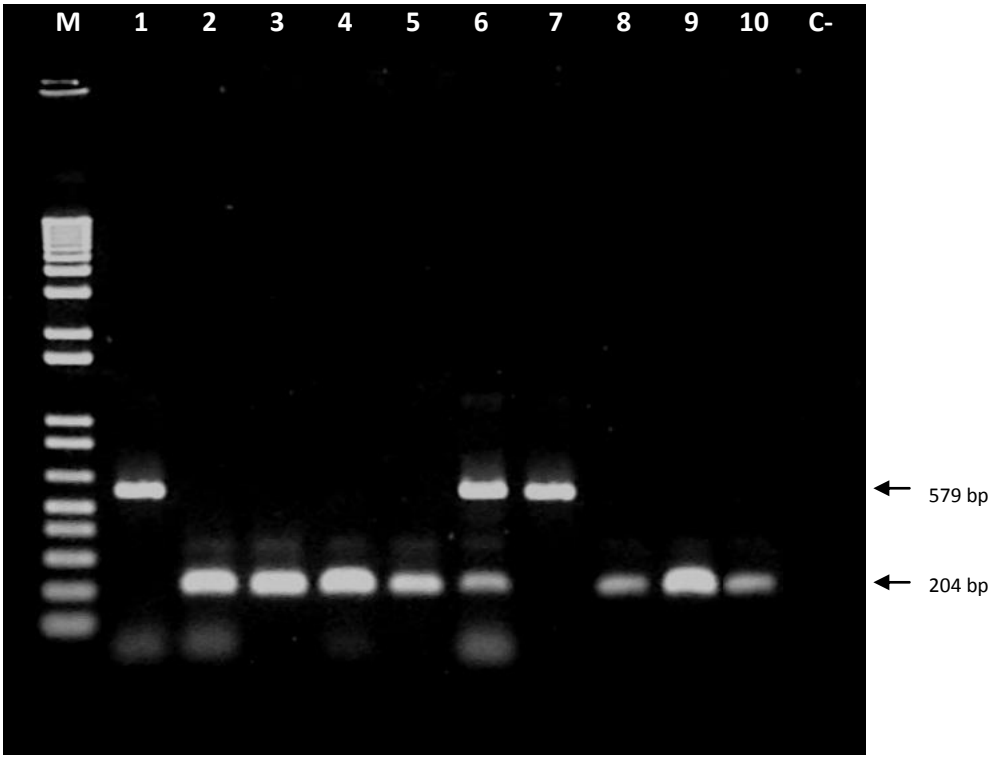


Figure 4.2: Amplicons generated in multiplex PCR assay using DNA extracted from roots of the bait plants growing in 8 soil samples of a high necrovirus infected olive orchard, with specific primers for *O. brassicae* and for *O. virulentus*, separated on a 1% agarose gel. Lane M: 1 kb plus DNA ladder (Invitrogen); Lane 1: DNA from *O. virulentus* (positive control); Lane 2: DNA from *O. brassicae* (positive control); Lane 3-10: DNA from the roots of the bait plants grown in 8 soil samples; Lane C-: healthy cabbage root. Size of generated amplicons are indicated on the right.

For the establishment of a single sporangial *O. brassicae* culture to be used in further studies (see next chapter), a single mature sporangium (Figure 4.3) was collected from roots of plants growing in a soil (tested in lane 4, Figure 4.2.) where only *O. brassicae* was detected by PCR.

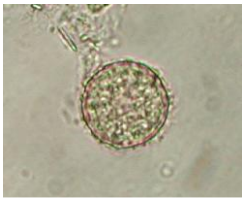


Figure 4.3: Single mature sporangium collected from bait plants growing in the soil of the olive orchard used to obtain a single sporangial *O. brassicae* culture

2 months after inoculation of cabbage seedlings with the single-sporangium, several rounded sporangia were observed in the roots (Figure 4.4) confirming the fungus presence.

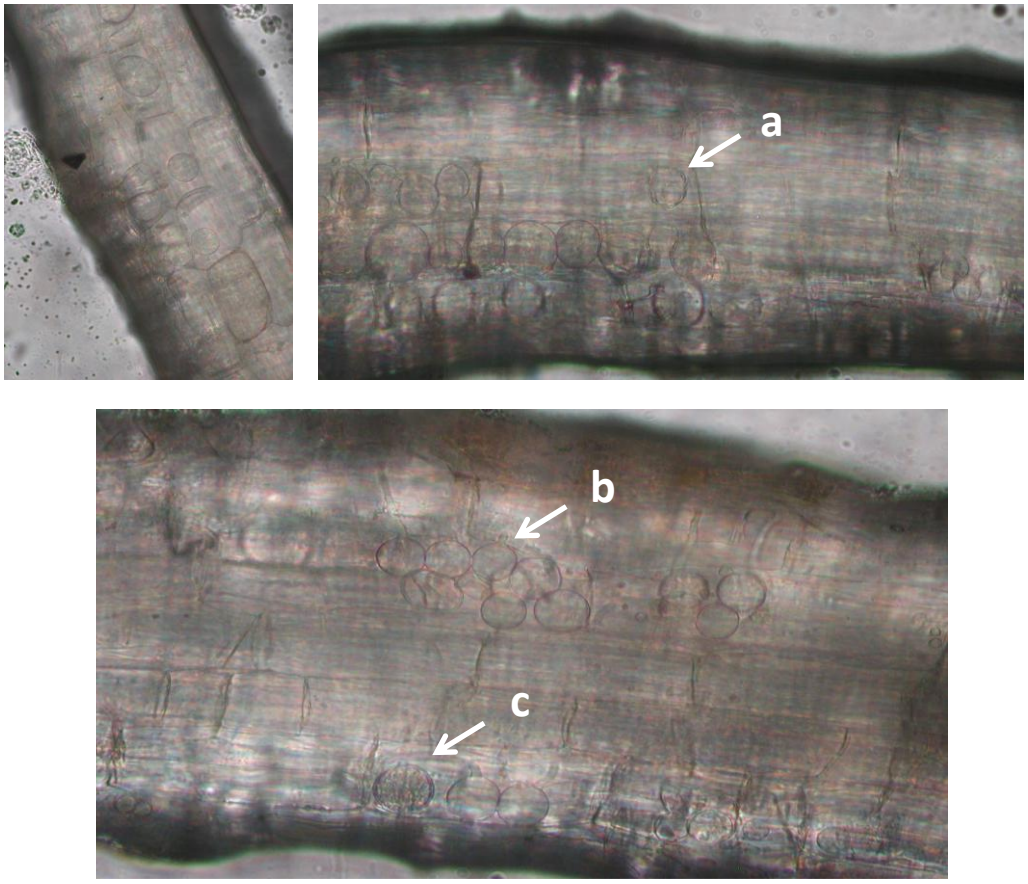


Figure 4.4: Cabbage roots containing sporangia previously inoculated with a single sporangium: a – resting spore; b – exit tube in sporangium; c – mature sporangium

The use of fungal DNA from the single sporangium culture as template in PCR based tests, resulted in the amplification of a single product *ca*. 204 bp (Figure 4.5), identifying it as an *O. brassicae* isolate.

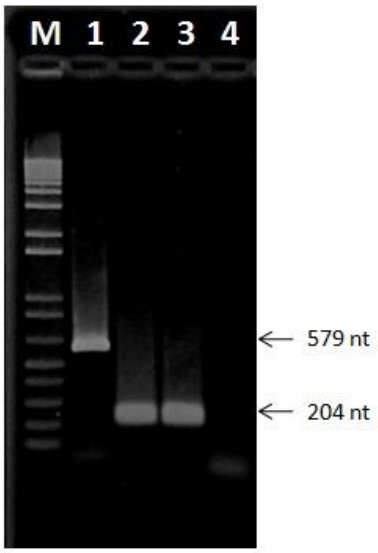


Figure 4.5: Products of multiplex PCR assay using DNA extracted from zoospores of the single sporangial culture, with specific primers for *O. brassicae* and for *O. virulentus*, separated on a 1% agarose gel. Lane M: 1 kb plus DNA ladder (Invitrogen); Lane 1: *O. virulentus* (positive control); Lane 2: *O. brassicae* (positive control); Lane 3: single sporangium culture; Lane 4: water (negative control). Size of generated amplicons are indicated on the right.

4.1.2. Molecular detection and identification of olive necroviruses

The use of primers complementary to OMMV, TNV-D and OLV-1 genomes in monospecific and multiplex PCR assays, using as template dsRNA extracted from herbaceous plants infected with OMMV, OLV-1 and plants infected with TNV-D, resulted in the amplification of cDNA fragments sized *ca*. 934 bp, 747 bp and 278 bp, respectively, as expected (Figure 4.6). Products with the expected size were easily distinguished in agarose gel electrophoresis. The sequencing of the generated fragments confirmed both the size and the corresponding genomic region sequence (see Appendix I and Appendix II).

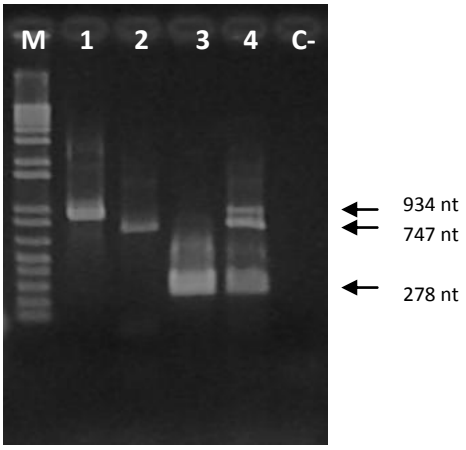


Figure 4.6: Products of monospecific and of multiplex RT-PCR applied to dsRNA extracted from infected herbaceous plants using specific primers for each of the three necroviruses, separated on a 1% agarose gel. Lane M: 1 Kb plus DNA ladder (Invitrogen); Lanes 1-3: OMMV, OLV-1 and TNV-D, respectively. Lane 4: mixture of OMMV, OLV-1, TNV-D. Lane C-: uninfected control. Size of generated amplicons are indicated on the right side.

The application of the optimized multiplex RT-PCR to the dsRNA fraction extracted from fruits and from stems of the sampled 54 olive trees, which molecules were not detectable in agarose gel following electrophoresis and apparently suggesting absence of RNA virus infection, revealed the amplification of fragments of *ca*. 934 bp, 747 bp and/or 278 bp in 31% of the analysed trees (Figure 4.7) using the same dsRNA as template. Using this assay, OMMV was found in 15 trees, OLV-1 in 12 trees and TNV-D in 4.

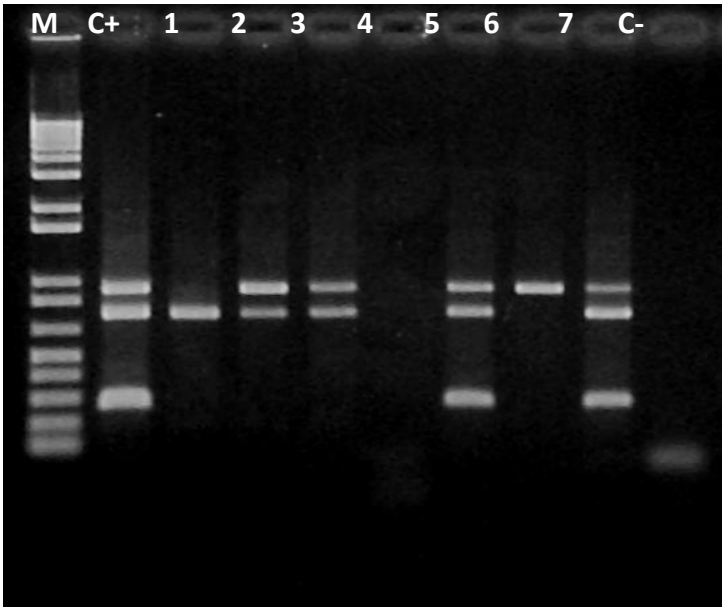


Figure 4.7: Products of multiplex RT-PCR using dsRNA fraction extracted from olive trees with specific primers for OMMV, OLV-1 and TNV-D, separated on a 1% agarose gel. Lane M: 1 Kb plus DNA ladder (Invitrogen); Lane C+: positive control obtained by using dsRNA extracted from an herbaceous plant multiple infected with OMMV, OLV-1 and TNV-D; Lane 1-7: trees tested; Lane C-: olive uninfected control. Size of generated amplicons are indicated on the right.

OMMV occurred either alone in five trees or in mixed infections with OLV-1 and with TNV-D. OLV-1 was also found either singly in two trees or together with the other olive necroviruses. TNV-D was detected in four trees all of which were co-infected with both OMMV and OLV-1. Double infections of OMMV and OLV-1 were demonstrated in six olive trees.

4.2. Discussion

This work showed that *Olpidium* sp. is very disseminated in the high necrovirus infected olive orchard. Application of PCR based tests revealed to be essential for the identification of Olpidium species. The observation of stellate resting spores and zoospores with a rapid and jerky movement (Lange and Insunza, 1977) in all samples is insuficient to differentiate *O. brassicae* from *O. virulentus*. Differentiation of these two species is only possible at a molecular level and the application of a multiplex PCR based test, using specific primers for each of the two Olpidium species showed the presence of *O. brassicae* in cabbage bait plants growing in 7 of the tested soil samples and *O. virulentus* in 2. In one of the samples tested, cabbage roots were found to be infected with both *O. brassicae* and *O. virulentus* and this finding was not anticipated as one of the criteria for separating these species is the host specificity (Koganezawa *et al*., 2005; Sasaya and Koganezawa, 2006). In this work they were both detected on crucifer cabbage plants. Indeed, natural mixed infections of *Olpidium* spp. have only been reported once, by Herrera-Vásquez *et al.* (2009).

This work also shows that dsRNA analysis, by itself, is not a sufficiently sensitive technique for the detection of olive necroviruses as compared to RT-PCR, reinforcing previous studies (Varanda *et al*., 2006). This may be due to the low concentration of replicating virus molecules in olive infected tissues, contrary to that observed in infected herbaceous hosts. However, the use of dsRNA as template for PCR is often a useful strategy as their isolation procedure eliminates much of virus non related nucleic acids present in the plant tissues as well as other components that may interfere with viral genome amplification (Saldarelli *et al*., 1994; Nolasco *et al*., 2000). On the other hand, the use of dsRNA in RT-PCR allows the use of a large sample (*ca*. 10 g), contrary to *ca*. 100 mg processed if total RNA were used, increasing the possibility of detecting viruses present in low concentrations and unevenly distributed in the plant, as happens with olive viruses (Bertolini *et al*., 2003). Their use in PCR based assays revealed a 31% level of necrovirus infection in the olive orchard tested.

In order to evaluate the potential role of Olpidium in transmitting OMMV, a molecular test enabling the discrimination of OMMV from TNV-D was needed because the high identity of the CP gene of these two viruses makes ambiguous all previous identification studies based either on serology or on PCR amplification with primers designed to amplify regions within the CP, which detects indistinctively TNV-D and OMMV. The specificity of the primers here described for discrimination of OMMV from TNV-D in RT-PCR assays, allowed for the first time to discriminate both viruses and its application allowed to investigate their distribution in olive

fields. The compatibility of these primers with those specific for OLV-1 allows their reliable use in multiplex RT-PCR assays.

The findings here reported on necrovirus infection reaching 31% on a site tallies with previous data revealing 21% of necrovirus infection in an olive orchard of cv. 'Negrinha de Freixo' (Mirandela, North of Portugal) and shows that necroviruses are frequently found in nature in this crop (Varanda *et al*., 2006). This has not been reported in other countries such as Italy where OLYaV is predominant. This further stresses the need for their accurate diagnosis in phytosanitary certification programmes in trading plants within the European Union, as required by directive 93/48 concerning the *Conformitas Agraria Communitatis*, as well as in improvement programs of important olive cultivars.

The much higher level of OMMV (28%) over TNV-D (7%) suggests that OMMV is either better adapted to the host or has some advantage over TNV-D in dissemination, or OMMV may be continuously arising from recombination events between OLV-1 and TNV-D, a mechanism that was proposed for the origin OMMV (Cardoso *et al*., 2005). TNV-D was the necrovirus found in lower number in olive trees, occurring always together with the other two necroviruses. This fact suggests that OLV-1 and OMMV may somehow facilitate TNV-D infection or dissemination.

The multiplex RT-PCR here optimized, is a rapid way of accurately diagnose those viruses. This allows to obtain and interpret data on mutual virus interaction, geographical distribution and means of field dissemination, which to a large extent remain unclear.

The finding of Olpidium species in all the sampled sites of a high necrovirus infected olive orchard together with previous studies showing that TNV sl is transmitted by *Olpidium brassicae* sl, suggest that these fungi may also act as vector of OMMV which is investigated here (see next chapter) and be responsible for its high level of infection in the olive orchards.

Chapter 5. *Olive mild mosaic virus* transmission by *Olpidium brassicae*

In chapter 4 it was shown that *O. brassicae* was present in almost all sampled sites of an olive orchard where trees had revealed to be highly infected with necrovirus. In addition, the application of multiplex PCR to another olive orchard revealed 31% of necrovirus infections, 28% of which were found to be OMMV-infected through the use of new designed specific primers to be used in RT-PCR assays. These facts, together with prior information gathered from the literature that show that TNV sl is soil transmitted through zoospores of *O. brassicae* (Temmink *et al*., 1970), led to the investigation of its role on OMMV transmission.

In this chapter, transmission of OMMV by *O. brassicae* was evaluated and possible determinants involved on transmission were studied. For that three strains of OMMV were used: 1) an OMMV wild type (WT) transcript, previously recovered from olive trees; 2) an OMMV variant (OMMVL11) obtained after 15 serial passages of single local lesions induced in *C. murale* plants by OMMV WT and 3) a construct OMMVWT/OMMVL11 in which the coat protein gene of OMMVL11 replaced that of the WT. OMMV WT and OMMVL11 CPs were sequenced, aligned and compared to search for mutations. The 3-dimensional location of each OMMVL11 mutation was determined, as well as its possible correlation with transmissibility loss through fungal zoospores.

5.1. Results

5.1.1. Search of domains in OMMV genome sequence

The search of the conserved domains of the necrovirus OMMV CP (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), revealed one single domain: the shell domain (53 – 270 aa) and Ca^{2+} binding sites in aa 153, 156, 212 and 269 (see Appendix III). The plant viruses icosahedral capsid protein 'S' region signature (134 – 159 aa) is composed of 26 aa ([FYW]-x-[PSTA]-x(7)-G-x-[LIVM]-x-[LIVM]-x-[FYWI]-x(2)-D-x(5)-P).

OMMV p6, retains the predicted transmembrane motif (AILILILAILVV) found in OLV-1 (Castellano *et al.*, 2005) preceeded by polar and proline residues (see appendix IV) which may have a role in virus replication in a plant cell.

5.1.2. Yield and infectivity of OMMV WT transcript, OMMVL11 and construct OMMV/OMMVL11

Plasmid DNA of pUC18OMMV containing the full-length cDNA of OMMV clone (Figure 5.1) (Appendix V) was extracted as described previously (3.9.), linearized with *Sma*I and transcribed (3.10).

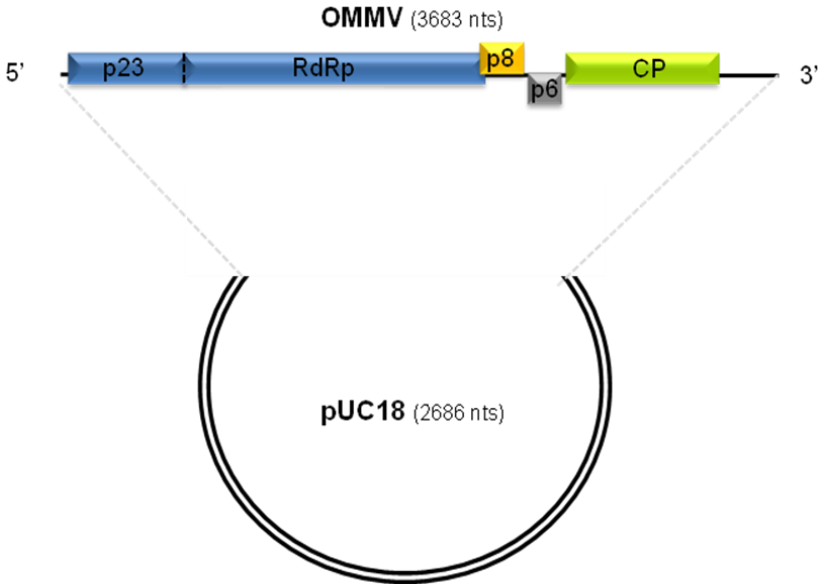


Figure 5.1: Schematic representation of pUC18OMMV

Linearized plasmid DNA was analyzed in agarose gel for quantification for *in vitro* transcription purposes and revealed to be *ca*. 6369 bp, as expected (Figure 5.2). Following transcription, the fragment revealed to be *ca*. 3683 nt when analyzed by agarose gel electrophoresis, as expected (Figure 5.3).

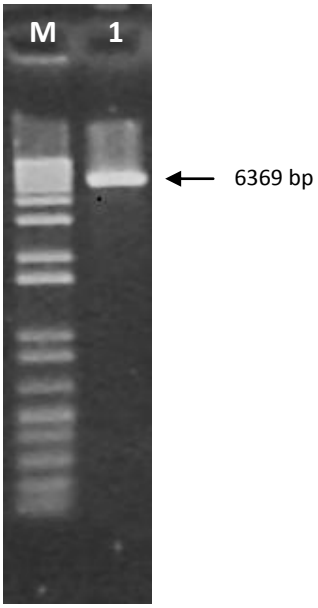


Figure 5.2: Analysis of pUC18OMMV plasmid DNA after linearization with *Sma*I in 1% agarose gel. M – 1 kb plus DNA ladder (Invitrogen); 1 – pUC18OMMV-*Sma*I fragment. Arrow indicates the position of the band.

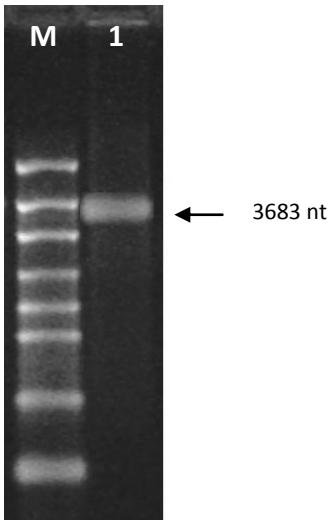


Figure 5.3: Analysis of OMMV *in vitro* transcript derived from the full-length pUC18OMMV cDNA in 1% agarose gel. M – RiboRuler high range RNA ladder (Fermentas); 1 – OMMV transcript. Arrow indicates the position of the band.

The infectivity of OMMV WT transcript was confirmed after inoculation with 2 µg of RNA in *C. murale* host plants. Symptoms began to appear three days after inoculation, which consisted of local necrotic lesions, as seen in Figure 5.4.. One of the local lesions produced by OMMV WT transcript in *C. murale* was used to produce an OMMV variant (OMMVL11) by 15 successive mechanical inoculations in *C. murale* plants. Symptoms observed after those passages were similar to the ones caused by OMMV WT in the same host (Figure 5.5).

Figure 5.4: *C. murale* plants showing local necrotic lesions after inoculation with transcript OMMV WT RNA

Figure 5.5: *C. murale* plants showing local necrotic lesions after inoculation with OMMVL11

5.1.2.1. Sequencing and alignment of OMMV and OMMVL11 CPs

The sequences of OMMV WT CP and OMMV L11 CP were determined and aligned and OMMVL11 CP was found to contain two nucleotide substitutions comparing to OMMV WT CP (Figure 5.6.): a A to T substitution at nucleotide 3200 and a G to A substitution at nucleotide 3281. These differences caused two amino acid changes, an Asn to Tyr at aa 189 and a Ala to Thr at aa 216 positions, respectively (Figure 5.7).

```
                                10        20        30        40        50
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      GACATTTACTATAACACCAAAACATGCCTAAGAGAGGACGAGTTGGACTC
OMMVL11coat5'-coat3'   ..................................................

                                60        70        80        90        100
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      GCTGAATCTTTTCAAGGAAAGACGAAGCAACAGAAACGGGCAGAGTACGA
OMMVL11coat5'-coat3'   ..................................................

                               110       120       130       140       150
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      AGCTGTAAAACGTGAGCAGCTCGAACGTGCATTCGCAAACAATTCCAAGG
OMMVL11coat5'-coat3'   ..................................................

                               160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      TAGCCAATCCTAGATCTTCTGGGCTAAGCTTCCGACCGTTGGTGGCACCA
OMMVL11coat5'-coat3'   ..................................................

                               210       220       230       240       250
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      ATCGCCGGGTCCGTTGTTTACAGCAGACCTCGCGTGCCACAGATCCGCAC
OMMVL11coat5'-coat3'   ..................................................

                               260       270       280       290       300
                       ....|....|....|....|....|....|....|....|....|....|
OMMVcoat5'-coat3'      GAACCAAATGTCAACTATTGTGGTAAACACTGAATTGGTAGCCAACATCA
OMMVL11coat5'-coat3'   ..................................................
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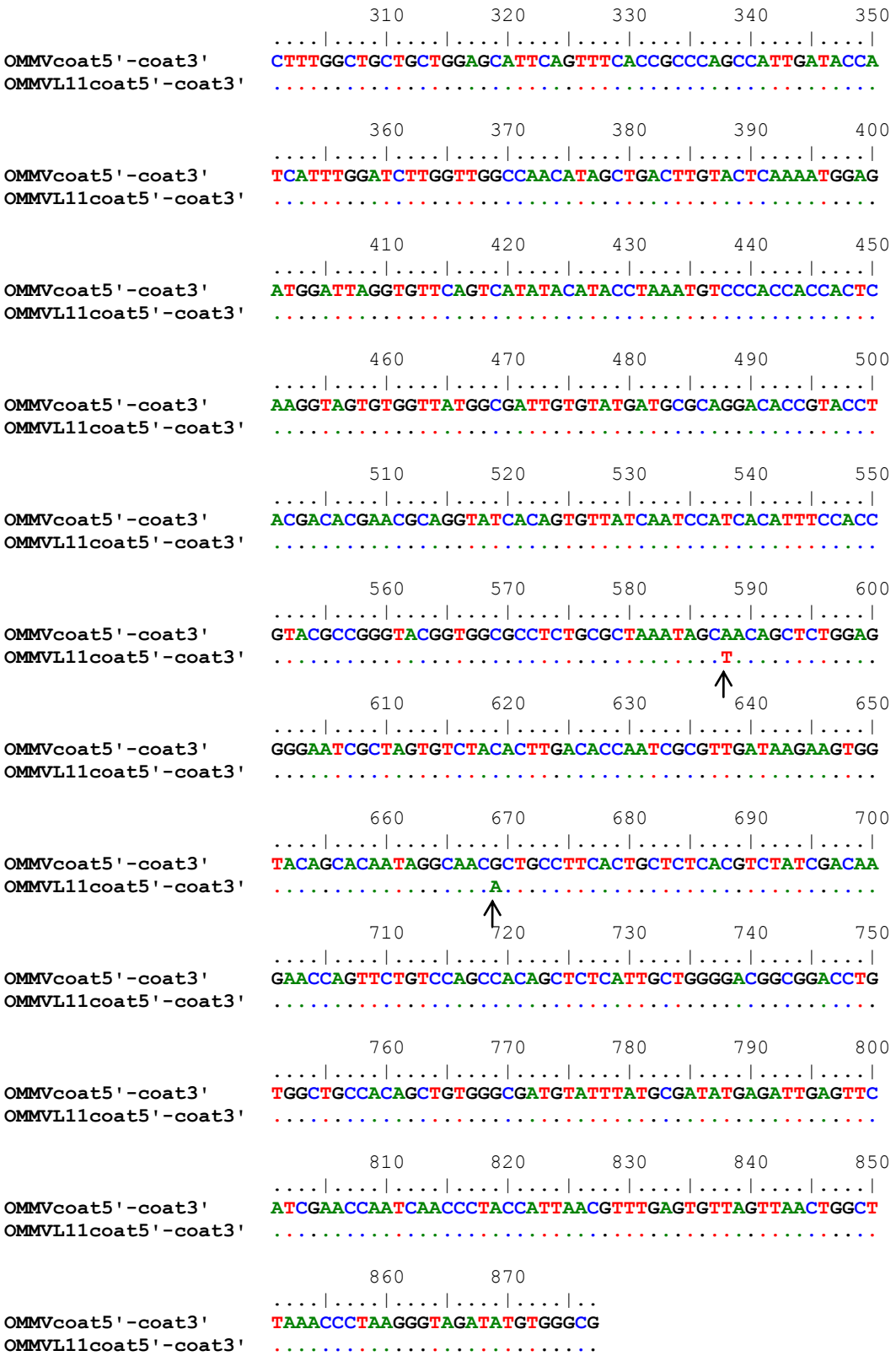

Figure 5.6.: CP nucleotide sequence alignment of OMMV and OMMVL11. The changes A to T at nt 3200 and G to A at nt 3280 are marked with arrows.

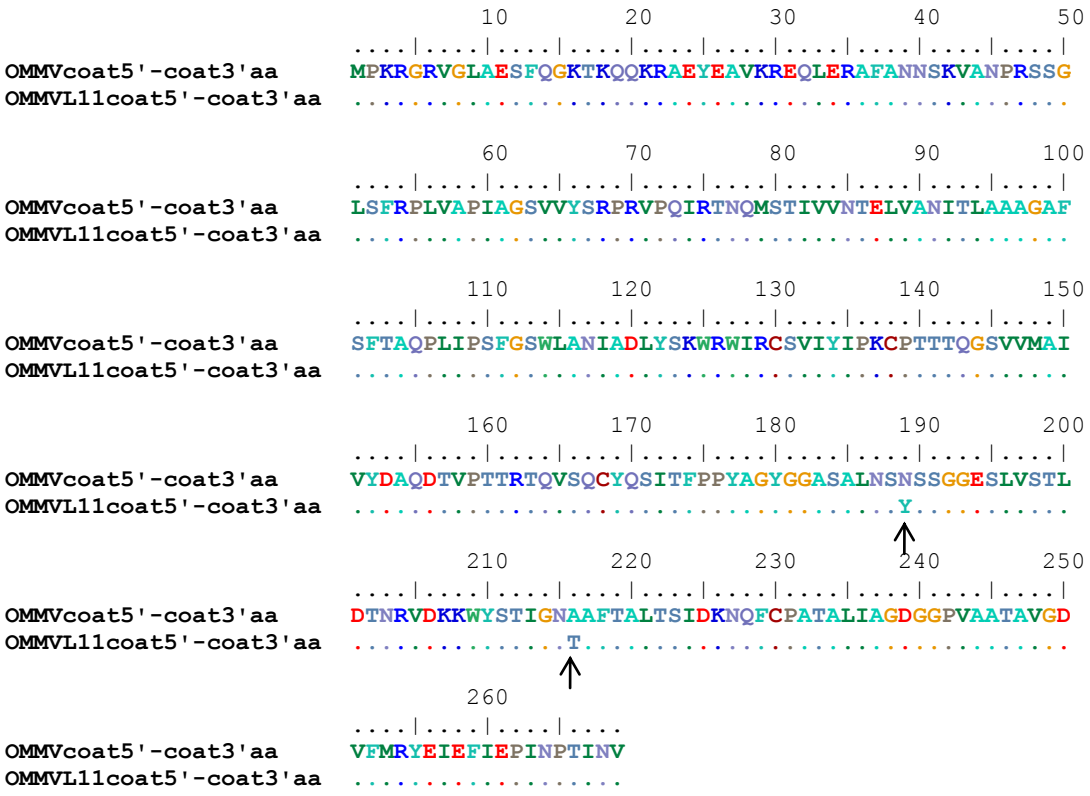

Figure 5.7.: CP aa sequence alignment of OMMV and OMMVL11. The changes Asn to Tyr at aa 189 and Ala to Thr at aa 216 are marked with arrows.

5.1.2.2. Construction of OMMVWT/OMMVL11

OMMVL11 RNA was extracted and used as template in RT-PCR assays using specific primers (OMMVcoat5' and OMMVcoat3', see section 3.5.1.) for the amplification of the CP gene and a product of *ca*. 877 bp was obtained (Figure 5.8) and sequencing confirmed the expected sequence (Appendix VII). This corresponds to cDNA comprising a region from 23 nucleotides upstream the CP to 44 nucleotides downstream the CP.

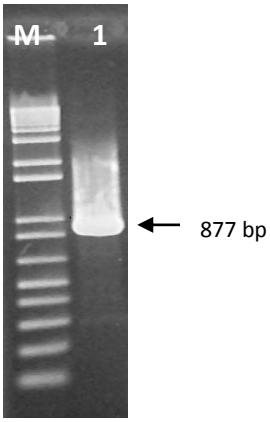

Figure 5.8.: Product of RT-PCR applied to OMMVL11 RNA using primers OMMVcoat5' and OMMVcoat3' that allow amplification of the CP gene, separated in a 1% agarose gel. M – 1 kb plus DNA ladder (Invitrogen); 1 – OMMVL11 fragment. Arrow indicates the position of the band.

The OMMVL11 CP product was ligated into pGEM®-T easy vector and plasmid DNA was extracted after transformation as described (section 3.8.). A fragment, pGEMOMMVL11CP, consisting of *ca*. 3892 bp was obtained (Figure 5.9).

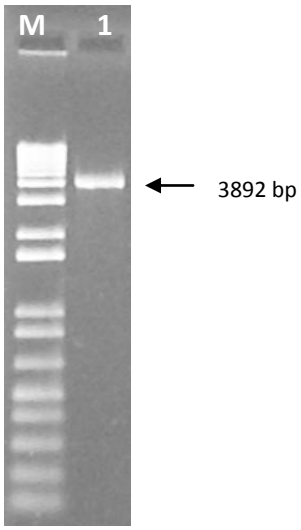

Figure 5.9.: Analysis of plasmid DNA fragment pGEMOMMVL11CP, separated in a 1% agarose gel. M – 1 kb plus DNA ladder (Invitrogen); 1 – pGEMOMMVL11CP fragment. Arrow indicates the position of the band.

DNA from clone pUC18OMMV and clone pGEMOMMVL11CP was digested with *EcoN*I and *HPa*I, which cut at unique sites flanking the CP ORF. In each case, two distinct fragments

were observed in gel electrophoresis (Figure 5.10.). The smaller obtained for pGEMOMMVL11CP (Figure 5.10., lane 1, green circle), of *ca*. 811 nt (fragment *Eco*NI-*HPa*I), corresponds to OMMVL11CP minus 8 nt downstream and plus 9 nt upstream the CP gene.

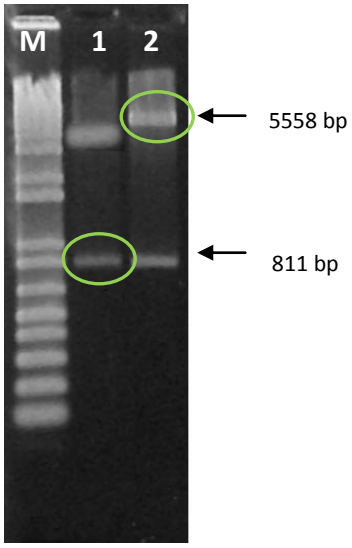

Figure 5.10.: Analysis of the digested products after restriction with EcoNI and HPaI, separated in a 1% agarose gel. M – 1 kb plus DNA ladder (Invitrogen); 1 – pGEMOMMVL11CP *Eco*NI-*HPa*I fragment. 2 – pUC18OMMV *Eco*NI-*HPa*I fragment. Arrows indicate the position of the bands. Green circles indicate the products extracted from gel and used for ligation.

Products were extracted from gel and purified as in 3.5.5. The *ca*. 811 nt product was ligated into the *ca*. 5558 nt pUC18OMMV larger fragment (Figure 5.10., lane 2, green circle), that corresponds to pUC18 (2686 nts) plus OMMV genome (3683 nts) minus fragment *Eco*NI-*HPa*I (811 nt), to produce pUC18OMMV/OMMVL11 as described (3.11.). After restriction with *Sma*I, linearized plasmid DNA was analyzed in agarose gel for quantification for *in vitro* transcription purposes and revealed to be *ca*. 6369 kb, as expected (Figure 5.11.). Following transcription, the fragment revealed to be *ca*. 3683 nt when analyzed by agarose gel electrophoresis, as expected (Figure 5.12).

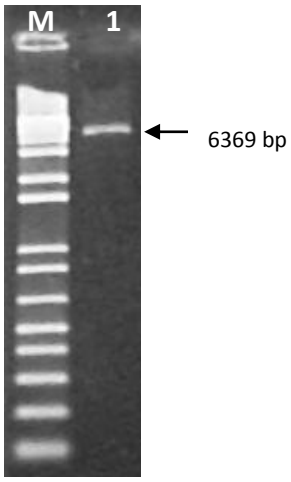

Figure 5.11.: Analysis of plasmid DNA fragment pUC18OMMV/OMMVL11, separated in a 1% agarose gel. M – 1 kb plus DNA ladder (Invitrogen); 1 – pUC18OMMV/OMMVL11 fragment. Arrow indicates the position of the band.

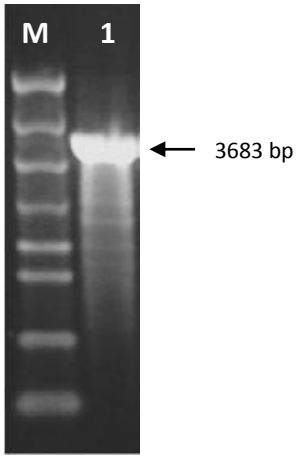

Figure 5.12.: Analysis of RNA, following *in vitro* transcription, of construct OMMVWT/OMMVL11 derived from pUC18OMMV/OMMVL11 cDNA in 1% agarose gel. M – RiboRuler high range RNA ladder (Fermentas); 1 – OMMVWT/OMMVL11 transcript. Arrow indicates the position of the band.

The infectivity of OMMVWT/OMMVL11 transcript was confirmed after inoculation with 2 µg of RNA in *C. murale* host plants. Symptoms started to appear three days after inoculation and consisted of local necrotic lesions as seen in Figure 5.13..

Figure 5.13.: *C. murale* plants showing local lesions three days after inoculation with OMMVWT/OMMVL11 RNA

5.1.2.3. Site-directed mutants OMMVN189Y and OMMVA216T properties

Plasmid DNA of pUC18OMMV containing the full-length cDNA of OMMV clone (Figure 5.1) was used as template for site-directed mutagenesis as described previously (3.12). Mutants OMMVN189Y and OMMVA216T were produced, containing, respectively, the single nucleotide substitution A to T at nt 3200 and G to A at nt 3281, corresponding to the amino acid changes Asn to Tyr at aa 189 and Ala to Thr at aa 216 (Figure 5.14). Mutants were sequenced to ensure that no other mutations occurred in the CP gene (Appendix VII).

After restriction with *Sma*I, linearized plasmid DNA of the two single aa mutants was used for *in vitro* transcription as described previously (3.10.). Both fragments revealed to be *ca*. 3683 nt when analyzed by agarose gel electrophoresis, as expected (Figure 5.15).

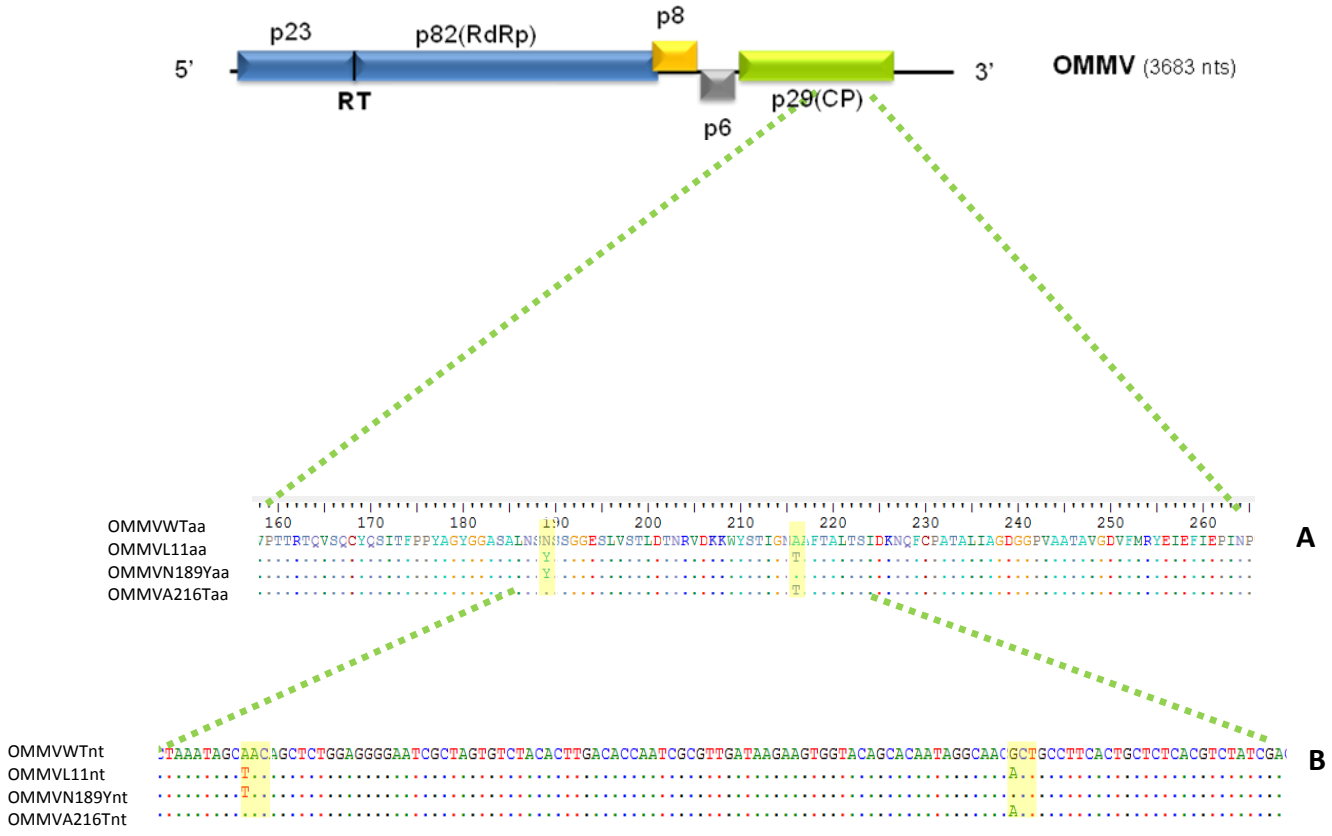

Figure 5.14: Schematic representation of OMMV mutants with location of mutations highlighted in yellow. Deduced amino acid sequences (A) and nucleotide sequences (B).

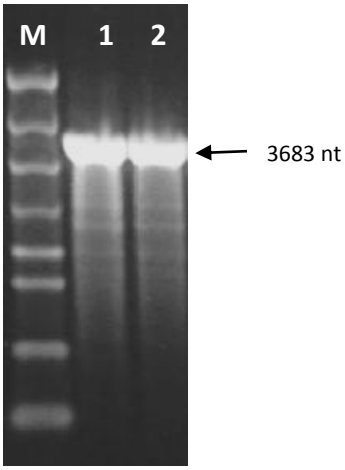

Figure 5.15: Analysis of OMMV *in vitro* site-directed mutants transcripts in 1% agarose gel. M – RiboRuler high range RNA ladder (Fermentas); 1 – OMMVN189Y transcript; 2 – OMMVA216T transcript. Arrow indicates the position of the band.

The infectivity of the transcripts of mutants OMMVN189Y and OMMVA216T was confirmed after inoculation with 2 µg of RNA in *C. murale* host plants. Symptoms started to appear three days later. Symptoms consisted of local necrotic lesions (Figure 5.16).

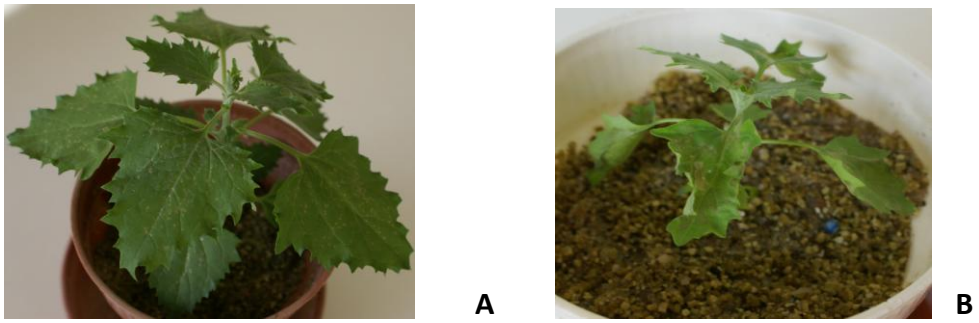

Figure 5.16: *C. murale* plants showing local lesions three days after inoculation with mutants OMMVN189Y and OMMVA216T RNA. A – OMMVN189Y; B – OMMVA216T

Using the previously described protocol (see section 3.1.) each virus purification, from 100 g of infected *C. murale* plants yielded approximately the same amount, *ca*. 0.3 mg of virus. These viral preparations produced a single band at the same tube position after ultracentrifugation in sucrose gradients.

5.1.3. Virus transmission by *O. brassicae*

The single sporangial *O. brassicae* culture (chapter 4) was used for transmission and binding assays. Cabbage roots inoculated with each virus, alone or previously incubated with zoospores of *O. brassicae* were pooled from each pot 5 days after inoculation and tested for the presence of virus by DAS-ELISA. Transmission efficiency was scored by assessing the number of pots containing virus-infected plants *versus* the total number of pots inoculated. Results of five transmission assays indicate on average that in 39 out of 100 pots (39%) plants became infected with OMMV after inoculation with OMMV alone, whereas in 87 of 100 pots (87%) plants became infected with OMMV after inoculation with OMMV/zoospore mixture (Table 5.1). These results demonstrate that OMMV transmission is highly facilitated in the presence of *O. brassicae* zoospores.

Transmission efficiency results of the OMMVL11 mutant and the OMMVWT/OMMVL11 in the absence of fungal zoospores were 32% and 29%, respectively. When zoospores were added to these viruses prior to inoculation to cabbage roots, no increase in transmission efficiency was observed, 31% (average of data of five experiments) in both (Table 5.1), suggesting that this fungus does not have a role in these viruses transmission.

In the case of the constructed single mutants, transmission assays indicated that plants became virus-infected in 35 of 100 pots (35% efficiency) after inoculation with OMMVN189Y alone, and in 33 of 100 pots (33% efficiency) plants became virus-infected after inoculation with OMMVN189Y/zoospore mixture. On the other hand, in 36 of 100 pots (36% efficiency) plants became virus-infected after inoculation with OMMVA216T alone whereas in 80 of 100 pots (80% efficiency) plants became virus-infected after inoculation with OMMVA216T/zoospore mixture (Table 5.1). These results suggest that the Asn to Tyr mutation is largely responsible for the loss of transmissibility of OMMVL11 by *Olpidium brassicae* and that the Ala to Thr mutation had no effect on transmissibility.

Table 5.1: Soil transmissibility of OMMV, OMMVL11, OMMVWT/OMMVL11, OMMVN189Y and OMMVA216T to cabbage roots in the absence and presence of *O. brassicae* zoospores

	Transmission efficiency (%) [a]					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Average
OMMV	35	35	45	40	40	39
OMMV + zoospores [b]	80	90	90	85	90	87
OMMV L11	30	35	30	30	35	32
OMMV L11 + zoospores [b]	35	30	30	30	30	31
OMMV/OMMVL11	30	30	25	30	30	29
OMMV/OMMVL11 + zoospores [b]	30	30	35	30	30	31
OMMVN189Y	33	37	36	36	32	35
OMMVN189Y + zoospores [b]	32	33	33	33	32	33
OMMVA216T	39	35	35	34	36	36
OMMVA216T + zoospores [b]	83	80	79	76	83	80

Virus root infection was determined by DAS-ELISA
[a]Percentage of pots containing infected plants. A hundred pots for each experiment were used
[b]Virus was incubated with zoospores for 15 min prior to adding to the plants in each pot

5.1.4. Virus-zoospores binding assays

Assays were carried out to determine if the reduced ability of OMMVL11 and OMMVWT/OMMVL11 particles to be transmitted by *O. brassicae* zoospores was at least partially due to a binding deficiency. It was found that only *ca*. 7 µg out of the 100 µg of OMMV initially incubated with zoospores became associated with them as the remaining 73 µg were found free in the supernatant. This suggests that 7 µg is the maximum amount of OMMV particles that 1 x 10^7 zoospores of *O. brassicae* can adsorb, reaching saturation of receptors above that level. The same values were obtained when mutant OMMVA216T was used. In the case of OMMVL11 and OMMVWT/OMMVL11, concentrations found in the supernatant, after incubation with zoospores, were similar to the initial amount used in the incubation step, indicating that no significant amount of virus remained adsorbed to zoospores. Also, OMMVN189Y concentrations found in the supernatant were similar to the initial amount used. In experiments where no spores were used, the amount of virus found in the supernatant was identical to that added initially, as expected. These results suggest that the lack of transmission of OMMVL11, OMMVWT/OMMVL11 and OMMVN189Y is due to their inability to stably attach to zoospores during the transmission process.

5.1.5. Homology modelling

Analysis of the structure of OMMV CP performed as in 3.15, showed that the OMMVA216T mutation occurs at the external face of the capsid (Figure 5.17,b and c) and the OMMVN189Y mutation occurs in the capsid interior (Figure 5.17, e and f). A more detailed analysis of the mutation locations shows that in OMMVA216T mutation occurs at the beginning of an helix in a very exposed region (Figure 5.17, b and c) and that in OMMVN189Y is located in a loop region not far from intersubunit contacts (Figure 5.17, e and f).

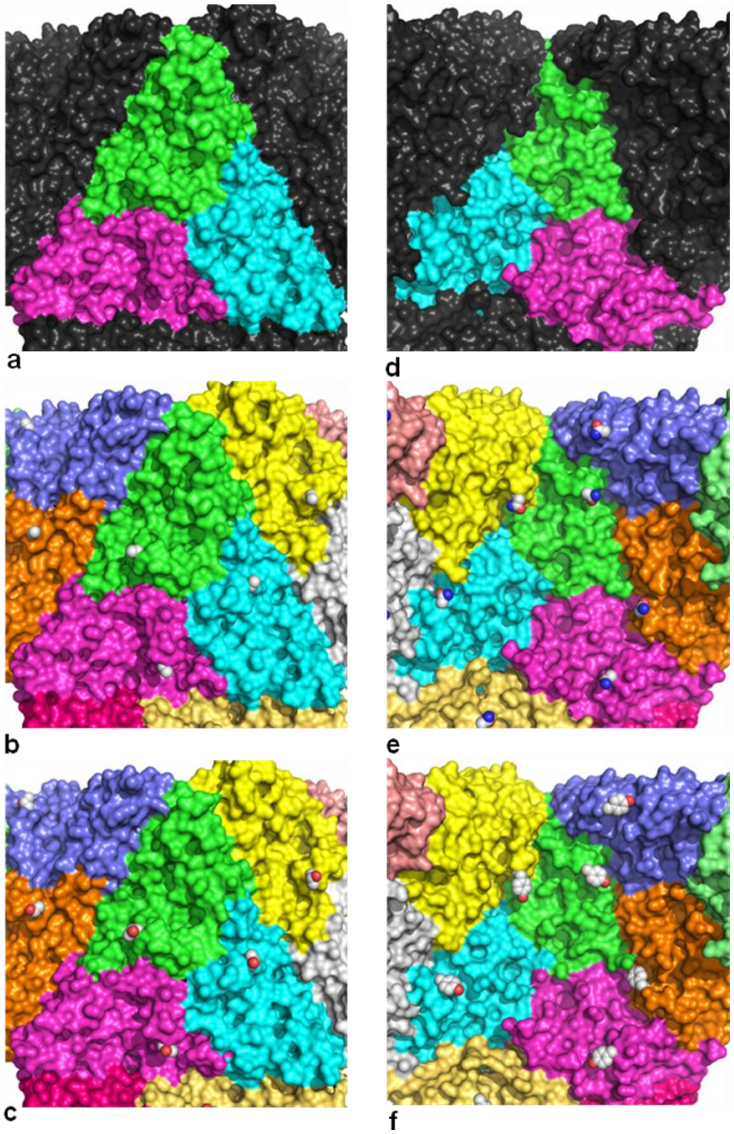

Figure 5.17: Three-dimensional representation of the surface of the OMMV minimum contact unit, in two different views: the external face of the capsid, at the left side, and the internal face, at the right side. The central trimer is coloured in green, cyan and magenta, corresponding to chains A, B and C, respectively.
a. The external face and d. the internal face, highlighting the central trimer, with other chains coloured in dark gray.
b. The external face, showing the A216 residue and c. the mutant A216T residue, with the sidechains rendered in spheres.
e. The internal face, showing the N189 residue and f. the mutant N189Y residue, with the sidechains rendered in spheres.
(Prepared with Pymol (DeLano, 2002) by Cláudio Soares, ITQB, Universidade Nova de Lisboa)

5.2. Discussion

In this chapter, the possibility of OMMV being soil transmitted by *Olpidium* sp. was examined, in view of the fact that both are common in olive orchards and that OMMV CP has a high amino acid identity to that of fungally transmitted TNV sl (Temmink *et al.*, 1970).

Work of Félix *et al.* (2006), using herbaceous hosts, has shown that OMMV was naturally transmitted to other plants when exposed to virus released from roots of a coinfected OMMV and OLV-1 plant or when a high concentrated suspension of OMMV (160 µg/10 mL) was poured around the roots of healthy plants. In this work it is shown that OMMV is naturally soil transmitted to roots of host plants with an efficiency of 39% when a 20 mL suspension of 5 µg of virus was used to irrigate cabbage seedlings and that its rate of transmission rises dramatically, more than two fold to an average of 87%, when the virus is previously incubated with a suspension of *O. brassicae* zoospores. The base levels of virus transmission (39%) recorded in the absence of the fungus is probably due to the high inocula level used. In nature, it is not likely to exist such a high amount of virus near the roots.

By comparison, the transmission efficiency of an infectious OMMV L11 natural variant did not increase in the presence of the fungus. Similarly when the infectious construct OMMVWT/OMMVL11 was tested, transmissibility remained almost unaltered, confirming that successive mechanically inoculated plant viruses frequently lose their vector transmissibility (Campbell, 1996).

OMMV L11 and OMMV/OMMVL11 did not bind *O. brassicae* zoospores at a detectable level suggesting that the lack of increased transmissibility is due to the failure of viral capsids to recognize or stably bind to zoospore receptors. This is in agreement with Robbins *et al*. (1997) who showed that the specificity of transmission lies at the level of *O. brassicae* recognition and binding.

Since the only difference between the construct and OMMV lies in the CP, it is plausible to assume that the loss of fungal transmissibility lies in alterations of certain domains of that peptide.

Through the construction of *in vitro* site-directed mutants, it was demonstrated that a single amino acid substitution in the OMMV CP gene of Asn to Tyr is the one largely responsible for the reduction in fungus transmissibility. OMMVN189Y particles, containing that single mutation are as highly infectious and stable as OMMV, OMMV L11, OMMV/OMMVL11, and OMMVA216T and accumulate to same levels in inoculated plants, indicating that the decreased transmissibility of OMMVN189Y is not due to loss of virus stability or infectivity. Viral particles containing N189Y mutation bind *O. brassicae* zoospores less efficiently,

suggesting that the decreased transmissibility is due to a decrease in the ability of viral capsids to recognize or stably bind zoospores.

This has been shown in experiments involving the reciprocal exchange of the CP gene of *Cucumber necrosis virus* (CNV) and that of a nontransmissible tombusvirus, the cherry strain of *Tomato bushy stunt virus* (TBSV-Ch). Particles containing a modified TBSV-Ch genome with the CNV CP gene were efficiently transmitted and those containing the TBSV-Ch CP gene were not (McLean *et al*., 1994). Similar observations were obtained when Robbins *et al*. (1997) performed the reciprocal exchange of the CP gene of CNV and that of a non transmissible mutant (LL5). In this study the mutated CP gene resulted in loss of recognition of the vector either by causing conformational changes on the virus particle affecting an efficient binding to the vector receptors or failure of the virus to stably interact with a receptor, perhaps by altering specific ionic interactions with a component of the zoospore membrane (Robbins *et al.*, 1997) or by altering putative attachment sites of the viral subunits thus failing to bind specific receptors in the zoospore outer membrane. An identical situation was recorded with several animal viruses, such as poliovirus, foot-and-mouth disease virus and influenza virus (Rossman, 1994; Fry *et al*., 1999; Skehel and Wiley, 2000).

With this work, previous studies on the role of virus CP in transmission are reinforced (Kakani *et al*., 2001; Mochizuki *et al*., 2008). What makes one virus transmissible by a vector but not by another may be explained in part by the requirement for a recognition event between the virion, or a viral coat protein motif, and a site of recognition in the zoospore vector (Kakani *et al*., 2001; Andret-Link & Fuchs, 2005; Mochizuki *et al*., 2008). In this work it is shown that a single aa change in the CP of OMMV, located in the interior of the capsid, is responsible for the loss of transmission by *O. brassicae*. Similar results have been shown by Mochizuki *et al*. (2008) when the single Ile to Phe substitution in *Melon necrotic spot virus* (MNSV) CP resulted in the loss of both binding and fungal transmission, and also by Robbins *et al.* (1997) where a Glu to Lys change in CNV CP affected vectoring capacity. A single Pro to Gly mutation in the interior of CNV, although not affecting virion binding to zoospores, resulted in the loss of transmissibility by its vector by affecting the ability of the virus to undergo a conformational change required for transmission (Kakani *et al*., 2004).

The comparative model of OMMV CP oligomers shows that the Asn to Tyr mutation is located in the capsid interior (Figure 5.17, e and f) and the Ala to Thr mutation is located in the capsid surface (Figure 5.17, b and c) and does not appear to substantially alter the folding of the protein subunit, nor does it affect intersubunit contacts. Apparently, introduction of this polar residue at this exposed location does not alter zoospore binding or other aspects of the

transmission process. On the other hand, the Asn to Tyr mutation is located in the capsid interior, in a loop region, not far from inter-monomer contacts. Figure 5.17, e and f show that, despite not being in van der Waals contact with any atom of the other monomers, the tyrosine residue is very near the interface between monomers. It consists in the creation of a considerably larger residue in a zone that may provide potential contacts with the nucleic acid in the capsid interior. Despite not appearing to affect the correct assembly of the capsid (as OMMVN189Y purified particles are highly infectious and accumulate in plants at the same levels as OMMV WT), this mutation may cause change particle conformation, generating a particle with a somewhat different surface, by the creation of a bulkier residue at this position and indirectly affecting virion attachment to zoospores and subsequent transmission, as was suggested for the LLK10 mutation in the *Olpidium bornovanus*-transmitted *Cucumber necrosis virus* (CNV) CP described by Kakani *et al*. (2001).

This study demonstrates for the first time that OMMV is transmitted by *Olpidium brassicae,* contributing to a better understanding of the features involved in fungal virus transmission, namely virion architecture, aiding in the identification of sites responsible for virus-vector interactions about which little was known. The knowledge concerning virus-vector relationships will aid to develop more efficient preventive measures against viruses.

Chapter 6. General Discussion

This work has shown that *Olpidium* sp. was very disseminated within a highly necrovirus infected olive orchard. *O. brassicae* and/or *O. virulentus* species were found in all the sampled sites of the orchard, being *O. brassicae* the most disseminated. These fungi are important vectors of several viruses (Rochon *et al*., 2004; Alfaro-Fernandez, 2009; Maccarone *et al*., 2010; Gonzalez *et al*., 2010) and their identification is essential to help to understand their role as viral vectors.

The high overall genome sequence identity between the 3 olive necroviruses (OLV-1, OMMV and TNV-D) has hampered their identification and discrimination (Cardoso *et al*., 2005). OLV-1 has been discriminated from the other two olive necroviruses either serologically or in RT-PCR assays, however TNV-D and OMMV have been detected indistinctively, thus inaccurately, either serologically or in RT-PCR assays using primers complementary to CP gene sequences. This work presents, for the first time, the design of two pairs of specific primers, for each OMMV and TNV-D and the optimization of a multiplex RT-PCR assay using these primers together with the ones specific for OLV-1 (Martelli *et al*., 1996). DsRNA analysis by itself has shown not to be sensitive enough for the detection of olive necroviruses, however it is a good template for RT-PCR assays allowing a large sample to be analyzed, a good advantage for the detection of olive necroviruses that are low concentrated and unevenly distributed in the olive tree. By allowing the detection and identification of olive necroviruses, this work represents an important contribution to the knowledge concerning each olive-infecting necrovirus, allowing to obtain and interpret data on mutual virus interaction, geographical distribution and means of field dissemination. In fact, the application of this method to an olive orchard revealed high levels of OMMV infection which led to the investigation of an efficient mean for its dissemination, namely if the widespread *O. brassicae* has a role in OMMV dissemination.

Although being naturally soil transmitted to host plants, OMMV transmission efficiency more than doubled in the presence of *O. brassicae*, showing that the fungus facilitates virus entry in the roots. Due to the fact that viruses may be present in soils in small amounts, the fungus may have an essential role for OMMV dissemination. Similar transmission assays performed with the natural mutant OMMVL11, obtained by serial inoculations through host plant, is also naturally soil transmitted but *O. brassicae* zoospores presence did not increase virus transmission efficiency. These results reinforce previous studies showing that viruses, after successive passages through a host, lose their ability to be transmitted by their vectors (Campbell, 1996). The role of the CP in transmission was confirmed when an infectious construct, OMMVWT/OMMVL11, obtained by artificially substituting the OMMV CP gene by

that of OMMVL11 also showed loss of transmissibility by the fungus. Further tests revealed that the loss of transmission was partly due to the failure of these viruses to bind to zoospores, showing that the specificity of transmission lies at the level of *O. brassicae* recognition and binding. This prompted the search for changes in the OMMV CP that might be responsible for the loss of transmissibility by the fungus. Comparison between the CP sequences of OMMV and OMMVL11 allowed to detect two amino acid changes and site-directed mutagenesis was used to induce each mutation in the full-length clone of OMMV producing two single mutants (OMMVN189Y and OMMVA216T). Transmission assays performed to these *in vitro* site-directed mutants, demonstrated that one of the amino acid substitutions, Asn to Tyr at position 189 of OMMV CP amino acid sequence, was largely responsible for the reduction in fungus transmissibility, and this was mostly due to a decrease in the ability of virus to bind zoospores. The 3-dimensional structure of OMMV showed that the Asn to Tyr mutation is located in the interior of the particle and although not in direct contact with zoospores, it may cause alterations in the particle conformation, generating a particle with a slightly different surface, turning inaccessible the residues essential for zoospore recognition. These findings are in line with those of other authors (Brown *et al*., 1995; Campbell, 1996; Gray, 1996; Gray and Rochon, 1999; Pirone and Blanc, 1996; van den Heuvel, 1999; Kakani *et al*., 2004)

The study presented in this work allowed to discriminate, for the first time, OMMV from TNV-D. In addition, it is shown, through molecular techniques, that the fungus *O. brassicae* and *O. virulentus* are present in a Portuguese olive orchard. Both Olpidium species were baited using crucifer plants (cabbage) which weakens the criteria of separating these two species according to host specificity (crucifer and non crucifer strains) as proposed by Koganezawa *et al.* (2005). OMMV transmission by *O. brassicae* is demonstrated for the first time. It was also shown that the determinants of transmission specificity reside in the CP and that transmission is very specific in that a single aa change in the CP of OMMV, located in the interior of the particle, rendered it non transmissible by the fungus.

The knowledge of the virus-vector specific interactions essential for the successful spread of most plant viruses, contribute to the development of effective measures for their control.

Future Perspectives

The virus-fungus binding and transmission assays optimized in this study together with the constructed single mutants, will allow to test other virus-vector interactions such as OMMV-*O. virulentus.* It would be interesting to check the effect of those mutations in OMMV transmission by *O. virulentus*. Interactions between TNV-D^P or OLV-1 isolates and *O. brassicae* or *O. virulentus* vectors are also interesting to be studied. Alignment between TNV-D or OLV-1 and OMMV mutants, will help in choosing the location of specific aa in these viruses genomes that can be mutated in order to determine specific aa sequences responsible for transmission by the fungus.

The knowledge of the proteins that assist transmission, will allow to use the viral genes that encode for proteins defective in that ability in transgenic plants.

The understanding of how viruses interact with their vectors, namely which amino acids are responsible for that interaction, will allow to develop preventive measures for virus control.

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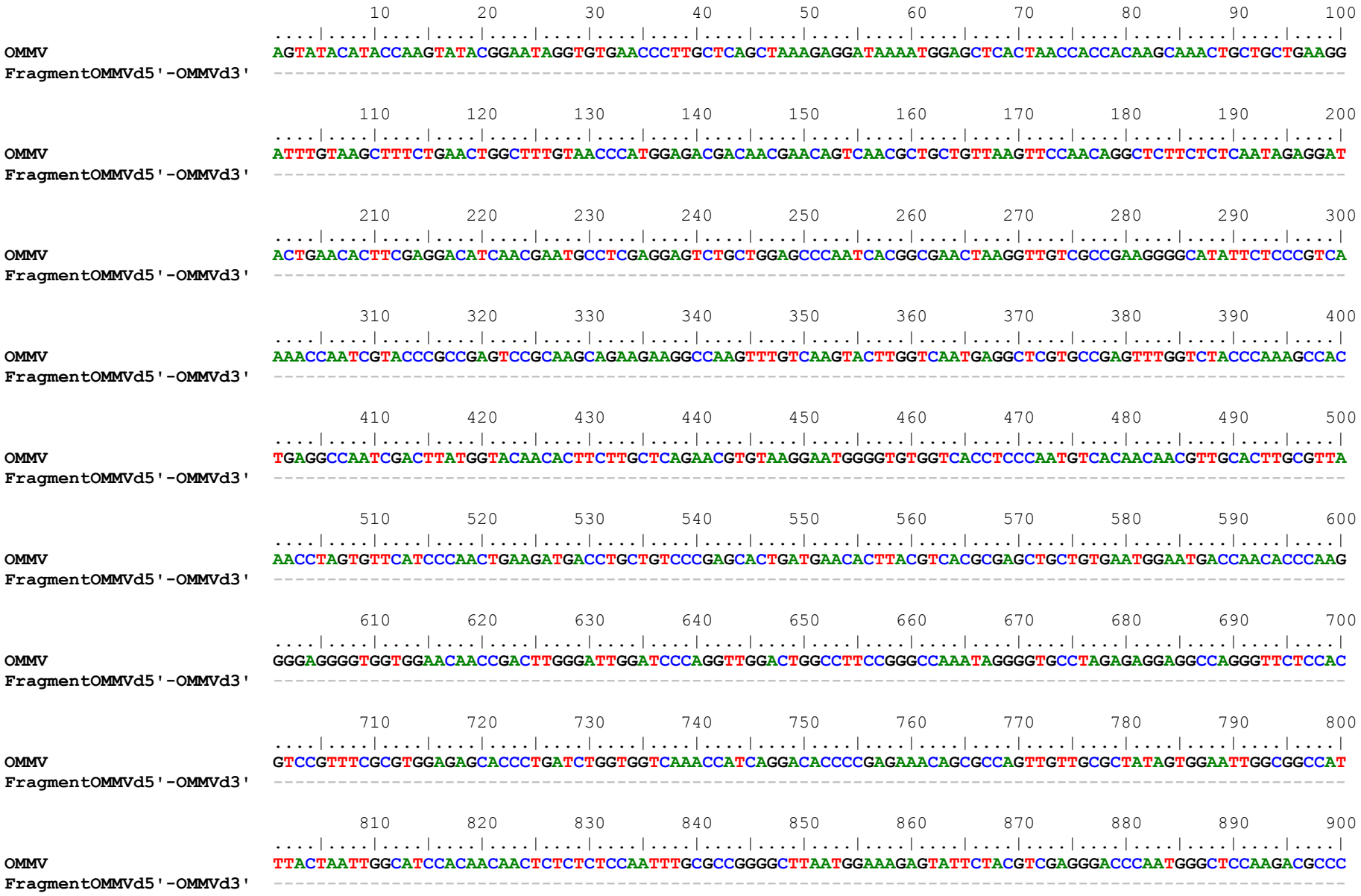
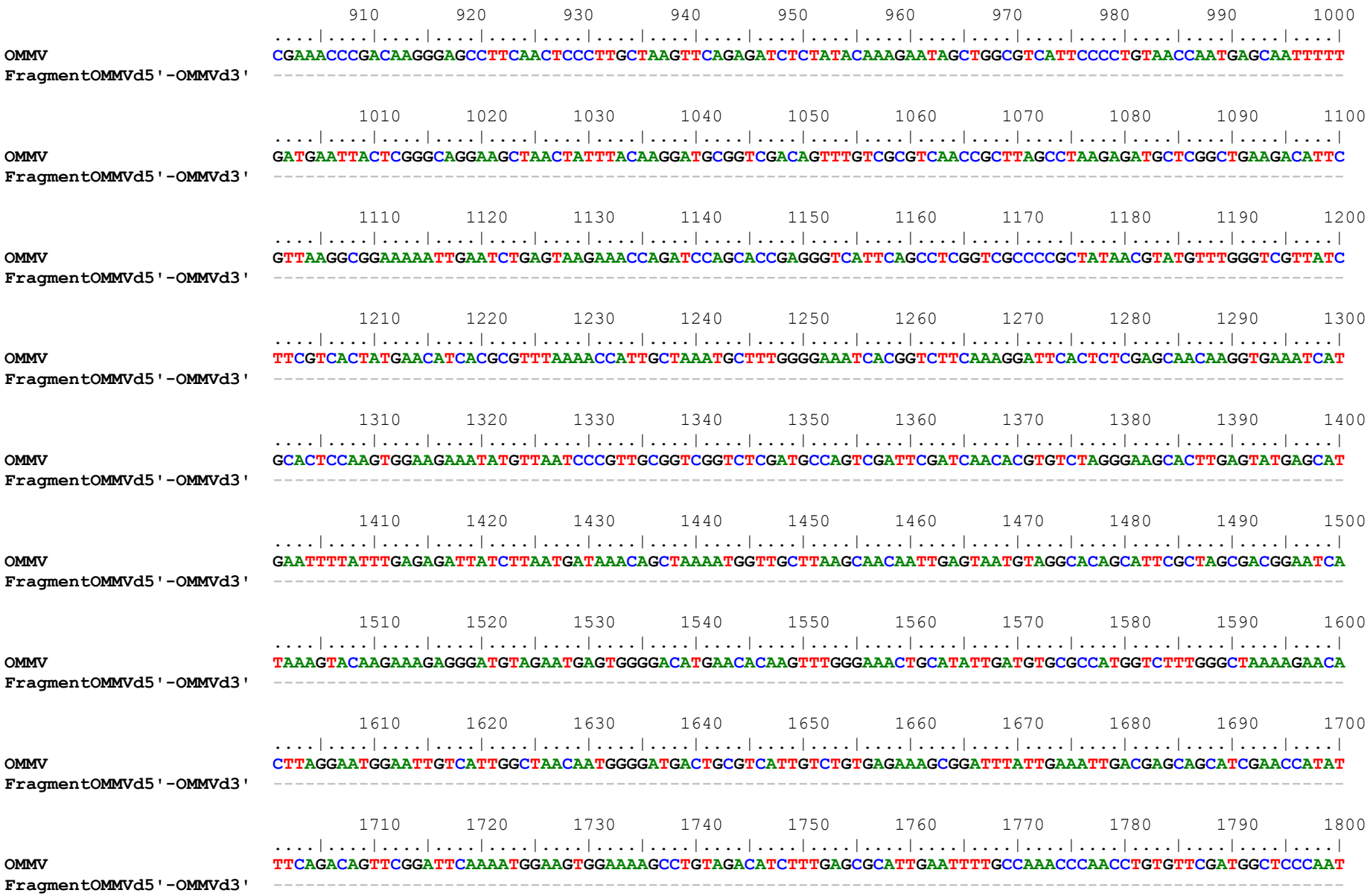
Appendix I: Nucleotide sequence alignment of fragment OMMVd5' – OMMVd3' with sequence of OMMV NC_006939

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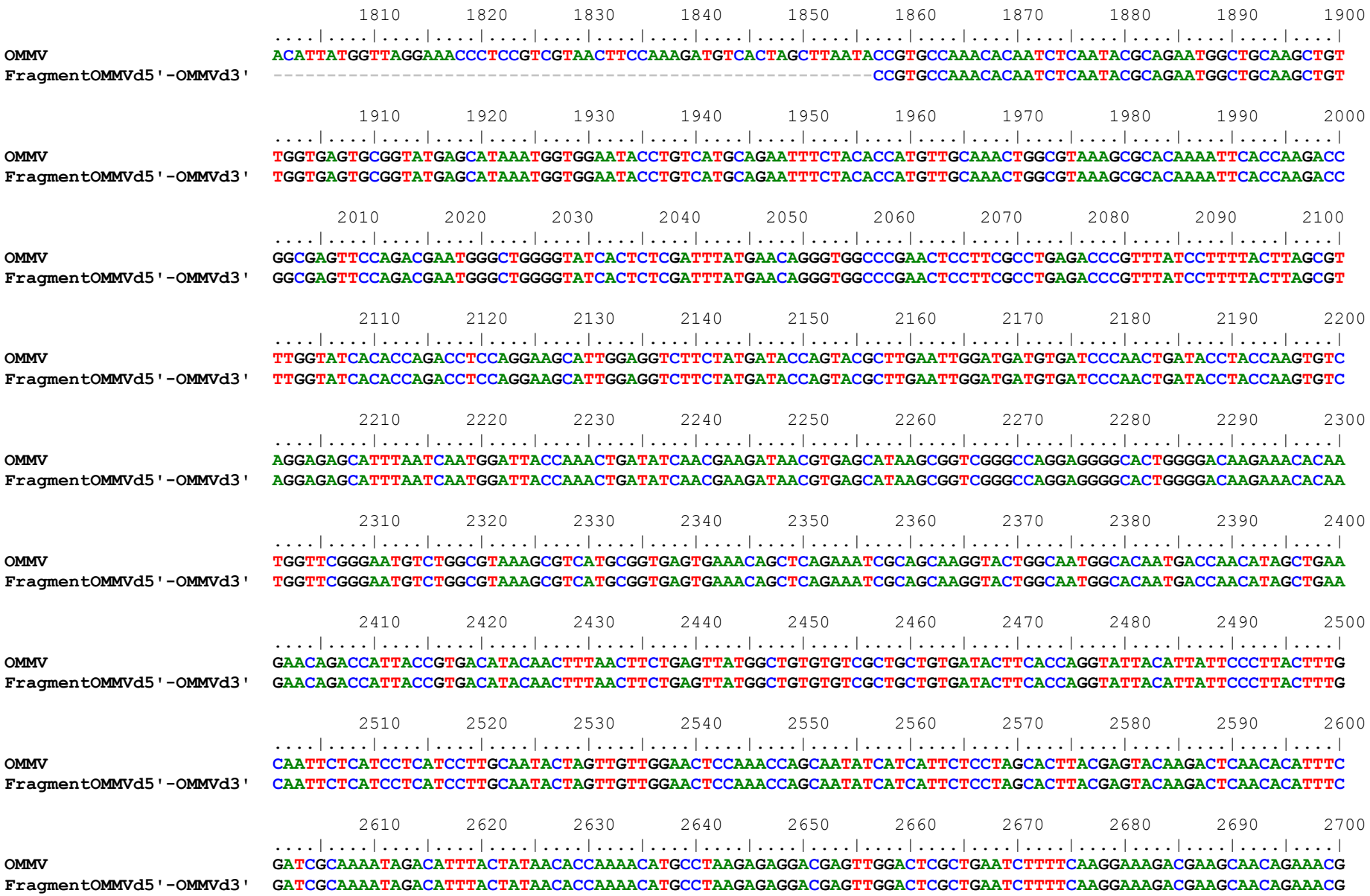
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2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
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2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
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2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
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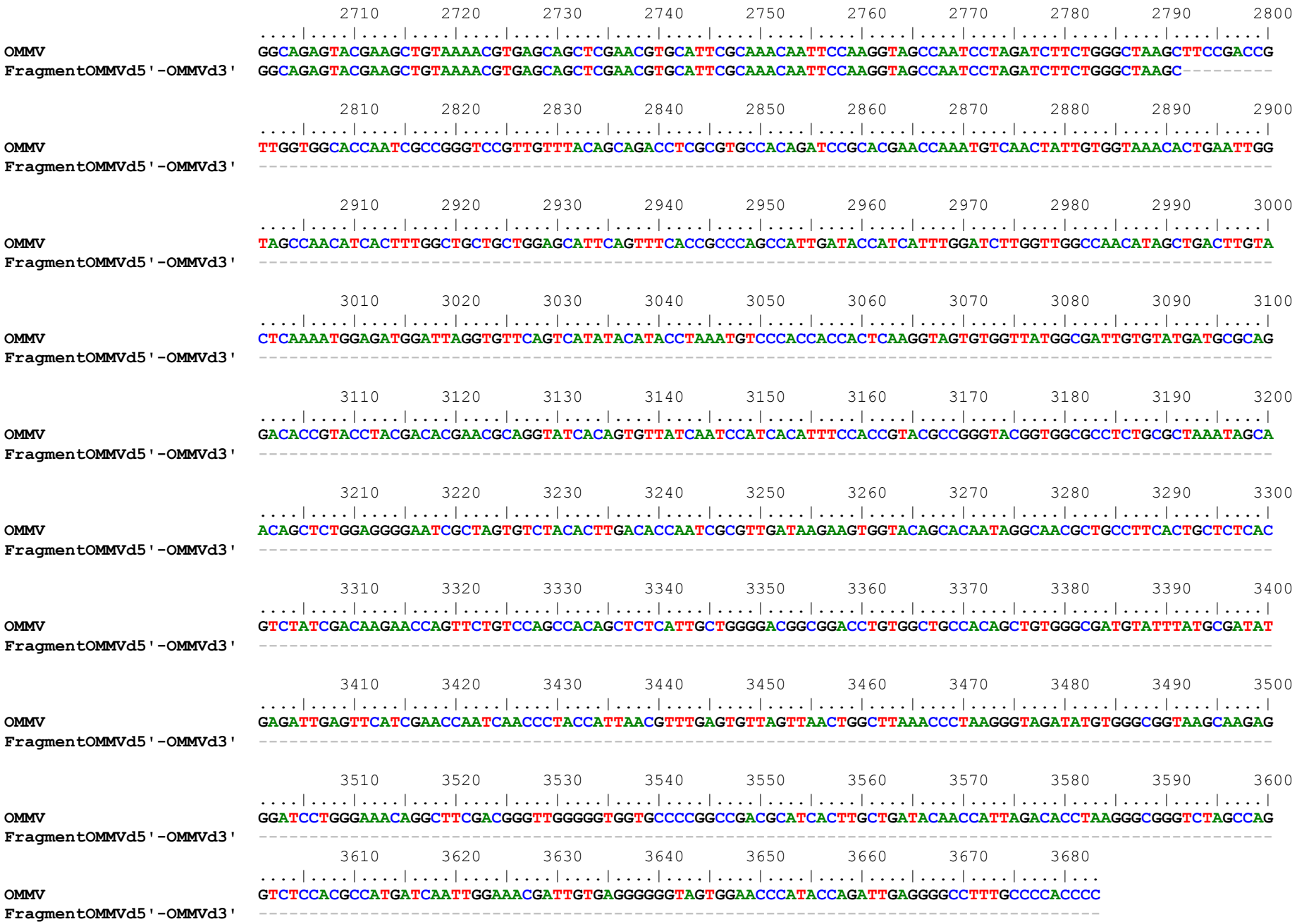
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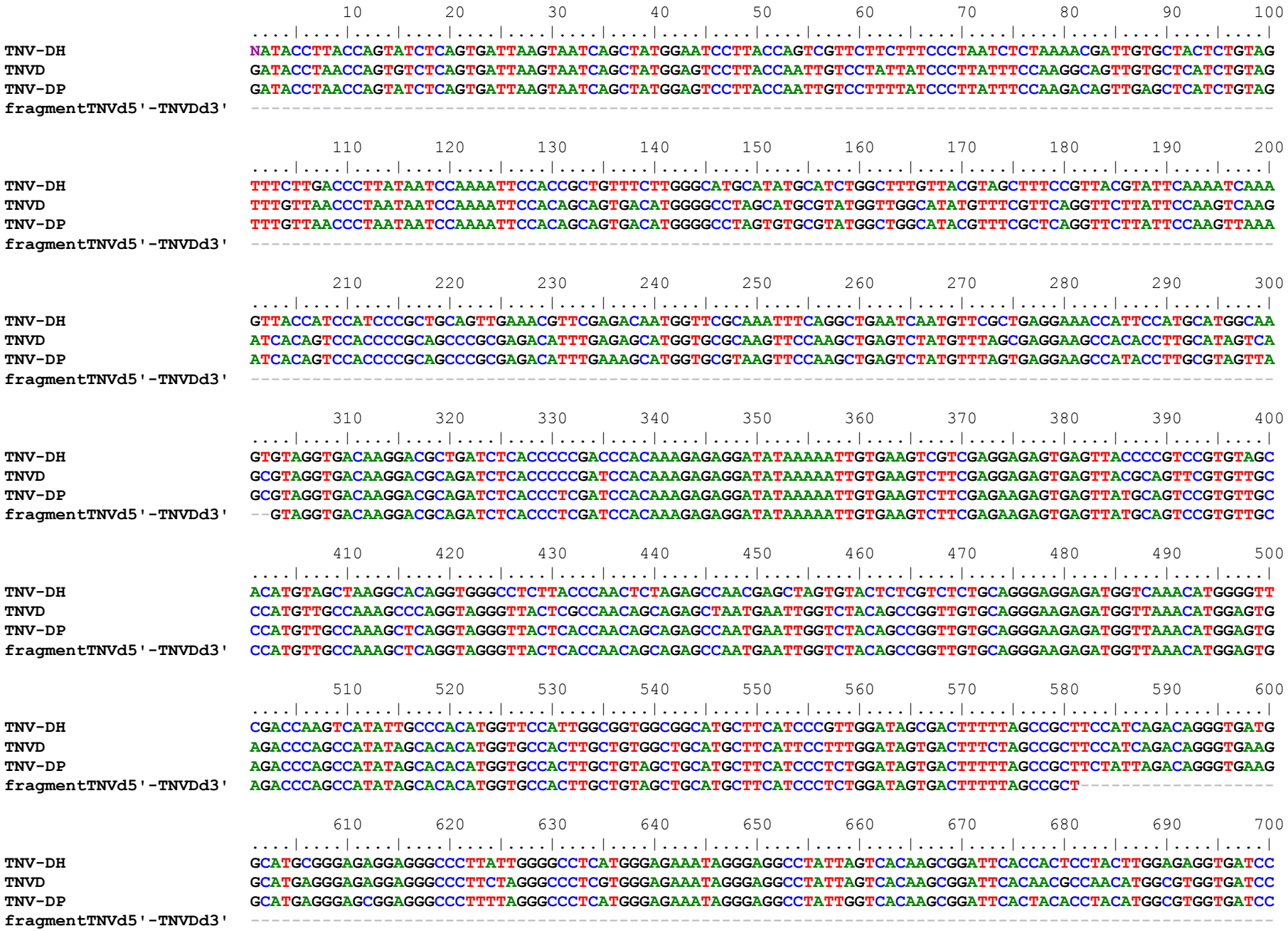
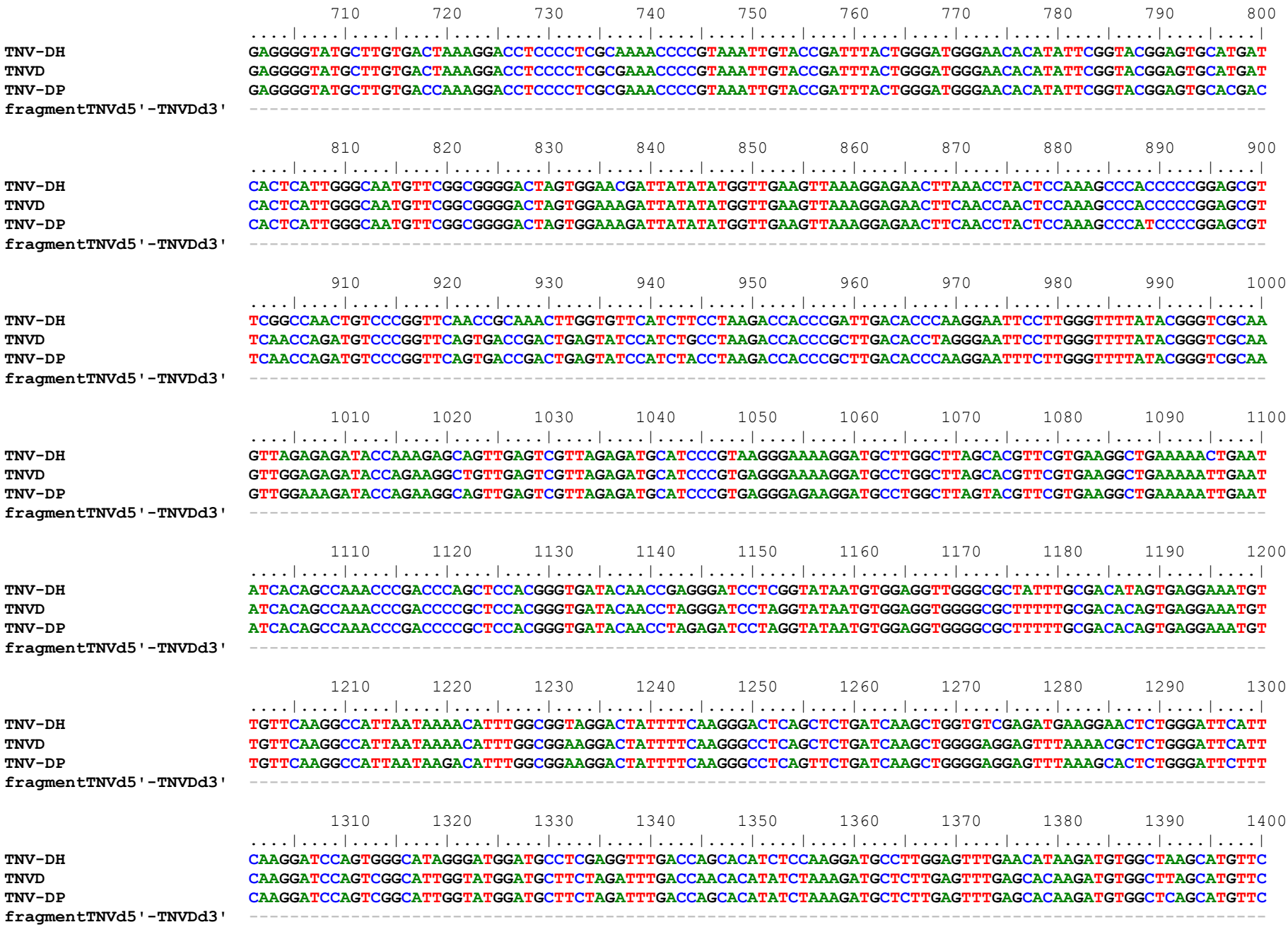
Appendix II: Nucleotide sequence alignment of fragment TNVDd5' – TNVDd3' with sequences: TNV-D^{H}, TNV-D, TNV-D^{P}

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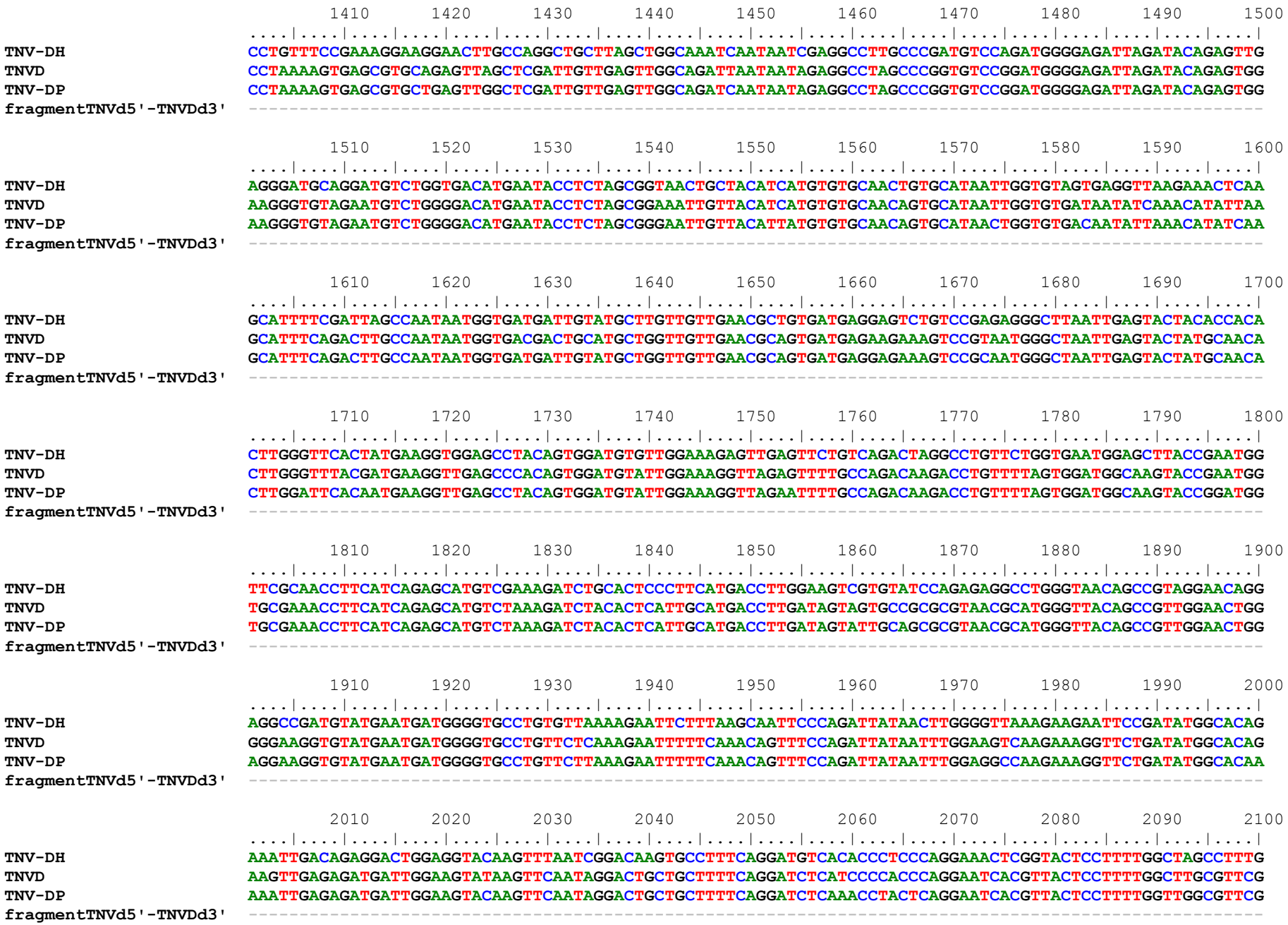
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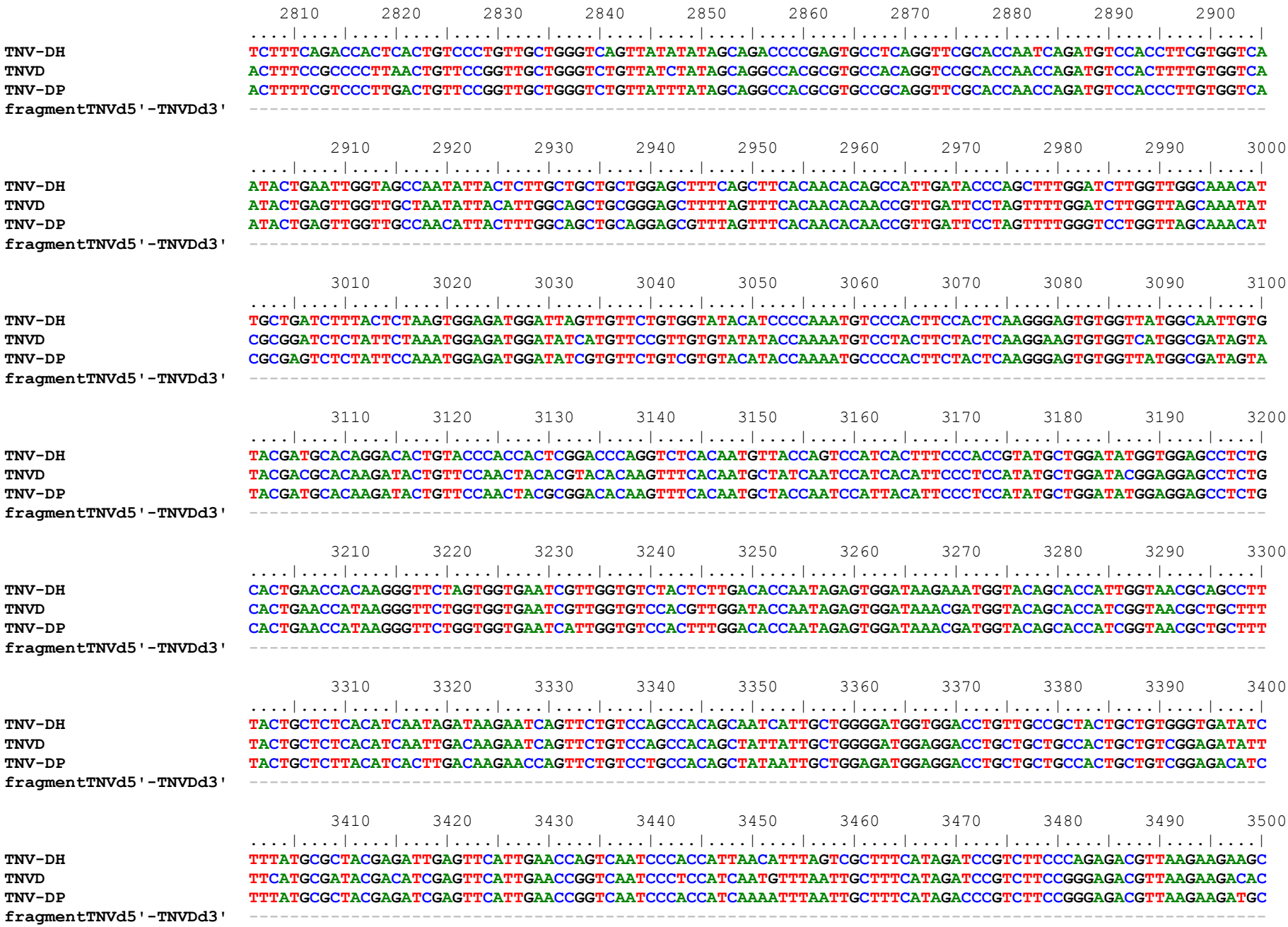
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fragmentTNVd5'-TNVDd3'    ------------------------------------------------------------------------------------------------------

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fragmentTNVd5'-TNVDd3'    ------------------------------------------------------------------------------------------------------

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fragmentTNVd5'-TNVDd3'    ------------------------------------------------------------
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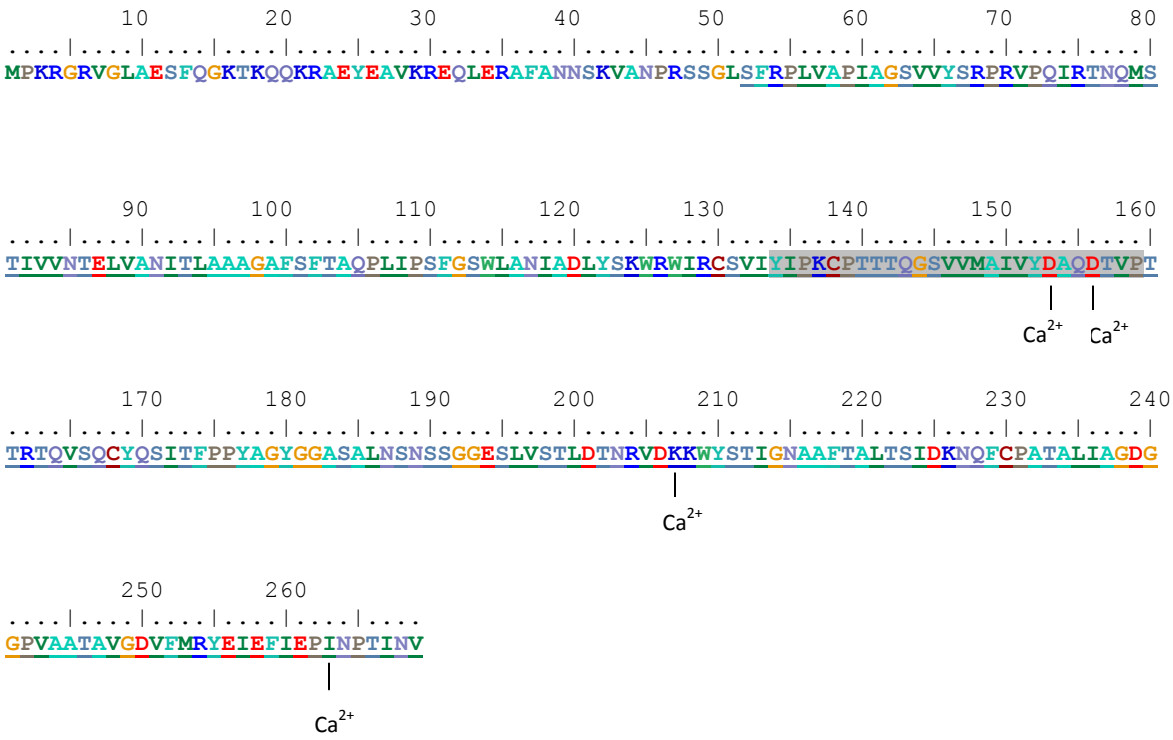
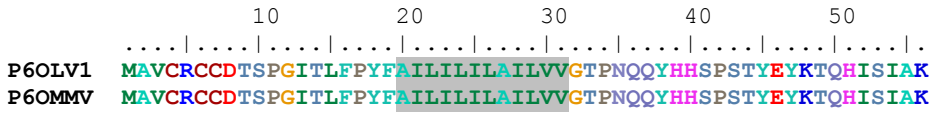
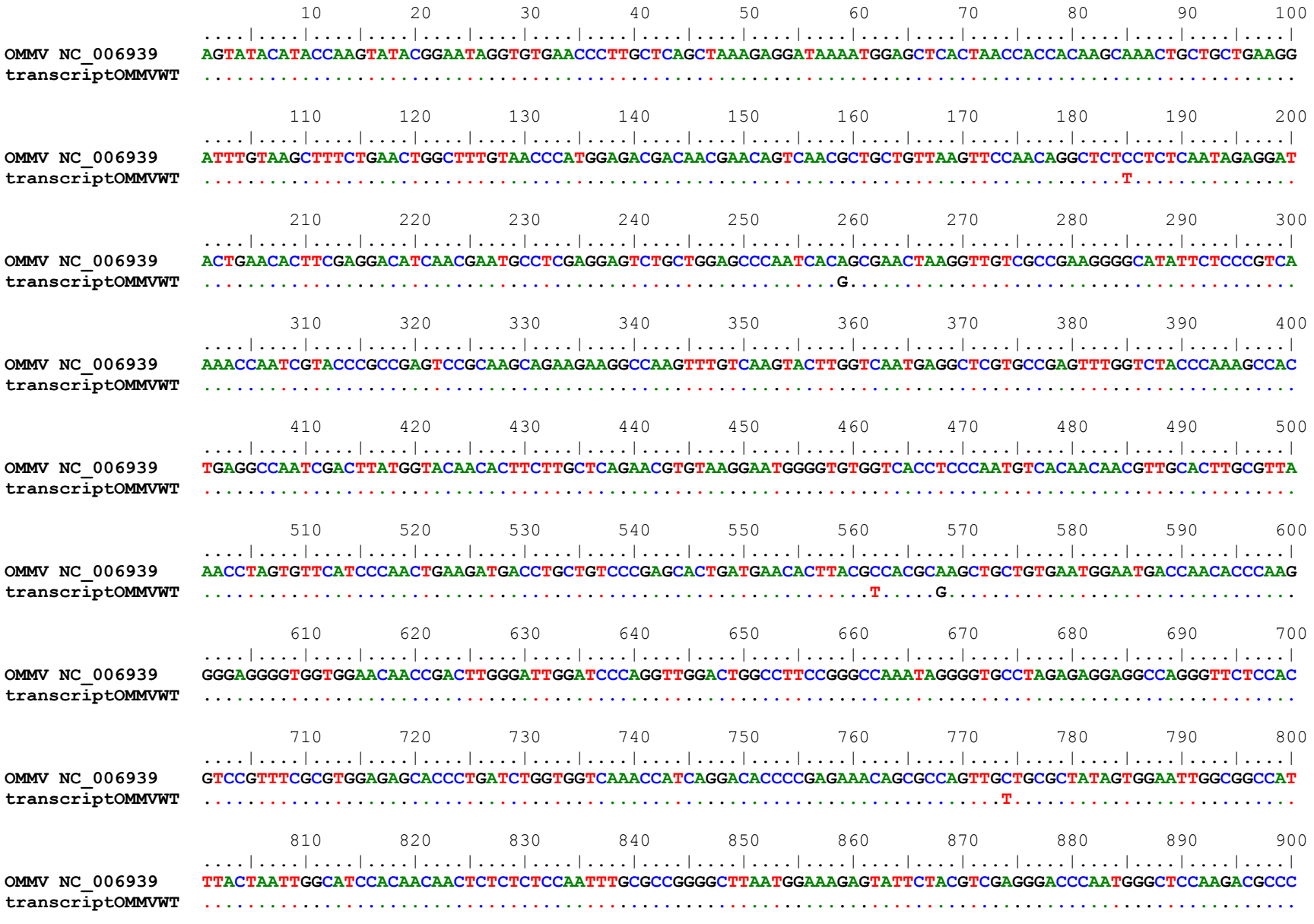
Appendix III: Deduced amino acid sequence of OMMV NC_006939 coat protein. The "S" region signature of small icosahedral plant viruses is highlighted in gray colour. Conserved aa residues identified as Ca^{2+} binding site are indicated below aa. The region corresponding to domain S is underlined.

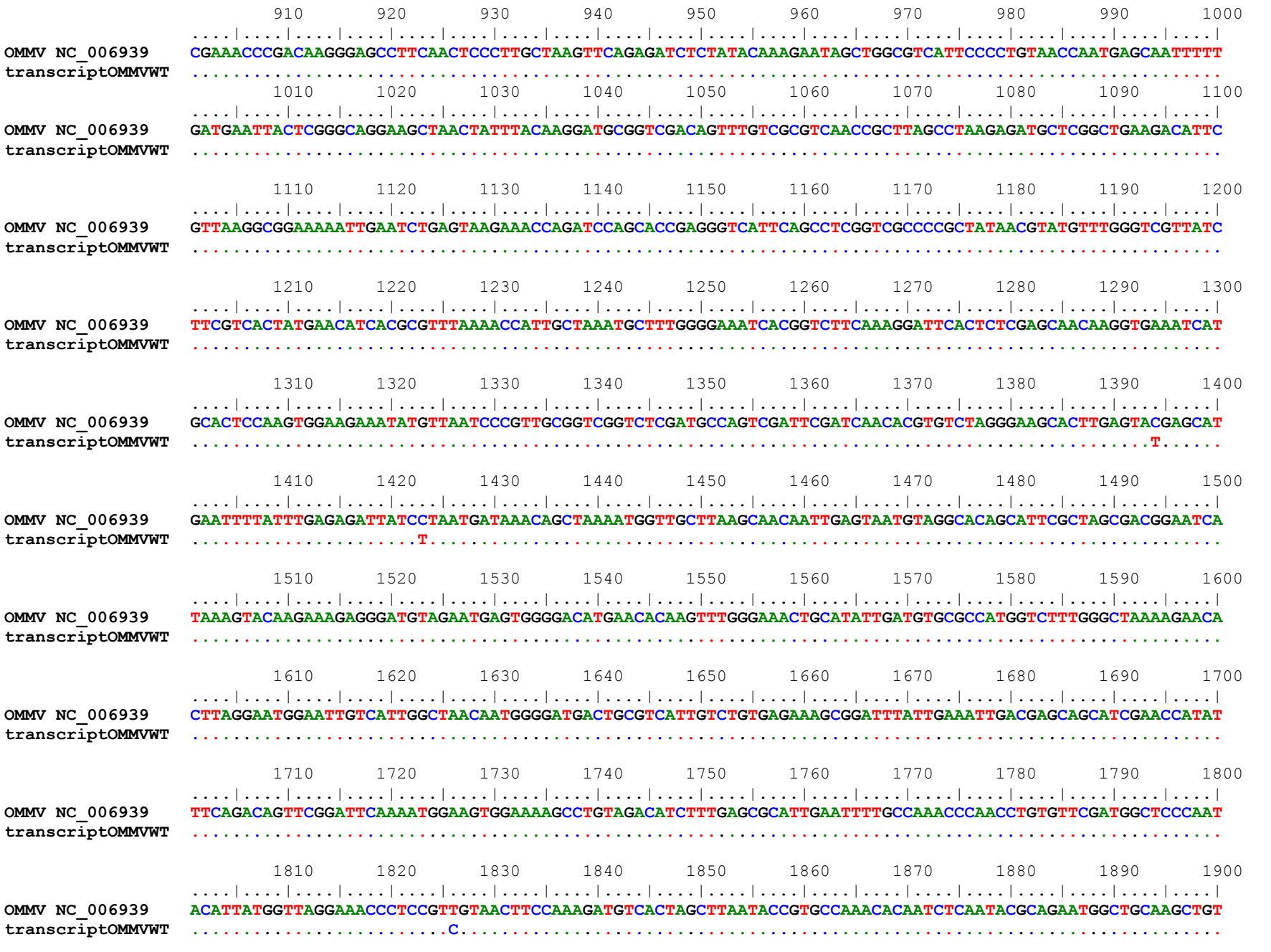


Appendix IV: Amino acid sequence alignment of OLV-1 and OMMV p6. The predicted transmembrane motif is highlighted in gray.



Appendix V: Nucleotide sequence alignment of OMMV NC_006939 with transcript OMMV WT

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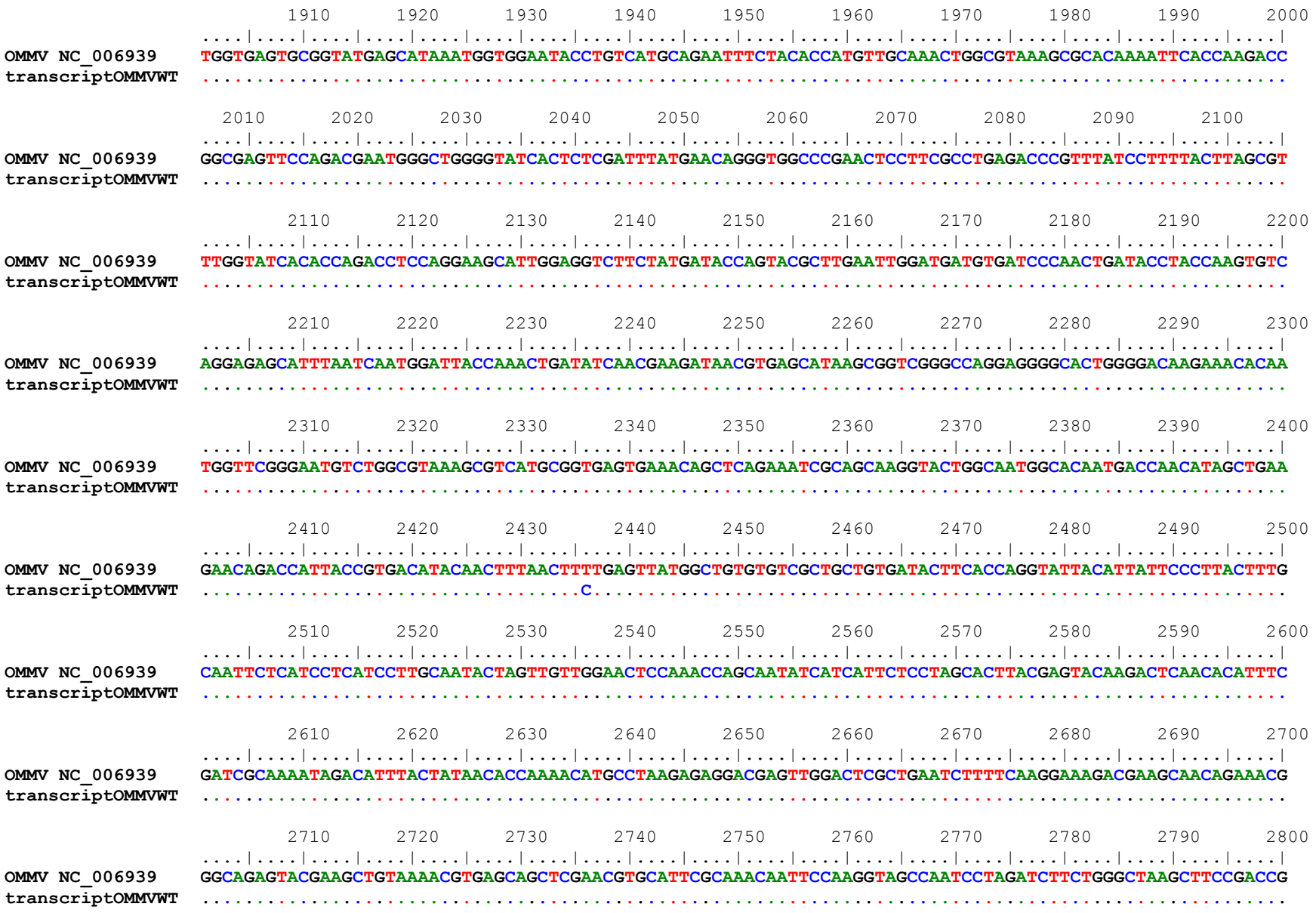
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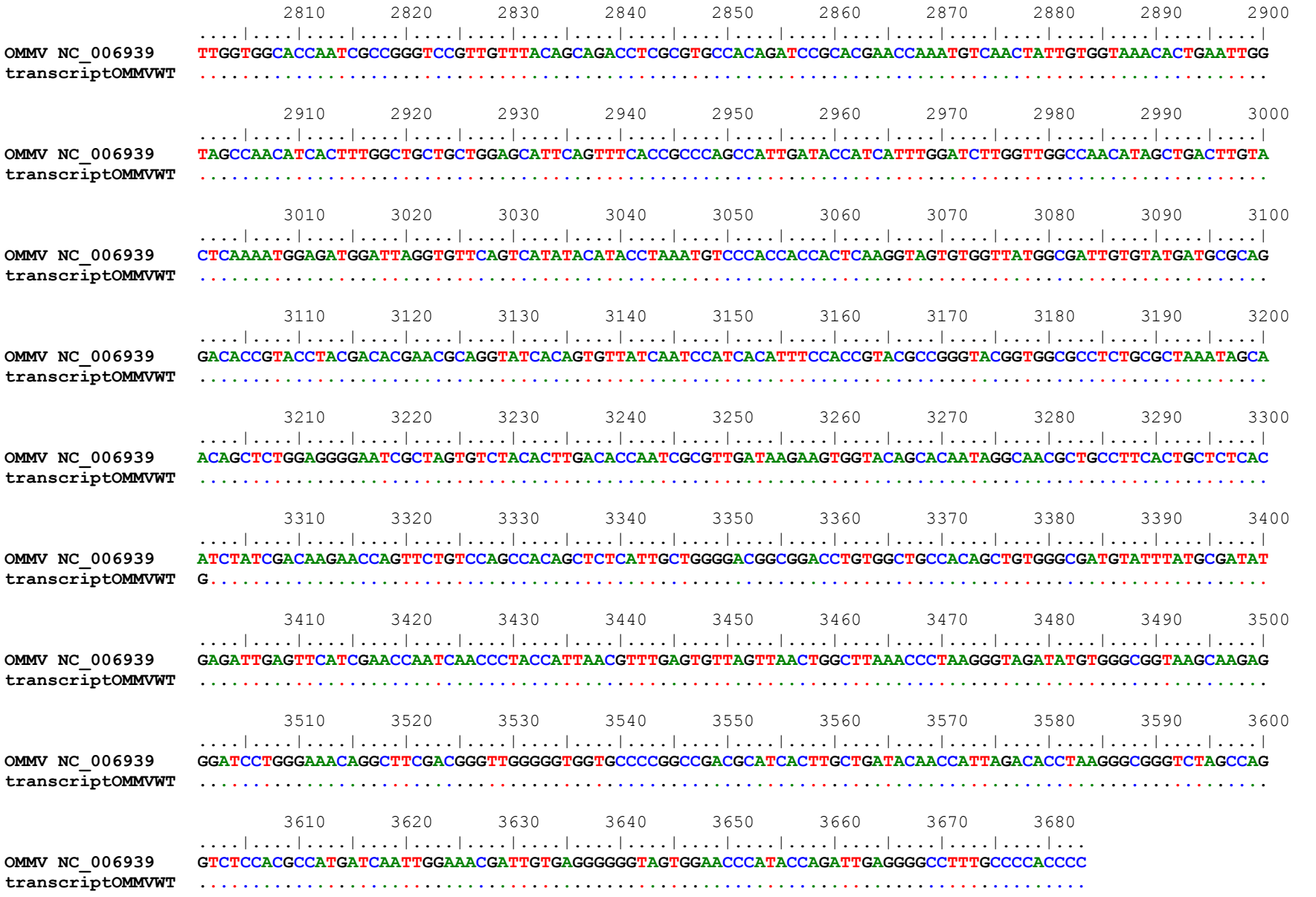
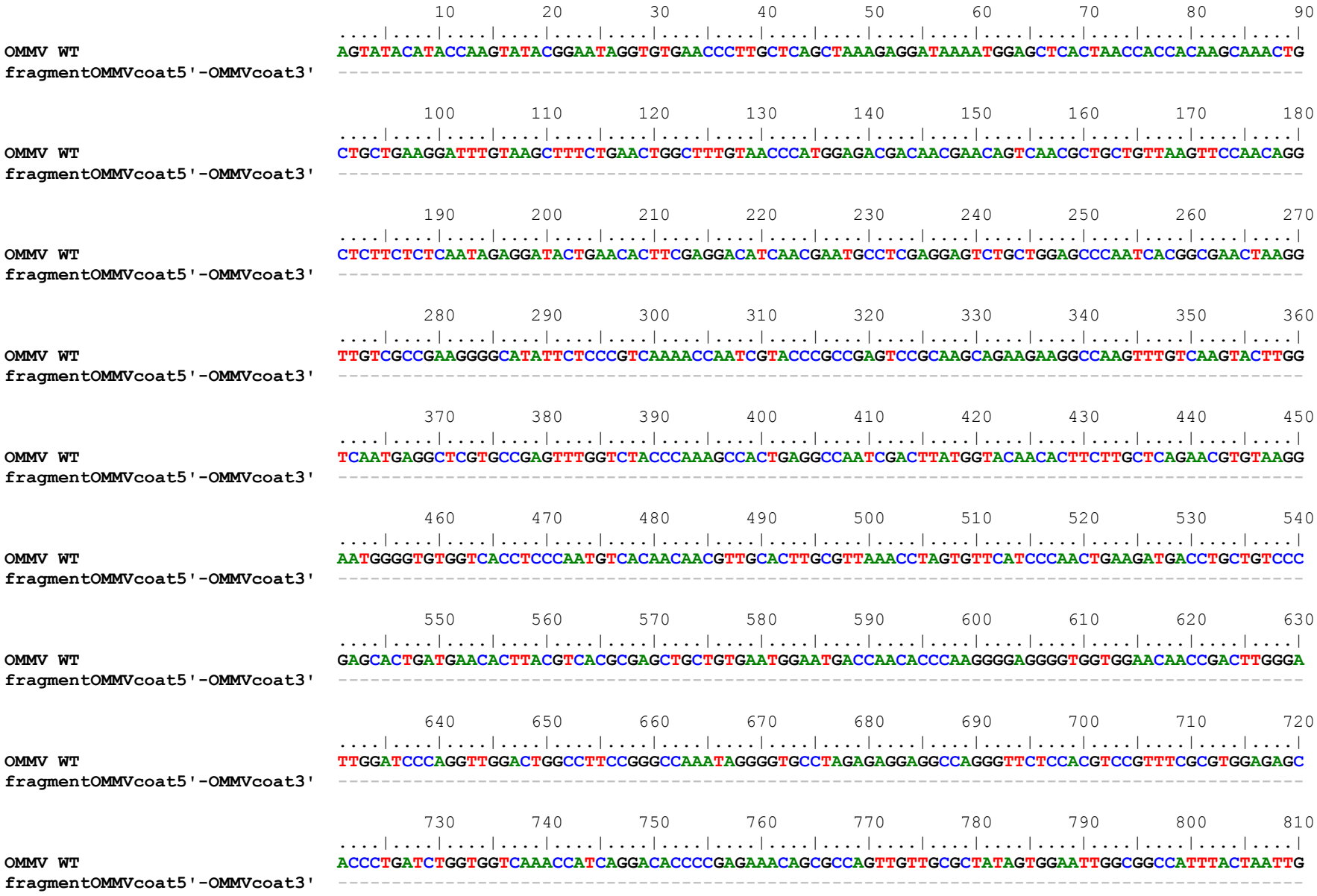
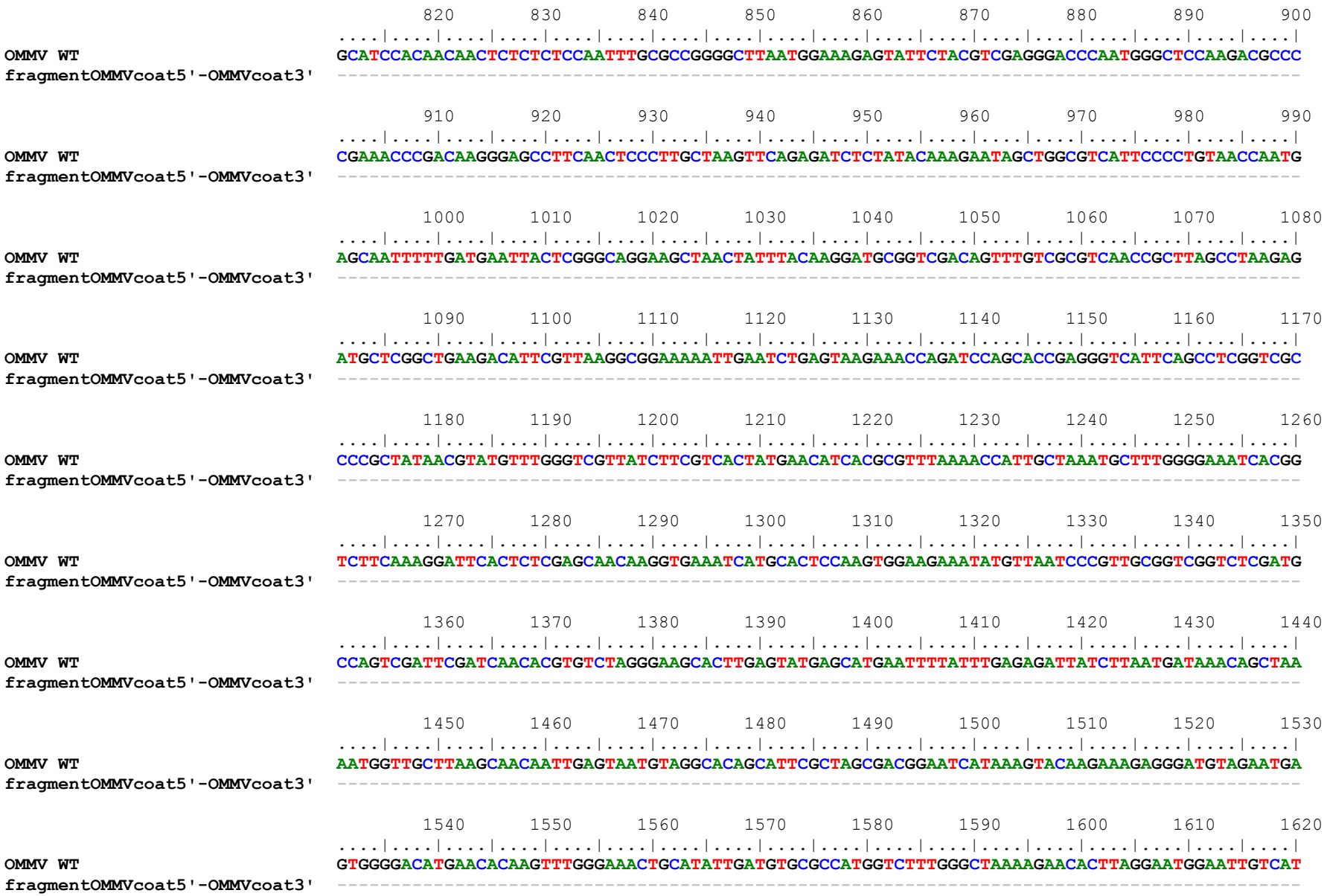
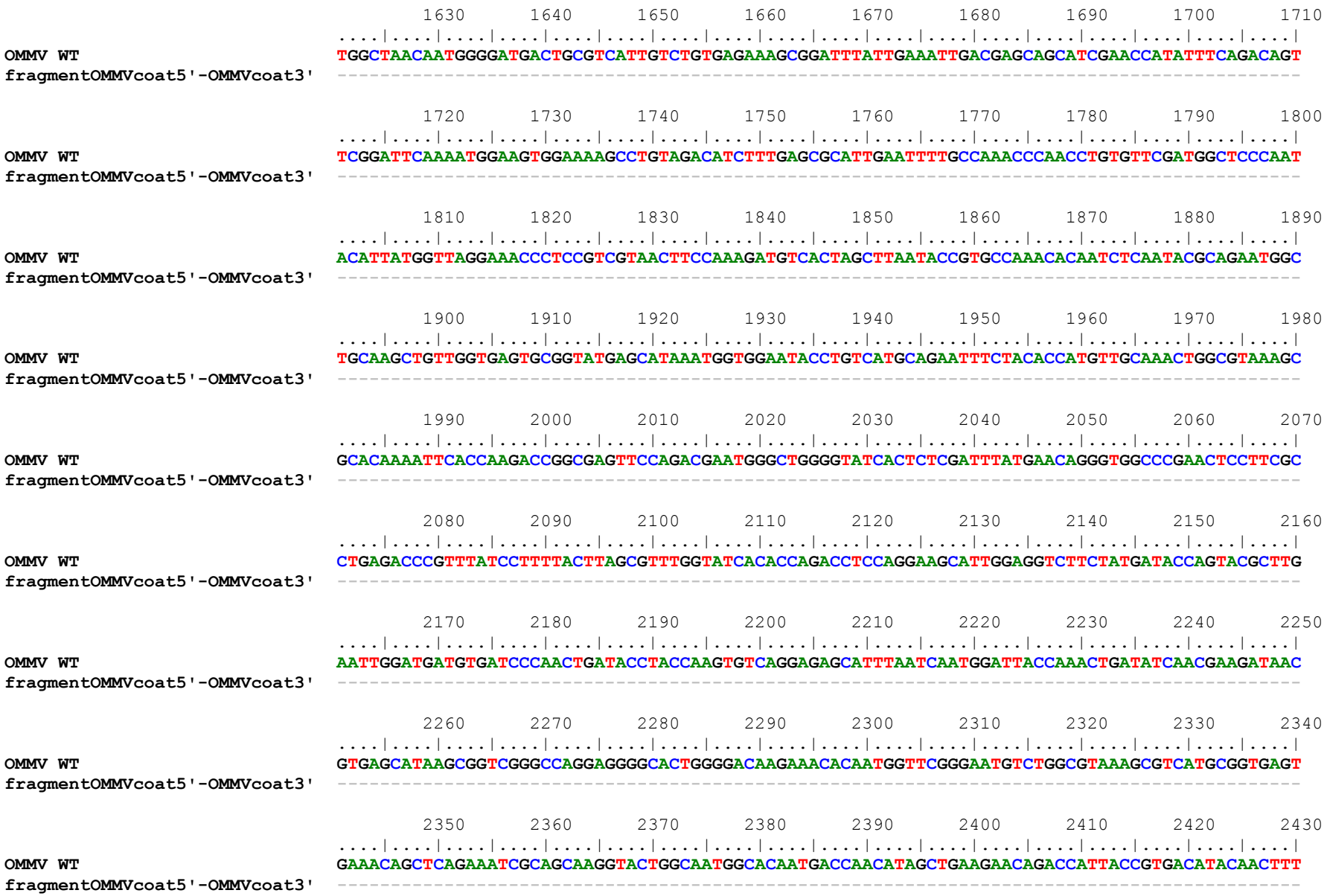
transcriptOMMVWT
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
OMMV NC_006939 GGCGAGTTCCAGACGAATGGGCTGGGGTATCACTCTCGATTTATGAACAGGGTGGCCCGAACTCCTTCGCCTGAGACCCGTTTATCCTTTTACTTAGCGT
transcriptOMMVWT
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
OMMV NC_006939 TTGGTATCACACCAGACCTCCAGGAAGCATTGGAGGTCTTCTATGATACCAGTACGCTTGAATTGGATGATGTGATCCCAACTGATACCTACCAAGTGTC
transcriptOMMVWT
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
OMMV NC_006939 AGGAGAGCATTTAATCAATGGATTACCAAACTGATATCAACGAAGATAACGTGAGCATAAGCGGTCGGGCCAGGAGGGGCACTGGGGACAAGAAACACAA
transcriptOMMVWT
2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
OMMV NC_006939 TGGTTCGGGAATGTCTGGCGTAAAGCGTCATGCGGTGAGTGAAACAGCTCAGAAATCGCAGCAAGGTACTGGCAATGGCACAATGACCAACATAGCTGAA
transcriptOMMVWT
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
OMMV NC_006939 GAACAGACCATTACCGTGACATACAACTTTAACTTTTGAGTTATGGCTGTGTGTCGCTGCTGTGATACTTCACCAGGTATTACATTATTCCCTTACTTTG
transcriptOMMVWT .....C.....
2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
OMMV NC_006939 CAATTCTCATCCTCATCCTTGCAATACTAGTTGTTGGAACTCCAAACCAGCAATATCATCATTCTCCTAGCACTTACGAGTACAAGACTCAACACATTTC
transcriptOMMVWT
2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
OMMV NC_006939 GATCGCAAAATAGACATTTACTATAACACCAAAACATGCCTAAGAGAGGACGAGTTGGACTCGCTGAATCTTTTCAAGGAAAGACGAAGCAACAGAAACG
transcriptOMMVWT
2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
OMMV NC_006939 GGCAGAGTACGAAGCTGTAAAACGTGAGCAGCTCGAACGTGCATTCGCAAACAATTCCAAGGTAGCCAATCCTAGATCTTCTGGGCTAAGCTTCCGACCG
transcriptOMMVWT


2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
OMMV NC_006939 TTGGTGGCACCAATCGCCGGGTCCGTTGTTTACAGCAGACCTCGCGTGCCACAGATCCGCACGAACCAAATGTCAACTATTGTGGTAAACACTGAATTGG
transcriptOMMVWT ....
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
OMMV NC_006939 TAGCCAACATCACTTTGGCTGCTGCTGGAGCATTCAGTTTCACCGCCCAGCCATTGATACCATCATTTGGATCTTGGTTGGCCAACATAGCTGACTTGTA
transcriptOMMVWT ....
3010 3020 3030 3040 3050 3060 3070 3080 3090 3100
OMMV NC_006939 CTCAAAATGGAGATGGATTAGGTGTTCAGTCATATACATACCTAAATGTCCCACCACCACTCAAGGTAGTGTGGTTATGGCGATTGTGTATGATGCGCAG
transcriptOMMVWT ....
3110 3120 3130 3140 3150 3160 3170 3180 3190 3200
OMMV NC_006939 GACACCGTACCTACGACACGAACGCAGGTATCACAGTGTTATCAATCCATCACATTTCCACCGTACGCCGGGTACGGTGGCGCCTCTGCGCTAAATAGCA
transcriptOMMVWT ....
3210 3220 3230 3240 3250 3260 3270 3280 3290 3300
OMMV NC_006939 ACAGCTCTGGAGGGGAATCGCTAGTGTCTACACTTGACACCAATCGCGTTGATAAGAAGTGGTACAGCACAATAGGCAACGCTGCCTTCACTGCTCTCAC
transcriptOMMVWT ....
3310 3320 3330 3340 3350 3360 3370 3380 3390 3400
OMMV NC_006939 ATCTATCGACAAGAACCAGTTCTGTCCAGCCACAGCTCTCATTGCTGGGGACGGCGGACCTGTGGCTGCCACAGCTGTGGGCGATGTATTTATGCGATAT
transcriptOMMVWT G.........
3410 3420 3430 3440 3450 3460 3470 3480 3490 3500
OMMV NC_006939 GAGATTGAGTTCATCGAACCAATCAACCCTACCATTAACGTTTGAGTGTTAGTTAACTGGCTTAAACCCTAAGGGTAGATATGTGGGCGGTAAGCAAGAG
transcriptOMMVWT ....
3510 3520 3530 3540 3550 3560 3570 3580 3590 3600
OMMV NC_006939 GGATCCTGGGAAACAGGCTTCGACGGGTTGGGGGTGGTGCCCCGGCCGACGCATCACTTGCTGATACAACCATTAGACACCTAAGGGCGGGTCTAGCCAG
transcriptOMMVWT ....
3610 3620 3630 3640 3650 3660 3670 3680
OMMV NC_006939 GTCTCCACGCCATGATCAATTGGAAACGATTGTGAGGGGGGTAGTGGAACCCATACCAGATTGAGGGGCCTTTGCCCCACCCC
transcriptOMMVWT ....

Appendix VI: Nucleotide sequence alignment of fragment OMMVcoat5’ – OMMVcoat3’ with sequence of OMMV NC_006939




820 830 840 850 860 870 880 890 900
OMMV WT GCATCCACAACAACTCTCTCTCCAATTTGCGCCGGGGCTTAATGGAAAGAGTATTCTACGTCGAGGGACCCAATGGGCTCCAAGACGCCC
fragmentOMMVcoat5'-OMMVcoat3' ----------
910 920 930 940 950 960 970 980 990
OMMV WT CGAAACCCGACAAGGGAGCCTTCAACTCCCTTGCTAAGTTCAGAGATCTCTATACAAAGAATAGCTGGCGTCATTCCCCTGTAACCAATG
fragmentOMMVcoat5'-OMMVcoat3' ----------
1000 1010 1020 1030 1040 1050 1060 1070 1080
OMMV WT AGCAATTTTTGATGAATTACTCGGGCAGGAAGCTAACTATTTACAAGGATGCGGTCGACAGTTTGTCGCGTCAACCGCTTAGCCTAAGAG
fragmentOMMVcoat5'-OMMVcoat3' ----------
1090 1100 1110 1120 1130 1140 1150 1160 1170
OMMV WT ATGCTCGGCTGAAGACATTCGTTAAGGCGGAAAAATTGAATCTGAGTAAGAAACCAGATCCAGCACCGAGGGTCATTCAGCCTCGGTCGC
fragmentOMMVcoat5'-OMMVcoat3' ----------
1180 1190 1200 1210 1220 1230 1240 1250 1260
OMMV WT CCCGCTATAACGTATGTTTGGGTCGTTATCTTCGTCACTATGAACATCACGCGTTTAAAACCATTGCTAAATGCTTTGGGGAAATCACGG
fragmentOMMVcoat5'-OMMVcoat3' ----------
1270 1280 1290 1300 1310 1320 1330 1340 1350
OMMV WT TCTTCAAAGGATTCACTCTCGAGCAACAAGGTGAAATCATGCACTCCAAGTGGAAGAAATATGTTAATCCCGTTGCGGTCGGTCTCGATG
fragmentOMMVcoat5'-OMMVcoat3' ----------
1360 1370 1380 1390 1400 1410 1420 1430 1440
OMMV WT CCAGTCGATTCGATCAACACGTGTCTAGGGAAGCACTTGAGTATGAGCATGAATTTTATTTGAGAGATTATCTTAATGATAAACAGCTAA
fragmentOMMVcoat5'-OMMVcoat3' ----------
1450 1460 1470 1480 1490 1500 1510 1520 1530
OMMV WT AATGGTTGCTTAAGCAACAATTGAGTAATGTAGGCACAGCATTCGCTAGCGACGGAATCATAAAGTACAAGAAAGAGGGATGTAGAATGA
fragmentOMMVcoat5'-OMMVcoat3' ----------
1540 1550 1560 1570 1580 1590 1600 1610 1620
OMMV WT GTGGGGACATGAACACAAGTTTGGGAAACTGCATATTGATGTGCGCCATGGTCTTTGGGCTAAAAGAACACTTAGGAATGGAATTGTCAT
fragmentOMMVcoat5'-OMMVcoat3' ----------


1630 1640 1650 1660 1670 1680 1690 1700 1710
OMMV WT TGGCTAACAATGGGGATGACTGCGTCATTGTCTGTGAGAAAGCGGATTTATTGAAATTGACGAGCAGCATCGAACCATATTTCAGACAGT
fragmentOMMVcoat5'-OMMVcoat3' ----------
1720 1730 1740 1750 1760 1770 1780 1790 1800
OMMV WT TCGGATTCAAAATGGAAGTGGAAAAGCCTGTAGACATCTTTGAGCGCATTGAATTTTGCCAAACCCAACCTGTGTTCGATGGCTCCCAAT
fragmentOMMVcoat5'-OMMVcoat3' ----------
1810 1820 1830 1840 1850 1860 1870 1880 1890
OMMV WT ACATTATGGTTAGGAAACCCTCCGTCGTAACTTCCAAAGATGTCACTAGCTTAATACCGTGCCAAACACAATCTCAATACGCAGAATGGC
fragmentOMMVcoat5'-OMMVcoat3' ----------
1900 1910 1920 1930 1940 1950 1960 1970 1980
OMMV WT TGCAAGCTGTTGGTGAGTGCGGTATGAGCATAAATGGTGGAATACCTGTCATGCAGAATTTCTACACCATGTTGCAAACTGGCGTAAAGC
fragmentOMMVcoat5'-OMMVcoat3' ----------
1990 2000 2010 2020 2030 2040 2050 2060 2070
OMMV WT GCACAAAATTCACCAAGACCGGCGAGTTCCAGACGAATGGGCTGGGGTATCACTCTCGATTTATGAACAGGGTGGCCCGAACTCCTTCGC
fragmentOMMVcoat5'-OMMVcoat3' ----------
2080 2090 2100 2110 2120 2130 2140 2150 2160
OMMV WT CTGAGACCCGTTTATCCTTTTACTTAGCGTTTGGTATCACACCAGACCTCCAGGAAGCATTGGAGGTCTTCTATGATACCAGTACGCTTG
fragmentOMMVcoat5'-OMMVcoat3' ----------
2170 2180 2190 2200 2210 2220 2230 2240 2250
OMMV WT AATTGGATGATGTGATCCCAACTGATACCTACCAAGTGTCAGGAGAGCATTTAATCAATGGATTACCAAACTGATATCAACGAAGATAAC
fragmentOMMVcoat5'-OMMVcoat3' ----------
2260 2270 2280 2290 2300 2310 2320 2330 2340
OMMV WT GTGAGCATAAGCGGTCGGGCCAGGAGGGGCACTGGGGACAAGAAACACAATGGTTCGGGAATGTCTGGCGTAAAGCGTCATGCGGTGAGT
fragmentOMMVcoat5'-OMMVcoat3' ----------
2350 2360 2370 2380 2390 2400 2410 2420 2430
OMMV WT GAAACAGCTCAGAAATCGCAGCAAGGTACTGGCAATGGCACAATGACCAACATAGCTGAAGAACAGACCATTACCGTGACATACAACTTT
fragmentOMMVcoat5'-OMMVcoat3' ----------

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                                2440      2450      2460      2470      2480      2490      2500      2510      2520
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        AACTTCTGAGTTATGGCTGTGTGTCGCTGCTGTGATACTTCACCAGGTATTACATTATTCCCTTACTTTGCAATTCTCATCCTCATCCTT
fragmentOMMVcoat5'-OMMVcoat3'  ------------------------------------------------------------------------------------

                                2530      2540      2550      2560      2570      2580      2590      2600      2610
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        GCAATACTAGTTGTTGGAACTCCAAACCAGCAATATCATCATTCTCCTAGCACTTACGAGTACAAGACTCAACACATTTCGATCGCAAAA
fragmentOMMVcoat5'-OMMVcoat3'  ------------------------------------------------------------------------------------

                                2620      2630      2640      2650      2660      2670      2680      2690      2700
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        TAGACATTTACTATAACACCAAAACATGCCTAAGAGAGGACGAGTTGGACTCGCTGAATCTTTTCAAGGAAAGACGAAGCAACAGAAACG
fragmentOMMVcoat5'-OMMVcoat3'  --GACATTTACTATAACACCAAAACATGCCTAAGAGAGGACGAGTTGGACTCGCTGAATCTTTTCAAGGAAAGACGAAGCAACAGAAACG

                                2710      2720      2730      2740      2750      2760      2770      2780      2790
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        GGCAGAGTACGAAGCTGTAAAACGTGAGCAGCTCGAACGTGCATTCGCAAACAATTCCAAGGTAGCCAATCCTAGATCTTCTGGGCTAAG
fragmentOMMVcoat5'-OMMVcoat3'  GGCAGAGTACGAAGCTGTAAAACGTGAGCAGCTCGAACGTGCATTCGCAAACAATTCCAAGGTAGCCAATCCTAGATCTTCTGGGCTAAG

                                2800      2810      2820      2830      2840      2850      2860      2870      2880
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        CTTCCGACCGTTGGTGGCACCAATCGCCGGGTCCGTTGTTTACAGCAGACCTCGCGTGCCACAGATCCGCACGAACCAAATGTCAACTAT
fragmentOMMVcoat5'-OMMVcoat3'  CTTCCGACCGTTGGTGGCACCAATCGCCGGGTCCGTTGTTTACAGCAGACCTCGCGTGCCACAGATCCGCACGAACCAAATGTCAACTAT

                                2890      2900      2910      2920      2930      2940      2950      2960      2970
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        TGTGGTAAACACTGAATTGGTAGCCAACATCACTTTGGCTGCTGCTGGAGCATTCAGTTTCACCGCCCAGCCATTGATACCATCATTTGG
fragmentOMMVcoat5'-OMMVcoat3'  TGTGGTAAACACTGAATTGGTAGCCAACATCACTTTGGCTGCTGCTGGAGCATTCAGTTTCACCGCCCAGCCATTGATACCATCATTTGG

                                2980      2990      3000      3010      3020      3030      3040      3050      3060
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        ATCTTGGTTGGCCAACATAGCTGACTTGTACTCAAAATGGAGATGGATTAGGTGTTCAGTCATATACATACCTAAATGTCCCACCACCAC
fragmentOMMVcoat5'-OMMVcoat3'  ATCTTGGTTGGCCAACATAGCTGACTTGTACTCAAAATGGAGATGGATTAGGTGTTCAGTCATATACATACCTAAATGTCCCACCACCAC

                                3070      3080      3090      3100      3110      3120      3130      3140      3150
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        TCAAGGTAGTGTGGTTATGGCGATTGTGTATGATGCGCAGGACACCGTACCTACGACACGAACGCAGGTATCACAGTGTTATCAATCCAT
fragmentOMMVcoat5'-OMMVcoat3'  TCAAGGTAGTGTGGTTATGGCGATTGTGTATGATGCGCAGGACACCGTACCTACGACACGAACGCAGGTATCACAGTGTTATCAATCCAT

                                3160      3170      3180      3190      3200      3210      3220      3230      3240
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                        CACATTTCCACCGTACGCCGGGTACGGTGGCGCCTCTGCGCTAAATAGCAACAGCTCTGGAGGGGAATCGCTAGTGTCTACACTTGACAC
fragmentOMMVcoat5'-OMMVcoat3'  CACATTTCCACCGTACGCCGGGTACGGTGGCGCCTCTGCGCTAAATAGCAACAGCTCTGGAGGGGAATCGCTAGTGTCTACACTTGACAC
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                                3250      3260      3270      3280      3290      3300      3310      3320      3330
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                         CAATCGCGTTGATAAGAAGTGGTACAGCACAATAGGCAACGCTGCCTTCACTGCTCTCACGTCTATCGACAAGAACCAGTTCTGTCCAGC
fragmentOMMVcoat5'-OMMVcoat3'   CAATCGCGTTGATAAGAAGTGGTACAGCACAATAGGCAACGCTGCCTTCACTGCTCTCACGTCTATCGACAAGAACCAGTTCTGTCCAGC

                                3340      3350      3360      3370      3380      3390      3400      3410      3420
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                         CACAGCTCTCATTGCTGGGGACGGCGGACCTGTGGCTGCCACAGCTGTGGGCGATGTATTTATGCGATATGAGATTGAGTTCATCGAACC
fragmentOMMVcoat5'-OMMVcoat3'   CACAGCTCTCATTGCTGGGGACGGCGGACCTGTGGCTGCCACAGCTGTGGGCGATGTATTTATGCGATATGAGATTGAGTTCATCGAACC

                                3430      3440      3450      3460      3470      3480      3490      3500      3510
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                         AATCAACCCTACCATTAACGTTTGAGTGTTAGTTAACTGGCTTAAACCCTAAGGGTAGATATGTGGGCGGTAAGCAAGAGGGATCCTGGG
fragmentOMMVcoat5'-OMMVcoat3'   AATCAACCCTACCATTAACGTTTGAGTGTTAGTTAACTGGCTTAAACCCTAAGGGTAGATATGTGGGCG--------------------

                                3520      3530      3540      3550      3560      3570      3580      3590      3600
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMV WT                         AAACAGGCTTCGACGGGTTGGGGGGTGGTGCCCCGGCCGACGCATCACTTGCTGATACAACCATTAGACACCTAAGGGCGGGTCTAGCCAG
fragmentOMMVcoat5'-OMMVcoat3'   ------------------------------------------------------------------------------------

                                3610      3620      3630      3640      3650      3660      3670      3680
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
OMMV WT                         GTCTCCACGCCATGATCAATTGGAAACGATTGTGAGGGGGGTAGTGGAACCCATACCAGATTGAGGGGCCTTTGCCCCACCCC
fragmentOMMVcoat5'-OMMVcoat3'   ----------------------------------------------------------------------------------
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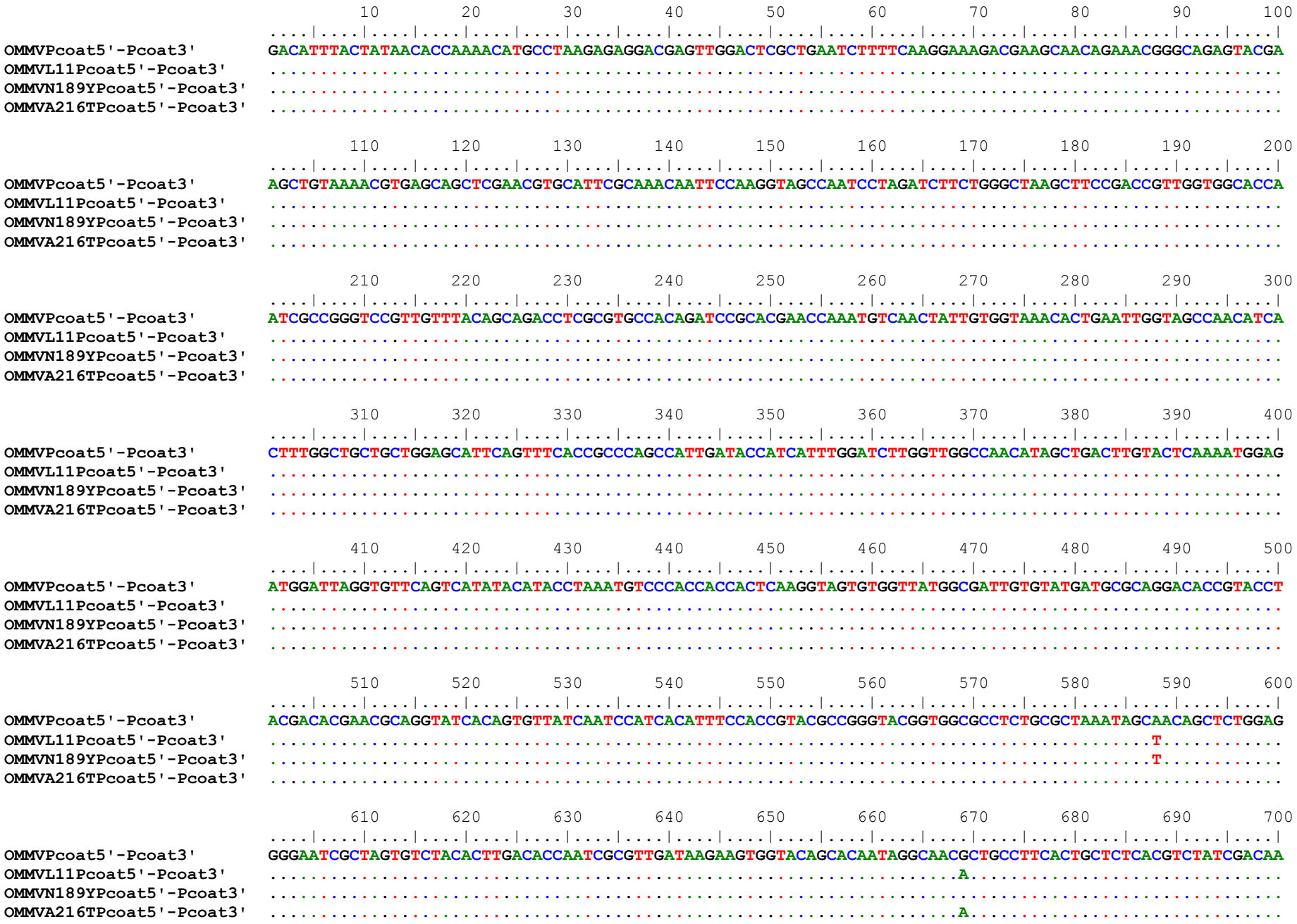
Appendix VII: Nucleotide sequence alignment of OMMV WT and OMMVL11 with site-directed mutants OMMVN189Y and OMMVA216T



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                          710       720       730       740       750       760       770       780       790       800
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OMMVPcoat5'-Pcoat3'       GAACCAGTTCTGTCCAGCCACAGCTCTCATTGCTGGGGACGGCGGACCTGTGGCTGCCACAGCTGTGGGCGATGTATTTATGCGATATGAGATTGAGTTC
OMMVL11Pcoat5'-Pcoat3'    ....................................................................................................
OMMVN189YPcoat5'-Pcoat3'  ....................................................................................................
OMMVA216TPcoat5'-Pcoat3'  ....................................................................................................

                                  810       820       830       840       850       860       870
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|..
OMMVPcoat5'-Pcoat3'       ATCGAACCAATCAACCCTACCATTAACGTTTGAGTGTTAGTTAACTGGCTTAAACCCTAAGGGTAGATATGTGGGCG
OMMVL11Pcoat5'-Pcoat3'    ...................................................................................
OMMVN189YPcoat5'-Pcoat3'  ...................................................................................
OMMVA216TPcoat5'-Pcoat3'  ...................................................................................
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