Specific amino acids of *Olive mild mosaic virus* coat protein are involved in transmission by *Olpidium brassicae*

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Transmission of *Olive mild mosaic virus* (OMMV) is facilitated by *Olpidium brassicae* (Wor.) Dang. An OMMV mutant (OMMVL11) containing two changes in the coat protein (CP), asparagine to tyrosine at position 189 and alanine to threonine at position 216, has been shown not to be *Olpidium brassicae*-transmissible owing to inefficient attachment of virions to zoospores. In this study, these amino acid changes were separately introduced into the OMMV genome through site-directed mutagenesis, and the asparagine-to-tyrosine change was shown to be largely responsible for the loss of transmission. Analysis of the structure of OMMV CP by comparative modelling approaches showed that this change is located in the interior of the virus particle and the alanine-to-threonine change is exposed on the surface. The asparagine-to-tyrosine change may indirectly affect attachment via changes in the conformation of viral CP subunits, altering the receptor binding site and thus preventing binding to the fungal zoospore.

Transmission of most plant viruses requires distinct invertebrate or fungal vectors to be efficient. Previous studies have shown that transmission is a specific process, suggesting that virus particles and vectors contain specific sites that mediate their recognition. The coat protein (CP) of several plant viruses has been shown to be responsible for transmission (Robbins *et al.*, 1997; Varanda *et al.*, 2011) and particular amino acids within the CP have been shown to be essential (Campbell, 1996; Kakani *et al.*, 2001; Mochizuki *et al.*, 2008).

The necrovirus *Olive mild mosaic virus* (OMMV) was isolated for the first time in 2005, from olive trees in Portugal (Cardoso *et al.*, 2005). The earlier existence of OMMV was recently revealed based on new sequence data (GenBank accession nos EF201608, EF201607, EF201606 and EF201605) found in the virus associated with Augusta disease, which was first observed in tulips in The Netherlands in 1928. OMMV is a 28 nm icosahedral virus, with a positive-sense ssRNA genome of 3683 nt, containing five ORFs. Its particle is an oligomer consisting of 180 copies of the CP (Cardoso *et al.*, 2005), which shows 43 % amino-acid sequence identity and 61 % amino-acid sequence similarity with the tobacco necrosis virus (TNV) CP, whose quaternary structure has been solved at 2.25 Å resolution (Oda *et al.*, 2000).

A recombination event between *Olive latent virus 1* (OLV-1) and *Tobacco necrosis virus D* (TNV-D) was proposed for the origin of OMMV, as its RNA-dependent RNA polymerase shares high amino acid identity with that of OLV-1 and its CP shares high amino acid identity with that of TNV-D (Cardoso *et al.*, 2005). RT-PCR assays using specific primers for the known necroviruses infecting olive (Varanda *et al.*, 2010) showed that OLV-1, TNV-D and OMMV exist in the same host, thus supporting the possibility of recombination events.

OMMV transmission through soil has recently been shown to be facilitated by zoospores of the chytrid fungus *Olpidium brassicae* (Wor.) Dang. (Varanda *et al.*, 2011). Zoospores and viruses are released from infected roots into the soil (Félix *et al.*, 2006) and virus particles are then thought to bind zoospore plasmalemma (Temmink *et al.*, 1970), encyst and enter plant roots. Exchange between the CP gene of wild type OMMV (WT) and that of a non-*Olpidium brassicae*-transmissible OMMV mutant (OMMVL11) produced particles that were no longer fungus-transmissible, indicating that the CP contains determinants that specify its interaction with the zoospores of *O. brassicae*. The CP sequence analysis of OMMVL11 revealed two amino acid substitutions relative to OMMV WT CP, asparagine to...
tyrosine at position 189 and alanine to threonine at position 216 (Varanda et al., 2011). In vivo binding studies demonstrated that OMMVL11 binds zoospores less efficiently than OMMV WT, indicating that specific regions of the OMMV CP mediate zoospore adsorption (Varanda et al., 2011).

In this work our aim was to determine the individual role of these alterations in fungus transmission and their implications for virion structure.

In vitro mutagenesis was used to individually introduce each mutation of OMMVL11 into OMMV WT cDNA in order to assess their possible independent roles in the non-transmissibility of OMMVL11 by O. brassicae. Mutants OMMVN189Y and OMMVA216T were produced; these contain the single nucleotide substitutions adenine to thymine at nt 3200 and guanine to adenine at nt 3281, respectively. Plasmid DNA containing the viral full-length cDNA of an OMMV clone (pUC18OMMV), was extracted using a DNA-spin Plasmid DNA Purification kit (Intron) and used as a template for site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The mutagenic oligonucleotide primers were designed using Stratagene’s web-based QuikChange primer design program. The primers (mutations shown underlined) used to produce OMMVN189Y were 5′-CTGGGCTAAATGCTACAGCTCTGGAGGGG-3′ (sense) and 5′-CCCCCTCA-GAGCTTAGCTATTTAGCAGCAG-3′ (antisense) (OMMV nt 3185–3215). The primers used to produce OMMVA216T were: 5′-AGAGCGATGAGCGACGTGTGCTG-3′ (sense) and 5′-AGAGCGATGAGCGACGTGTGCTG-3′ (antisense) (OMMV nt 3265–3297). All of these are located in the CP region of OMMV. The mutants were sequenced to ensure that no other mutations occurred (not shown).

Plasmid DNA containing full-length cDNA of the two single amino acid mutants of OMMV, was linearized by digestion with Smal, which cuts at a unique restriction site within pUC18OMMV. Approximately 2 μg of linear plasmid DNA were used for in vitro transcription by using a RiboflMax Large Scale RNA Production System – T7 (Promega) according to the manufacturer’s instructions. DNA template was removed by digestion with DNase I [1 U DNase I (μg of template DNA) –1] and the transcript was purified by extraction with citrate-saturated phenol (pH 4.7)/chloroform/isooamyl alcohol (in the ratio 125: 24: 1, respectively) and ethanol precipitated.

To test the infectivity of the in vitro transcripts, approximately 2 μg of synthesized virus was mechanically inoculated into two Chenopodium murale plants. OMMV WT and OMMVL11 RNA were also inoculated as controls. Three days after inoculation, plants began to develop similar local lesions. Total RNA was extracted from C. murale leaves 3 days after inoculation with each virus, and agarose gel electrophoresis was used to show that there were no major differences in the levels of the genomic RNAs for the different viruses, suggesting that viruses accumulate to identical levels (not shown).

OMMV WT, OMMVL11, OMMVN189Y and OMMVA216T were purified from infected C. murale leaves as in Varanda et al. (2011). Virus concentration was determined by measuring A260 in a UV/visible spectrophotometer (DU 530 Life Sciences; Beckman) using the extinction coefficient E1%1cm=5.0. To test virus stability, virus particles were incubated in transmission buffer [50 mM glycine/NaOH (pH 7.6)] for 20 h and then used as inocula. No decrease in the number of lesions was observed, suggesting the virus particles were stable. For each virus, typically 100 g of tissue infected with each virus yielded approximately the same amount of virus, approximately 0.3 mg. Purified viruses were tested for in vitro acquisition and transmission to cabbage seedlings, as described in previous assays (Robbins et al., 1997; Kakani et al., 2001; Varanda et al., 2011). Five micrograms of each purified virus was added to 100 ml of transmission buffer containing 1 × 10⁶ zoospores ml⁻¹ of O. brassicae, or to transmission buffer alone as a control. After a 20 min period to allow virus acquisition, 1 ml of the suspension was poured into 100 ml pots containing 5-day-old cabbage seedlings growing in sterile sand. Six days later, the roots were carefully washed with a 1% SDS solution, placed under running tap water for 3 h and tested for the presence of virus using double antibody sandwich-ELISA (DAS-ELISA) (Clark & Adams, 1977). Final A405 readings were considered positive if they showed greater than twice the mean A405 of negative control samples. Control experiments were conducted in the absence of fungal zoospores. A hundred pots containing ten plants each were used in each experiment, which was repeated five times, giving a total of 500 pots. Results showed that each site-directed mutant could infect plant roots in the absence of fungus at similar levels (30–40%) (Table 1). The presence of O. brassicae did not increase the transmissibility of OMMVN189Y, which was 33 %, or of OMMVL11, which was 34 %. In contrast, the transmission efficiencies of OMMVA216T and OMMV WT increased markedly, to 80 % and 86 %, respectively, indicating that the asparagine-to-tyrosine mutation is largely responsible for the loss of transmissibility of OMMVL11 by O. brassicae.

To test whether the loss of transmissibility was due to inefficient binding of virions to zoospores, a binding assay was optimized based on that previously described by Robbins et al. (1997). Different amounts of virus were incubated with different amounts of zoospores to optimize the assay (data not shown). The optimized assay consisted of 100 μg of each of purified OMMV, OMMVL11, OMMVN189Y and OMMVA216T, separately incubated with 1 × 10⁶ zoospores ml⁻¹ in 10 ml 50 mM glycine/NaOH (pH 7.6) for 20 min. Lower initial amounts of virus resulted in a decrease in the amount of virus associated with zoospores; higher initial amounts of virus did not result in any increase, which was only observed when a higher number of zoospores were used. After incubation,
Virus was incubated with zoospores for 15 min prior to inoculation of the plants in each pot.

O. brassicae zoospores, determined by DAS-ELISA

Transmission efficiency is given as the percentage of pots containing infected plants. A hundred pots were used for each treatment.

Table 1. Soil transmissibility of OMMV, OMMVL11, OMMVN189Y and OMMVA216T to cabbage roots in the presence of O. brassicae zoospores, determined by DAS-ELISA

Transmission efficiency (%) | Expt 1 | Expt 2 | Expt 3 | Expt 4 | Expt 5 | Mean
---|---|---|---|---|---|---
OMMV | 40 | 44 | 39 | 42 | 42 | 41
OMMV + O. brassicae zoospores* | 84 | 82 | 88 | 88 | 90 | 86
OMMV L11 | 34 | 34 | 33 | 33 | 34 | 34
OMMV L11 + O. brassicae zoospores* | 33 | 36 | 33 | 34 | 33 | 34
OMMVN189Y | 33 | 37 | 36 | 36 | 32 | 35
OMMVN189Y + O. brassicae zoospores* | 32 | 33 | 33 | 33 | 32 | 33
OMMVA216T | 39 | 35 | 35 | 34 | 36 | 36
OMMVA216T + O. brassicae zoospores* | 83 | 80 | 79 | 76 | 83 | 80

*Virus was incubated with zoospores for 15 min prior to inoculation of the plants in each pot.

zoospores were pelleted by centrifugation at 2800 g for 7 min. Supernatant containing unbound virus was centrifuged at 117 000 g for 3 h and the pellet was resuspended in 30 μl of 0.02 M sodium phosphate buffer (pH 6.0). The amount of virus in the pellet was determined spectrophotometrically, as described above. Each binding experiment included a virus-only control. Only 7 μg, approximately, of the 100 μg of OMMV WT and of OMMVA216T initially incubated with zoospores became associated with them, as the remaining amount of virus was found free in the supernatant after low-speed centrifugation. In contrast, the OMMVL11 and OMMVN189Y virus concentrations found in the supernatant were similar to the initial amount used in the incubation step, indicating a lack of significant adsorption to the spores. In experiments where no spores were used, the amount of virus found in the supernatant was identical to that used initially, as expected. These results suggest that the inefficient transmission of OMMVL11 and OMMVN189Y is partly because of their inability to recognize or stably bind zoospores.

Owing to the significant similarity of the OMMV CP polypeptide chain with that of TNV, it is plausible that the two virus CPs display similar three-dimensional structural arrangements, opening the way for deriving the quaternary structure of the OMMV coat arrangement by using comparative modelling approaches. The MODELLER package (version 9v6) (Sali & Blundell, 1993) was used for this task. The TNV CP structure was solved as a repetition (60 times) of a trimer unit. This was composed of the same protein, but presented 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). Owing to these invisible parts within the crystal, one can only align the OMMV protein sequence with each one of these chains by considering the segments consisting of residues 79–269, 80–269 and 57–269, as chains A, B and C, respectively. Modelling the whole virus capsid, which contains 180 polypeptide chains, is beyond the reach of MODELLER. Therefore, we decided to model a ‘minimum contact unit’, consisting of one central trimer surrounded by three other trimers, to give a total of 12 chains. This arrangement has most of the multimer contacts of the central trimer unit [shown in Fig. 1(a), (d), where the central trimer is rendered in colour and the rest of the multimer being modelled is rendered in grey], and is, therefore, useful for analysing the effects of mutations. The alignment made with the align2d procedure of MODELLER was used to derive 40 models and we chose the model with the lowest value for the objective function. The Ramachandran plot of this model showed that 95.7 % of the residues are found in the most-favoured regions and 4.3 % found in additional allowed regions. No residues were found in the generously allowed or disallowed regions, indicating the good quality of the model. Given that we were interested in the effect of two mutations, we also built a double mutant containing them by using the same procedure as was used for the WT.

While the OMMVA216T mutation occurs at the external face of the capsid (Fig. 1b, c), the OMMVN189Y mutation occurs in the capsid interior (Fig. 1e, f). A more careful analysis of the mutations shows that the OMMVA216T mutation occurs at the beginning of a helix (not shown) in a very exposed zone (Fig. 1b, c). Mutation OMMVN189Y is located in the capsid interior and is found within a loop region (not shown), which is not far from the intersubunit contacts (Fig. 1e–h).

Through site-directed mutagenesis and transmission assays we have demonstrated that the asparagine-to-tyrosine substitution in the OMMVL11 CP gene is largely responsible for the reduction in fungus transmissibility. OMMVN189Y particles containing that single mutation are highly infectious, stable and accumulate to WT levels in inoculated plants, indicating that the reduced transmissibility of OMMVN189Y is not because of loss of virus stability or infectivity.

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Viral particles containing this mutation bind *O. brassicae* zoospores less efficiently, suggesting that the decreased transmissibility is because of a decrease in the ability of viral capsids to recognize or stably bind zoospores.

Our comparative model of OMMV CP oligomers shows that the asparagine-to-tyrosine mutation is located in the capsid interior (Fig 1e, f) and the alanine-to-threonine mutation is located on the capsid surface (Fig. 1b, c). The alanine-to-threonine mutation (Fig. 1b, c) 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. In contrast, the asparagine-to-tyrosine mutation is located in the capsid interior, in a loop region, not far from the inter-monomer contacts. Fig. 1(e, f) shows 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 the monomers. Despite not being so evident at all interfaces, given the non-equivalence between monomers, this is particularly evident at the interface between the monomer coloured in yellow and the monomer coloured in green, or between the latter and the monomer coloured in magenta.

**Fig. 1.** Three-dimensional representation of the surface of the OMMV virus minimum contact unit, from two different views: the external face of the capsid (left) and the internal face (right). The central trimer is coloured green, cyan and magenta, corresponding to chains A, B and C, respectively. (a) The external face and (d) the internal face, show the central trimer highlighted and coloured as for the other panels. The other chains are coloured in dark grey in order to show the repeating unit. (b) The external face, showing the A216 residue and (c) showing the mutated A216T residue; the side chains are rendered in spheres to distinguish them from the molecular surface. (e) The internal face, showing the N189 residue and (f) showing the mutated N189Y residue; the side chains are rendered in spheres to distinguish them from the molecular surface. The figure was prepared by using PyMOL (DeLano, 2002).
coloured in darker blue. This mutation results in the presence of a considerably larger residue in a zone that may provide potential contacts with the nucleic acid in the capsid interior, although this cannot be proven at this stage. Despite not appearing to affect the correct assembly of the capsid (as purified OMMVN189Y particles are highly infectious and accumulate in plants at the same levels as OMMV WT), this mutation, by the introduction of a bulkier residue at this position, may affect the interfaces between monomers. Thus it might generate a particle with a somewhat different surface and indirectly affect virion attachment 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). Obviously, while this speculation is possible at this stage, this matter cannot be solved without having experimental structural data for the OMMV virus capsid.

With this work we reinforce previous studies on the role of virus CP in transmission (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 recognition site in the vector (Kakani et al., 2001; Andret-Link & Fuchs, 2005; Mochizuki et al., 2008). Owing to the high transmission specificity sometimes observed, in that one virus is only transmitted by one vector, it is not surprising that few changes in the CP of a vector-transmissible virus would render it non-transmissible. In this work we show that a single amino acid 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 were shown by Mochizuki et al. (2008) when the single isoleucine-to-phenylalanine 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) with a glutamic acid-to-lysine change in CNV CP. A single proline-to-glycine mutation in the interior of CNV, although not affecting virion binding to zoospores, resulted in loss of transmissibility by its vector by affecting the ability of the virus to undergo conformational change, which is required for the transmission process (Kakani et al., 2004).

This work contributes to a better knowledge of the features of virion architecture that are required for attachment to a vector, aiding in the identification of sites responsible for virus–vector interactions about which little is known and which have important implications in the control of virus-associated diseases.

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### References


