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The protruding domain of the coat protein of *Melon necrotic spot virus* is involved in compatibility with and transmission by the fungal vector *Olpidium bornovanus*

Takehiro Ohki *, Fusamichi Akita, Tomofumi Mochizuki [1], Ayami Kanda, Takahide Sasaya, Shinya Tsuda

National Agricultural Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

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ABSTRACT

The Chi and W strains of *Melon necrotic spot virus* (MNSV) are efficiently transmitted by isolates Y1 and NW1, respectively, of the fungal vector *Olpidium bornovanus*. Analysis of chimeric viruses constructed by switching the coat protein (CP) gene between the two strains unveiled the involvement of the CP in the attachment of MNSV to zoospores of a compatible isolate of *O. bornovanus* and in the fungal transmission of the virus. Furthermore, analysis of the chimeric virus based on the Chi strain with the protruding domain of the CP from strain W suggested the involvement of the domain in compatibility with zoospore. Comparison of the three-dimensional structures between the CP of the two MNSV strains showed that many of the differences in these amino acid residues are present on the surface of the virus particles, suggesting that these affects the recognition of fungal vectors by the virus.

Introduction

Melon necrotic spot virus (MNSV) is an isometric particle, ~30 nm in diameter and belongs to the genus *Carmovirus* in the family *Tombusviridae* (Hibi and Furuki, 1985). MNSV is transmitted by the soil-inhabiting fungus *Olpidium bornovanus* (Furuki, 1981; Campbell and Sim, 1994). MNSV causes necrotic lesions on leaves, stems and fruits of infected melon plants, resulting in severe deterioration in fruit quality and economic damage (Furuki, 1981; Matsuo et al., 1991). The MNSV genome consists of a 4.3-kb, positive-sense, single-stranded RNA containing five open reading frames (ORFs), including p29, p89, p7A, p7B and p42 (Riviere and Rochon, 1990; Genovés et al., 2006). The coat protein (CP) is encoded on p42 (Riviere et al., 1989).

Several isometric viruses belonging to the family *Tombusviridae* are transmitted by *O. bornovanus* and *O. virulentus* (Adams, 2002; Rochon et al., 2004). These fungi are obligate, intracellular parasites and form motile zoospores and enduring resting spores in infected root tissues. These fungus-borne viruses attach to the surface of zoospores and are transmitted externally by *in vitro* acquisition (Adams, 2002; Rochon et al., 2004). The CP is indispensable for the attachment to zoospores and fungal transmission of MNSV (Mochizuki et al., 2008) and *Cucumber necrosis virus* (CNV) (McLean et al., 1994).

The three-dimensional structures of the CPs in the family *Tombusviridae* have been studied with X-ray structural analysis (Harrison et al., 1978; Olson et al., 1983; Morgunova et al., 1994; Hogle et al., 1986; Ke et al., 2004; Oda et al., 2000; Wada et al., 2008). These viruses have an icosahedral symmetry with a triangulation number of $T=3$. These are composed of 180 identical CP subunits (Lommel et al., 2005), which consist of three subunits (A, B and C) related to an icosahedral asymmetric unit. The CP is divided into three domains, designated as the RNA-binding domain (R), the shell domain (S), and the protruding domain (P). The arm region is located between the R and S domains, and the hinge region is between the P and S domains. The P domain projects outward from the virus particle and has a characteristic anti-parallel β-sheet called a jellyroll conformation, which has been found in a variety of proteins having ligand-binding functions (Richardson, 1981).

To clarify the specific domain of the CP that is involved in fungal transmission, several mutagenesis experiments using infectious clones of CNV were conducted. A comparison of amino acid sequences of CPs among transmissible and nontransmissible mutants of CNV showed that several amino acid residues in the S and P domains were correlated with fungal transmission (Robbins et al., 1997; Kakani et al., 2001). These amino acid mutations in the S and P domains of CP generally decreased the attachment of virus particles to the surface of the zoospores, resulting in reduced fungal transmissibility of virus. Most of these mutations are exposed on the surface of the virus particle, and several of the mutated sites are located near the quasi-threefold axis of the particle, although it is unclear whether these mutations directly affect attachment to a zoospore or indirectly affect attachment by changing the structure of the CP. The arm region of the R domain of CNV is also involved in fungal transmission (Kakani et al., 2004; Hui and Rochon, 2006); CNV particles on zoospores have an altered conformational state (the swelling condition). Furthermore, a

* Corresponding author. Fax: +81 29 838 7845.
E-mail address: take2001@affrc.go.jp (T. Ohki).
[1] Present address: Graduate School of Life and Environmental Science, Osaka Prefecture University, Osaka 599-8531, Japan.

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substitution and deletion in the arm region of the R domain inhibits the conformational change of the CNV particles into the swelling condition, resulting in a defect of fungal transmissibility. These results indicated that the conformational change of CNV particles on the surface of zoospores is an indispensable step for fungal transmission.

On the other hand, a structural analysis of the P domain of MNSV and *Tomato bushy stunt virus*, which is not fungal borne, predicted that the CP of MNSV had specific amino acid insertions within three loops of the P domain, which might be involved in fungal transmission (Wada et al., 2008). In addition, we showed that an amino acid substitution at position 300 in the P domain of the CP of MNSV influenced fungal transmission similar to the case of CNV (Mochizuki et al., 2008).

Recently, we reported that the watermelon strain of MNSV (MNSV-W) has characters that differ from the wild type MNSV-Chi (Ohki et al., 2008). Interestingly, the Y1 isolate of *O. bornovanus* efficiently transmits MNSV-Chi but not MNSV-W. In contrast, the NW1 isolate of *O. bornovanus* efficiently transmits MNSV-W but not MNSV-Chi (Ohki et al., 2008). The amino acid identity of the CP between the two strains of MNSV is about 75%. In this study, we constructed chimeric viruses by replacing whole or part of the CP gene between the two strains of MNSV and comparing their transmissibility by the two isolates of *O. bornovanus*. Furthermore, we compared the three-dimensional structures of the CP from the two strains of MNSV and from the chimeric viruses to elucidate the key domains.

Results

Fungal transmission and attachment to zoospores of chimeric viruses with the CP from other strains

The CP genes of MNSV-Chi and MNSV-W were switched with each other, and chimeric viruses, designated as Chi-WCP and W-ChiCP, were constructed (Fig. 1). These two chimeric viruses caused necrotic spots on inoculated leaves of watermelon. In a transmission test using isolates Y1 and NW1 of *O. bornovanus* (Table 1), W-ChiCP, which has the CP from MNSV-Chi, was transmitted by isolate Y1 similar to MNSV-Chi, and Chi-WCP, which has the CP from MNSV-W, was efficiently transmitted by isolate NW1 similar to MNSV-W. Thus, the transmission of the two chimeric viruses by Y1 and NW1 isolates of *O. bornovanus* was controlled by the CP.

By indirect fluorescence microscopy, we compared the attachment of MNSV-W, MNSV-Chi, and the two chimeric viruses to the surface of zoospores (Fig. 2). Strong signals were obtained on the zoospores of isolate Y1 for MNSV-Chi and W-ChiCP and on zoospores of isolate NW1 for MNSV-W and Chi-WCP in correspondence with their transmissibility (Fig. 2A). The western blot analysis also showed attachment of MNSV-Chi and W-ChiCP to Y1 zoospores and of MNSV-W and Chi-WCP to NW1 zoospores (Fig. 2B). The amount of attachment was almost the same between MNSV-Chi and W-ChiCP to Y1 zoospores and between MNSV-W and Chi-WCP to NW1 zoospores (Fig. 2B). These results indicated that the amount of virus particles that attached to the surface of *O. bornovanus* zoospores correlated positively with fungal transmissibility of the virus.

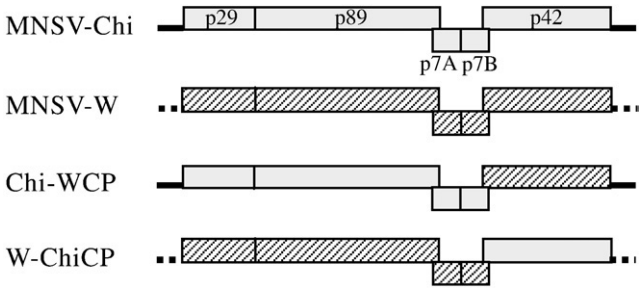

Fig. 1. Schematic representation of MNSV-Chi, MNSV-W and their chimeric viruses with CP from other strains. Solid boxes show the genomic sequence of MNSV-Chi, and hatched boxes show that of MNSV-W. P42 encodes the CP.

Table 1
Transmission by *Olpidium bornovanus* of MNSV-Chi, MNSV-W and their chimeric viruses, which had the CP from other strains (see Fig. 1 for chimeric viruses) to watermelon seedlings.

Virus	Isolate of *Olpidium bornovanus*		
	Y1	NW1	Mock
MNSV-Chi	13/13[a]	2/13[a]	0/9[a]
MNSV-W	1/13	13/13	0/9
Chi-WCP	1/13	13/13	n.t.[b]
W-ChiCP	13/13	1/13	n.t.
Mock	0/9	0/9	n.t.

[a] Seedlings were inoculated with 1 ml of solution containing 0.1 μg virus and 5.0×10^5 zoospores. MNSV-Chi and W-ChiCP were detected with anti-MNSV-Chi IgG, and MNSV-W and Chi-WCP were detected with anti-MNSV-W IgG. The results show total number of plants infected/total plants tested in three experiments.
[b] n.t., not tested.

Fungal transmission and attachment to zoospores of chimeric viruses with replacement at the P domains of CP

To determine the role of the P domain of the CP in fungal transmission of MNSV, we constructed two chimeric viruses, Chi-WP and W-ChiP, with replacement at the P domain of CP (Fig. 3). Between the two viruses, only Chi-WP with the P domain from MNSV-W was transmitted by isolate NW1 similar to MNSV-W but with a lower degree of transmissibility (Table 2). Furthermore, a high level of Chi-WP is attached to the zoospores of isolate NW1 compared to those of isolate Y1 (Fig. 4). These results suggested that the P domain of the CP has an important role in the recognition of the fungal vector. On the other hand, W-ChiP with the P domain from MNSV-Chi was not transmitted by either isolate (Table 2). The transcripts from W-ChiP infected watermelon plants and caused necrotic spots on inoculated leaves; however, sap of leaves that were inoculated with the transcripts did not cause infection (Table 2). W-ChiP particles seemed to be unstable or constituted incorrectly. For the same reason, we could not obtain purified particles of W-ChiP to confirm attachment to zoospores.

By analyzing the three-dimensional crystal structures of the CP of MNSV (KS isolate), Wada et al. (2008) predicted that three loops (loop 1 [273–279 aa], loop 2 [288–292 aa] and loop 8 [356–361 aa]) of the P domain may be involved in the fungal transmissibility of MNSV. Chi-WP_{L28} with the sequence of loop 2 and loop 8 from MNSV-W was able to infect watermelons. However, Chi-WP_{L28} was not transmitted by either the Y1 or the NW1 isolate and did not attach to zoospores (Table 2, Fig. 5).

Three-dimensional structures of CPs of strains of MNSV and chimeric viruses

Because the CP of MNSV plays an important role in the compatibility of MNSV with specific isolates of *O. bornovanus*, the amino acid sequences and three-dimensional structures of two strains of MNSV were compared in detail based on homology modeling of the two strains. The R and arm, S and P domains had amino acids identity of 76.6%, 81.4% and 64.3%, respectively. Different amino acid residues between the two strains are distributed over the surface of the S and P domains (Fig. 5B; see yellow and blue boxes). These amino acid residues were largely exposed toward the 5-fold and 3-fold axis, which symmetrically consist of five A subunits, and three B/C subunits, respectively (Fig. 5B, left and center). However, several amino acid residues of the P domain differed between the two strains exposed toward the cavity of the quasi-3-fold axis, which