RNA Helicase Domain of Tobamovirus Replicase Executes Cell-to-Cell Movement Possibly through Collaboration with Its Nonconserved Region

Kyotaro Hirashima1,2 and Yuichiro Watanabe1*

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153-8902,1 and R&D Group, ASPEX Division, Asahi Glass Co., Ltd., Kanagawa-ku, Yokohama-shi, Kanagawa 221-8755,2 Japan

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Tobacco mosaic virus (TMV), a positive-stranded plant RNA virus, is the type member of the tobamoviruses in the alphavirus-like superfamily. The genomic RNA of TMV is 6,395 nucleotides (nt) long (6) and encodes at least four proteins. The full-length RNA is used to produce 126-kDa and 6,395 nucleotides (nt) long (6) and encodes at least four proteins. The full-length RNA is used to produce 126-kDa and 183-kDa replicase proteins (14), while the 30-kDa movement protein (MP) and the 17.5-kDa coat protein are translated from 3’-coterminal subgenomic mRNAs (9, 13, 25). These coding regions are flanked by the 5’ and 3’ untranslated regions, both of which are required for viral replication (20, 21). Systemic infection of plant viruses proceeds through three steps: intracellular replication, cell-to-cell movement, and long-distance movement (3). It is well known that TMV MP is indispensable for and involved in cell-to-cell movement (5, 17).

In a previous study, we found that tobamovirus replicase is also involved in cell-to-cell movement (11). The 126-kDa replicase protein contains two domains showing similarities to the methytransferase and RNA helicase domains commonly recognized in replicases of various RNA viruses (1, 7, 8, 15, 22). There is a relatively nonconserved region between the two domains (8, 22). The 183-kDa replicase is translated by read-through of the amber termination codon of the 126-kDa protein (2, 19). The read-through region contains so-called RNA polymerase domains, which can also be found in the replicase proteins of various RNA viruses (22).

UR-hel, a chimera virus obtained by replacement of the RNA helicase domain of tobacco mosaic virus (TMV)-U1 replicase with that from the TMV-R strain, could replicate similarly to TMV-U1 in protoplasts but could not move from cell to cell (K. Hirashima and Y. Watanabe, J. Virol. 75:8831-8836, 2001). It was suggested that TMV recruited both the movement protein (MP) and replicase for cell-to-cell movement by unknown mechanisms. Here, we found that a recombinant, UR-hel/V, in which the nonconserved region was derived from TMV-R in addition to the RNA helicase domain of replicase, could move from cell to cell. We also analyzed revertants isolated from UR-hel, which recovered cell-to-cell movement by their own abilities. We found amino acid substitutions responsible for phenotypic reversion only in the nonconserved region and/or RNA helicase domain but never in MP. Together, these data show that both the nonconserved region and the RNA helicase domain of replicase are involved in cell-to-cell movement. The RNA helicase domain of tobamovirus replicase possibly does not interact directly with MP but interacts with its nonconserved region to execute cell-to-cell movement.

UR-hel could replicate to a level similar to that of TMV-U1 in protoplasts but could not move from cell to cell (11). The defect could not be rescued by MP supplied in trans by coinoculation with wild-type virus or expression in MP-positive transgenic plants (11). These results demonstrated that the cell-to-cell movement of TMV requires not only MP but also replicase, although the mechanism remains unknown. To address whether the defect in cell-to-cell movement of UR-hel is caused by its replicase alone and whether only the RNA helicase domain is involved in the cell-to-cell movement, we analyzed various chimeric viruses constructed from TMV-U1 and TMV-R and movement-competent revertants isolated from UR-hel.

Cognate combination of the RNA helicase domain and MP could not rescue the defect in cell-to-cell movement of UR-hel. A fragment covering nt 2354 to 3332 from TMV-U1 was replaced with the counterpart of TMV-R to obtain UR-hel (Fig. 1A). UR-hel had a sequence identical to that of TMV-U1 except for 75 nucleotide differences located in the RNA helicase domain. Of these, 12 substitutions led to amino acid changes (see Fig. 3, legend) Even so, UR-hel replicated to levels similar to those of TMV-U1 during the time analyzed in the previous study (11). Meanwhile, the replication efficiency in protoplasts of the other parental virus, TMV-R, was lower than that of TMV-U1 (data not shown). However, when we inoculated TMV-R on Nicotiana tabacum cv. Xanthi-nc, many local lesions appeared, which were approximately half the size of those caused by TMV-U1. This result indicated that TMV-R could move from cell to cell (Fig. 2A, upper left) while UR-hel could not and, therefore, that replication efficiency was not correlated with the capacity for cell-to-cell movement.

After the earlier study, we considered the cause of the defect in the cell-to-cell movement of UR-hel. It has been shown that
MP is involved in cell-to-cell movement and that there are 12 amino acid differences between the MP coding regions of TMV-U1 (or UR-hel) and TMV-R. Therefore, we first suspected that the RNA helicase domain of TMV-R interacted directly with MP to perform the function. If this was the case, cognate combination of the origins of MP and the RNA helicase domain would restore cell-to-cell movement.

We had already created a construct, UR-MP, by replacing MP of TMV-U1 with the corresponding region from TMV-R (4) (Fig. 1A). This time, we constructed a new chimeric virus, UR-hel/MP, by replacing the RNA helicase domain of UR-MP, the BamHI (nt 3332)-KpnI (nt 6400) fragment, with that of TMV-R (Fig. 1A). We compared the replication levels of TMV-U1, UR-hel, UR-MP, and UR-hel/MP in BY-2 protoplasts. All the viruses showed similar replication levels in the protoplasts (Fig. 2B). We checked the cell-to-cell movement ability of plants by observing local lesions on N. tabacum cv. Xanthi-nc leaves, a local lesion host. Contrary to our expectations, neither UR-hel/MP nor UR-hel caused any local lesions, whereas UR-MP caused as many local lesions as TMV-U1 caused (Fig. 2A, upper right). These results indicated that the combination of the RNA helicase domain and MP coding region was not the determinant of whether or not the chimeric viruses could move from cell to cell.

The nonconserved region is involved in the movement function as well as the RNA helicase domain. Since TMV-R MP could not restore the capacity of UR-hel for cell-to-cell movement, it was suggested that replicase is involved independently from MP in cell-to-cell movement. Thus, we next focused on replicase and tried to dissect replicase genes to identify the domain(s) responsible for cell-to-cell movement. We constructed and analyzed various chimeric viruses of replicase. First, we compared UR-hel with UR-183K (formerly described as URSmH [4]), which was constructed by replacing almost the whole TMV-U1 183-kDa replicase with the counterpart of TMV-R (Fig. 1B). There were 40 amino acid differences in the 183-kDa replicases between TMV-U1 and TMV-R. UR-183K, which contains 39 of the 40 amino acid differences, was reported to be able to multiply on inoculated leaves (4). We checked the capacity of UR-183K for cell-to-cell movement compared with that of UR-hel on Xanthi-nc leaves. UR-183K caused local lesions with sizes similar to those caused by TMV-U1 at 4 days postinfection (dpi), whereas UR-hel did not (Fig. 2A, lower left). This result showed that UR-183K could move from cell to cell, although the sequence of the RNA helicase domain of replicase is identical to that of UR-hel. Thus, it was conceivable that the defect in cell-to-cell movement of UR-hel was not caused by the RNA helicase domain alone, indicating that a certain genomic element derived from

FIG. 1. Schematic representation of the genomic organization of the viruses studied. (A) Parental TMV-U1 and TMV-R viruses and their derivatives, UR-hel, UR-MP, and UR-hel/MP. (B) Viruses in which each part of the replicase was derived from TMV-R in addition to the RNA helicase domain of TMV-R. Shaded portions indicate those parts that were derived from the TMV-R genome. Vertical white lines indicate the locations of amino acid differences between TMV-U1 and TMV-R. The cell-to-cell movement ability of each virus is indicated (as positive or negative) to the right of each construct diagram. The in vitro transcription reaction was performed with T7 RNA polymerase (Invitrogen) and mGpppG (New England Biolabs) as a cap analog, as described previously (12).
FIG. 2. Analysis of UR-183K and relevant recombinants. (A) Assay of cell-to-cell movement ability of viruses on Xanthi-nc leaves, a local lesion host (at 4 dpi). The arrows indicate local lesions caused by revertants of UR-hel (see text). (B and C) Comparison of genomic RNA accumulation in protoplasts at 18 hpi by Northern blotting. rRNAs were detected by ethidium bromide as loading controls. BY-2 protoplast isolation and inoculation of viral RNA were performed as described previously (24). Total RNA was extracted from protoplasts at 18 hpi with an RNeasy plant minikit (QIAGEN). Each 0.2-μg RNA sample was subjected to formaldehyde-agarose gel electrophoresis and blotted onto Hybond-N+ membranes (Amersham). For RNA detection, digoxigenin-labeled hybridization probes encompassing the sequence of nt 5713 to 6191 (11) to detect genomic RNA were generated with a DIG-RNA labeling kit (Roche). Bands were detected with antidigoxigenin-alkaline phosphatase-conjugated Fab fragments (Roche) and a BCIP-NBT (5-bromo-4-chloro-3-indolyl-phosphate–nitroblue tetrazolium) phosphatase substrate system (Kirkegaard & Perry Laboratories).
TMV-R might complement the defect in cell-to-cell movement of UR-hel in the same genome.

To reveal the genomic element for recovering the cell-to-cell movement of UR-hel, we constructed various chimeric viruses of UR-183K and UR-hel (Fig. 1B): UR-hel/M, UR-hel/V, and UR-hel/P, having an R-type methyltransferase domain, an R-type nonconserved region, and an R-type RNA polymerase domain, respectively, together with the R-type RNA helicase domain in each genome. cDNA clones of UR-hel/M, UR-hel/V, and UR-hel/P were constructed by replacing the Smal (nt 258)-MluI (nt 811), MluI-NsiI (nt 2354), and BamHI-HindIII (nt 5080) fragments of UR-hel with the corresponding fragments from UR-183K (Fig. 1B).

We compared the replication levels of these chimeric viruses in BY-2 protoplasts. All the viruses showed similar replication levels in the protoplasts on the basis of genomic RNA accumulation at 18 h postinfection (hpi) (Fig. 2C). Next, we checked the infectivity of each chimeric virus on Xanthi-nc leaves. Similar to UR-hel (data not shown), UR-hel/M and UR-hel/P caused no local lesions on Xanthi-nc leaves. Only UR-hel/V caused local lesions at 4 dpi, and these were similar in number and size to those caused by TMV-U1 (Fig. 2A, lower right). UR-hel/V has an R-type nonconserved region and the RNA helicase domain. This result suggested that the nonconserved region is also involved in the cell-to-cell movement function of the replicate together with the RNA helicase domain.

Analysis of revertants of UR-hel. We sometimes found that local lesions appeared sporadically after UR-hel was inoculated on Xanthi-nc leaves (Fig. 2A, arrows on an upper left leaf). Some of the local lesions were isolated from the leaves, and the saps were extracted separately. Fresh Xanthi-nc leaves were inoculated with each sap, and all caused as many local lesions as wild-type TMV-U1, indicating that they were revertant viruses.

Revertants were identified by their ability to form local lesions on UR-hel-inoculated leaves. Isolated local lesions were homogenized in 10 mM sodium phosphate buffer (pH 7.0), and each sap was inoculated onto new leaves of the local lesion host. This process was repeated at least three times for each revertant. To propagate the virus for further analysis, homogenized local lesions were inoculated onto Nicotiana tabacum cv. Samsun-nn, a systemic host plant. After 2 weeks, the infected leaves were harvested, and viruses were purified as described previously (18). To analyze these viruses, we isolated and purified 10 revertants. Each independently isolated revertant was assigned a number from 1 to 10.

To map and determine the genetic changes responsible for the reversion, we avoided whole-genome sequencing. Instead, we selected a strategy to characterize and focus on the RNA helicase domain and nonconserved region. Reverse transcription was performed with each purified revertant RNA as a template and oligonucleotides 6172 to 6191 (TCAAGTTGCG AGGACCAGGG) as a reverse primer. Then, DNA fragments spanning each nonconserved region and RNA helicase domain of the replicate were amplified by high-fidelity PCR with two primers, a forward primer spanning nucleotides 474 to 491 (ATGCCGACGAAGGCCGAG) and a reverse primer spanning nucleotides 3341 to 3360 (GATCTCTAATGATAC TAACT). Each PCR fragment was digested with MluI and BamHI and then ligated to a larger fragment of pUR-hel, which had been compatibly digested with MluI and BamHI (Fig. 1). If the recombinant caused local lesions on Xanthi-nc tobacco plants, the MluI-BamHI region of the template plasmids of each recombinant were subjected to nucleotide sequence analysis with an ABI 310 sequence analyzer.

All the recombinants that caused local lesions exchanged only the genomic elements covering the nonconserved region and RNA helicase domain (Fig. 3). These results reinforced our previous hypotheses that the nonconserved region and RNA helicase domain of replicase are deeply involved in the cell-to-cell movement. The local lesions caused by all the revertants were 90 to 100% of the size of those caused by TMV-U1. These results might be explained by our procedures for isolating and purifying the revertants. We performed three rounds of single-lesion isolation on Xanthi-nc leaves and then selected phenotypically stable, nearly wild-type revertants which had regained the capacity for movement at a level similar to that of the wild type.

The sequences of these segments revealed that each revertant carried different and various mutations in the nonconserved region and/or RNA helicase domain, with at least one amino acid substitution (Fig. 3). These results strongly suggested that the replicate coding region is involved in the cell-to-cell movement as a protein product and not as a cis element of the genomic segment.

It is interesting that we observed common mutations located at nt 3024 in both revertants 4 and 8. Furthermore, this nucleotide position coincided with 1 of the 12 missense substitutions between TMV-U1 (Met) and UR-hel (Val). This location could be very important, although not exclusively responsible, for the functioning of tobamovirus replicase in cell-to-cell movement.

The interaction between the RNA helicase domain and nonconserved region is essential for the function of replicase. Revertants 1, 3, 5, 6, 9, and 10 (Fig. 3) acquired responsible substitution(s) only in the nonconserved region but not in the RNA helicase domain. This suggests that the nonconserved region interacts with the RNA helicase domain by a certain mechanism and that this interaction in replicase may be important for cell-to-cell movement. Watanabe et al. showed that the TMV 126-kDa and read-through 183-kDa replicase proteins interact with each other (23). Goregaoker et al. showed that the RNA helicase domain interacts with the nonconserved region by using a yeast two-hybrid system (8) and electron microscopy (7). Most mutations which abolished the two-hybrid interaction prevented viral replication. However, the one mutation which abolished the two-hybrid interaction but did not prevent viral replication lost infectivity in planta (8). Thus, it is conceivable that the interaction between the RNA helicase domain and nonconserved region is involved not only in replication but also in cell-to-cell movement. We are planning to investigate the interaction between the nonconserved region and RNA helicase domain and its relationship with the cell-to-cell movement function.

Tobamovirus replicase and MP work together for cell-to-cell movement. It has been well established that MP is essential for cell-to-cell movement (5, 17). No evidence of a direct interaction between replicase and MP has been reported to date.
However, we suspect that MP and replicase have a relationship for the execution of intercellular movement of progeny viral RNA by an unknown mechanism. Some researchers reported that MP and replicase colocalized in protoplasts (10, 16). In this study, we constructed a virus, UR-hel/MP, which included the R-type RNA helicase domain and R-type MP in genome that was otherwise identical to that of TMV-U1. The R-type MP was functional because the virus could not move from cell to cell, whereas UR-MP could (Fig. 2B). Moreover, no revertants were found that had a responsible mutation(s) mapped in MP during the analysis of revertants isolated from UR-hel (Fig. 3). These results indicate that replicase and MP work together for cell-to-cell movement but do not interact directly with each other. It is possible that replicase and MP associate with viral RNA and/or with a host factor(s) and collaborate indirectly. We are now characterizing the localizations of replicase and MP of UR-hel compared with those of TMV-U1 by immunostaining with antibodies or green fluorescent protein tagging to test such possibilities.

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