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Identification and study of tobacco mosaic virus movement function by complementation tests

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The phenomenon of *trans*-complementation of cell-to-cell movement between plant positive-strand RNA viruses is discussed with an emphasis on tobamoviruses. Attention is focused on complementation between tobamoviruses (coding for a single movement protein, MP) and two groups of viruses that contain the triple block of MP genes and require four (potato virus X) or three (barley stripe mosaic virus) proteins for cell-to-cell movement. The highlights of complementation data obtained by different experimental approaches are given, including (i) double infections with movement-deficient (dependent) and helper viruses; (ii) infections with recombinant viral genomes bearing a heterologous MP gene; (iii) complementation of a movement-deficient virus in transgenic plants expressing the MP of a helper virus; and (iv) co-bombardment of plant tissues with the cDNAs of a movement-dependent virus genome and the MP gene of a helper virus.

Keywords: plant virus complementation; cell-to-cell movement; hybrid viruses

1. COMPLEMENTATION OF TMV *TS*-MUTANTS. CELL-TO-CELL MOVEMENT IS A GENETICALLY ACTIVE PROCESS

Complementation experiments with temperature-sensitive (*ts*) mutants of tobacco mosaic virus (TMV) were initiated at Moscow University in 1968 using *ts* strains obtained from Professor H. G. Wittmann (Schaskolskaya *et al*. 1968). It was shown that the coat protein (CP) *ts*-mutants could be complemented by the temperature-resistant helper strains of TMV and some other tobamoviruses (Atabekov *et al*. 1970, 1972; Atabekova *et al*. 1975; Taliansky *et al*. 1977). Shortly thereafter, the laboratories of S. Sarkar (Sarkar 1969) and B. Kassanis (Kassanis & Bastow 1971*a*,*b*; Kassanis & Conti 1971) also engaged in this line of research. These experiments indicated that the specificity of viral RNA–CP interactions *in vivo* was rather high.

In analysing the Tübingen collection of TMV mutants in 1966, H. Jockusch (a student of Professor H. G. Wittmann at that time) found that the mutant Ni2519 is *ts* in cell-to-cell movement (Jockusch 1966). Another TMV mutant, Lsl, had the same phenotype (Nishiguchi *et al*. 1978). However, both mutants accumulated normally in protoplasts at non-permissive temperatures (Bosch & Jockusch 1972; Nishiguchi *et al*. 1978). The authors suggested that the TMV genome contains a gene controlling the cell-to-cell movement of the virus. In 1979, we proposed (i) that the non-structural 30K protein encoded by TMV is the most probable candidate for the role of a transport protein mediating the transfer of the infective viral genome from cell to cell, and (ii) that the cell-to-cell spread of a plant virus infection is a genetically active process (Atabekov & Morozov 1979). In agreement with this, we found that the cell-to-cell movement of the *ts*-mutants Lsl and Ni2519 could be complemented by TMV U1 in double infections at the non-permissive temperature. These pilot complementation experiments supported the hypothesis that the TMV genome codes for a distinct protein responsible for the transport function. These results were reported in a series of papers (Taliansky *et al*. 1982*a*–*c*). We also proposed that a transport protein can mediate the spread of virus infection from cell to cell by 'opening' plasmodesmata (PD) (Taliansky *et al*. 1982*b*). At the same time Leonard & Zaitlin (1982) and Zimmern & Hunter (1983) found that mutations in the TMV 30K protein are associated with the *ts*-movement of Lsl and Ni2519. Taken together, these results strongly indicated that the 30K protein served as a TMV transport protein (i.e. a movement protein, MP), and this function was later directly proved by the groups of R. N. Beachy and Y. Okada (Deom *et al*. 1987; Meshi *et al*. 1987).

2. COMPLEMENTATION BETWEEN UNRELATED VIRUSES FOR CELL-TO-CELL MOVEMENT

Whereas the complementation of cell-to-cell movement between the TMV mutants and between different tobamoviruses was not a surprise, it was rather unexpected that the unrelated virus, potato virus X (PVX),

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was also able to complement the movement of Lsl in *N. glutinosa* (Taliansky *et al*. 1982*b*,*c*). In succeeding years it was well documented by different authors, including our group, that complementation for cell-to-cell movement is non-specific. In many cases, RNA viruses belonging to disparate taxonomic groups and having different genome structures, morphologies, and host ranges may complement each other in mediating the transport function (TF). Apparently, the phenomenon of *trans*-complementation of TF has a direct bearing on the control of virus host range. In many instances, a virus can replicate in the inoculated cells or in isolated protoplasts of a 'resistant' plant to cause a 'subliminal symptomless infection'. It was supposed that the establishment of a subliminal infection in the 'resistant' plants might be due to incompetence of the MP(s) of a given virus to function in this plant species. However, a plant that is 'resistant' to one virus, typically can be infected by other viruses whose MP(s) are functionally active in that species. Under the conditions of a mixed infection, such a helper virus may provide the TF for the movement-deficient virus to complement its transport through the 'resistant' plant. In other words, a non-host plant in which resistance to a virus is due to blockage of the TF can be infected by this virus via complementation by an unrelated helper virus (for a review, see Atabekov & Taliansky 1990).

It is well known that the principles of TF coding and expression are different in different viruses. For example, TMV encodes a single MP with distinct functional domains for RNA binding, plasmodesmata targeting, and increasing the plasmodesmatal size exclusion limit (SEL), whereas some viruses require three to four individual proteins to mediate the TF. In the following sections, we consider cell-to-cell movement complementation between tobamoviruses and two viruses that contain so-called 'triple gene block' (TGB) TF, namely a potexvirus (PVX) and a hordeivirus (barley stripe mosaic virus, BSMV). At least four different experimental approaches have been used to analyse this and other such complementations, namely (i) double infection with a TF-deficient virus and a helper virus (Taliansky *et al*. 1982*a*–*c*); (ii) complementation of the TF-deficient virus in transgenic plants expressing a functional MP (Deom *et al*. 1987; Holt & Beachy 1991); (iii) expression of an MP gene transferred to a foreign virus genome in order to restore its TF (Nejidat *et al*. 1991; Hilf & Dawson 1993); and (iv) co-bombardment of plant tissues with infectious cDNA of a dependent virus genome and individual MP gene cDNAs (Morozov *et al*. 1997).

3. INTERCHANGEABILITY BETWEEN THE TOBAMOVIRUS 30K MP AND PVX TGB AND/OR CP GENES

(a) *Mixed infections*

In contrast to TMV, cell-to-cell movement of PVX is mediated by the three TGB-coded proteins (the 25K protein, having an NTPase activity, and the 12K and 8K hydrophobic proteins). No sequence similarities were found between the TMV 30K and the three PVX MPs. In addition, PVX requires CP for cell-to-cell movement (Chapman *et al*. 1992). The exact roles of the PVX MPs are not completely understood. The 25K MP plays a role in increasing the plasmodesmatal SEL (Angell *et al*. 1996), but it hardly functions as an RNA-binding MP. Indeed, the level of RNA-binding activity of the 25K MP under physiological conditions, if there is any, is extremely low (Kalinina *et al*. 1996; Karpova *et al*. 1997). The CP of PVX does not modify the SEL of PD, but, for intercellular movement of PVX virions through PD, it performs an essential function as a specific PVX RNA-binding protein (Oparka *et al*. 1996; Santa Cruz *et al*. 1998). In contrast to PVX, TMV does not require CP for cell-to-cell translocation, and the TMV 30K MP is known to be an efficient sequence-independent RNA-binding protein. It is generally accepted that TMV moves from cell to cell in the form of a ribonucleoprotein complex containing genomic RNA, 30K MP and possibly additional component(s) (for a review, see Ghoshroy *et al*. 1997).

Table 1. *Complementation by sunn hemp mosaic tobamovirus (SHMV) of PVX mutants accumulation in inoculated leaves of* V. unguiculata

(Note: the leaves were pre-inoculated with the helper SHMV (20 μg ml^{-1}) and super-inoculated two days later with the 35S promoter-based cDNA constructs (600–800 μg ml^{-1}) of a dependent virus. Accumulation of dependent virus was detected by DAS-ELISA.)

dependent virus	helper virus	accumulation of PVX (ng g^{-1} tissue)[a]
wt PVX	—	300
wt PVX	SHMV	2, 475
25K$^-$PVX	—	282
25K$^-$PVX	SHMV	5, 725
12K$^-$PVX	—	356
12K$^-$PVX	SHMV	3, 865
8K$^-$PVX	—	186
8K$^-$PVX	SHMV	2, 920
TGB$^-$PVX	—	94
TGB$^-$PVX	SHMV	4, 462
CP$^-$PVX[b]	—	±
CP$^-$PVX	SHMV	+++
CP$^-$25K$^-$PVX[b]	—	±
CP$^-$25K$^-$PVX	SHMV	+++

[a] Mean values for four to six plants inoculated, except for the CP$^-$ frameshift mutants.
[b] Accumulation of RNA of frameshift PVX (CP$^-$) mutants was detected by slot-blot hybridization (see figure 1).

The results of complementation experiments with a helper tobamovirus (sunn hemp mosaic virus, SHMV) and PVX mutants in *Vigna unguiculata* are presented in table 1. These data clearly demonstrate that the cell-to-cell movement of PVX can be complemented efficiently by the tobamovirus in cowpea (a non-host for PVX), suggesting that the 30K MP can substitute functionally for the PVX MPs and PVX CP (figure 1). It is reasonable to propose that, in the absence of PVX CP, the tobamovirus 30K acts as an RNA-binding protein to give a transportable form of PVX, and that PVX then uses, partially or even totally, the movement system of the tobamovirus. In other words, there is at least a 'unidirectional' complementation of cell-to-cell movement in plants doubly infected with PVX and TMV. Remarkably,

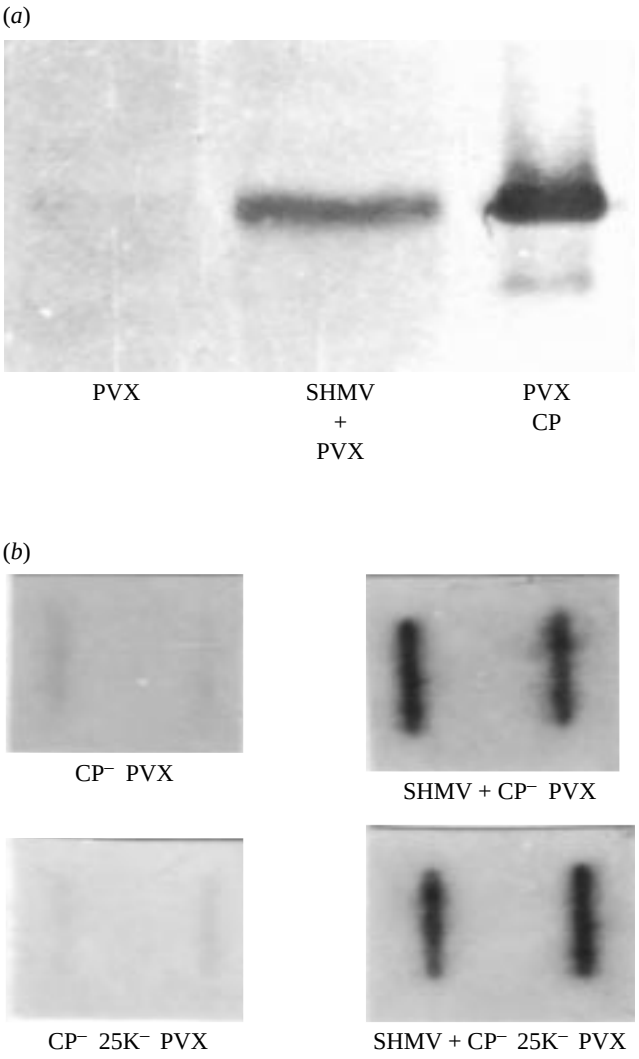

Figure 1 (*a*) Western blot analysis of proteins extracted from *V. unguiculata* leaves with PVX CP-specific antiserum. Leaves inoculated with PVX alone or with SHMV plus PVX were analysed eight days post-inoculation. Right track contains PVX CP as a marker. (*b*) Slot-blot hybridization analysis, with PVX replicase gene-specific probes, of total RNA isolated from *V. unguiculata*. The leaves were inoculated with the movement-defective viruses CP⁻PVX or CP⁻25K⁻PVX, each with or without SHMV.

in a series of experiments we failed to show complementation of cell-to-cell movement of the movement-deficient PVX mutants (25K⁻PVX and TGB⁻PVX) inoculated onto tobacco plants expressing the TMV 30K MP (J. G. Atabekov, unpublished data). This is in agreement with the results of Ares *et al.* (1998).

The situation becomes more complicated when the reverse experiment on complementation of TMV by PVX is performed. If this were to occur, cell-to-cell movement complementation would be of a 'bidirectional' type, i.e. the MPs of either virus would mediate transport of the heterologous virus. However, in contrast to that of TMV MP, the RNA-binding function performed by PVX CP is RNA-specific. It has been found (our unpublished data; Goodman & Ross 1974) that TMV and PVX particles are assembled independently in mixedly inoculated cells. No phenotypic mixing or genome masking was detected; therefore, it is unlikely that PVX CP could interact with TMV RNA in the experiments on TMV movement complementation. It is reasonable to propose that the mutations in TMV 30K, which result in the loss of its RNA-binding ability, should not be complemented by PVX, because PVX is unable to provide the RNA-binding MP to movement-deficient TMV. Consequently, the 30K MP of a movement-deficient TMV would need to retain its RNA-binding ability to ensure complementation of the movement by PVX helper virus. This idea is consistent with our observations that movement of *ts* mutant TMV Ls1 (where Ser replaces Pro at position 153 of the MP) could be complemented by wild-type PVX (Taliansky *et al.* 1982*b*,*c*). It is improbable that this point mutation would have abolished the RNA-binding ability of Ls1 MP, because an MP deletion mutant lacking amino acids 130–185 retained its RNA-binding ability (Karpova *et al.* 1997). No complementation occurred when a TMV mutant lacking a major part of the MP gene (deletion of nucleotides 4923–5402) was used in a double infection with PVX (Ares *et al.* 1998). This result was presumably due to an absence of RNA-binding by the mutant TMV MP that could not be substituted for any PVX-coded products. We presume that when movement-deficient TMV is complemented by the PVX helper, the mutant TMV still provides an RNA-binding MP, whereas the PVX provides some TGB-coded proteins needed to mediate cell-to-cell movement of the TMV RNA.

(b) *Co-bombardment with cloned MP genes*

Recently, we used co-bombardment of plant tissues with CaMV 35S promoter-driven cDNAs of a movement-deficient virus genome and of the MP gene of a helper virus as a fast and simple method to test for *trans*-complementation (Morozov *et al.* 1997). This method allows the function of an MP and putative MP genes to be studied in the absence of expression of other genes of a helper virus. It has been shown that the cell-to-cell movement of a movement-deficient, GUS gene-tagged PVX (25K⁻PVX.GUS) was complemented upon co-bombardment with a cDNA expressing the 30K MP of TMV. Analogous results were obtained with the 30K MP genes of tomato mosaic tobamovirus (ToMV) and a crucifer-infecting tobamovirus (crTMV), with a dianthovirus MP gene and with the 25K MP gene of PVX (Morozov *et al.* 1997). When movement was monitored by histochemical analysis of GUS, large blue foci were detected in *Nicotiana benthamiana* leaves bombarded by parental PVX.GUS, whereas the infection foci produced by 25K⁻PVX.GUS were confined to individual cells; the mutant PVX was not able to move. However, when the PVX mutant cDNA was co-bombarded with the cDNAs expressing the wt 25K protein of PVX, the 30K protein of ToMV or crTMV, or the MP of red clover necrotic mosaic dianthovirus (RCNMV), large infection foci were produced, showing that cell-to-cell movement of the 25K⁻PVX mutant was readily *trans*-complemented (Morozov *et al.* 1997). Similar results were obtained when a double mutant (25K⁻CP⁻PVX.GUS) was complemented by the 30K MP gene of ToMV in co-bombardment experiments (S. Yu. Morozov, unpublished data). These data indicate that 30K TMV MP can substitute

Table 2. *Reciprocal complementation of cell-to-cell movement by TMV–BSMV and PVX–BSMV in mixed infections*

helper virus	dependent virus	non-host plant in which dependent virus moves	reference
TMV	BSMV	*Nicotiana tabacum*	Malyshenko *et al.* (1989)
BSMV	TMV	*Hordeum vulgare*	Hamilton & Dodds (1970)
BSMV	Ls1 TMV	*Triticum aestivum*	Taliansky *et al.* (1982*c*)
PVX	BSMV	*Nicotiana clevelandii*	Prody & Jackson (1993)
BSMV	PVX	*Hordeum vulgare*	Malyshenko *et al.* (1989)

functionally for the PVX MPs in the absence of expression of other TMV genes.

An additional advantage of this method is its potential application in identifying putative movement-protein genes. Thus, it has been shown recently that the 65K gene of beet yellows closterovirus (BYV) mediates limited transport of the movement-defective 25K^{-}PVX.GUS upon co-bombardment (Agranovsky *et al.* 1998).

4. INTERCHANGEABILITY BETWEEN TMV 30K AND BSMV TGB GENES

(a) *Mixed infections*

Table 2 shows that TMV–BSMV and PVX–BSMV complement each other reciprocally. This means that at least part of the TMV 30K MP functions can be substituted by BSMV TGB-coded MPs. In contrast to PVX, BSMV does not need its CP for cell-to-cell or systemic movement (Petty & Jackson 1990). It could be proposed that when TMV is complemented by BSMV, the hordeivirus may provide its 58K MP as an RNA-binding protein. It is probable that the 58K MP of BSMV is also involved in BSMV movement complementation upon mixed infection with PVX since the only RNA-binding protein coded by PVX is the CP, a protein that could hardly be expected to bind to BSMV RNAs because of its strict specificity for PVX RNA. Therefore, the complementations of cell-to-cell movement between PVX and BSMV may represent a 'bidirectional' type of interaction.

(b) *Biological properties of a BSMV–TMV 30K MP recombinant*

To study the compatibility between the BSMV TGB-encoded MPs and the unrelated TMV 30K MP, the TGB in RNAβ of BSMV ND18 was replaced by the TMV 30K MP gene. The ability of the BSMV–TMV hybrid to infect host and non-host plants of the parental viruses was assayed by inoculation with infectious T7 transcripts of RNAα, RNAγ, and the chimeric RNAβ (Solovyev *et al.* 1996). Table 3 shows that the BSMV–TMV 30K MP hybrid was able to infect the inoculated leaves of *N. benthamiana* and *C. amaranticolor*, the common hosts for the parental viruses. However, the hybrid failed to infect either tobacco or barley, the selective hosts. It is noteworthy that the size and morphology of local lesions produced by hybrid virus on *C. amaranticolor* were influenced by the foreign 30K MP gene (Solovyev *et al.* 1996). Thus, the TMV 30K MP was able to substitute functionally for BSMV TGB-coded MPs to promote the cell-to-cell (but not systemic) movement of the hybrid. Apparently, some kind of host-specific adaptation of MP and/or the presence of some other genomic elements of TMV (e.g. replicase) are needed for the hybrid virus to move in selective hosts. It is remarkable that our attempts to complement cell-to-cell movement of BSMV in transgenic tobacco plants expressing the TMV 30K MP were unsuccessful (data not presented).

Table 3. *Cell-to-cell movement of parents and a recombinant BSMV containing the TMV 30K MP gene*

plant inoculated	BSMV–TMV 30K MP[a]	BSMVND18	TMV
Nicotiana benthamiana	+	+	+
Chenopodium amaranticolor	+	+	+
N. tabacum	−	−	+
N. clevelandii	−	−	+
Hordeum vulgare	−	+	−

[a] Transcripts of BSMV RNAα and RNAγ, together with the hybrid RNAβ (BSMV–TMV 30K MP) transcript were used for inoculation (Solovyev *et al.* 1996); (+) and (−) denote the presence and absence of cell-to-cell movement, respectively.

5. THE NON-MP ELEMENTS IN CELL-TO-CELL MOVEMENT

Various hybrid genomes have been constructed in several laboratories using tobamoviruses as parental viruses. Typically, the parental tobamoviruses differed in their ability to infect certain plant species systemically, whereas both partners were able to move from cell to cell in the inoculated leaf, although not necessarily with the same efficiency (e.g. TMV and *Odontoglossum* ringspot virus, ORSV, in tobacco leaves; Hilf & Dawson 1993). Important results from the groups of W. O. Dawson and R. N. Beachy based on this type of experiment allowed them to conclude that (i) CP and MP of tobamoviruses may interact specifically with host factors to mediate long-distance movement (Hilf & Dawson 1993; Fenczik *et al.* 1995); and (ii) ability of a tobamovirus to infect a plant systemically may be a function of viral non-MP elements (Nejidat *et al.* 1991; Deom *et al.* 1994). Similar results were obtained for BSMV recombinant viruses in which the TGB was replaced by foreign MP genes (Solovyev *et al.* 1996). Table 4 gives some examples of recombinants that were able to infect plant species that were 'resistant' to the parental virus donating the MP gene.

It should be noted that the effects of double infection on complementation may depend on numerous factors, e.g. the effectiveness of replication, possible interference between the viruses, concentration of dependent and

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Table 4. *Role of non-MP elements of recombinant viruses in host range control*

(Note: IL and S correspond to inoculated leaf and non-inoculated tip leaves, respectively; (+) and (−) denote the presence or absence of virus accumulation.)

inoculum	*Lycopersicon esculentum* IL	S	*Nicotiana tabacum* IL	S	*Nicotiana benthamiana* IL	S	*Hordeum vulgare* IL	S	reference
TMV	+	+							
TMGMV(U2)	−	−							
TMV–TMGMV 30K MP	+	+							Nejidat *et al*. (1991)
TMV			+	+					
RCNMV			+	−					
TMV–RCNMV 35K MP			+	+					Giesman-Cookmeyer *et al*. (1995)
BSMV					+	+	+	+	
PSLV					−	−	−	−	
BSMV–PSLV TGB[a]					+	+	+	+	
BSMV–RCNMV 35K MP[b]							+	−	Solovyev *et al*. (unpublished data) Solovyev *et al*. (1996)

[a,b]Inoculum contained transcripts of BSMV ND18 RNAα and RNAγ, and a recombinant RNAβ in which BSMV TGB was replaced by that of *Poa* semilatent hordeivirus (PSLV)[a] or by RCNMV 35K MP gene[b].

Table 5. *Complementation of cell-to-cell movement between tobamoviruses in non-host plants by mixed infection*

dependent virus	helper virus	plant resistant to dependent virus	cell-to-cell movement of dependent virus[a]
CGMMV	—	*Nicotiana tabacum*	−
CGMMV	TMV	*N. tabacum*	+
TMV U1	—	*Cucumis sativus*	−
TMV U1	CGMMV	*C. sativus*	+
CGMMV	—	*N. tabacum*	−
CGMMV	SHMV	*N. tabacum*	+
SHMV	—	*Lycopersicon esculentum*	−
SHMV	TMV	*L. esculentum*	+
tobamovirus from orchids	—	*C. sativus*	−
tobamovirus from orchids	CGMMV	*C. sativus*	+
CGMMV	—		
CGMMV	tobamovirus from orchids	*C. bowringiana*	−
		C. bowringiana	+

[a] (+) and (−) denote the accumulation or the lack of accumulation, respectively, of the dependent virus as determined by DAS-ELISA (Malyshenko *et al*. 1989).

helper viruses in the inoculum, the interval between pre-inoculation and super-inoculation, and the interval between super-inoculation and determination of virus content. Consequently, the conditions of tests for complementation should be selected carefully to obtain a reliable level of accumulation of the dependent virus. When this is done, movement of different tobamoviruses within inoculated leaves of non-host plants can be mediated by various helper tobamoviruses (table 5). Thus, cucumber green mottle mosaic virus (CGMMV) could be complemented by TMV in tobacco, and TMV could be complemented by CGMMV in cucumber plants. Similarly, TMV and CGMMV complemented the cell-to-cell movement of SHMV in its non-host plants, tomato and cucumber, respectively.

Complementation for the cell-to-cell movement between unrelated viruses usually proceeds more readily under conditions of mixed infections than by the other experimental approaches mentioned above. For example, when a hybrid virus carrying the MP gene of a foreign virus is used, only common hosts for both parental viruses, but not their selective hosts, can be infected (e.g. see Mise *et al*. 1993; Solovyev *et al*. 1996). It has also been observed that while an unrelated helper virus may complement movement in double infections, the complementation can fail in transgenic plants expressing the MP of the helper virus. As mentioned above, complementation of cell-to-cell movement of BSMV or PVX movement-deficient mutants was unsuccessful in tobacco plants expressing the 30K MP of TMV, whereas the complementation was efficient in mixedly infected leaves. Another example of unsuccessful complementation of cell-to-cell movement of an unrelated virus in transgenic plants expressing the TMV 30K MP is described by

Taliansky *et al*. (1992). Despite the significant differences between the transport mechanisms of tobamo- and comoviruses, TMV efficiently complemented the cell-to-cell movement of red clover mottle comovirus (RCMV) in doubly infected tobacco plants normally resistant to RCMV (Malyshenko *et al*. 1988, 1989). However, RCMV was not complemented in tobacco plants expressing the TMV 30K MP, unless they were co-inoculated with movement-deficient TMV. It was suggested that the presence of TMV 30K MP was essential, but insufficient, for complementation and that some additional TMV-specific elements or TMV-induced host proteins were needed (Taliansky *et al*. 1992). Apparently, MPs are not the only factors involved in virus movement and the control of host range.

It is known that replication-associated proteins (RPs) of different viruses may be involved, either directly or indirectly, in virus movement (Traynor *et al*. 1991; Gal-On *et al*. 1994; Weiland & Edwards 1996; Deom *et al*. 1997). The role of RPs in movement may be independent of their functions in RNA replication. One can speculate that different host factors are needed by RPs in RNA replication and in movement. Therefore, the blockage of virus movement in a non-host plant may be due to the lack of cellular factors needed when replicative proteins act as MPs, despite the fact that RPs can replicate viral RNA in primary inoculated cells of this non-host plant. This phenomenon may be important for complementation to occur between distantly related or unrelated viruses.

Understanding the molecular mechanisms of the process of movement complementation between viruses and, in particular, between unrelated viruses is therefore complicated by the involvement of many players in the game. Apparently, a number of virus-coded proteins (MPs, CPs, replicases) and putative host factors, presumably specific for each of these proteins, may contribute to cell-to-cell movement and long-distance transport. Therefore, *trans*-complementation of the TF between unrelated viruses may represent a distinct type of complementation that depends not only on the functional interchangeability between the MPs of the partner viruses, but also on compatibility between the helper virus-coded MP(s) and specific host factors (putative cytoplasmic and plasmodesmatal receptors).

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