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# The mechanism(s) of coat protein-mediated resistance against tobacco mosaic virus

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*The resistance of transgenic plants that express genes encoding viral coat proteins to infection by the viruses from which the genes are derived was termed coat protein-mediated resistance (CP-MR) and has been demonstrated for a variety of virus/host combinations. The mechanism of CP-MR is perhaps best understood in the tobacco/TMV system. CP-MR against TMV requires accumulation of CP and does not seem to involve the induction of plant defense mechanisms. The resistance appears to be mainly based on the inhibition of virion disassembly in transgenic cells although there is evidence that a later step of infection is also affected. CP-MR of tobacco to TMV shares some features with classical cross-protection and with CP-MR in some, but not all other host/virus combinations.*

**Key words:** tobacco / tobacco mosaic virus / coat protein-mediated resistance

THE FIRST EXAMPLE of genetically engineered disease resistance in transgenic plants was the resistance of transgenic tobacco plants that accumulated the tobacco mosaic virus (TMV) coat protein to TMV infection.[1] The successful application of this strategy to a variety of host/virus combinations has been recently reviewed;[2] selected examples are discussed elsewhere in this issue. Coat protein-mediated resistance (CP-MR) was defined as resistance caused by the expression of a gene encoding virus coat protein (CP) in transgenic plants. Such plants are resistant to infection and/or disease development by the virus from which the CP gene was derived and by closely related viruses.[3] The molecular and cellular mechanisms of CP-MR have been most thoroughly investigated in the tobacco/TMV system. These studies have been facilitated by the extensive knowledge of the process of infection of tobacco by TMV, the structure of the virion and the genome organization and expression of the viral genome. Here we describe the main features of CP-MR of tobacco to TMV and discuss the evidence for interference with different steps in TMV infection.

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## The infection of tobacco with TMV

TMV, the type member of the tobamovirus group, is a rigid rod that consists of a positive sense, single stranded RNA genome encapsidated in 2130 molecules of the coat protein (CP). The genome encodes at least four proteins: the replicase; the 30 kDa 'movement protein' (MP) whose function is required for cell-to-cell spread;[4] the 17 kDa CP; and a 54 kDa protein of unknown function. The replicase is translated directly from the genomic RNA. During the replication cycle subgenomic mRNAs are synthesized that encode the other three viral proteins. MP and CP accumulate in infected cells but the 54 kDa protein has not been detected.

The infection of tobacco with TMV proceeds in three steps: the initial infection of isolated cells and replication therein; local movement from cell to cell through the plasmodesmata; and systemic spread through the sieve elements. Figure 1 summarizes the different events leading to systemic infection. Most of the events that occur in the initially infected cells are well characterized. Local and systemic spread of infection, however, are not yet completely understood. The MP probably facilitates cell-to-cell movement of the infection in two ways: it modifies the size exclusion limit of the plasmodesmata[5] and changes the secondary structure of the viral RNA by binding to it.[6] Rapid spread of TMV throughout the infected plant occurs through the phloem, and virions have been observed in sieve elements of diseased plants.[7] TMV mutants that cannot assemble to form virions lack the ability to rapidly move systemically.[8] These results suggest that TMV moves in the form of virions. However, it is not known how virus enters and exits the sieve elements and if assembly to virions occurs within the sieve elements or in the adjoining cells.

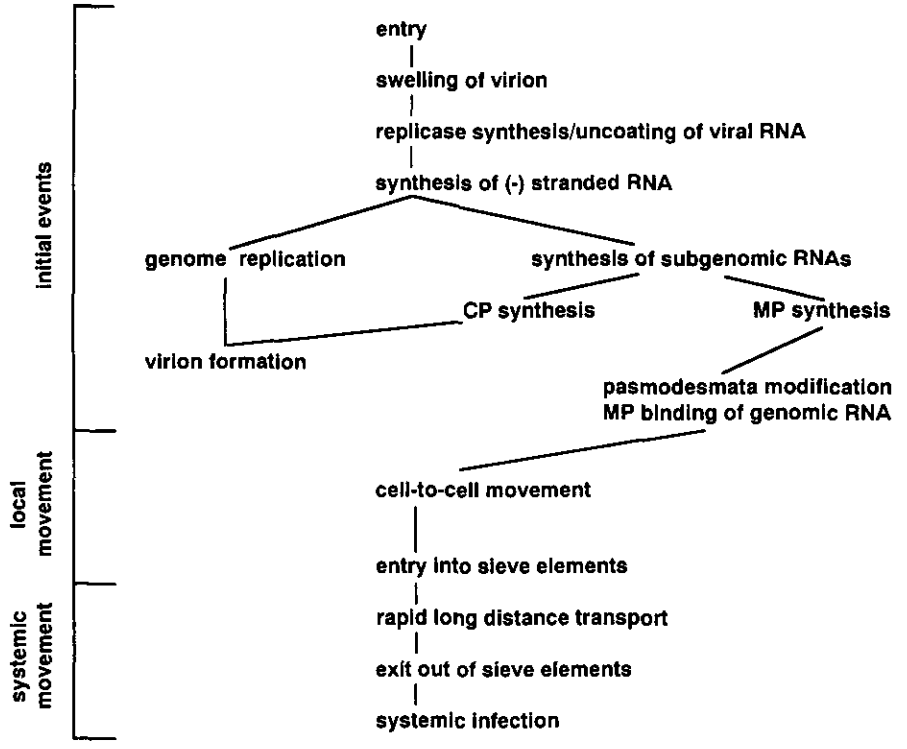


**Figure 1.** Proposed events in the replication and spread of TMV in tobacco.

## Manifestation of CP-MR in tobacco

Transgenic tobacco plants that accumulate high levels of TMV CP (up to 0.2% of total soluble protein) do not develop systemic disease symptoms after inoculation with up to 1 $\mu g\,ml^{-1}$ TMV. In contrast plants that lack CP [i.e. CP(-)] can be infected with as little as 0.001 $\mu g\,ml^{-1}$ TMV. With increasing inoculum concentrations a gradual breakdown of CP-MR occurs and the percentage of plants that accumulate CP [CP(+)] and that develop symptoms increases with the inoculum concentration.[9] Systemic symptom development proceeds at a slower rate in those transgenic plants that become diseased compared to control plants.[1] There is no accumulation of virus in upper leaves of transgenic plants that do not develop symptoms.[10] TMV CP accumulation in transgenic tobacco plants not only inhibits or delays systemic infection but also results in a reduced number of local infection sites (necrotic local lesions) on tobacco plants that carry the N hypersensitivity gene from *Nicotiana glutinosa*.[10] Accumulation of the TMV CP provides resistance to TMV and related members of the tobamovirus group. However, the level of resistance against other tobamoviruses is positively correlated with the degree of amino acid sequence homology of the CP of the challenging viruses with the sequence of TMV CP.[11]

## CP-MR of tobacco to TMV is a direct protein effect

Interference with virus infection in transgenic plants that contain gene constructs encoding viral coat proteins can be due to effects caused by the mRNA or by the protein encoded therein. The effect of accumulating CP gene transcripts in the absence of CP accumulation on resistance was investigated in different ways. Powell *et al* generated transgenic tobacco plants that expressed a gene construct that lacked the ATG initiation codon of the TMV CP.[12] These plants accumulated the gene transcript but not the CP. When these plants were inoculated with very low concentrations of TMV there was no escape from infection or delay of symptom development. The effect of elevated temperatures on the accumulation

of CP in transgenic plants was used by Nejidat and Beachy[13] to demonstrate the requirement of CP accumulation for resistance. When transgenic plants were placed at continuous 35°C, CP mRNA continued to accumulate but the level of CP was reduced. The effect was probably due to poor stability of the CP in the plants at high temperatures. Under these conditions there was a significant reduction of CP-MR. When transgenic plants were returned from elevated temperatures to 22°C, prior to inoculation, CP accumulation and resistance were restored. The data show that CP-MR of tobacco to TMV is due to the effect of the coat protein rather than mRNA.

Tobacco plants carrying the N gene react in a hypersensitive manner to TMV infection, limiting the spread of the virus to necrotic local lesions at the sites of initial infection.[14] Furthermore, fewer and smaller lesions are formed following a second inoculation on leaves distant from the primary inoculated leaf. This is described as 'systemic acquired resistance' (SAR).[15] SAR can be induced by different factors,[16-19] is not limited to TMV[20] and is accompanied by the accumulation of a group of 'pathogenesis-related proteins' (PR proteins).[21] A low level of constitutive expression of the PR 1a protein was detected in some, but not all, transgenic CP( + ) tobacco plant lines that did or did not harbor the N gene.[22] Only in the tobacco cultivar that carries the N gene was there a correlation between PR 1a accumulation and expression of the CP gene in the absence of TMV infection. There was, however, resistance in all CP accumulating plants regardless of whether or not they accumulated PR 1a. It therefore appears likely that expression of the TMV CP gene in transgenic tobacco plants results in direct interference of the CP with TMV infection rather than in triggering plant defense responses that lead to resistance. Furthermore the fact that expression of the TMV CP gene provides resistance to TMV and related tobamoviruses with CPs of similar primary structure but not other viruses[11] further supports this conclusion.

## Evidence for interference with an early step in TMV infection

CP-MR against TMV in tobacco is largely, but not fully, overcome when purified TMV-RNA is used as inoculum. The number of necrotic local lesions formed on inoculated leaves or the rate at which systemic symptoms developed on transgenic tobacco plants that express the TMV CP gene were only slightly reduced compared to non-transgenic plants following inoculation with TMV-RNA.[10] The decreased level of protection to infection by TMV-RNA shows that CP-MR involves the inhibition of an event prior to the release of the viral RNA from the viron. Register and Beachy[23] demonstrated that protoplasts prepared from transgenic plants did not support virus replication after electroporation with TMV. However, electroporation with TMV-RNA or TMV which had been pre-incubated at pH 8.0 led to virus infection. Treatment of TMV at pH 8.0 greatly enhances the translation of TMV *in vitro* but does not cause obvious structural changes.[24,25] It was postulated that treatment at pH 8.0 results in removal of a small number of CP molecules from the 5′ end of the viral RNA exposing it to binding by ribosomes and subsequent co-translational uncoating.[26] Such structures, called 'striposomes' were isolated from infected leaf tissue.[27] Wu *et al*[28] reported that the number of striposomes in transgenic CP( + ) protoplasts after electroporation with TMV was greatly reduced compared to CP( − ) protoplasts. This suggests that the modification of TMV that leads to co-translational virion disassembly is affected in CP accumulating transgenic plants.

Replication was not inhibited in protoplasts prepared from CP( + ) transgenic plants following inoculation with hybrid virus containing genomic RNA of sunn hemp mosaic virus (SHMV) that was *trans*-capsidated *in vitro* by TMV CP.[29] This may reflect either that inhibition of virion disassembly is not sufficient to provide protection to the hybrid virus or that *trans*-capsidation was not complete, leaving a short portion of the 5′ end of viral RNA exposed for binding by ribosomes and subsequent translation.

Further evidence that whole plant resistance is the result of inhibition of an early event of TMV infection was obtained by tissue-specific gene expression studies. Transgenic plants expressing the CP gene from the petunia ribulose bisphosphate carboxylase small subunit (*rbc*S),[30] the bean phenylalanine ammonia lyase (*pal*2) (U. Reimann-Philipp, R.N. Beachy, submitted), and the *Agrobacterium rhizogenes rol*C (U. Reimann-Philipp, R.N. Beachy, unpublished) promoters were tested for resistance to TMV infection. The *rbc*S promoter is active mainly in the mesophyll tissue.[31] Plants that contained *rbc*S/CP construct showed only very limited protection to systemic infection compared to plants containing the CaMV 35S/CP gene construct

although the levels of CP accumulation were similar. However, mesophyll protoplasts prepared from either plant line were equally resistant.[30] Transgenic plants that expressed the CP gene from the *pal*2 promoter accumulated CP mainly in the upper epidermis and stems but not in the mesophyll (U. Reimann-Philipp, R.N. Beachy, submitted). Although these plants contained much less CP than plants harboring the CaMV 35S/CP gene construct they were partially resistant to systemic infection at low inoculum concentrations. CP gene expression from the *pal*2 promoter in tobacco containing the N gene resulted in a reduction of lesion numbers that were formed after TMV inoculation compared to non-transgenic control plants. No inhibition of systemic or local infection was found in plants that were transformed with the *rol*C/CP gene construct. The activity of the *rol*C promoter in transgenic tobacco is restricted to the phloem[32,33] and the tips of the leaf hair (U. Reimann-Philipp, R.N. Beachy, submitted). The results of these studies indicate that CP must accumulate in the initially infected tissue in order to interfere efficiently with TMV infection.

## Inhibition of virion disassembly

The mechanism(s) which leads to loss of CP molecules in the initial stage of TMV infection is not yet known. One possibility is that the chemical environment in the cell alone is sufficient to cause dissociation of CP from the RNA. It is also possible that partial disassembly occurs at specific sites within the cell and that it might involve binding to a receptor. A model by which transgenic CP might inhibit virion disassembly was proposed recently.[34] Results of protoplast assays were taken as an indication that reconstruction of partially disassembled virions treated at pH 8.0 might not contribute to CP-MR.[35] From double transgenic plants that expressed genes encoding the TMV CP and a transcript consisting of the TMV 5′ leader sequence, the chloramphenicol acetyltransferase (CAT) coding sequence, and the TMV origin of assembly sequence pseudovirions consisting of the CAT transcript and TMV CP could not be isolated.[36] Furthermore, there was no repression of CAT activity, as would be expected if the CAT transcript was encapsidated.

If reconstruction of virus particles (as contrasted with interference with disassembly) is not the primary mechanism of CP-MR one might suggest that the CP in transgenic plant cells occupies specific sites of virion modification. In this case the CP that accumulates in the transgenic plant cells would mimic the virion structure. The capability of TMV CP to self-aggregate to different structures depending on the pH conditions[37,38] was used in transient protection assays in which tobacco protoplasts were co-inoculated with TMV and isolated TMV CP in different aggregation states. Virus replication was more efficiently inhibited by large, virion-like CP aggregates than by smaller aggregates or individual CP molecules,[35] which might indicate the involvement of a receptor-like site that predominantly binds virion-like structures. However, it is not known whether the aggregation state of the CP in the inoculum reflected the aggregation state of the CP inside the protoplasts. Although it is not known how the CP that accumulates in transgenic plants is aggregated, low concentrations of virion like particles were found in extracts of these plants.[39]

In a recent study, the infectivity of several TMV mutants on CP(+) and CP(−) plants was tested (W.G. Clark, A. Nejidat, R.N. Beachy, unpublished results). The mutations resulted in changes in the amino acid sequences of either the N terminus, the C terminus, or both termini of the TMV CP sequence to the SHMV CP sequence. Since the C and N termini of the TMV CP are located on the surface of TMV[40] the mutations resulted in virions with a surface structure similar to SHMV. TMV CP accumulating plants are resistant to TMV but not SHMV.[11,41] It was hypothesized that if TMV CP accumulating in transgenic plants occupied receptor-like structures that specifically recognize the TMV virion surface structure resistance should be overcome by the mutant viruses whose virion surface structures resemble that of SHMV. This was not the case, perhaps indicating that virion modification prior to co-translational disassembly does not involve receptor-like structures. Given these results an alternative hypothesis is that the chemical environment in the host cell favors dissociation of CP from the virion. Resistance might be a result of an equilibrium between the dissociation of CP with the virion that stabilizes virion structure and inhibits ribosome binding to the viral RNA. In *in vitro* translation assays the addition of TMV CP reduces the efficiency of translation of pH 8.0 treated virions and, to some extent, the TMV-RNA.[42] This suggests that CP bound to the 5′ end of the viral RNA prevents ribosome binding. The lack of resistance

to infection by pH 8.0 treated TMV[23] suggests that virion stabilization occurs by exchange of low numbers of CP molecules (less than 20) from the virion rather than re-constitution of virions that have undergone the modification that leads to the binding of a ribosome. From these studies we concluded that there is little evidence for the involvement of receptor-like sites in virion modification and that CP-MR is rather a result of virion stabilization.

## Evidence for interference with a later step of TMV infection

There are several indications that TMV CP in transgenic plants not only inhibits virion disassembly but also interferes with a subsequent step in infection. When transgenic CP( + ) plants were inoculated with a high concentration of TMV-RNA virus accumulated in inoculated leaves at the same rate as in control plants. However, systemic symptom development and virus accumulation in non-inoculated leaves were delayed indicating that the CP interfered with the long distance transport of the infection.[43] In the same study, grafting experiments were used to investigate TMV movement through stem sections. Sections derived from CP( + ) plants were grafted between rootstock and apical portions of plants that did not accumulate CP [CP( − )]. After inoculation of the rootstock development of systemic disease symptoms and virus accumulation in leaves of the apical section were monitored. Grafted plants that contained CP( − ) stem sections or CP( + ) stem sections without a leaf developed systemic symptoms at the same rate. However, grafted plants that contained a CP( + ) stem section with a leaf developed or symptoms after a delay did not become systemically infected. The requirement for a leaf on the grafted stem section suggests that it might function as a sink for infectious units and that CP accumulation in the leaf interferes with further spread of the virus. It appears unlikely that there is interference of CP in the sieve elements with virus translocation.

The local spread of TMV in inoculated leaves is delayed in transgenic CP( + ) plants. Virus accumulation within 2.5 mm of the point of inoculation occurred equally in CP( + ) and CP( − ) leaves after inoculation with TMV-RNA. Outside this area TMV accumulated significantly more slowly in leaves of CP( + ) plants.[43] The delay could be due either to a reduced rate of replication or interference with cell-to-cell spread.

Protoplasts prepared from CP( + ) plants showed a reduced percentage of infected protoplasts after infection and accumulated less TMV compared to controls.[23] Infection with a very high concentration of TMV-RNA ($200\,\mu g\,ml^{-1}$) resulted in equal percentages of infected protoplasts. This suggests that CP not only reduces the removal of CP from the virion but also affects another step prior to cell-to-cell spread.

CP-MR is based on different mechanisms in different host/virus combinations. Chimeric genes encoding the CPs of a number of different viruses have been introduced into different host plants often leading to resistance.[2] Although extensive studies on the mechanism(s) of CP-MR have only been carried out in the tobacco/TMV, system it is apparent that there are different mechanisms of CP-MR in other systems. A common feature of CP-MR is that virus accumulation is absent or greatly reduced in transgenic CP( + ) plants indicating that the expression of CP genes results in interference with virus infection rather than with symptom development. In contrast to the TMV/tobacco system CP-MR is not overcome when tobacco or potato plants accumulating the potato virus X CP[44] or tobacco plants accumulating the alfalfa mosaic virus CP[45] are inoculated with viral RNA. This suggests that while virion disassembly may be affected, a later step in virus infection is also affected. Tobacco etch virus (TEV) replication is inhibited in transgenic plants that express a gene encoding an untranslatable TEV CP transcript.[46] Equal levels of resistance to tomato spotted wilt virus (TSWV) were found in tobacco plants that accumulated either wild-type or translation-deficient TSWV CP transcripts.[47] These results show that resistance in transgenic plants harboring selected CP genes can be the result of an RNA effect. Studies on CP-MR in novel host/virus combinations should include plants transformed with genes encoding untranslatable CP transcripts and, if possible, studies with purified viral nucleic acids in order to better understand the mechanisms of disease resistance.

## Similarities between cross-protection and CP-MR

Classical cross-protection[48] and CP-MR of tobacco to TMV have some features in common, suggesting

that similar mechanisms may be involved in the two reactions. Tobacco, tomato and squash plants which are infected with the mild strain S of cucumber mosaic virus (CMV-S) are protected against superinfection by CMV-P. However, when viral CMV-P RNA was used as challenge inoculum the protection was overcome.[49] The same effect was found in TMV infected *Nicotiana sylvestris* plants after challenging with purified RNA or bentonite-treated virions of a necrotizing TMV strain.[50] The bentonite treatment removes CP from TMV. The results indicate that, as in CP-MR to TMV, the primary mechanism of cross protection is inhibition of disassembly. Virus concentration in TMV infected tobacco leaves is much higher in light green than in dark green areas and the susceptibility to superinfection by heterologous TMV strains was greater in the dark green than in light green areas.[51] The correlation between high concentrations of protecting virus and resistance in cross-protection, and of high levels of CP accumulation and resistance in CP-MR to TMV might indicate that in both cases resistance is a direct effect of the presence of high amounts of CP.

TMV can superinfect tobacco that is infected with SHMV. However, TMV-RNA that was transcapsidated with SHMV CP was far less infectious. When either SHMV CP or TMV CP was added to the inoculum, SHMV CP but not TMV CP reduced infectivity on SHMV infected tobacco.[52] This might indicate that an exchange of CP from the virion with CP that accumulates in infected cells occurs more efficiently between homologous or closely related CP molecules than less related CP molecules. TMV mutants generated *in vitro* in which the N and C termini of the CP were changed to the SHMV sequences did not overcome CP-MR of TMV CP accumulating tobacco plants (W.G. Clark, A. Nejidat, R.N. Beachy, unpublished results). Since the termini of the CP are located on the surface of the virion[40] and thus are probably not involved in the subunit aggregation it is likely that exchange of mutant and wild-type CP was not affected in this case.

Although CP-MR of tobacco to TMV appears to be based on similar mechanisms as cross-protection this may not generally be the case. Cross-protection may be a result of either direct interaction(s) of the challenging virus with the protecting virus or its CP, or plant defense responses that lead to systemic acquired resistance, or a combination of both, whereas CP-MR of tobacco to TMV does not seem to involve either specific or general plant responses.

## Conclusions

Many studies have contributed to a better understanding of the mechanisms that are involved in the resistance of transgenic TMV CP accumulating tobacco plants to TMV infection. The main effect appears to be inhibition of virion disassembly by a direct protein effect. This is similar to cross-protection. Virion stabilization might occur through exchange of CP molecules from the virion with CP molecules that accumulate in transgenic plant cells. However, it remains to be shown that such an exchange does occur. A receptor-like structure in tobacco that specifically interacts with TMV to modify the virion structure prior to co-translational disassembly has not been identified, and there is little evidence that it exists.

Local and systemic spread of TMV is delayed in transgenic CP( + ) tobacco. This could either be a consequence of a reduced replication rate alone, or in combination with delayed phloem transport. The later effects alone are not sufficient to confer resistance. Further studies on the mode in which TMV enters and leaves the sieve elements will help to better understand CP-MR. Although the TMV/tobacco system has proven to be a powerful tool to study the mechanisms of CP-MR and other strategies to genetically engineer virus resistance it is apparent that different mechanisms are effective in other host/virus combinations. The inclusion of gene constructs that encode antisense RNA and untranslatable transcripts has the potential to improve the effectivity of future attempts to utilize CP-MR and will contribute to greater understanding of resistance.

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