The Biological Properties of Recombinant CMV Strains Relevant to the Biosafety of Transgenic Virus-Resistant Plants

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Abstract

Over the last ten years, numerous laboratories have introduced viral coat protein (cp) genes into plant genomes as a means of creating novel sources of virus resistance. More recently, there has been concern about the possibility that large scale cultivation of plants expressing viral genes could lead to ecological risks. One of the most problematic potential risks is that recombination between the viral sequence expressed by the plant and the RNA of the genome of an infecting virus could result in the creation of a novel virus genome. It has been shown experimentally that recombination in transgenic plants can occur, and thus one of the key questions concerning recombinant virus genomes is whether recombinants can have a competitive advantage or aggravate disease symptoms, in comparison to the parent strains. Using cucumber mosaic virus (CMV) and the closely related tomato aspermy virus (TAV), we have created recombinant viral genomes, including those in which the CMV *cp* gene has been replaced by the TAV *cp* gene, and vice versa. Such recombinants theoretically could arise by recombination upon infection of plants expressing a CMV or TAV *cp* gene. We present here an evaluation of the biological characteristics of the recombinant viruses as compared to the parent strains, including their ability to replicate, to spread within host plants, and to induce symptoms.

Introduction

Since CMV and TAV are both members of the genus Cucumovirus, the two viruses share numerous characteristics. The genome of cucumoviruses is composed of three messenger-sense RNA molecules. RNAs 1 and 2 encode part of the viral replicase complex (Hayes and Buck, 1990), and a subgenomic RNA derived from RNA 2 codes for a protein involved in long distance movement of CMV in certain hosts (Ding *et al.*, 1995a). RNA 3 encodes two proteins, the movement protein (mp), which is necessary for movement of the virus from the initially infected cells into neighboring cells (Suzuki *et al.*, 1991; Boccard and Baulcombe, 1993; Ding *et al.*, 1995b), and the cp, which is translated from a subgenomic RNA. Both the *mp* and the *cp* genes are required for systemic spread of the virus throughout the plant (Boccard and Baulcombe, 1993), and are major determinants of host range and symptom induction (see for instance Habili and Francki, 1974; Shintaku and Palulaitis, 1990; Shintaku, 1991; Perry *et al.*, 1994;

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Zhang *et al.*, 1994; Taliansky and Garcia-Arenal, 1995). Although both viruses have a broad host range, there are host range differences, for instance TAV does not infect cucumber, as well as differences in the symptoms induced on certain plants. Thus, CMV and TAV constitute a particularly interesting system for studying recombinant viruses, and how this may affect both host range and symptoms of infection on host plants.

Results

Using either gel-purified viral RNAs or RNAs transcribed *in vitro* from cDNA clones of the different segments of the genomes of the R strain of CMV and the P strain of TAV, we have created pseudorecombinant viruses composed of RNAs 1 and 2 of one of the viruses and RNA 3 of the other (C1C2T3 and T1T2C3). We have also created recombinant RNA 3 molecules, one of which is composed of the 5' portion from CMV, including the *mp* gene, and the 3' portion from TAV, including the *cp* gene (designated as RT3). The other recombinant is the converse of RT3, composed of the 5' portion of a TAV RNA 3 and the 3' portion of a CMV RNA 3, and is thus designated TR3. The recombinant RNA 3 molecules were mixed with CMV RNAs 1 and 2, in order to create the recombinant strains C1C2RT3 and C1C2TR3. Thus, six strains were studied further: the two parental strains (R-CMV and P-TAV), two pseudorecombinant strains (T1T2C3 and C1C2T3) and two recombinant strains (C1C2RT3 and C1C2TR3).

In protoplasts, cucumoviral RNAs 1 and 2 can replicate in the absence of RNA 3, showing that the *mp* and *cp* genes are not necessary for constituting the viral replicase complex. Thus one can use protoplast transfection as a means of testing the ability of an RNA 3 to be replicated, in the absence of limitations in virus multiplication in entire plants that can be due to defects in virus movement or encapsidation. We have synthesized by transcription in an acellular *in vitro* system RNAs corresponding to RNA 3 of R-CMV, P-TAV, and the two recombinants RT3 and TR3, as well as RNAs corresponding to RNAs 1 and 2 of R-CMV. By transfecting into protoplasts mixtures of the appropriate RNAs we were able to test the ability of RNA 3 to be replicated in the following four strains: C1C2C3, C1C2T3, C1C2RT3, C1C2TR3. Analysis of the viral RNAs synthesized 24 hours after transfection showed that all the four RNA 3 molecules were replicated to similar levels, and in all cases the subgenomic RNA 4, which encodes the *cp* gene, was able to be synthesized from RNA 3.

However, when tobacco plants were inoculated with the same artificial strains, as well as the pseudorecombinants and wild-type strains, a somewhat different picture emerged. Of the six strains tested (T1T2T3, C1C2C3, T1T2C3, C1C2T3, C1C2RT3, C1C2TR3), all gave rise to normal infections that spread systemically, except for the C1C2TR3 recombinant strain. This was tested in several inoculation experiments, and similar results were obtained in tests with other host species.

Viral RNAs were purified from plants infected with the five strains that were viable on whole plants, and used in further tests on plants, in order to evaluate symptom expression. Infection of different *Nicotiana* species has allowed us to observe quite dif-

ferent properties in these five strains. In tobacco (*N. tabacum*), all strains caused similar symptoms, a variable diffuse mosaic, occasionally presenting linear patterns. In contrast, in *N. glutinosa*, TAV induced a pronounced mosaic, while CMV induced not only mosaic but severe leaf wrinkling and plant stunting. Plants inoculated with C1C2T3 expressed symptoms similar to those induced by P-TAV, while those inoculated with T1T2C3 expressed symptoms like those induced by R-CMV. These findings indicate that in *N. glutinosa* the major determinants of extreme stunting occur on RNA 3. Plants infected with the recombinant C1C2RT3 displayed mild symptoms without stunting, which suggests that the CMV coat protein gene is a major determinant of stunting.

In *N. benthamiana*, the situation was different again, since CMV and TAV induced identical symptoms of mild mosaic and slight stunting. However, evaluation of the pseudorecombinant and recombinant strains showed that all the induced symptoms were similar to those of the parental strains, except for the T1T2C3 pseudorecombinant, which aggravated stunting compared with the other viruses. In addition, when the five strains were inoculated on tomato, similar symptoms were observed in all plants, except for those inoculated with T1T2C3, where severe stunting was observed.

Cucumber is a particularly interesting species for testing recombinants between CMV and TAV, since TAV does not infect this species, whereas, as its name indicates CMV readily does. When the five strains (P-TAV, R-CMV, T1T2C3, C1C2T3, C1C2RT3) were used in inoculation tests on cucumber plants, as expected, plants inoculated with P-TAV did not show any signs of infection, while those inoculated with R-CMV were clearly infected, with severe symptoms on both inoculated and systemic leaves. Similarly to what was observed with P-TAV, no symptoms and no viral RNA were observed in inoculated or systemic leaves of plants infected with the T1T2C3 pseudorecombinant. In plants infected with the converse pseudorecombinant, C1C2T3, symptoms and virus were clearly present in the inoculated leaves, but were absent in systemic leaves. Similar limitation of symptoms and virus to inoculated leaves was observed with the recombinant strain, C1C2RT3. When the five strains were transfected into cucumber protoplasts, all were replicated in a similar manner, showing that the differences observed on whole plants are due to differences in the ability to spread from the initially infected cells, or to the ability to spread systemically.

Discussion and conclusions

Plant virus infection is a complex process consisting of at least three distinct steps: 1) introduction of virus into a small number of cells, followed by translation of viral proteins and replication of viral RNAs in the initially infected cells; 2) movement from the initially infected cells into adjacent non-infected cells, and by this cell-to-cell movement, which is often *mp*-mediated, spread throughout the infected leaf; 3) long-distance movement through the vascular system of the plant to achieve systemic infection of the entire plant. Competence for each of these three steps can be evaluated by:

1) the ability to replicate in protoplasts; 2) the ability to infect the inoculated leaf; 3) the ability for infection to spread to uninoculated systemic leaves.

When the complete collection of six strains (P-TAV, R-CMV, T1T2C3, C1C2T3, C1C2RT3, C1C2TR3) was tested for the ability to replicate in protoplasts, all were replication-competent. However, one of the recombinant strains, C1C2TR3, was unable to infect the inoculated leaves of plants of several species, suggesting that the virus was blocked at the first stage, and was unable to move from the initially infected cells. This is surprising, since the strain is essentially a CMV in which the TAV *mp* gene had replaced the corresponding CMV gene, and it had been shown that a *mp* of one virus can function for cell-to-cell movement of even relatively distantly related viruses (De Jong and Ahlquist, 1992; Hilf and Dawson, 1993; Mise *et al.*, 1993). We do not at this time have an explanation for the inability of C1C2TR3 to infect inoculated leaves, but it is of interest that a recombinant that could occur either in doubly infected plants or in transgenic ones by homologous double crossing-over was not viable. This observation suggests that there are other constraints, at this time unknown, on virus viability in plants that are not based on an inability to replicate. However, it is not unlikely that other recombinant RNA 3 molecules with the *mp* from TAV and the *cp* from CMV will be more viable than that tested here, if they are based on other parental strains, or are constructed somewhat differently.

All of the five strains that were able to cause infection on intact plants (P-TAV, R-CMV, T1T2C3, C1C2T3, C1C2RT3) were able to infect tobacco (*N. tabacum*) systemically and induced very similar symptoms. In contrast, comparison of the response in cucumber to infection by the same five strains provides an excellent example of the complexity of the genetic basis for the ability to infect a given host. Since all of these strains replicated in a similar manner in cucumber protoplasts, and yet both strains with RNAs 1 and 2 from P-TAV were unable to induce infection spread throughout the inoculated leaves, it is suggested that RNAs 1 and 2 contain an essential determinant of cell-to-cell spread. Comparison of the response to the three strains in which RNAs 1 and 2 were derived from R-CMV shows clearly that the *cp* gene is essential for systemic spread, since the strain R1R2RT3 could infect the inoculated leaf, but was unable to spread to systemic leaves.

In *N. glutinosa*, all the five strains were able to infect and spread systemically, and there were striking differences between the symptoms induced by CMV and by TAV, since the former caused severe stunting not observed in plants infected with TAV. Evaluation of the pseudorecombinant and recombinant strains suggests that the CMV *cp* is a major determinant of plant stunting. There are several other studies in which it has been shown that the CMV *cp* gene is important in symptom expression, for instance the striking yellow mosaic described by Shintaku (1991).

When inoculated on the species discussed above (*N. tabacum*, cucumber, *N. glutinosa*), none of the pseudorecombinant or recombinant strains aggravated symptoms compared with the parental viruses. In contrast, with *N. benthamiana*, and also tomato, the T1T2C3 pseudorecombinant caused stunting that was more severe than that

by either the parental strains or the converse pseudorecombinant, C1C2T3. Since the recombinant strain, C1C2RT3, caused symptoms that were identical with those of the parental strains, it is suggested that the 3' part of CMV RNA 3, which contains the *cp* gene and the 3' non-coding region, plays a role in symptom aggravation induced by T1T2C3.

Over the past five years, significant progress has been made in our understanding of potential risks associated with plants expressing viral genes. Initially, all of the publications in this area were limited to predictions based on knowledge of plant-virus interactions, which made it possible to describe mechanisms by which transcapsidation or recombination in plants expressing *cp* genes, or mutational drift in those expressing a satellite RNA gene, could potentially lead to undesirable effects (see for instance Palukaitis, 1991; Tepfer, 1993, Tepfer, 1995; Jacquemond and Tepfer, 1997). More recently, it has been shown in the laboratory that indeed transcapsidation (Lecoq *et al.*, 1993) and recombination (Schoelz and Wintermantel, 1993; Greene and Allison, 1994) do occur in transgenic plants, and that a recombinant TAV/CMV RNA 3 can confer a competitive advantage over the parental strains (Fernandez-Cuartero *et al.*, 1994). We believe that evaluation under controlled conditions of the biological properties of recombinant viruses created in the laboratory, such as those studied here, is relevant to our future understanding of the potential impact of recombination in transgenic plants in the field.

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