*European Journal of Plant Pathology* **103:** 235–243, 1997.



# Pepper resistance-breaking tobamoviruses: Can they co-exist in single pepper plants?

F. Tenllado, I. García-Luque, M.T. Serra and J.R. Díaz-Ruíz*
*Departamento de Biología de Plantas, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain (Fax: 34-1-5627518); *Author for correspondence*

Accepted 24 October 1996

*Key words:* pepper tobamoviruses, coexistence, cross-protection, pepper resistance, pathotypes, restrictopatterns

**Abstract**

We previously reported a procedure for identifying pathotypes of the tobamoviruses infecting the L-resistant genotypes of pepper based on the polymerase chain reaction (PCR) (Tenllado et al., 1994). We have now used this method to assess pathotype incidence in virus isolates from field pepper samples representing three successive epidemic episodes in southeastern Spain, and to analyse the interaction of pathotypes during experimental infections in pepper plants. The majority of virus isolates corresponded to the $P_{1,2}$ pathotype, and only three out of twenty behaved as $P_{1,2,3}$ pathotypes. Interestingly, restriction enzyme analysis of the PCR-amplified products distinguished two restriction subgroups on each $P_{1,2}$ and $P_{1,2,3}$ pathotype, referred to as restrictopatterns $I_1$ and $I_2$ or $II_1$ and $II_2$, respectively. Experimental coinoculations of pepper plants with mixtures of two different pathotypes showed coexistence between them when inoculations proceeded simultaneously. However, reciprocal cross-protection was observed between $P_{1,2}$ and $P_{1,2,3}$ pathotypes when they were successively inoculated, while no cross-protection was observed between $P_1$ and either $P_{1,2}$ or $P_{1,2,3}$ pathotypes. The potential of the PCR-based method for detecting heterogeneity within $P_{1,2}$ and $P_{1,2,3}$ pathotypes, and the possibility of genetically engineered resistance to those viruses by genetic transformation with viral coat protein genes are briefly discussed.

*Abbreviations:* CP – coat protein; PaMMV – Paprika mild mottle virus; PMMoV – Pepper mild mottle virus; RT-PCR – reverse transcription-polymerase chain reaction; TMV – Tobacco mosaic virus.

## Introduction

Different members of the *Tobamovirus* genus have been reported as disease agents in pepper crops (Martelli and Quacquarelli, 1982). Of special interest are the so-called tobamoviruses of pepper or pepper strains, which are able to infect commercial pepper cultivars with genetic resistances against tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) introduced by classical breeding (Boukema, 1980; Tobias et al., 1982). These specialized strains have been isolated all over the world, and produce important losses in pepper crops grown under plastic (Wetter et al., 1984; Pares, 1985; Avgelis, 1986; Rast, 1988; Alonso et al., 1989).

In *Capsicum* spp. the resistance against tobamoviruses is conferred by an allelic series of genes at the locus L, known as $L^1$, $L^2$ and $L^3$. Based upon their ability to overcome each of these genes, the pepper strains of the tobamoviruses have been designated by breeders as pathotypes $P_1$, $P_{1,2}$, and $P_{1,2,3}$, respectively (Boukema, 1980). In every case, the resistance is mediated by a hypersensitive reaction, expressed through the induction of necrotic local lesions.

Two tobamoviruses infecting TMV-resistant pepper crops isolated in Spain and Italy were identified as strains of pepper mild mottle virus (PMMoV), based upon biological, serological and nucleotide sequence data (Alonso et al., 1989; García-Luque et al., 1993), and were termed PMMoV-S and PMMoV-I, respec-

tively. The S strain (Alonso et al., 1991), which belongs to the $P_{1,2}$ pathotype, induces a hypersensitive reaction in *C. chinense* ($L^3L^3$) plants, while the I strain (Wetter et al., 1984), which corresponds to a $P_{1,2,3}$ pathotype, systemically infects this host. PMMoV is transmitted through seeds (Avgelis, 1986) and direct contact between plants. A pepper tobamovirus isolated in Holland, identified as a $P_1$ pathotype, appeared to be a distinct species in the *Tobamovirus* genus for which the name paprika mild mottle virus (PaMMV) has been proposed and accepted (García-Luque et al., 1993; Fauquet and Martelli, 1995). PMMoV-S, PMMoV-I and PaMMV, are able to systemically infect *C. annuum* cv. 'Dulce Italiano' ($L^1L^1$) plants. However, it is not known whether different pathotypes can coinfect a single pepper plant in nature. In a survey of virus-infected pepper crops in Southeastern Spain, it was found that tobamoviruses were the main disease causing agents of these crops grown under plastic (Alonso et al., 1989). The disease induced mild mosaic on the leaves and fruit malformation and necrosis which severely affected yield. In an early work (Tenllado et al., 1994), we developed a procedure, based on reverse transcription and polymerase chain reaction (RT-PCR) followed by restriction enzyme analysis of the amplified products, which allows detection and molecular distinction of tobamovirus pathotypes infecting TMV-resistant genotypes of pepper. Moreover, the procedure provides a simple way to detect mixed-pathotype infections in single field samples. In the present work, we apply this procedure for the screening of pathotypes in field isolates representing three successive epidemic episodes in peppers grown under plastic. In addition, we study the interaction of pathotypes during experimental coinfections in pepper plants and investigate cross-protection between the three different pathotypes, with a view to providing a practical control of virus disease either by protective inoculation or by transferring viral coat protein genes to pepper plants by genetic transformation.

## Materials and methods

### *Virus sources*

The origin of PaMMV, PMMoV-S and PMMoV-I, used as representative of the $P_1$, $P_{1,2}$ and $P_{1,2,3}$ pathotypes respectively, has been previously reported (Wetter et al., 1984; García-Luque et al., 1990, 1993). Viruses were propagated and purified from systemically infected *Nicotiana clevelandii* Gray plants as described by García-Luque et al. (1990).

Nineteen virus isolates from field pepper samples were analysed. They were obtained during a three year (1983–1985) survey of tobamoviruses infecting TMV-resistant commercial pepper cultivars in southeastern Spain, and named AL isolates (Alonso et al., 1989) (Table 1). In addition, a Dutch isolate from pepper, termed P14 and identified previously as a $P_{1,2,3}$ pathotype, was also used (Tobias et al., 1982). Field isolates were stored as dried leaf over calcium chloride at 4 °C. All isolates had been previously checked by electron microscopy and tested on different indicator plants for possible virus contaminations (Alonso et al., 1989). The Spanish isolates AL78/84 and AL132/85, and the Dutch isolate P14 were mechanically transmitted to *N. benthamiana* plants by macerating desiccated leaf tissue in 20 mM sodium phosphate buffer pH 7.0, and were purified as indicated above.

### *Virus inoculation and RNA extraction*

*C. annuum* cv. 'Dulce Italiano' ($L^1L^1$) plants at the 3–4 leaf stage were mechanically inoculated with 40 $\mu$g ml$^{-1}$ of each of the purified viruses in mixtures of two pathotypes, either simultaneously or at an inoculation interval of 15 days, as described by Tenllado et al. (1995). Single virus inoculations were carried out as controls. When plant sap was used as inoculum, it was prepared by grinding desiccated leaf tissue from field isolates AL78/84 and AL132/85 in 20 mM sodium phosphate buffer (pH 7.0) at a ratio of 1 g:10 ml. Equivalent infectivity of the sap inocula was established by inoculating each homogenate onto the local lesion host *N. tabacum* 'Xanthi nc'. At least two trials with three replicates per trial were performed. With simultaneous inoculations, viruses were applied onto the first pair of true leaves. With successive inoculations, the second inocula were applied onto the next upper, uninoculated leaves. Samples were taken from inoculated leaves at 7 days postinoculation (dpi) or from systemically infected upper leaves at 15 and 30 dpi, and maintained at −70 °C. *N. tabacum* cv. 'Xanthi nc', a host exhibiting chlorotic or necrotic local lesions after either PaMMV or PMMoV infection, was used to determine the presence of infectious virus in mixed infections containing PaMMV. Total RNA was extracted from 50 mg of desiccated leaf tissue from field isolates and from 0.1 g of mechanically infected plant tissue (Logemann et al., 1987) and tested by RT-PCR amplification.

*C. chinense* 'PI 159236' plants carrying the *Capsicum* $L^3$ resistance gene were mechanically inoculated with extracts from desiccated leaf tissue of field isolates as indicated above. Systemic infections in these plants were assayed by back inoculation onto *N. tabacum* cv. 'Xanthi nc'.

*RT-PCR amplification, restriction digests and PCR sequencing*

The pair of primers CP1 and CP2 were designed from the PMMoV-S nucleotide sequence to amplify a PMMoV-I and a PMMoV-S CP gene fragment of 395 bp. The pair of primers MP1 and MP2 were used for PaMMV-specific amplification, producing a major fragment of 800 bp long and several minor ones, which contained part of the movement protein gene sequence. All these primers, as well as the RT-PCR procedures using total RNA extracts as templates, have been previously described (Tenllado et al., 1994). *Taq*I restriction digestion were done on aliquots (10–30 $\mu$l, depending on the concentration of product as judged from the initial agarose gel analysis) of the CP1 and CP2 primers-amplified PCR products, using 6–8 U of enzyme (Boehringer), and analysed on 6% polyacrylamide slab gels (Tenllado et al., 1994). For determining the sensitivity of the RT-PCR procedure, series of log dilutions ($10^{-1}$–$10^{-5}$) were prepared from total RNA extract of AL78/84-infected pepper plants at 2.4 mg $ml^{-1}$. The dilutions were mixed in equal volumes with a fixed concentration of total RNA extract from AL132/85-infected pepper plants (3.5 mg $ml^{-1}$), i.e., decreasing concentrations of AL78/84 RNA extract were combined with a fixed concentration of AL132/85 RNA extract.

CP1 and CP2 primers-amplified PCR products from the field isolates AL66/83, AL80/83, AL132/85 and AL141/85, and the Dutch isolate P14 were purified by the Magic PCR preps DNA purification system (Promega) and resuspended in 50 $\mu$l of $H_2O$. Typically, 4 $\mu$l aliquots (approx. 1 $\mu$g) of the purified PCR products were sequenced with the fmol DNA sequencing kit (Promega), using oligonucleotides CP1 and CP2 as 5′ and 3′ sequencing primers, respectively. The PCR sequencing protocol was carried out following the manufacturer's indications; after an initial 2 min denaturation at 95 °C, 30 cycles of 95 °C for 30 s, 45 °C for 30 s and 70 °C for 1 min were performed, followed by a 30 min final extension with 3.6 U of Terminal transferase at 37 °C.

## Results

*Analysis of incidence and coexistence of pathotypes of the tobamoviruses in naturally infected pepper plants*

For the nineteen Spanish field isolates and the Dutch isolate P14 tested (Table 1), RT-PCR amplified a product of expected size (395 bp) when using CP1 and CP2 primers (not shown). No PCR-amplified products were detected when employing MP1 and MP2 primers, indicating that $P_1$ pathotype was not present in the field samples analysed (not shown). Digestion of the amplified cDNAs with *Taq*I restriction endonuclease revealed four different restriction patterns. Restriction maps were based on fragment size in comparison with the known sequence of PMMoV-S (Alonso et al., 1991). The first pattern consisted of two fragments with the approximate size of 356 bp and 40 bp, respectively (Figure 1, lane 2). It was undistinguishable from that described for PMMoV-S ($P_{1,2}$ pathotype) (Tenllado et al., 1994), which will be referred to as restrictopattern $I_1$. This restrictopattern was produced by six field isolates (Table 1). The second restrictopattern ($II_1$) consisted of four fragments with the approximate size of 135 bp, 113 bp, 107 bp and 40 bp, respectively (Figure 1, lane 4). It was obtained from two isolates (Table 1) and coincided with the pattern described for PMMoV-I ($P_{1,2,3}$ pathotype) (Tenllado et al., 1994).

Unexpectedly, amplified products from the remaining eleven Spanish isolates produced three DNA fragments when digested with *Taq*I. This new restriction map, referred to as restrictopattern $I_2$ (Figure 1, lane 3), differed from the PMMoV-S $I_1$ restrictopattern because of an additional restriction site, cleaving the 356 bp PMMoV-S fragment into two fragments with the expected size of 220 bp and 135 bp, respectively. Bases 5897 to 5900 of the PMMoV-S genome have the sequence 5′ CCGA 3′, one nucleotide different from the *Taq*I recognition site (5′ TCGA 3′). Hence, it seems likely that the additional site in those isolates involved the conversion of the C residue found in PMMoV-S to a T residue, thereby generating the observed restrictopattern. This was confirmed for four different field isolates that showed *Taq*I restrictopattern $I_2$ (isolates AL66/83, AL80/83, AL132/85 and AL141/85) by sequencing PCR-amplified products using either CP1 or CP2 primer. Sequence comparisons showed that these isolates were almost identical in sequence to the 310 nucleotides of the CP central region (nts 5807-6116) from PMMoV-S RNA (Alonso et al., 1991). The differences between these four field

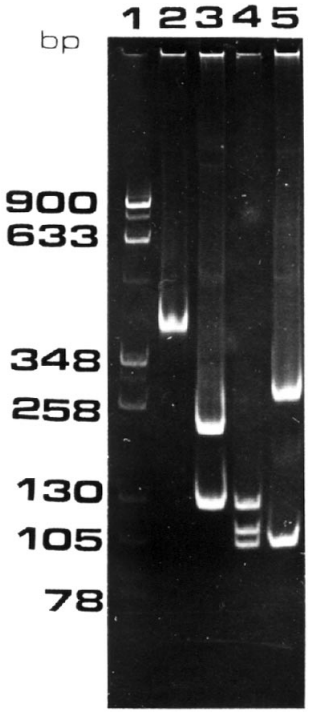


*Figure 1*. *Taq*I restriction enzyme analysis of RT-PCR-amplified products from virus isolates representative of the four different restrictopatterns shown by the pathotypes of the tobamoviruses of pepper. Samples were analysed on 6% polyacrylamide gels and stained with ethidium bromide. Lane 1, *Sau*3A+pUC18 DNA size markers; lane 2, Spanish isolate AL78/84 (restrictopattern $I_1$); lane 3, Spanish isolate AL132/85 (restrictopattern $I_2$); lane 4, Spanish isolate AL130/85 (restrictopattern $II_1$); lane 5, Dutch isolate P14 (restrictopattern $II_2$); The small restriction fragment of 40 bp was not clearly visible. Amplification of cDNA products were carried out using CP1 and CP2 primers.

*Table 1*. Pathotypes of the tobamoviruses in naturally infected pepper plants differentiated by RT-PCR analysis and biological test in *C. chinense*

| Isolate name/year of isolation | *Taq*I Restrictopattern[1] | Response of *C. chinense*[2] | Pathotype[3] |
|---|---|---|---|
| AL37/83 | $II_1$ | SI | $P_{1,2,3}$ |
| AL61/83 | $I_2$ | NLL | $P_{1,2}$ |
| AL66/83 | $I_2$ | NLL | $P_{1,2}$ |
| AL67/83 | $I_1$ | NLL | $P_{1,2}$ |
| AL69/83 | $I_2$ | NLL | $P_{1,2}$ |
| AL73/83 | $I_2$ | NLL | $P_{1,2}$ |
| AL75/83 | $I_1$ | NLL | $P_{1,2}$ |
| AL77/84 | $I_2$ | NLL | $P_{1,2}$ |
| AL78/84 | $I_1$ | NLL | $P_{1,2}$ |
| AL80/84 | $I_2$ | NLL | $P_{1,2}$ |
| AL82/84 | $I_2$ | NLL | $P_{1,2}$ |
| AL84/84 | $I_1$ | NLL | $P_{1,2}$ |
| AL89/84 | $I_1$ | NLL | $P_{1,2}$ |
| AL129/85 | $I_2$ | NLL | $P_{1,2}$ |
| AL130/85 | $II_1$ | SI | $P_{1,2,3}$ |
| AL132/85 | $I_2$ | NLL | $P_{1,2}$ |
| AL133/85 | $I_2$ | NLL | $P_{1,2}$ |
| AL138/85 | $I_1$ | NLL | $P_{1,2}$ |
| AL141/85 | $I_2$ | NLL | $P_{1,2}$ |
| P14 | $II_2$ | SI | $P_{1,2,3}$ |

[1] Classification of pathotypes of the tobamovirus field isolates according to *Taq*I restriction mapping of the RT-PCR-amplified coat protein gene.
[2] Abbreviations: SI, systemic infection on noninoculated leaves as assayed by back inoculation on *N. tabacum* 'Xanthi nc'; NLL, necrotic local lesions.
[3] Classification of pathotypes of the tobamovirus field isolates according to their biological response in *C. chinense* ($L^3L^3$) plants.

isolates and PMMoV-S RNA involved two C to T substitutions at positions 5897 and 6030 of PMMoV-S RNA. In addition, isolate AL141/85 showed a C to T transition at nucleotide 5909 of PMMoV-S RNA. None of these nucleotide changes alter the CP amino acid sequence of the field isolates analysed.

PCR-amplified cDNA from the Dutch isolate P14 ($P_{1,2,3}$ pathotype) also produced a new restrictopattern ($II_2$) of three fragments with approximate size of 248 bp, 107 bp and 40 bp, respectively (Figure 1, lane 5; Table 1). Restrictopattern $II_2$ differed from restrictopattern $II_1$ (PMMoV-I) by the loss of one restriction site (nt 5897 in PMMoV-S RNA). In fact, sequencing of isolate P14 CP-amplified fragment revealed only one T to C and one C to T transition from the corresponding sequence of the PMMoV-I RNA at nucleotide positions 5897 and 5903, respectively (García-Luque et al., 1993). As with the Spanish field isolates sequenced, the observed nucleotide changes in P14 do not modify the amino acid sequence of the coat protein with regard to PMMoV-I CP.

When *C. chinense* ($L^3L^3$) plants were inoculated with sap homogenates from Spanish field isolates, 17 out of 19 showed a hypersensitive response in the inoculated leaves, including all isolates showing *Taq*I restrictopatterns $I_1$ and $I_2$ (Table 1). Systemic infections were not detected in the upper leaves of these pepper plants as assayed on *N. tabacum* 'Xanthi nc'. Therefore, they are $P_{1,2}$ pathotypes (Boukema, 1980). The other two Spanish isolates (isolates AL37/83 and AL130/85) showing restrictopattern $II_1$ and the Dutch isolate P14 showing restrictopattern $II_2$ failed to induce the hypersensitive reaction int he indicator pepper plant, but produced a systemic infection in the upper

leaves, confirmed by back inoculation onto *N. tabacum* 'Xanthi nc' (Table 1). Therefore, they are $P_{1,2,3}$ pathotypes (Boukema, 1980). *Taq*I digestion of the CP1 and CP2 primer-amplified PCR products resulted in two restriction subgroups for each $P_{1,2}$ and $P_{1,2,3}$ pathotype, here called restrictopatterns $I_1$ and $I_2$, and $II_1$ and $II_2$, respectively. No mixed restrictopatterns from single samples was observed in the present survey. In addition, when individual field isolates displaying one of the four restrictopatterns described above were inoculated onto *N. benthamiana* plants and then processed by RT-PCR and *Taq*I analysis, restrictopatterns identical to those observed in pepper plants were obtained (data not shown).

As shown in Table 1, a correlation was observed between the field isolates classified as pathotype $P_{1,2}$ or $P_{1,2,3}$ and their *Taq*I restrictopatterns. Thus, the biological typing of pathotypes $P_{1,2}$ and $P_{1,2,3}$ corresponds to that shown by our RT-PCR procedure in all the field isolates tested so far (Tenllado et al., 1994; this report).

*Analysis of coexistence of pathotypes of the tobamoviruses in experimentally infected pepper plants*

To test whether paired combinations of $P_1$, $P_{1,2}$ and $P_{1,2,3}$ pathotypes are able to systemically coinfect a single pepper plant, and evaluate the relative degree of virulence and adaptability of these pathotypes, we carried out experimental coinoculations in a susceptible pepper cultivar (*C. annuum* cv. 'Dulce Italiano'). For this purpose, we employed PaMMV, representing the $P_1$ pathotype, and PMMoV-S and AL132/85, representing restrictopatterns $I_1$ and $I_2$, respectively, within the $P_{1,2}$ pathotype, as well as PMMoV-I and P14, representing restrictopatterns $II_1$ and $II_2$, respectively, within the $P_{1,2,3}$ pathotype.

Table 2 summarises the results obtained in the coinoculation experiments when total RNA extracts from doubly-inoculated plants were analysed by RT-PCR. In each of the six combinations, both viruses could be detected in the upper leaf tissue at 15 dpi, when systemic symptoms became apparent. Analysis of the new, uppermost leaves at 30 dpi confirmed that the systemic spread of any binary combination of pathotypes $P_1$, $P_{1,2}$, or $P_{1,2,3}$ may occur when the inoculation is simultaneous. Infections were also obtained when homogenates of mixed-infected pepper were back-inoculated onto *N. tabacum* cv. 'Xanthi nc'. Both chlorotic and necrotic local lesions were observed in these tobacco plants indicating that PaMMV and either PMMoV-I or PMMoV-S infections had occurred. No synergistic effect on systemic symptoms was observed in mixed-infected pepper plants when compared to the singly-infected, control plants.

*Analysis of coexistence of viral restrictopatterns within pathotypes $P_{1,2}$, and $P_{1,2,3}$*

To study mutual relationships between tobamovirus isolates displaying the two different restrictopatterns within $P_{1,2}$ and $P_{1,2,3}$ pathotypes, we also investigated their ability to coinfect a single pepper plant in mixtures of two viruses. To do so, pepper plants were doubly inoculated with either isolate pairs AL78/84 and AL132/85, representing $P_{1,2}$ pathotype restrictopatterns $I_1$ and $I_2$, respectively, or PMMoV-I and P14, representing $P_{1,2,3}$ pathotype restrictopatterns $II_1$ and $II_2$, respectively. When isolates PMMoV-I and P14 were coinoculated, *Taq*I-digested PCR product from upper leaves of individual plants showed a mixture of both restrictopatterns $II_1$ and $II_2$ at 15 and 30 dpi (Figure 2, lane 2 and data not shown). This result indicates that those virus isolates displaying different restrictopatterns within the $P_{1,2,3}$ pathotype are capable of replication and coexistence within individual host plants. Unexpectedly, when the inoculum combination was AL78/84 plus AL132/85, the PCR-amplified product from systemically infected leaf tissue yielded only *Taq*I restrictopattern $I_2$ (isolate AL132/85) (Figure 2, lane 3). None of the individual samples collected at 15 and 30 dpi showed evidence of restrictopattern $I_1$ (isolate AL78/84). Furthermore, when samples from inoculated leaves were taken 7 days after the coinoculation, *Taq*I analysis showed only the diagnostic pattern of isolate AL132/85 (not shown), suggesting that a kind of interference phenomenon was operating within the inoculated leaf. As many as five independent experiments with three to five plants per treatment confirmed the above results, regardless of whether the origin of the inoculum was purified virus or desiccated leaf tissue. However, when pepper plants were coinoculated with five other combinations of tobamovirus field isolates displaying the two different restrictopatterns described within the $P_{1,2}$ pathotype, *Taq*I digestion analysis of the PCR products at 15 and 30 dpi produced the parental mixture of restrictopatterns (Figure 2, lane 4 and data not shown). Therefore, with the exception of the combination of isolates AL78/84 and AL132/85, no general interference occurred between tobamovirus isolates displaying restrictopatterns $I_1$ and $I_2$ within the $P_{1,2}$ pathotype. The nature of the interference mecha-

*Table 2*. Coexistence of the different pathotypes of tobamoviruses in experimentally infected *C. annuum* cv. 'Dulce Italiano' plants

| Simultaneous coinoculation[1] | Virus detected by RT-PCR | |
|---|---|---|
| | 15 dpi | 30 dpi |
| PaMMV + PMMoV-S | PaMMV, PMMoV-S | PaMMV, PMMoV-S |
| PaMMV + PMMoV-I | PaMMV, PMMoV-I | PaMMV, PMMoV-I |
| PMMoV-S + PMMoV-I | PMMoV-S, PMMoV-I | PMMoV-S, PMMoV-I |
| PMMoV-S + P14 | PMMoV-S, P14 | PMMoV-S, P14 |
| PMMoV-I + AL132/85 | PMMoV-I, AL132/85 | PMMoV-I, AL132/85 |
| AL132/85 + P14 | AL132/85, P14 | AL132/85, P14 |

[1] PaMMV, representing the $P_1$ pathotype, PMMoV-S and AL132/85, representing the two restrictopatterns $I_1$ and $I_2$ of $P_{1,2}$ pathotype, and PMMoV-I and P14, representing the two restrictopatterns, $II_1$ and $II_2$ of $P_{1,2,3}$ pathotype.

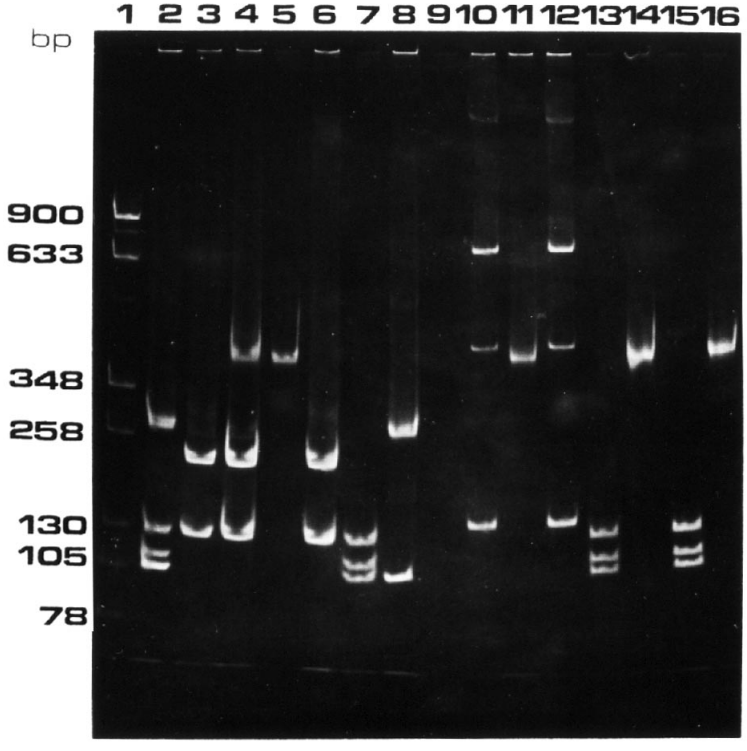


*Figure 2*. *Taq*I restriction enzyme analysis of RT-PCR-amplified products from pepper plants inoculated with the different pathotypes of the tobamoviruses. Samples were analysed on 6% polyacrylamide gels and stained with ethidium bromide. Lane 1, *Sau*3A+pUC18 DNA size markers; lanes 2–4, plants coinoculated with isolates P14 + PMMoV-I, AL78/84 + AL132/85, and AL84/84 + AL80/84 respectively, at 30 dpi; lanes 5–8, plants singly inoculated with isolates AL78/84, AL132/85, PMMoV-I and P14 respectively at 30 dpi, used as controls; lane 9, healthy plant; lanes 10–16, plants inoculated initially with PaMMV (10–13), PMMoV-S (14), PMMoV-I (15) or AL78/84 (16), and challenge inoculated 15 days later with PMMoV-S (10 and 11), PMMoV-I (12 and 13), PMMoV-I (14), PMMoV-S (15) or AL132/85 (16), at 30 days after second inoculation. The small restriction fragment of 40 bp was not clearly visible. Samples from lanes 10–11 and 12–13 correspond to the same inoculated plants. Amplification of cDNA products were carried out using CP1 and CP2 primers, except those on lanes 10 and 12, which used MP1 and MP2 primers.

nism between isolates AL78/84 and AL132/85 has not been determined.

To determine the sensitivity of the RT-PCR procedure in detecting mixed restrictopatterns in doubly-inoculated pepper plants, serial dilutions of total RNA from AL78/84-infected plants and a fixed concentration of total RNA from AL132/85-infected plants were reverse-transcribed and amplified by PCR. A mixture of restrictopatterns corresponding to isolates AL132/85 and AL78/84 was obtained in samples containing total RNA from AL78/84-infected plants diluted to $1.46 \times 10^{-4}$ the concentration of total RNA from AL132/85-infected plants (not shown). This limit corresponds to a value of 240 pg of total RNA from AL78/84-infected plants, equivalent to 0.13 mg of fresh leaf tissue. Thus, the virus population in pepper plants inoculated with isolates AL78/84 and AL132/85 consisted of nearly all AL132/85 with only a trace ($<1.46 \times 10^{-4}$), if any, of AL78/84.

*Analysis of cross-protection between pathotypes of the tobamoviruses*

To test the ability of each pathotype to cross-protect pepper plants against each of the others, we evaluated $P_1$, $P_{1,2}$, and $P_{1,2,3}$ pathotypes in reciprocal cross-protection experiments. When PaMMV ($P_1$ pathotype) was tested as the protecting virus (Table 3), both PMMoV-S ($P_{1,2}$ pathotype) and PMMoV-I ($P_{1,2,3}$ pathotype) were able to individually replicate and spread in the symptomatic plants inoculated 15 days previously with PaMMV, as assayed by RT-PCR. Both MP1-MP2- and CP1-CP2- amplified PCR products were detected in total RNA extracts from these doubly inoculated plants (Figure 2, lanes 10–13). In the reciprocal tests in which pepper plants systemically infected with either PMMoV-S or PMMoV-I were challenged with PaMMV, both PMMoV- and PaMMV-specific PCR products were also amplified form total RNA extracts of the uppermost leaves at 15 and 30 days after the second inoculation (not shown). Biological evidence of mixed infections in these plants was obtained when pepper homogenates were back-inoculated onto *N. tabacum* cv. 'Xanthi nc'. Therefore, there is no evidence of cross-protection or interference between $P_1$ and either $P_{1,2}$ or $P_{1,2,3}$ pathotypes (Table 3). In contrast, complete cross-protection was observed in pepper plants systemically infected with either PMMoV-S ($P_{1,2}$ pathotype) or PMMoV-I ($P_{1,2,3}$ pathotype) when challenged with PMMoV-I or PMMoV-S, respectively. Digestion of the PCR-amplified products with *Taq*I showed the restrictopattern corresponding to the protecting pathotype, while the challenger restrictopattern was not detected in any of the plants tested at 15 and 30 days after the second inoculation (Figure 2, lanes 14 and 15).

In an attempt to determine if this cross-protection phenomenon applies to tobamovirus isolates showing the two different $P_{1,2}$ pathotype restrictopatterns, pepper plants were inoculated as above, first with AL78/84 (restrictopattern $I_1$) and then with AL132/85 (restrictopattern $I_2$). The PCR-amplified product from noninoculated, infected leaf tissue yielded only *Taq*I restrictopattern $I_1$ at 15 and 30 days postchallenge inoculation (Figure 2, lane 16). This result indicates that prior systemic infection with isolate AL78/84 prevents or inhibits efficient replication and/or spread of isolate AL132/85 in pepper plants, even though coinoculation assays between these two variants resulted in complete exclusion of isolate AL78/84.

## Discussion

Restriction enzyme analysis of PCR-amplified products (Tenllado et al., 1994) and study of the biological response in *C. chinense* plants with the $L^3$ resistance gene (Boukema, 1980) have enabled the pathotype characterisation of nineteen tobamovirus isolates from southeastern Spain, plus one isolate from Holland. From the epidemiological point of view it is interesting to note that the majority of virus isolates corresponds to the $P_{1,2}$ pathotype, and only three out of twenty behave as $P_{1,2,3}$ pathotypes. In a previous work (García-Luque et al., 1993), it was suggested that the pathotype $P_{1,2,3}$ (PMMoV-I) could have evolved from pathotype $P_{1,2}$ (PMMoV-S) by positive selection in pepper crops carrying the $L^3$ resistance gene. The limited distribution of commercial pepper cultivars possessing $L^3$ gene (Gil Ortega and Luis Arteaga, 1992) may be the reason for the low proportion of the $P_{1,2,3}$ pathotype found in the field samples analysed. However, in our survey, one of the two Spanish $P_{1,2,3}$ isolates found (AL37/83) came from a pepper cultivar lacking the $L^3$ gene, while the other (AL130/85) was from a experimental cultivar carrying this gene.

Interestingly, digestion of the amplified cDNA with *Taq*I revealed polymorphism amongst the isolates biologically characterised as pathotypes $P_{1,2}$ and $P_{1,2,3}$, with two different restriction patterns being detected in each of the two pathotypes. The relationships between these two restrictopatterns were simple, involving

*Table 3.* Cross-protection between the different pathotypes of the tobamoviruses in experimentally infected *C. annuum* cv. 'Dulce Italiano' plants

| Successive inoculation[1] at 15 days interval | | Virus detected by RT-PCR after the second inoculation | |
|---|---|---|---|
| First | Second | 15 dpi | 30 dpi |
| PaMMV | PMMoV-S | PaMMV, PMMoV-S | PaMMV, PMMoV-S |
| PaMMV | PMMoV-I | PaMMV, PMMoV-I | PaMMV, PMMoV-I |
| PMMoV-S | PaMMV | PMMoV-S, PaMMV | PMMoV-S, PaMMV |
| PMMoV-I | PaMMV | PMMoV-I, PaMMV | PMMoV-I, PaMMV |
| PMMoV-I | PMMoV-S | PMMoV-I | PMMoV-I |
| PMMoV-S | PMMoV-I | PMMoV-S | PMMoV-S |

[1] PaMMV, representing the $P_1$ pathotype, PMMoV-S, representing the $P_{1,2}$ pathotype, and PMMoV-I, representing the $P_{1,2,3}$ pathotype.

the addition or deletion of single restriction sites in the PCR-amplified CP gene region as determined by sequence comparisons. Moreover, the type of host plant used does not seem to affect *Taq*I restrictopatterns. Electrophoretic analysis of restriction digests carried out on the amplified CP gene of tobamovirus field isolates may, therefore, provide a rapid method for detecting heterogeneity within $P_{1,2}$ and $P_{1,2,3}$ pathotypes, as has been reported for other virus groups using similar procedures (Rizos et al., 1992; Guillings et al., 1993). Previously, a single *Taq*I restrictopattern for each $P_{1,2}$ and $P_{1,2,3}$ pathotype was reported, corresponding to those displayed by PMMoV-S (restrictopattern $I_1$) and PMMoV-I (restrictopattern $II_1$), respectively (Tenllado et al., 1994). In the present work, however, new restrictopatterns were observed in the PCR-amplified CP gene region (restrictopatterns $I_2$ and $II_2$). In all twenty isolates typed according to *Taq*I restrictopattern, there was complete agreement with the results of typing based on bioassay with pepper indicator plants (*C. chinense*). Thus, the method described appears to be a rapid and conclusive technique for classifying tobamoviruses infecting the L-resistant genotypes of pepper into pathotypes $P_{1,2}$ and $P_{1,2,3}$.

We have also shown that no interfering mechanism precludes the coinfection of *C. annuum* cv. 'Dulce Italiano' with any mixture of two different pathotypes when inoculations are simultaneous. However, prevention of detectable multiplication of the challenge virus was observed in cross-protection tests between $P_{1,2}$ and $P_{1,2,3}$ pathotypes, while no interference was apparent between $P_1$ and either $P_{1,2}$ or $P_{1,2,3}$ pathotypes. This confirms observations by Rast (1979) who reported mixtures of both $P_1$ and $P_{1,2}$ pathotypes in Dutch glasshouse crops of sweet pepper. The use of RT-PCR analysis described here is a new approach for analysing the degree and extension of cross-protection, a phenomenon usually found only between virus strains which are closely related. PMMoV-S and PMMoV-I, representative of $P_{1,2}$ and $P_{1,2,3}$ pathotypes, respectively, share a higher degree of aminoacid sequence identity on the CP gene (98.1%) than does PaMMV ($P_1$ pathotype) with either PMMoV-S (69.2%) or PMMoV-I (67.2%) (García-Luque et al., 1993). Therefore, effective cross-protection between the tobamovirus pathotypes is correlated with the degree of relatedness in CP sequence. Time course studies of cross-protection with different viruses indicated that the challenge virus eventually became established in some initially protected plants (Cassells and Herrick, 1977; Wen et al., 1991), suggesting that protection was progressively overcome in those cases. In our system, cross-protection between pathotypes $P_{1,2}$ and $P_{1,2,3}$ was not time-dependent, at least up to 70 dpi. This could explain why our screening of virus isolates from TMV-resistant pepper cultivars did not show mixed restrictopatterns in single samples. In this sense, systemic infections of pepper plants by any of the viruses belonging to either pathotype $P_{1,2}$ or $P_{1,2,3}$, which are non-vector-transmitted and thus unlikely to infect together by contact a single plant, would prevent or interfere with a subsequent superinfection by another virus of the pathotype $P_{1,2}$ or $P_{1,2,3}$.

A primary condition for the successful control of pepper disease by protective inoculation is that the protecting virus does not affect fruit quality. However, none of the naturally occurring pepper tobamoviruses described so far can be considered for the purpose, as they all cause severe fruit symptomatology. It remains

possible to engineer artificially attenuated viruses unable to induce fruit damage but capable of interfering with the multiplication of pepper tobamoviruses. Strategies based on transferring CP viral genes into crop plants have established the considerable potential of genetically engineered cross-protection as a new tool for the control of plant virus diseases (Fuchs and Gonsalves, 1995). Our results suggest that PMMoV CP genes from the Italian and Spanish isolates may be capable of eliciting a cross-protection-like effect in transgenic plants against closely related pepper strains of tobamoviruses, as it was reported for other virus groups (Fitchen and Beachy, 1993).

## Acknowledgements

F. T. was recipient of a fellowship from the Subprograma de Intercambio de Personal entre Industrias (S & G semillas, S.A.) y Centros Públicos de Investigación (CIB, CSIC). The contribution of S & G semillas, S.A. is gratefully acknowledged. This work was supported by grant PB94-0023 from the Dirección General de Investigación Científica y Técnica (DGICYT).

## References

Alonso E, García-Luque I, Avila-Rincón MJ, Wicke B, Serra MT and Díaz-Ruíz JR (1989) A tobamovirus causing heavy losses in protected pepper crops in Spain. J Phytopathol 125: 67–76

Alonso E, García-Luque I, de la Cruz A, Wicke B, Avila-Rincón MJ, Serra MT, Castresana C and Díaz-Ruíz JR (1991) Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. J Gen Virol 72: 2875–2884

Avgelis AD (1986) A pepper strain of TMV who is new in Crete (Greece). Phytopath medit 25: 33–38

Boukema IW (1980) Strains of TMV and genes for resistance. In: Capsicum (pp. 44–48).Eucarpia Capsicum Working Gp. Synopses of the IVth meeting, Wageningen (Holland)

Cassells AC and Herrick CC (1977) Cross protection between mild and severe strains of tobacco mosaic virus in doubly inoculated tomato plants. Virology 78: 253–260

Fauquet C and Martelli G (1995) Updated ICTV list of names and abbreviations of viruses, viroids and satellites infecting plants. Arch Virol 140: 393–413

Fitchen J and Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. Annu Rev Microbiol 47: 739–763

Fuchs M and Gonsalves D (1995) Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyvirus. BioTechnology 13: 1466–1473

García-Luque I, Serra MT, Alonso E, Wicke B, Ferrero ML and Díaz-Ruíz JR (1990) Characterization of a Spanish strain of pepper mild mottle virus (PMMV-S) and its relationship to other tobamoviruses. J Phytopathol 129: 1–8

García-Luque I, Ferrero ML, Rodríguez JM, Alonso E, de La Cruz A, Sanz AI, Vaquero MT, Serra MT and Díaz-Ruíz JR (1993) The nucleotide sequence of the coat protein genes and 3′ non-coding regions of two resistance-breaking tobamoviruses in pepper shows that they are different viruses. Arch Virol 131: 75–88

Gil Ortega R and Luis Arteaga M (1992) Resistencia al virus del moteado suave del pimiento (PMMV) en pimiento. Phytoma 37: 15–19

Guillings M, Broadbent P, Indsto J and Lee R (1993) Characterization of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. J Virol Methods 44: 305–317

Logemann J, Schell J and Willmitzer L (1987) Improved method for the isolation of RNA from plant tissue. Anal Biochem 163: 16–20

Martelli GP and Quacquarelli A (1982) The present status of tomato and pepper viruses. Acta Hortic 127: 39–95

Pares RD (1985) A tobamovirus infecting *Capsicum* in Australia. Ann appl Biol 106: 469–474

Rast AThB (1988) Pepper tobamovirus and pathotypes used in resistance breeding. Capsicum Newsletter 7: 20–23

Rast AThB (1979) Pepper strains of TMV in the Netherlands. Med Fac Landbouww Rijksuniv Gent 44: 617–622

Rizos H, Gunn LV, Pares RD and Gillings MR (1992) Differentiation of cucumber mosaic virus isolates using the polymerase chain reaction. J Gen Virol 73: 2099–2103

Tenllado F, García-Luque I, Serra MT and Díaz-Ruíz JR (1994) Rapid detection and differentiation of tobamovirus infecting L-resistant genotypes of pepper by RT-PCR and restriction analysis. J Virol Methods 47: 165–174

Tenllado F, García-Luque I, Serra MT and Díaz-Ruíz JR (1995) *Nicotiana benthamiana* plants transformed with the 54 kDa region of the pepper mild mottle tobamovirus replicase gene exhibit two types of resistance responses against viral infection. Virology 211: 170–183

Tobias I, Rast AThB and Maat DZ (1982) Tobamoviruses of pepper, eggplant and tobacco: Comparative host reactions and serological relationships. Neth J Plant Path 88: 257–268

Wen F, Lister RM and Fattouh FA (1991) Cross-protection among strains of barley yellow dwarf virus. J Gen Virol 72: 791–799

Wetter C, Conti M, Altschuh D, Tabillion R and Van Regenmortel MHV (1984) Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily. Phytopathology 74: 405–410