The coat protein of tobamovirus acts as elicitor of both \( L^2 \) and \( L^4 \) gene-mediated resistance in \textit{Capsicum}

P. Gilardi,† I. García-Luque and M. T. Serra

Department of Plant Biology, CIB, CSIC, C/Ramiro de Maeztu 9, Madrid 28040, Spain

In \textit{Capsicum}, the resistance conferred by the \( L^2 \) gene is effective against all of the pepper-infecting tobamoviruses except \textit{Pepper mild mottle virus} (PMMoV), whereas that conferred by the \( L^4 \) gene is effective against them all. These resistances are expressed by a hypersensitive response, manifested through the formation of necrotic local lesions (NLLs) at the primary site of infection. The \textit{Capsicum} \( L^2 \) gene confers resistance to \textit{Paprika mild mottle virus} (PaMMV), while the \( L^4 \) gene is effective against both PaMMV and PMMoV. The PaMMV and PMMoV coat proteins (CPs) were expressed in \textit{Capsicum frutescens} (\( L^2 \)) and \textit{Capsicum chacoense} (\( L^4 \)) plants using the heterologous \textit{Potato virus X} (PVX)-based expression system. In \textit{C. frutescens} (\( L^2 \)), the chimeric PVX virus containing the PaMMV CP was localized in the inoculated leaves and produced NLLs, whereas the chimeric PVX containing the PMMoV CP infected the plants systemically. Thus, the data indicated that the PaMMV CP is the only tobamovirus factor required for the induction of the host response mediated by the \textit{Capsicum} \( L^2 \) resistance gene. In \textit{C. chacoense} (\( L^4 \) plants, both chimeric viruses were localized to the inoculated leaves and produced NLLs, indicating that either PaMMV or PMMoV CPs are required to elicit the \( L^4 \) gene-mediated host response. In addition, transient expression of PaMMV CP into \textit{C. frutescens} (\( L^2 \)) leaves and PMMoV CP into \textit{C. chacoense} (\( L^4 \)) leaves by biolistic co-bombardment with a \( β\)-glucuronidase reporter gene led to the induction of cell death and the expression of host defence genes in both hosts. Thus, the tobamovirus CP is the elicitor of the \textit{Capsicum} \( L^2 \) and \( L^4 \) gene-mediated hypersensitive response.

INTRODUCTION

The hypersensitive response (HR) is a generalized active response in plants against pathogens (Klement, 1982; Goodman & Novacky, 1994), resulting in rapid cell death and restriction of pathogen growth at the site of infection. Usually the HR is a highly specific event that depends on the matching specificity between a disease resistance gene (\( R \)) in the plant and an avirulence gene (\( avr \)) in the pathogen, a concept referred to as gene-for-gene resistance (Flor, 1971). A ligand–receptor model has been postulated to explain the molecular basis of gene-for-gene interactions (Keen, 1990; Gabriel & Rolfe, 1990). Molecular characterization of matched pairs of plant R proteins and pathogen Avr proteins has provided support for this model in different plant–pathogen systems (Scofield \textit{et al}., 1996; Tang \textit{et al}., 1996; Leister & Katagiri, 2000; Jia \textit{et al}., 2000). Another model, the so-called guard hypothesis (van der Biezen & Jones, 1998; Nimchuk \textit{et al}., 2003), postulates an indirect interaction between the Avr and the R proteins, in which other host factor(s) might be involved (Dangl & Jones, 2001; Schulze-Lefert, 2004). In this model, the Avr proteins correspond to virulence factors, such that the R proteins protect the key cellular targets of pathogen virulence factors. Thus, the R protein ‘guards’ the plant against pathogen attack, by detecting modifications in the host target of the pathogen virulence factor. Independent of the interaction mechanism between the elicitor and the R gene product, the recognition of a pathogen-derived elicitor and an R gene product is thought to trigger the signal transduction pathways leading to the HR defence response activation (Hammond-Kosack & Jones, 1996; Martin \textit{et al}., 2003). Recent work has shown that specific elicitor recognition is a more general event than expected and it has been postulated as an initial component of the extreme resistance conferred by either potato \( Rx \) or \( Ry \) genes against \textit{Potato virus X} (PVX) (Bendahmane \textit{et al}., 1999) or \textit{Potato virus Y} (Mestre \textit{et al}., 2000), respectively.

In \textit{Capsicum} \textit{spp}., the resistance against tobamoviruses conferred by the \( L \) genes is expressed as an HR that results in the induction of necrotic local lesions (NLLs) and virus confinement at the primary infection site (reviewed by...
Gilardi et al., 1999). It is governed by four seemingly allelic genes (L¹–L⁴), numbered in order of increasing effectiveness at the locus L (Boukema, 1980, 1982). Correspondingly, tobamoviruses have been classified in terms of increased pathogenicity as pathotypes P₁, P₁,₂ and P₁,₂,₃, based upon their ability to infect systemically Capsicum L¹, L² and L³ resistant plants, respectively (Rast, 1979).

In previous studies, it was shown that the elicitation of the L¹ and L² gene-mediated resistances required a functional coat protein (CP) from Tobacco mosaic virus (TMV) (P₀ pathotype) and Pepper mild mottle virus strain S (PMMoV-S) (P₁,₂ pathotype), respectively (Dardick et al., 1999; Berzal-Herranz et al., 1995). In addition, the PMMoV-S CP was identified as the elicitor of the L³ gene-mediated HR (Gilardi et al., 1998).

To provide further insight into Capsicum resistance mechanisms against tobamoviruses, we have extended our previous studies to identify the virus elicitors of Capsicum L² and L⁴ gene-mediated resistance.

The L² gene from Capsicum frutescens was the first virus resistance gene described in plants (Hollins, 1934). It is a dominant gene active against pepper tobamoviruses belonging to either P₀ or P₁ pathotypes. In contrast, viruses behaving as pathotypes P₁,₂ or P₁,₂,₃ are not recognized by the L² gene product and they infect L² plants systemically. In previous work, we showed that the functional CP of Paprika mild mottle virus (PaMMV) (P₁ pathotype) (García-Luque et al., 1993) was required for the elicitation of L² gene-mediated resistance in C. frutescens (de la Cruz et al., 1997). However, it remained an open question whether the induction of L² gene-mediated resistance by PaMMV CP required the presence of other tobamovirus genes.

The nature of the tobamovirus factors involved in activation of the Capsicum L⁴ resistance gene has not been established. This resistance gene from Capsicum chacoense Hunz. plants (Boukema, 1982) is active against all tobamoviruses known to infect pepper plants, resulting in the induction of NLLs. Here we report evidence showing that tobamovirus CPs are the elicitors in both Capsicum L² and L⁴ gene-mediated resistance. The identification of the tobamovirus-encoded Avr factor of Capsicum resistance genes may contribute to a better understanding of the recognition events required for the induction of the HR.

METHODS

Plant material, virus sources and inoculation procedure. Capsicum L.cv. Tabasco (L¹L²), C. chacoense Hunz. Cha2 (L¹L⁴) and Nicotiana benthamiana Domin were used as host plants. Plants were maintained in growth chambers at 25°C, with a 16 h photoperiod and a light intensity of 11 000 lux.

In vitro-growing plants were obtained from surface-sterilized seeds germinated on Murashige & Skoog (MS) medium. Plants were maintained in growth chambers at 23°C, with a 16 h photoperiod and a light intensity of 10 000 lux.

The origins of the tobamoviruses PaMMV, PMMoV-1 and PMMoV-S have been described previously (García-Luque et al., 1990, 1993; Wetter et al., 1984).

Carborundum-dusted leaves of plants were mechanically inoculated with purified virion preparations diluted in 20 mM sodium phosphate buffer, pH 7-0 (inoculation buffer). The first pair of true leaves at the two fully expanded leaf stage was inoculated. PaMMV was inoculated at a concentration of 15 μg ml⁻¹ and PMMoV at 1 μg ml⁻¹. For each inoculum, four to eight plants were used and experiments were repeated twice.

**Virus constructs and virus propagation.** The PVX-derived vector pPC2S (Chapman et al., 1992) as well as its derivatives pPVX-CP5 and pPVX-CP1 containing the CP gene of the S and I strains of PMMoV, respectively, have been described previously (Chapman et al., 1992; Gilardi et al., 1998).

For cloning PaMMV CP into the pPC2S vector, the 5'-flanking region of the PaMMV CP open reading frame was mutated by site-directed PCR mutagenesis so that no extra amino acids were present in the CP construct. The PaMMV CP gene was amplified from the plasmid pTHG-1 containing the 3′-terminal 1146 nt of PaMMV (de la Cruz et al., 1997) using oligonucleotides CPPa5 and CPPa3 as 5′ and 3′ primers, respectively. Oligonucleotide CPPa5′ (5′-TCTAGAATGCGTTATACGTGATCTTTG-3′) corresponded to the first 19 nt of the CP gene including the ATG start codon (underlined) to which a XbaI restriction site (bold) was added. Oligonucleotide CPPa3′ (5′-CCCTTGGATTAAGTGGGAAGGATAAACACT-3′) was complementary to nt 56–83 of the 3′ non-coding region. The amplified fragment was treated with the Klenow fragment of DNA polymerase and the 575 bp long fragment was cloned into the Xhol blunt site of plasmid pMJD82, thus obtaining the intermediate construct pT-CPpa. Correct nucleotide sequence and fragment orientation were corroborated by DNA sequence analysis. To make construct pPVX-CPpa, the Xbhl–blunt-SalI fragment of plasmid pT-CPpa was cloned into the unique EcoRV and Sall sites of plasmid pPC2S.

Viral RNAs were synthesized by in vitro transcription of Spel-linearized constructs with T7 RNA polymerase (Roche) and propagated directly in N. benthamiana plants as described previously (Berzal-Herranz et al., 1995).

At 5 days post-inoculation (p.i.), sap extracts from the systemically infected leaves were diluted to 1 μg PVX CP ml⁻¹ in inoculation buffer and used to inoculate carborundum-dusted leaves of C. frutescens L cv. Tabasco (L¹L²) and C. chacoense (L¹L⁴) plants. PVX CP content was determined by double-antibody sandwich (DAS)-ELISA, using a commercial kit (Roche).

**Constructs for transient expression.** Plant expression vector pMJD82 and the constructs pMJD82(GUS) containing the β-glucuronidase (GUS) reporter gene and pMJD82(ΔP) and pMJD82(ΔPS) containing the PMMoV-I and PMMoV-S CP genes, respectively, have been described previously (Dowson Day et al., 1994; Gilardi et al., 1998). To express the PaMMV CP transiently, the plasmid pMJD82(CPPa) was created by inserting the Xbhl–blunt-PstI fragment of plasmid pT-CPpa into the Ncol–blunt-PstI sites of plasmid pMJD82.

Expression of the inserted nucleotide sequences in the pMJD82 derivatives was controlled by the enhanced 35S RNA promoter of Cauliflower mosaic virus (CaMV).

The plasmid pDE1001gm1/GUS, which contains the GUS reporter gene under the control of an HR-inducible promoter from the 1.3–β-glucanase gm1 gene of Nicotiana plumbaginifolia (Castresana et al., 1990), was also used.
Viral CP detection. The accumulation of the viral CPs in plants was determined by DAS-ELISA and/or immunoblot analysis using specific antisera against PaMMV (Tenllado et al., 1995), PMMoV (Alonso et al., 1989) and PVX CPs (Roche). DAS-ELISA and immunoblot assays were carried out on total plant protein extracted from the inoculated and upper uninoculated leaves as described previously (Tenllado et al., 1995; Berzal-Herranz et al., 1995; Ruiz del Pino et al., 2003).

Viral RNA analysis. The accumulation of tobamovirus CP RNA in the inoculated and upper uninoculated leaves was assayed by RT-PCR. Total leaf RNA was extracted from the inoculated and upper uninoculated leaves at 5 and 15 days p.i., respectively, as described previously (Logemann et al., 1987) and subjected to RT-PCR amplification as described in Tenllado et al. (1994). The primers CPPa3’, and CPPa5’, and CP1 and CP2 (Tenllado et al., 1994), were used for the amplification of PaMMV and PMMoV RNAs, respectively. The resulting PCR products were electrophoresed in 0.8% agarose gels and stained with ethidium bromide.

Biologic transient expression assay. Five-week-old leaves from C. frutescens and C. chacoense plants grown in vitro were bombarded under the conditions described in Gilardi et al. (1998). After each bombardment, leaves were maintained on water-soaked filter paper in Petri dishes and kept in growth chambers at 25 °C with a 16 h photoperiod and a light intensity of 5000 lux. At 8, 16 and 24 h post-bombardment (p.b.), GUS activity was assayed histologically according to the method of Jefferson et al. (1987).

RESULTS

The PaMMV coat protein is an elicitor of L2-mediated resistance

To determine whether the PaMMV CP protein is the only tobamovirus factor responsible for the induction of L2 gene-mediated resistance, the CPs from PaMMV, PMMoV-S and PMMoV-I were expressed in C. frutescens (L2L2) plants using the PVX-derived vector pPC2S.

RNA transcripts generated in vitro from the three hybrid PVX constructs, pPVX-CPPa, pPVX-CPS and pPVX-CPI, as well as from the PVX control vector pPC2S, were initially propagated in N. benthamiana plants. The three chimeric viruses induced similar symptoms and accumulated to the same extent as wild-type PVX, as determined by DAS-ELISA using specific PVX CP antiserum.

By DAS-ELISA, it was also determined that the three tobamovirus CPs accumulated in both the inoculated and upper uninoculated leaves, albeit at lower levels than PVX CP: 30–60 ng PMMoV CP (μg PVX CP)−1 and 20–40 ng PaMMV CP (μg PVX CP)−1.

Sap extracts from N. benthamiana systemically infected leaves were inoculated in C. frutescens L. cv Tabasco (L2L2). At 5 days p.i., all Tabasco pepper plants inoculated with PVX-CPPa developed NLLs in the inoculated leaves that were similar to those induced by PaMMV. No symptoms developed in the uninoculated leaves of these plants during the time period assayed (up to 15 days p.i.). In contrast, inoculation with PVX-CPS, PVX-CPI and PVX did not result in the development of any visible symptoms in the inoculated leaves, but by 10 days p.i. the upper uninoculated leaves had developed mosaic symptoms, similar to those induced by PVX infection (summarized in Fig. 1a).

The presence of viral CPs was examined in these plants at 5 days p.i. in the inoculated leaves and at 15 days p.i. in the upper uninoculated leaves by immunoblot analysis. In PVX-CPPa-infected plants, both PaMMV and PVX CPs could be detected only in the inoculated leaves, whereas in PVX-CPS- and PVX-CPI-infected plants, the CPs from either PMMoV-S or PMMoV-I as well as from PVX were detected in both types of assayed leaves (Fig. 1b).

The presence of tobamovirus sequences in the C. frutescens L. cv Tabasco (L2L2) plants was further corroborated by RT-PCR analysis on total leaf RNA extracts. In PVX-CPPa-infected plants, a PaMMV-specific cDNA fragment of 575 bp was amplified from the RNA extracted from the inoculated leaves, but not from that extracted from the uninoculated upper leaves (Fig. 1b). However, in plants infected with either PVX-CPS or PVX-CPI, a PMMoV-specific cDNA fragment of 395 bp was amplified from both the inoculated and uninoculated leaves of the infected plants (Fig. 1b).

These data showed that the expression of the PaMMV CP in the genome context of an unrelated virus (PVX) conferred avirulence to the chimeric virus towards the Capsicum L2 resistance gene. Thus, the inoculation of PaMMV and PVX-CPPa in C. frutescens (L2L2) plants resulted in the development of NLLs and confinement of the chimeric virus to the inoculated leaves. Thus, the PaMMV CP can activate the Capsicum L2 gene-mediated resistance HR in the absence of other tobamovirus factors.

To analyse further whether the PaMMV CP was by itself sufficient to trigger the plant defence response mediated by the L2 resistance gene in the absence of other viral factor(s) and/or processes, biologic transient expression assays were conducted. The transient expression method employed was similar to those reported previously (Mindrinos et al., 1994; Gopalan et al., 1996; Gilardi et al., 1998; Tao et al., 2000). This assay makes use of the observation that induction of HR-associated cell death by the expression of an elicitor will reduce the accumulation of a GUS reporter gene co-expressed within the same plant cell when this gene is expressed under the control of the constitutive 35S promoter. In turn, the induction of defence gene expression associated with the HR will increase the accumulation of the GUS gene co-expressed with the elicitor within the same plant cell when the expression of the GUS gene is driven by a plant defence gene promoter. In this work, the HR-inducible promoter from the 1,3-β-glucanase gnl1 gene of N. plumbaginifolia (Castresana et al., 1990) was used to drive expression of the GUS gene.

To assess whether the PaMMV CP was able to activate cell death associated with the HR in C. frutescens (L2L2) leaves, plasmid pMJD82(GUS), expressing the GUS reporter gene
under the control of the 35S promoter, plus plasmid pMJJD82(CPPa), expressing the PaMMV CP, were co-introduced into *Capsicum* *frutescens* leaves by biolistic bombardment. As controls, *C. frutescens* leaves were also co-bombarded with plasmids pMJJD82(GUS) or pMJJD82(CPS) expressing the CP from PMMoV-S (the *L*2 virulent virus), GUS activity was assayed histologically at 8, 16 and 24 h p.b.

Blue spots (GUS activity) were observed at 16 and 24 h p.b. in leaves co-bombarded with either pMJJD82(GUS) or pMJJD82(CPS) (Fig. 2). However, no blue spots could be observed in leaves co-bombarded with constructs pMJJD82(GUS) plus pMJJD82(CPPa) at any time analysed (Fig. 2). Thus, the CP of PaMMV (the *L*2 avirulent virus), but not that of PMMoV-S (the *L*2 virulent virus), was able to activate cell death when produced inside cells of *L*2 resistant plants.

To analyse further the defence gene activation by PaMMV CP expression, we performed a similar co-bombardment experiment using the pDE1001 gn1/GUS plasmid, which contains the GUS reporter gene under the control of an HR-inducible promoter from the *N. plumbaginifolia* gn1 gene (Castresana et al., 1990; Gilardi et al., 1998). In these assays, GUS activity could only be observed on *C. frutescens* leaves co-transfected with pDE1001 gn1/GUS and either pMJJD82(CPPa) at 16 and 24 h p.b., and not in leaves co-bombarded with pDE1001 gn1/GUS plus either pMJJD82 or pMJJD82(CPS) (Fig. 2). These results indicated that only the expression of PaMMV CP activated the expression of the defence gene(s).

Thus, the above data are consistent with the conclusion that PaMMV CP is an elicitor of *Capsicum* *L*2 gene-mediated resistance and is able to induce a defence response when expressed in *C. frutescens* (*L*2) plants.

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**Fig. 1.** (a) Pathogenicity in *C. frutescens* cv. Tabasco (*L*2*)* of PVX and PVX-derived chimeric constructs containing CPs from PaMMV (PVX-CPPa) and PMMoV strains S (PVX-CPS) and I (PVX-CPI). A schematic representation of the hybrid viral genomes used in this study is shown on the left. The shadowed boxes correspond to PaMMV (CPPa), PMMoV-S (CPS) and PMMoV-I (CPI) CP genes. The symptom types induced on the inoculated plants are shown at the right of each construct. NLL, necrotic local lesions; S, systemic mosaic. (b) Detection of viral CPs and CP RNA sequences in *C. frutescens* cv. Tabasco (*L*2*) plants inoculated with PaMMV, PMMoV-S, PMMoV-I and PVX hybrid viruses (PVX-CPPa, PVX-CPS and PVX-CPI). For immunodetection of PaMMV, PMMoV and PVX CPs in the inoculated (I) and upper uninoculated (S) leaves of inoculated *C. frutescens* plants, plant proteins were extracted at 5 days p.i. for the inoculated leaves and 15 days p.i. for the upper uninoculated leaves and assayed for the presence of viral CPs. PaMMV and PMMoV CPs were detected with PaMMV- and PMMoV-specific antisera, respectively, and PVX CP was detected with a commercial antiserum (Roche). CP bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). The positions of tobamovirus (cp) and PVX (PVX cp) CPs are indicated on the left. For RT-PCR analysis of PaMMV and PMMoV-S and -I CPs, total leaf RNA was extracted at 5 or 15 days p.i. as above and amplified using PaMMV- and PMMoV-specific primers. Amplified DNA fragments (*) were electrophoresed on 0.8% agarose gels and visualized under UV light after ethidium bromide staining.
These results were in agreement with those reported previously on the identification of the tobamovirus CP as the elicitor of the Capsicum L^2 gene-mediated resistance (Gilardi et al., 1998) and prompted us to analyse whether the tobamovirus CP is also an elicitor of the resistance conferred by the Capsicum L^4 gene.

The PaMMV and PMMoV coat proteins are elicitors of L^4-mediated resistance

Capsicum L^4 plants are resistant against all tobamoviruses tested so far. As there are no tobamoviruses able to infect systemically L^4 plants, it is not possible to use chimeric viruses formed from HR-eliciting and HR-non-eliciting tobamovirus strains, an approach used previously to identify the viral factor(s) required to activate Capsicum L^2 and L^3 gene-mediated resistance (de la Cruz et al., 1997; Berzal-Herranz et al., 1995; Tsuda et al., 1998). However, the use of PVX-derivatives and transient expression systems allowed the identification of the tobamovirus avr gene(s) corresponding to the Capsicum L^4 resistance gene.

Thus, C. chacoense Hunz. cv. Cha2 (L^4L^4) plants were inoculated with sap extracts from N. benthamiana plants infected with the PVX derivatives PVX-CPPa, PVX-CPS and PVX-CPI, as well as with PVX. In addition, to compare the symptoms induced by the chimeric viruses with their parental tobamoviruses (PaMMV, PMMoV-S and PMMoV-I, respectively), plants were inoculated on opposite halves of the leaves with each chimeric virus and with the corresponding purified parental tobamovirus.

At 5 days p.i., plants inoculated with each of the seven viruses except PVX developed NLLs on the inoculated leaves. Although phenotypically similar, NLLs induced by the PVX derivatives were more defined and regular in shape than those produced by their parental tobamoviruses (summarized in Fig. 3a, and data not shown). No systemic symptoms were visible at this time in any of the infected plants. By 15 days p.i., only plants infected with PVX and PVX-CPI displayed systemic mosaic symptoms.

The presence of viral proteins and RNAs was examined in the inoculated and upper uninoculated leaves of these plants at 5 and 15 days p.i., respectively, by immunoblot and RT-PCR assays. Immunoblot analysis using specific CP antisera revealed the presence of the tobamovirus CPs only in the inoculated leaves of plants inoculated with either the chimeric viruses or the parental tobamoviruses. No tobamovirus CP was detected in the upper leaves (Fig. 3b). However, PVX CP was detected in the upper leaves of plants infected with either PVX or PVX-CPI, although PMMoV-I CP was not detected in these upper uninoculated leaves (Fig. 3b).

These results were confirmed by RT-PCR analysis on total leaf RNA extracted from the inoculated and upper uninoculated leaves (Fig. 3b). As in the immunoblot assay, no PMMoV-I sequences were detected in the upper uninoculated leaves of plants infected with the chimeric PVX-CPI, although the PVX CP was readily detected in these leaves.
These data indicated that the PVX derivative had lost the PMMoV-I inserted sequence, probably as a result of recombination events that had taken place during virus propagation in *N. benthamiana* and/or *C. chacoense* (Chapman et al., 1992).

From these data, it could be inferred that tobamovirus CPs expressed through the PVX vector conferred the same behaviour on the hybrid viruses as the corresponding tobamovirus towards the *Capsicum L*4 gene-mediated resistance. In addition, they showed that only the chimeric viruses expressing the tobamovirus CPs were localized at the infection sites. In contrast, PVX molecules that had lost these sequences were not affected by the *L*4 gene-mediated resistance, which in turn indicated the specificity of this host resistance response.

Thus, the tobamovirus CP is a factor in the tobamovirus genome required to activate the *L*4 gene-mediated resistance against tobamoviruses. Thus, the PaMMV, PMMoV-S and PMMoV-I CP genes correspond to the *avr* genes of the *Capsicum L*4 resistance gene. Indeed, the finding that the three tobamovirus CPs, when expressed through the PVX vector, induced similar NLLs suggests that the three proteins are equally effective as elicitors.

To examine further whether the tobamovirus CPs by themselves are sufficient to trigger the plant defence response mediated by the *L*4 gene in *C. chacoense* plants, the PMMoV-I CP was transiently expressed in this host. PMMoV-I (pathotype *P*1,2,3) is the most virulent tobamovirus against the *Capsicum L* resistance genes, since it systemically infects *L*1–*L*3 *Capsicum* spp. resistant plants (Wetter et al., 1984; García-Luque et al., 1993).

In these experiments, leaf cells of 5-week-old *C. chacoense* plants were co-bombarded with plasmids pMJD82(CPI), expressing the PMMoV-I CP, and pMJD82(GUS). As a control, leaves were co-transfected with plasmids pMJD82(GUS) and pMJD82. GUS activity was assayed histologically at 8, 16 and 24 h p.b. In this assay, blue spots were observed at both 16 and 24 h p.b. in leaves co-bombarded with pMJD82(GUS) and pMJD82 (Fig. 4). However, no blue spots were observed in leaves co-bombarded with pMJD82(GUS) and pMJD82(CPI) at any time point analysed (Fig. 4). Thus, transient expression of PMMoV-I CP was sufficient to induce cell death when produced inside cells of *L*4 resistant plants.

Finally, to assess defence gene activation by PMMoV-I CP expression in *C. chacoense* cells, leaves were co-transfected with plasmid pDE1001gnl/GUS plus pMJD82(CPI) or pDE1001gnl/GUS plus pMJD82. GUS activity could only be observed in *C. chacoense* leaves co-transfected with pDE1001gnl/GUS plus pMJD82(CPI), at 16 and 24 h p.b. (Fig. 4). These data revealed that the expression of the PMMoV-I CP alone leads to transcriptional activation of the reporter gene when expressed under the control of a defence gene promoter.
Thus, the data showed that PMMoV-I CP induces cell death and transcriptional activation of defence genes in *Capsicum L*² plants, acting as an elicitor of the *Capsicum L*⁴ gene-mediated resistance.

**DISCUSSION**

The expression of the PaMMV CP in a heterologous virus (PVX) allowed identification of the CP as the only tobamovirus factor required for the induction of the *Capsicum L*² gene-mediated resistance HR. Thus, the data corroborate and expand previous work (de la Cruz et al., 1997), ruling out the possibility that another viral factor encoded by the tobamovirus genome is required to activate *L*² gene-mediated resistance.

Similarly, the heterologous expression of the CPs from either PaMMV or PMMoV in the context of the PVX genome showed that either tobamovirus CP could activate the HR associated with the resistance conferred by the *Capsicum L*⁴ resistance gene.

In both hosts, expression of the tobamovirus CP conferred on the chimeric virus the pathogenic behaviour of the parental tobamovirus towards the *L*² and *L*⁴ resistance genes. Thus, in *Capsicum L*²⁴ plants, infection with either the avirulent virus PaMMV or its PVX-derivative, PVX-CPPa, resulted in the development of NLLs, with confinement of the chimeric virus to the inoculated leaves. In contrast, infection by the virulent viruses – the S and I strains of PMMoV and their corresponding chimeric viruses, PVX-CPS and PVX-CPI – was systemic in this host. In *Capsicum L*²⁴⁴ plants, infection with the PVX-derivatives containing the PaMMV and PMMoV CPs was associated with the formation of NLLs and restriction of the viruses to the inoculated leaves, thus reproducing the avirulence properties of their parental tobamovirus.

In addition, biolistic transient expression of the avirulent PaMMV CP in *Capsicum L*²⁴ leaves allowed its identification as an elicitor of the HR mediated by the *L*² resistance gene. Its expression, but not that from the virulent PMMoV-S, induced cell death and transcriptional activation of defence genes. Similarly, the CP from the avirulent PMMoV-I was identified as an elicitor of the *Capsicum L*⁴ gene-mediated resistance. In previous work, it was shown that the transient expression of PMMoV-I CP in *C. chineense* leaf cells did not activate the *Capsicum L*² gene-mediated resistance (Gilardi et al., 1998). Taken together with the data shown here, it is possible to conclude that PMMoV-I CP activates the host resistance response in a gene-for-gene-specific manner, depending upon the presence in the plant of the *Capsicum L*⁴ resistance gene. Therefore, PMMoV-I CP acts as a specific elicitor of the *Capsicum L*⁴ gene-mediated resistance.

The transient expression assay employed here has been used successfully for the functional analysis of *avr* genes from bacteria (Gopalan et al., 1996; Leister et al., 1996), fungi (Jia et al., 2000) and viruses (Gilardi et al., 1998).

In accordance with previous work (Mindrinos et al., 1994; Gopalan et al., 1996; Gilardi et al., 1998; Leister et al., 1996; Jia et al., 2000), the co-expression of the elicitor in cells from non-compatible hosts repeatedly resulted in a failure of or decrease in GUS activity when the GUS reporter gene was expressed under the control of the CaMV 35S promoter. The decrease in GUS activity has been associated with activation of cell death associated with the HR induction. A non-specific inhibition of protein accumulation during HR activation has been proposed to account for the decline in GUS activity (Tao et al., 2000). However, in incompatible interactions, an increase in GUS activity is observed in co-expression assays where the GUS reporter gene is expressed in the presence of the elicitor CP (Gilardi et al., 1998; this work) but under the control of a defence gene promoter (gene gn1; Castresana et al., 1990). Therefore, neither a non-specific degradation of GUS mRNA nor a non-specific degradation of GUS protein due to HR induction are seemingly responsible for the observed inhibition of GUS activity. Instead, transcriptional inhibition of the CaMV 35S promoter could explain the reduced GUS activity observed, as a consequence of the elicitation of the HR. Thus, it is possible that HR-associated cell death
activation could lead to the depletion or inactivation of transcription factors required for the CaMV 35S promoter-driven transcription.

Our data show that the tobamovirus CPs are elicitors of the HR mediated by the \( I^2 \) and \( L^4 \) genes independent of other viral factors and/or processes. Thus, the four \( L \) gene-mediated resistances are activated by the viral CP. Since none of these genes has yet been cloned, it is not known whether triggering of the host defence response requires a direct or indirect interaction of the elicitor CP with its matching \( L \) resistance gene. The specific features of the tobamovirus CP required for eliciting the \textit{Capsicum L} gene-mediated resistances are currently unknown (Berzal-Herranz et al., 1995; de la Cruz et al., 1997; Gilardi et al., 1998; Tsuda et al., 1998; Dardick et al., 1999). The finding that distinct tobamovirus CPs – sharing overall amino acid sequence identities of \( \sim 60\% \) scattered along the entire protein – activate the resistance conferred by the same resistance gene, \( L^i \), could be indicative that in \textit{Capsicum} the \( L \) resistance genes have evolved to recognize an amorphous structural configuration of the tobamovirus CPs. Recognition of structural elements in the tobamovirus CP seems to be a common phenomenon for both \textit{Nicotiana} \( N^p \) and \textit{Capsicum L} resistance genes (Culver, 2002). This type of recognition would lead to a more durable and efficient resistance.

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