Chimeras between *Oilseed rape mosaic virus* and *Tobacco mosaic virus* highlight the relevant role of the tobamoviral *RdRp* as pathogenicity determinant in several hosts

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SUMMARY

Oilseed rape mosaic virus (ORMV) is a tobamovirus taxonomically distinct from the type member of the genus, *Tobacco mosaic virus* (TMV). Both viruses display a specific host range, although they share certain hosts, such as *Arabidopsis thaliana*, *Nicotiana benthamiana* and *N. tabacum*, on which they induce different symptoms. Using a gain-of-symptom approach, we generated chimeric viruses, starting from a TMV infectious clone, over which different regions of ORMV were exchanged with their corresponding regions in the TMV genome. This approach allowed the association of pathogenicity determinants to certain genes within the ORMV genome. A general trend was observed associating the viral origin of the RNA-dependent RNA-polymerase (*RdRp*) gene and the gain of symptoms. In *A. thaliana* and *N. benthamiana*, chimeric viruses were unable to reproduce the symptoms induced by the parental viruses, leading to disease states which could be described as intermediate, and variable in some cases. In contrast, a hypersensitive reaction caused by both of these viruses on *N*-gene-bearing tobaccos could be found in resistance reactions to all chimeric viruses, suggesting that the avirulence determinant maps similarly in both viruses. A systemic necrotic spotting typical of non-*N*-gene tobaccos infected with ORMV was associated with the polymerase domain of *RdRp*. To our knowledge, this is the first time that this controversial portion of the tobamovirus genome has been identified directly as a pathogenicity determinant. None of the reactions of the chimeric viruses could be correlated with increases or decreases in virus titres in the infections.

INTRODUCTION

When a virus interacts with a plant, the infection often produces visible changes in the plant. Symptoms are variable and depend on the virus, the plant and the environment (Culver and Padmanabhan, 2007). The interaction is a complex process, but sometimes a simple association of certain symptoms with certain viral structures is possible. For example, transgenic tobacco plants that express protein VI of *Cauliflower mosaic virus* reproduce the symptoms of the viral infection in this host (Baughman *et al.*, 1988); moreover, there is a direct relationship between a non-coding region of the *Tobacco vein mottle virus* and the symptoms produced by this virus (Rodríguez-Cerezo *et al.*, 1991), and also between the coat protein of *Tobacco mosaic virus* (TMV) and the appearance of a hypersensitivity response (HR) in eggplant (Culver and Dawson, 1991). Therefore, on occasions, the molecular characterization of a specific virus–plant interaction is possible by means of the presence or absence of a viral pathogenicity determinant and the visual observation of the characteristic associated symptoms.

Tobamoviruses are mechanically transmissible rod-shaped viruses. The genomic RNA consists of three open reading frames (ORFs) from which at least four protein products are made: viral components of the polymerase (the largest formed by readthrough of a leaky termination codon) or *RdRp* (126k and 183k), the movement protein or MP (30k), and the coat protein or CP (17.5k) (Hull, 2002). MP and CP are translated from their corresponding subgenomic RNAs. The type member of the genus is TMV (Gibbs, 1977).

Three phylogenetic subgroups have been differentiated in the tobamoviruses depending on the amino acid composition of MP: subgroup 1, with viruses isolated from solanaceous plants, such as TMV or *Tomato mosaic virus* (ToMV); subgroup 2, with viruses isolated from several dicotyledonous plants, such as *Cucumber green mild mottle virus* (CGMMV) or *Sunn-hemp mosaic virus* (SHMV); and subgroup 3, with viruses isolated from cruciferous plants, such as *Oilseed rape mosaic virus* (ORMV) or *Turnip*

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vein-clearing virus (Lartey *et al.*, 1996; Melcher, 2003). With regard to the ORF array in completely sequenced viruses, in subgroup 1 there are viruses with ORFs separated by small intergenic regions, such as *Pepper mild mottle virus*, or with an overlapping region between *RdRp* and MP, such as TMV and ToMV. In subgroup 3, there is always an overlap between MP and CP ORFs, as in ORMV. Finally, ORF organization in subgroup 2 is variable, from the absence of overlap, as in *Cucumber fruit mottle mosaic virus*, to double overlap, as in CGMMV or SHMV. In this study, we used two tobamoviruses with different ORF organization: TMV from subgroup 1, with an overlap between *RdRp* and MP, and ORMV from subgroup 3, in which MP and CP overlap.

ORMV is a tobamovirus able to infect cruciferous and solanaceous plants, giving rise to very characteristic symptoms (Aguilar *et al.*, 2000; Martín Martín *et al.*, 1997). The structural similarity between ORMV (Aguilar *et al.*, 1996) and TMV, and the availability of shared hosts between the two viruses, such as *Arabidopsis thaliana*, *Nicotiana tabacum* and *N. benthamiana* (inducing different symptoms), open the way to the characterization of tobamovirus pathogenicity determinants through the use of molecular chimeras between the two virus genomes.

RESULTS

Different disease symptoms are induced by TMV and ORMV in their common hosts

An infectious clone of TMV (TMV-KK1) was used throughout this work. No symptom differences were found between plants infected with this infectious clone and TMV-U1-infected *Nicotiana* sp. plants (results not shown; Lehto and Dawson, 1990). The TMV sequence in this clone includes a *XhoI* restriction site in position 4904, just upstream from the MP ORF (Lehto and Dawson, 1990). ORMV reference symptoms were evaluated in plants inoculated with sap from ORMV-infected *Nicotiana* sp. plants or viral RNA.

In order to compare the disease symptoms induced by these viruses, several host plants common to both viruses were inoculated with crude sap from *N. benthamiana* plants infected with the corresponding *in vitro*-transcribed (TMV) or viral (ORMV) RNA (Table 1). Both viruses induced necrotic local lesions, typical of the resistance reaction governed by the *N*-gene (Dinesh-Kumar *et al.*, 2000), on the inoculated leaves of *N. tabacum* cv. Xanthi nc 3–4 days post-inoculation (dpi), and the early death of young plants of *N. benthamiana* (four- to six-leaf developmental stage) or systemic mosaic in older plants. The necrotic local lesions induced by ORMV were similar to those produced by TMV, but with a diameter of approximately 0.5 mm larger (Fig. 1A, 1/2). The inoculation of *N. tabacum* cv. Samsun with TMV induced a systemic mosaic with green islands around 7 dpi. ORMV symptoms in this cultivar consisted of a systemic mosaic, always accompanied by systemic necrotic spotting, developing around 7 dpi. In *A. thaliana*, ecotypes RLD and Can-0, inoculation with TMV did not induce any visible symptoms (Aguilar *et al.*, 2000; Dardick *et al.*, 2000). However, ORMV induced chlorosis and dwarfism in RLD, and systemic necrosis and early death in Can-0 (Martín Martín *et al.*, 1997).

Construction of chimeric TMV/ORMV viruses

The identification of ORMV-specific pathogenicity determinants was approached through a systematic replacement strategy of ORMV genomic regions over TMV-KK1. No ORMV infectious clone was available during this work, and so it was decided that only the coding and intergenic regions of ORMV would be used for replacement in this study, leaving all original regulatory signals present in the non-coding 5' and 3' untranslated regions (UTRs). Consequently, UTRs in the chimeric constructs were always of TMV origin.

We first generated two reciprocal chimeras, named MC and R (Fig. 2A,B). The names of the chimeras are intended to inform

Table 1 Symptoms induced by *Oilseed rape mosaic virus* (ORMV), *Tobacco mosaic virus* (TMV) and chimeras MC and R (see 'Experimental procedures') in the assayed plants. Plants were inoculated with the infectious transcripts or with sap from *Nicotiana benthamiana* plants previously inoculated with the appropriate virus.

Plant	Symptoms			
	ORMV	TMV	Chimera MC	Chimera R
<i>N. tabacum</i> cv. Xanthi nc	Necrotic local lesions in the inoculated leaves	Necrotic local lesions in the inoculated leaves	Necrotic local lesions in the inoculated leaves	Necrotic local lesions in the inoculated leaves
<i>N. tabacum</i> cv. Samsun	Systemic mosaic and necrotic spotting	Systemic mosaic	Systemic mosaic, emergence delayed with respect to ORMV and TMV	Systemic necrotic spotting, emergence delayed with respect to ORMV
<i>N. benthamiana</i>	Early plant death	Early plant death	Systemic mosaic	Systemic mosaic, development delayed with respect to MC
<i>A. thaliana</i> RLD ecotype	Yellowing and dwarfism	No symptoms	Variable symptoms: yellowing in some inoculated leaves, plants without symptoms	Systemic symptoms ORMV-like, delayed systemic emergence
<i>A. thaliana</i> Can-0 ecotype	Systemic necrosis and early plant death	No symptoms	Variable symptoms: light rosette dwarfism in some plants	Systemic yellowing evolving to necrosis, emergence delayed with respect to ORMV

A. *N. tabacum* cv. Xanthi nc **B. *N. tabacum* cv. Samsun** **C. *N. benthamiana*** **D. *A. thaliana* ecotype RLD** **E. *A. thaliana* ecotype Can-0**

Fig. 1 Symptoms induced by the inoculation of *Oilseed rape mosaic virus* (ORMV) (1), *Tobacco mosaic virus* (TMV) (2) and chimeras MC (3) and R (4) in *Nicotiana tabacum* cv. Xanthi nc (A), *N. tabacum* cv. Samsun (B), *N. benthamiana* (C) and RLD (D) and Can-0 (E) ecotypes of *Arabidopsis thaliana* plants. B4 is a close-up view of necrotic lesions. Bars in panels A1/2, A3/4 and B4 correspond to 5 mm. Photographs were taken in the week after symptoms appeared, between 7 and 50 days post-inoculation depending on the host–virus combination. See text for details.

Fig. 2 Genetic diagram of parental (A) and chimeric (B, C) viruses. Genes encoding for viral proteins: CP, coat protein; MP, movement protein; RdRp, viral replicase. Regions from *Tobacco mosaic virus* (TMV) are in light grey and regions from *Oilseed rape mosaic virus* (ORMV) are in dark grey.

about the ORMV genomic sequence replacing the corresponding sequence of TMV (MC, movement protein and coat protein; R, RNA-dependent RNA-polymerase). In chimera MC, the overlap between MP and CP, originally present in ORMV, was maintained. In chimera R, an intergenic region of six nucleotides between RdRp and MP ORFs, not present in TMV (Lehto and Dawson, 1990), was created (see 'Experimental procedures').

Infectivity of chimeras MC and R

The ability to infect common hosts was assayed for chimeras MC and R in parallel with the parental viruses under the same conditions.

Molecular detection

The detection of chimeras in non-inoculated leaves showed that these viruses could replicate, move and accumulate in the assayed plants, and so the chimeric viruses were infectious.

Molecular detection of progeny viruses derived from chimeric inocula was performed by immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR). The amplicons selected for identification are shown in Fig. S1 (see 'Supporting information'). Primers T1 and T2 amplified a 407-bp fragment present in the RdRp pre-readthrough region in TMV and in chimera MC. An ORMV-specific fragment of this cistron was amplified with primers O1 and O2 (784-bp amplicon). An amplification reaction with primers T1 and O3 of a 1113-bp fragment allowed chimera MC (positive amplification) to be distinguished from TMV in infected plants (negative amplification). Amplification of a 1423-bp fragment with primers O1 and T3 allowed chimera R (positive amplification) to be differentiated from ORMV in infected plants (negative amplification) (see Fig. S1).

Symptoms produced by chimeric viruses

Both chimeras maintained their ability to produce necrotic local lesions in the inoculated leaves of *N*-gene tobacco plants. The diameters of the lesions were smaller than for parental viruses [1–2 mm (Fig. 1A, 3/4) compared with 3–4 mm of TMV and

ORMV (Fig. 1A, 1/2)]. Lesions were first observed as chlorotic spots (4–5 dpi), later evolving to necrotic lesions (7–8 dpi). The induced necrotic local lesions were also a symptom of virus resistance to the chimeras, as no systemic lesions or symptoms of any kind were detected in these plants, which were shown to be virus free in non-inoculated leaves at the temperature used for the experiments (21–23 °C, results not shown).

The inoculation of non-*N*-gene *N. tabacum* cv. Samsun with chimera MC induced a systemic mosaic (Fig. 1B, 3; Table 1), whose manifestation was delayed by about 15 days with respect to TMV or ORMV (Fig. 1B, 1 and 2). Plants inoculated with chimera R developed necrotic spotting similar to ORMV (Fig. 1B, 4; Table 1), which was also similarly delayed with respect to that induced by the parental virus.

In the case of young plants of *N. benthamiana*, the chimeras induced a systemic mosaic (Fig. 1C, 3 and 4), chimera MC being delayed with respect to chimera R (around 35 and 25 dpi, respectively). Unlike TMV and ORMV, the chimeras did not induce the early death of young plants (Fig. 1C, 1a and 2a; Table 1). The mosaic was similar to that produced by the parental viruses when inoculated onto plants at the developmental stage of 10–12 leaves (Fig. 1C, 1b and 2b).

Arabidopsis plants, RLD and Can-0 ecotypes, gave non-uniform responses when inoculated with the chimeras (Table 1, Fig. 1). In the case of chimera MC, symptoms were milder, with a large number of non-symptomatic plants (Fig. 1D, 3a), as with TMV (Fig. 1D, 2). Symptoms included chlorosis of the inoculated leaves in RLD plants (Fig. 1D, 3b) and light dwarfism in some Can-0 plants (Fig. 1E, 3). When chimera R was assayed, necrosis was observed in inoculated and some systemic leaves (Fig. 1E, 4a and 4b). This symptom was characteristic of ORMV infection (Martín Martín *et al.*, 1997). Chimeras showed delayed symptom development in both ecotypes, symptoms appearing 15–25 days later.

The gain of symptoms associated with ORMV infection was observed with chimera R in both *Nicotiana* and *Arabidopsis*. Thus, overall, symptoms produced by these viral infections seem to be associated with the RdRp origin.

Systemic necrotic spotting induced by ORMV in tobacco maps to the *RdRp* polymerase domain

As the systemic necrotic spotting symptom produced by ORMV in non-*N*-gene tobacco plants is associated with the presence of *RdRp* of this virus, we decided to extend our analysis into the different domains of this large viral cistron, and developed a partial R chimera. The R-54k chimera created had a hybrid TMV–ORMV *RdRp* gene, and *MP* and *CP* genes of TMV (Fig. 2A,C). In other words, it is a TMV, with an ORMV polymerase domain instead. The intergenic regions are as in chimera R.

This chimera was inoculated onto *Nicotiana* plants of the same cultivars as used previously. In *N. tabacum* cv. Xanthi nc, R-54k induced necrotic local lesions on the inoculated leaves, similar to the parental viruses and chimeras MC and R. The sizes of these lesions were 2–3 mm in diameter, thus returning to the normal size of the lesions produced by the individual parental viruses separately (Fig. 3A). In *N. tabacum* cv. Samsun, systemic necrotic spotting was induced by this chimera (Fig. 3B, 1 and 2), with delayed development (around 20–30 days later) in all infected plants. Finally, in *N. benthamiana*, R-54k produced a delayed systemic mosaic (Fig. 3C, 1 and 2), similar to that obtained with chimeras MC and R in this plant.

Viral titres by quantitative RT-PCR

The symptoms produced by infection with the chimeric viruses were systematically milder than those caused by the parental viruses. An immediate question was whether this general decrease in symptom intensity was related to the viral titres attained. To approach this question, a relative quantification of parental and chimeric viruses was carried out in the inoculated leaves using quantitative RT-PCR. The quantification assay was performed in extracts of inoculated *N. tabacum* and *N. benthamiana* leaves at 14 dpi (Fig. 4). Quantification using PCR was based on the threshold cycle (Ct) value, the cycle at which a significant

increase in fluorescence takes place. Thus, the lower the Ct value, the higher the viral concentration in the sample (Lunello *et al.*, 2004; Mumford *et al.*, 2000).

No significant differences were found in the accumulation of ORMV or TMV in *N. benthamiana* or *N. tabacum*. The accumulation of chimera MC was lower than that of the rest of the assayed viruses, whereas chimera R presented a higher accumulation level. The accumulation of chimera R-54k was closer than the accumulation of the other chimeras to that of the parental viruses, slightly lower in *N. tabacum*, but only significantly different with respect to TMV. In all assayed viruses, the accumulation level was higher in *N. benthamiana* than in *N. tabacum* plants.

DISCUSSION

ORMV and TMV are two different tobamoviruses sharing common hosts, such as *N. benthamiana*, *N. tabacum* and *A. thaliana*. In some of these plants, they produce different symptoms, as in *N. tabacum* cv. Samsun and some *A. thaliana* ecotypes. Thus, we aimed to identify the pathogenicity determinants associated with the symptoms induced during the development of infection with these viruses.

In order to associate the different symptoms produced by both viruses with specific viral genetic regions, we prepared chimeric viruses, an approach extensively used in tobamoviruses, mostly by the exchange of TMV fragments with other tobamoviruses (Arce-Johnson *et al.*, 2003; Chen *et al.*, 1996; Deom *et al.*, 1994; Padgett *et al.*, 1997; Saito *et al.*, 1987; Zhang *et al.*, 1999). All chimeras tested were infectious in the assayed hosts.

The inoculation of the chimeras in *A. thaliana* showed that the symptoms induced by chimera R were more similar to those of ORMV than those of chimera MC. A similar result has been reported for TMV in experiments interchanging the MP gene between TMV and a cruciferous tobamovirus (named TMV-Cg, a misleading name for a virus that is not TMV) (Arce-Johnson *et al.*,

A. *N. tabacum*
cv. Xanthi nc

B. *N. tabacum*
cv. Samsun

C. *N. benthamiana*

Fig. 3 Symptoms produced by the chimera R-54k in *Nicotiana tabacum* cv. Xanthi nc (A), *N. tabacum* cv. Samsun (B) and *N. benthamiana* (C) plants.

Fig. 4 Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) in inoculated leaves of *Nicotiana tabacum* and *N. benthamiana* 14 days post-inoculation with Oilseed rape mosaic virus (ORMV), Tobacco mosaic virus (TMV) and chimeras MC, R and R-54k. Ct, threshold cycle.

2003). Therefore, it seems that, in this host plant, symptoms are more strongly influenced by the *RdRp* origin than by the *MP* and *CP* origins. However, the origin of this viral ORF is not an exclusive determinant of the symptoms associated with ORMV infection in *A. thaliana*, because chimera R did not reproduce exactly ORMV-induced symptoms.

In the infection of *N. tabacum* cv. Xanthi nc, no differences were found between the symptoms produced by chimeras MC and R. All chimeras retained the parental ability to produce necrotic lesions in the inoculated leaves. In TMV, the elicitor for the hypersensitive response to the *N*-gene has been mapped to the viral helicase domain (Erickson *et al.*, 1999; Padgett and Beachy, 1993; Padgett *et al.*, 1997). All our chimeras reproduced this response; therefore, it is probable that the ORMV *N*-gene elicitor also maps in the polymerase domain, as in TMV.

The chimeras produced a systemic mosaic in young plants of *N. benthamiana*, instead of plant death caused by parental viruses. Quantitative measurements of the levels of virus accumulation in the inoculated leaves did not support a somewhat trivial explanation of an increased fitness of parental viruses with respect to chimeras. Thus, although chimera MC showed substantially less accumulation than any of the parental viruses, chimera R accumulated more extensively, and no significant differences could be established for chimera R-54k. We believe that the explanation may be found by exploring the notion of potential complex disease elicitors whose components could map to different regions in the viral genome, all being required for the induced phenotype.

The appearance of necrotic spotting, characteristic of ORMV infection, in *N. tabacum* cv. Samsun was only reproduced with chimera R, but not with chimera MC. These results are consistent with those obtained using *A. thaliana* as a host, in that the symptoms produced by the chimeras were significantly more influenced by the *RdRp* viral origin. In tobacco plants, the presence of this ORF is necessary and sufficient to produce the different symptoms associated with each parental virus.

As ORMV symptom similarity in non-*N*-gene tobacco correlated with the presence of the ORMV *RdRp* gene in the chimeras, a partial chimera of chimera R was made: chimera R-54k. The tobamoviral *RdRp* gene has three different domains: the methyltransferase domain, corresponding to the protein region with guanylyl-transferase activity required for CAP 5' synthesis, the helicase domain, with conserved helicase motifs, and the polymerase domain (54k or Pol), proposed as the RNA-dependent RNA-polymerase domain (Goregaoker and Culver, 2003). Chimera R-54k is actually a hybrid TMV–ORMV *RdRp*, in which the TMV polymerase domain is interchanged with the corresponding region of ORMV. The systemic necrotic spotting found with chimera R, and with the parental virus ORMV, was also obtained with chimera R-54k, and so the genetic determinant for this symptom was localized at the polymerase domain.

Another cruciferous tobamovirus, TMV-Cg, is able to induce symptoms similar to those found for ORMV and chimeras R and R-54k (Stange *et al.*, 2004). However, these TMV-Cg symptoms are induced in *N. tabacum* cv. Xanthi, not in cv. Samsun, like ours. TMV-Cg symptoms in cv. Xanthi have been proposed to be an HR of the plant (Stange *et al.*, 2004). These authors have identified a resistance-like gene to tobamoviruses (*NH*), a homologue of the *N*-gene. Their proposal is that the HR could correspond to a response not associated with the ability to limit virus spread, possibly because of the absence of alternative RNA splicing in plants carrying the *NH* gene. If the necrotic spotting observed in *N. tabacum* cv. Samsun with ORMV is considered to be an HR similar to that produced by TMV-Cg in cv. Xanthi, these situations could, in fact, be similar. The CP of TMV-Cg has been proposed as an elicitor of the HR-like response (Ehrenfeld *et al.*, 2008). In our case, it was clear that the polymerase domain of ORMV *RdRp* was the elicitor of systemic necrotic spotting.

The gene encoding ORMV *RdRp* codes for two proteins: 125k, including the methyltransferase and helicase domains, and 182k, including the polymerase domain. In TMV, the polymerase domain has been the subject of long controversy about its possible existence as an individual protein (54k). The protein itself has never been found in infected plants, but the presence in infected tissue of a subgenomic RNA (*I*₁) encoding the protein (Palukaitis *et al.*, 1983), and the fact that *I*₁ produces 54k when translated *in vitro* (Sulzinski *et al.*, 1985), provide a basis to support its existence. Such an analysis has not been performed for ORMV, and so we cannot formally eliminate the possibility that a

54k-like protein (or its encoding RNA) could be involved in the induction of necrotic spotting.

The polymerase domain of TMV, within the 183k protein, has been proposed to be part of a higher structure: an oligomer of the 126k and 183k proteins, with a 5 : 1 stoichiometric ratio (Goregaoker and Culver, 2003). In this structure, the polymerase domain of the 183k protein would protrude, and would be easily accessible to interaction with other viral or host structures. In addition to its own function as an RNA-polymerase, this accessibility could favour the recognition by R-like products, triggering an HR, observed as necrotic spotting. As in the case of the *NH* gene, the ORMV HR would not be mediated by real resistance to the virus that can be found systemically, inducing further necrotic local lesions.

To our knowledge, this is the first time in which a tobamoviral pathogenicity determinant has been ascribed to the polymerase domain (54k) of the gene encoding viral *RdRp*. This finding expands the complexity of the network of interactions taking place in the infected plant, but also provides new research avenues for the study of HRs, regardless of whether or not they are associated with virus resistance.

EXPERIMENTAL PROCEDURES

TMV/ORMV chimeric virus constructions

Chimera MC

The genetic region coding for the ORMV MP and CP was amplified by PCR (O-MC fragment, 1226 bp), introducing restriction sites for *XhoI* in 5' and *NsiI* in 3'. The O-MC fragment was cloned in the pCR[®]2.1 plasmid with the TOPO-TA cloning kit (Invitrogen) using the manufacturer's conditions. From this construction, the fragment corresponding to MP and CP (O-MC_XN) was obtained by restriction with *XhoI* and *NsiI* restriction enzymes.

pTMV-KK1 was digested with *XhoI* (position 4904 in TMV-KK1) and *KpnI* (position out of the viral genome), with unique restriction sites in pTMV-KK1. This fragment, corresponding to MP, CP and 3' UTR of TMV, was cloned in the pBS-SK+ plasmid, previously digested with the same restriction enzymes. The subclone obtained presented a unique restriction site for *NsiI* (position 6209 in TMV-KK1), downstream of the viral CP. This subclone was digested with *XhoI* and *NsiI*, replacing the fragment with an O-MC_XN fragment. By restriction with *XhoI* and *KpnI*, the OT-XK fragment was obtained, with MP and CP of ORMV close to TMV 3' UTR. The ligation of OT-XK with the fragment of pTMV-KK1 previously digested with *XhoI* and *KpnI*, corresponding to TMV *RdRp* and the plasmid, allowed chimera MC to be obtained.

In this chimera, the RdRp–MP overlapping region was reduced to one nucleotide with respect to TMV (10 nucleotides; Lehto and Dawson, 1990), and the overlap between MP and CP, originally present in ORMV, was maintained. A silent a → g change was

introduced at the third position of the codon encoding R1614 of the *RdRp* gene.

Chimera R

The genetic regions corresponding to the beginning and end of ORMV *RdRp* were amplified by PCR. In the 5' fragment (O-5', 823 bp), a restriction site for *Tth111I* in 5' and a stop codon were introduced in order to avoid the transcription of the remnant TMV *RdRp* nucleotides in the chimera. In the 3' fragment (O-3', 1028 bp), a restriction site for *XhoI* was introduced. Both fragments were independently cloned into the pCR[®]2.1 plasmid with the TOPO-TA cloning kit (Invitrogen) using the manufacturer's conditions. Fragment O-5'_T (783 bp) was obtained by digestion of O-5' with *Tth111I*, and O-3'_NX (960 bp) by digestion of O-3' with *NgoMI* and *XhoI*. The intermediate fragment (O_TX, 3119 bp) of *RdRp* was obtained by restriction of pORMV with *Tth111I* and *XhoI*.

pTMV-KK1 was digested with *Tth111I* (position 108 in TMV-KK1) and *XhoI* (position 4904 in TMV-KK1). The ligation of this fragment with O-5'_T, O_TX and O-3'_NX allowed the replacement of TMV *RdRp* by ORMV *RdRp* in chimera R.

In chimera R, an intergenic region of six nucleotides between *RdRp* and MP ORFs, not present in TMV (Lehto and Dawson, 1990), was created. Upstream of the *RdRp* gene of this chimera, there were 48 nucleotides corresponding to the TMV-KK1 *RdRp* gene, not included in the translated chimeric *RdRp* protein.

Chimera R-54k

The genetic region corresponding to the ORMV 54k domain was amplified by PCR (O-54k fragment, 1576 bp), introducing restriction sites for *BamHI* in 5' and *XhoI* in 3'. The O-54k fragment was cloned in the pCR[®]2.1 plasmid with the TOPO-TA cloning kit (Invitrogen) using the manufacturer's conditions. The fragment corresponding to 54k (O-54k_BX) was obtained by restriction with *BamHI* and *XhoI*.

pTMV-KK1 was digested with *BamHI* (position 3332 in TMV-KK1) and *XhoI* (position 4904 in TMV-KK1). The ligation of this fragment with O-54k_BX allowed the replacement of TMV 54k by ORMV 54k in the chimera R-54k.

Infectious transcripts

The plasmid DNAs, with the viral sequences of TMV-KK1 and the chimeras under the control of the T7 promoter, were purified with a Plasmid Midi Kit (Qiagen). The infectious transcripts were obtained by *in vitro* transcription of the corresponding plasmids with the T7 mMessage mMachine[™] kit (Ambion).

Plant material and plant inoculation

Arabidopsis thaliana (RLD and Can-0 ecotypes) and *N. tabacum* (cv. Samsun and Xanthi nc) seeds were surface sterilized [10 min in sodium hypochlorite 3% (v/v); Triton X-100 0.02% (v/v)], washed

five to six times in sterile distilled water and sown in Murashige and Skoog (MS) medium. Plates were kept for 24 h at 4 °C. Plants were transplanted 10–12 days after sowing to soil substrate in individual pots, and were maintained in a growth chamber (16 h photoperiod; temperature: 22–24 °C day and 20 °C night).

Infectious transcripts from the *in vitro* transcription and sap from infected *N. benthamiana* plants were used as inocula. With transcripts obtained from 1 µg of each plasmid, approximately four *Nicotiana* or eight *Arabidopsis* plants were inoculated. Purified and quantified ORMV was used, instead of transcripts. Infectivity was compared with TMV by inoculation onto *N. tabacum* plants, followed by the counting of necrotic local lesions. *N. benthamiana* was used as intermediate host to replicate parental and chimeric viruses, in order to be used as inocula. Previously, all transcripts had been inoculated directly onto the different hosts used in this study to ensure that they were infective. Two leaves per plant were mechanically inoculated: in *Nicotiana* sp. at the six- to eight-leaf stage and in *Arabidopsis* at the eight- to 10-leaf stage.

In all inoculations, two to four plants were inoculated with buffer as negative controls and with each parental virus as positive controls. The symptoms of infection with ORMV and TMV have been observed and evaluated over a number of years in our laboratory, in growth chambers or glasshouses (Aguilar *et al.*, 2000; Lunello *et al.*, 2007; Mansilla *et al.*, 2006; Martín Martín *et al.*, 1997; A. Martín Martín *et al.*, INTA, Madrid, unpublished data). For chimeras MC and R, five to seven different inoculation experiments were performed; 15–30 *Nicotiana* plants and 30–60 plants of each *Arabidopsis* ecotype were inoculated with each chimera. For chimera R-54k, five different experiments were performed; 12–30 *Nicotiana* plants of each assayed host were inoculated.

The photographs shown in Fig. 1 were taken at different times after inoculation, depending on particular combinations of host and inoculum, choosing the times at which the infection phenotypes were more evident.

In the case of the necrotic spotting produced by ORMV and chimera R-54k in tobacco (Figs 1 and 3), no measurement of lesion size is shown because the lesions did not grow. However, they increased in number and tended to become confluent. The final outcome is the impression of larger lesions, but this is inaccurate.

PCR

O-MC fragment amplification

Amplification from ORMV DNA with primers 5'-CTCGAGGTGGCTTAATGTCTTACGAGCCTA-3' and 5'-ATGCATCTTGACTACCTATGTAGCTGGCGCAGT-3'. Annealing temperature: 52 °C.

O-5' fragment amplification

Amplification from ORMV DNA with primers 5'-GACACTGTCTAAATGGCACAATTC-3' and 5'-GGGTGTAATTGAGTGCTCTCG-3'. Annealing temperature: 55 °C.

O-3' fragment amplification

In the same conditions as the O-5' fragment with primers 5'-GGCTCTCCAAACAAGAGTCG-3' and 5'-CTGGAGTCAAACAAAAACAAATC-3'.

O-54k fragment amplification

Amplification from ORMV DNA with primers 5'-GGATCCGATGGTGAATGTGAT-3' and 5'-CTGGAGTCAAACAAAAACAAATC-3'. Annealing temperature: 54 °C.

IC-RT-PCR

The presence of the virus was assessed by IC-RT-PCR as described in Mansilla *et al.* (2003) and Nolasco *et al.* (1993) with minor modifications. The immunocapture was performed in a final volume of 50 µL. The antibodies used were anti-*Ribgrass mosaic virus* (anti-RMV; Adgia), which gives cross-reaction with ORMV, and anti-TMV (Adgia). The annealing temperature used was 50 °C. The primer sequences are described in Fig. S1.

Quantitative PCR/RT-PCR

Primers and probes were designed using the Primer Express program (PE Biosystems). The TMV probe was marked with the VIC fluorochrome (emission wavelength, 558 nm) and the ORMV probe with the 6-carboxyfluorescein (6-FAM) fluorochrome (531 nm). The sequences (5' → 3') were as follows: TMV1, CCTACAC-CAGTCTCCATCATTG; TMV2, CGAACAGGTGCTTGACA; TMV-VIC, AGACAGCCCACATGTTTGGTCGCA; ORMV1, GAAAGAACGGCCG-GTTTC; ORMV2, CCAGCAGTTCGTGGCATT; ORMV-FAM, TGAAAC-CTAAATTGAGGACGGCGGC. Primers for TMV-KK1 amplified a region of 73 nucleotides (3236–3308) located in the helicase domain of the *RdRp* gene. The same region was present in chimeras MC and R-54k, and so they could be detected with TMV primers and probe. Primers for ORMV amplified a region of 66 nucleotides (3596–3661) located in the 54k domain of the *RdRp* gene. The same region was present in chimeras R and R-54k, and so they could be detected with ORMV primers and probe. Chimera R-54k could be detected with both probes. Therefore, the variations caused by the use of two different fluorochromes were corrected using the corresponding DNA controls, in order to compare the values obtained.

Quantitative PCR

Five nanograms of plasmid DNA of each viral construction were independently resuspended in 50 µL of PCR mixture containing 1 × Master Mix (Applied Biosystems), 100 nM of each primer and 50 nM of each probe. Quantitative PCR standard conditions were used in an AbiPrism 7700 (Applied Biosystems).

Quantitative RT-PCR

The samples were prepared by homogenizing inoculated *N. tabacum* and *N. benthamiana* leaves at 14 dpi. Plants were

inoculated with equivalent quantities of infectious transcripts of each construction. In the case of ORMV, plants were inoculated with purified virus diluted to an amount giving a similar number of local lesions in a bioassay. Samples were ground in a 1 : 3 weight (mg)/volume (μ L) ratio in extraction buffer and diluted 100–1000-fold for detection. The mix was the same as that used for quantitative PCR with 0.4 U of RNase inhibitor and 0.25 U of MultiScribe retrotranscriptase (Applied Biosystems) in a final volume of 25 μ L.

Sap from plants inoculated with buffer and non-template controls were used as negative controls.

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REFERENCES

- Aguilar, I., Sánchez, F., Martín Martín, A., Martínez-Herrera, D. and Ponz, F. (1996) Nucleotide sequence of Chinese rape mosaic virus (oilseed rape mosaic virus), a crucifer tobamovirus infectious on *Arabidopsis thaliana*. *Plant Mol. Biol.* **30**, 191–197.
- Aguilar, I., Sánchez, F. and Ponz, F. (2000) Different forms of interference between two tobamoviruses in two different hosts. *Plant Pathol.* **49**, 659–665.
- Arce-Johnson, P., Medina, C., Padgett, H.S., Huanca, W. and Espinoza, C. (2003) Analysis of local and systemic spread of the crucifer-infecting TMV-Cg virus in tobacco and several *Arabidopsis thaliana* ecotypes. *Funct. Plant Biol.* **30**, 401–408.
- Baughman, G.A., Jacobs, J.D. and Howell, S.H. (1988) Cauliflower mosaic virus gene VI produces a symptomatic phenotype in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA*, **85**, 733–737.
- Chen, J.B., Watanabe, Y., Sako, N., Ohshima, K. and Okada, Y. (1996) Mapping of host range restriction of the rakkyo strain of tobacco mosaic virus in *Nicotiana tabacum* cv bright yellow. *Virology*, **226**, 198–204.
- Culver, J.N. and Dawson, W.O. (1991) Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. *Mol. Plant–Microbe Interact.* **4**, 458–463.
- Culver, J.N. and Padmanabhan, M.S. (2007) Virus-induced disease: altering host physiology one interaction at a time. *Annu. Rev. Phytopathol.* **45**, 221–243.
- Dardick, C.D., Golem, S. and Culver, J.N. (2000) Susceptibility and symptom development in *Arabidopsis thaliana* to tobacco mosaic virus is influenced by virus cell-to-cell movement. *Mol. Plant–Microbe Interact.* **13**, 1139–1144.
- Deom, C.M., He, X.Z., Beachy, R.N. and Weissinger, A.K. (1994) Influence of heterologous tobamovirus movement protein and chimeric-movement protein genes on cell-to-cell and long-distance movement. *Virology*, **205**, 198–209.
- Dinesh-Kumar, S.P., Tham, W.H. and Baker, B.J. (2000) Structure–function analysis of the tobacco mosaic virus resistance gene N. *Proc. Natl. Acad. Sci. USA*, **97**, 14 789–14 794.
- Ehrenfeld, N., Gonzalez, A., Canon, P., Medina, C., Perez-Acle, T. and Arce-Johnson, P. (2008) Structure–function relationship between the tobamovirus TMV-Cg coat protein and the HR-like response. *J. Gen. Virol.* **89**, 809–817.
- Erickson, F.L., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C. and Baker, B. (1999) The helicase domain of the TMV replicase proteins induces the N-mediated defence response in tobacco. *Plant J.* **18**, 67–75.
- Gibbs, A. (1977) *Tobamovirus Group. CMI/AAB Descriptions of Plant Viruses*. No 184: 6pp.
- Goregaoker, S.P. and Culver, J.N. (2003) Oligomerization and activity of the helicase domain of the tobacco mosaic virus 126- and 183-kilodalton replicase proteins. *J. Virol.* **77**, 3549–3556.
- Hull, R. (2002) *Matthew's Plant Virology*, 4th edn. Norwich: Academic Press.
- Lartey, R.T., Voss, T.C. and Melcher, U. (1996) Tobamovirus evolution: gene overlaps, recombination, and taxonomic implications. *Mol. Biol. Evol.* **13**, 1327–1338.
- Lehto, K. and Dawson, W.O. (1990) Changing the start codon context of the 30K gene of tobacco mosaic virus from 'weak' to 'strong' does not increase expression. *Virology*, **174**, 169–176.
- Lunello, P., Mansilla, C., Conci, V. and Ponz, F. (2004) Ultra-sensitive detection of two garlic potyviruses using a real-time fluorescent (Taqman) RT-PCR assay. *J. Virol. Methods*, **118**, 15–21.
- Lunello, P., Mansilla, C., Sánchez, F. and Ponz, F. (2007) A developmentally linked, dramatic, and transient loss of virus from roots of *Arabidopsis thaliana* plants infected by either of two RNA viruses. *Mol. Plant–Microbe Interact.* **20**, 1589–1595.
- Mansilla, C., Sánchez, F. and Ponz, F. (2003) The diagnosis of the tomato variant of pepino mosaic virus: an IC-RT-PCR approach. *Eur. J. Plant Pathol.* **109**, 139–146.
- Mansilla, C., Aguilar, I., Martínez-Herrera, D., Sánchez, F. and Ponz, F. (2006) Physiological effects of constitutive expression of Oilseed Rape Mosaic Tobamovirus (ORMV) movement protein in *Arabidopsis thaliana*. *Transgenic Res.* **15**, 761–770.
- Martín Martín, A., Martínez-Herrera, D., Cabrera y Poch, H.L. and Ponz, F. (1997) Variability in the interactions between *Arabidopsis thaliana* ecotypes and oilseed rape mosaic tobamovirus. *Aust. J. Plant Physiol.* **24**, 275–281.
- Melcher, U. (2003) Turnip vein-clearing virus, from pathogen to host expression profile. *Mol. Plant Pathol.* **4**, 133–140.
- Mumford, R.A., Walsh, K., Barker, I. and Boonham, N. (2000) Detection of potato mop top virus and tobacco rattle virus using a multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. *Phytopathology*, **90**, 448–453.
- Nolasco, G., de Blas, C., Torres, V. and Ponz, F. (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J. Virol. Methods*, **45**, 201–218.
- Padgett, H.S. and Beachy, R.N. (1993) Analysis of a tobacco mosaic virus strain capable of overcoming N gene-mediated resistance. *Plant Cell*, **5**, 577–586.
- Padgett, H.S., Watanabe, Y. and Beachy, R.N. (1997) Identification of the TMV replicase sequence that activates the N gene-mediated hypersensitive response. *Mol. Plant–Microbe Interact.* **10**, 709–715.
- Palukaitis, P., García-Arenal, F., Sulzinskia, M.A. and Zaitlin, M. (1983) Replication of tobacco mosaic virus VII. Further characterization of single- and double-stranded virus-related RNAs from TMV-infected plants. *Virology*, **131**, 533–545.

- Rodríguez-Cerezo, E., Klein, P.G. and Shaw, J.G. (1991) A determinant of disease symptom severity is located in the 3'-terminal noncoding region of the RNA of a plant virus. *Proc. Natl. Acad. Sci. USA*, **88**, 9863–9867.
- Saito, T., Meshi, T., Takamatsu, N. and Okada, Y. (1987) Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant. *Proc. Natl. Acad. Sci. USA*, **84**, 6074–6077.
- Stange, C., Matus, J.T., Elorza, A. and Arce-Johnson, P. (2004) Identification and characterization of a novel tobacco mosaic virus resistance N gene homologue in *Nicotiana tabacum* plants. *Funct. Plant Biol.* **31**, 149–158.
- Sulzinski, M.A., Gabard, K.A., Palukaitis, P. and Zaitlin, M. (1985) Replication of tobacco mosaic virus VIII. Characterization of a third subgenomic TMV RNA. *Virology*, **145**, 132–140.
- Zhang, Y., Lartey, R.T., Hartson, S.D., Voss, T.C. and Melcher, U. (1999) Limitations to tobacco mosaic virus infection of turnip. *Arch. Virol.* **144**, 957–971.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1 (A) Diagram of the genomic array of parental and chimerical viruses. Arrows show the annealing zone of the primers used for the molecular detection, listed in the table. Names and colours like in Figure 2. (B) Electrophoretic distinction of viruses and chimeras. With primers O1 and O2 a 784 bp fragment of ORMV or chimera R was amplified (a), while with primers T1 and T2 a 407 bp fragment of TMV or Chimera MC RdRp was amplified (b). The amplification with primers T1 and O3 of a 1113 bp fragment (c) allowed the differentiation between chimera MC infected plants (positive amplification) and TMV infected plants (negative amplification), while the amplification with primers O1 and T3 of a 1423 bp fragment (d) allowed the differentiation between chimera R infected plants (positive amplification) of ORMV infected plants (negative amplification).

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