FUNCTIONAL ANALYSIS OF THE FIVE MELON NECROTIC SPOT VIRUS GENOME-ENCODED PROTEINS

A. Genovés, J. A. Navarro and V. Pallás

Instituto de Biología Molecular y Celular de Plantas (IBMCP), UPV-CSIC, Avda de los Naranjos s/n, 46022 Valencia, Spain

Function of the melon necrotic spot virus (MNSV) genome-encoded proteins (p29, p89, p7A, p7B and p42) has been studied. Protein-expression mutants of an infectious, full-length cDNA clone of a Spanish MNSV-Al isolate and a recombinant green fluorescent protein (GFP)-expressing virus were used in infection bioassays on melon plants. Results revealed that p29 and p89 are both essential for virus replication, whereas small proteins p7A and p7B are sufficient to support viral movement between adjacent cells operating in trans. It is also demonstrated that, in addition to its structural role as coat protein, p42 is an important factor controlling symptoms and is required for systemic transport. Moreover, both p42 and p7B, among all of the MNSV-encoded proteins, were able to delay RNA silencing in transient-expression assays on GFP-transgenic Nicotiana benthamiana plants. Finally, the presence of p42 also produced an enhancing effect on local spread similar to that of potyviral helper component proteinase (HC-Pro), probably due to its RNA silencing-suppression ability.

INTRODUCTION

Melon necrotic spot virus (MNSV) is a small (~30 nm), isometric plant virus that has been classified in the genus Carmovirus within the family Tombusviridae (Hibi & Furuki, 1985; Riviere & Rochon, 1990). MNSV has a narrow range of host plants that is almost restricted to species in the family Cucurbitaceae. Characteristic symptoms of diseased plants include necrotic spots on leaves and stem necrosis. MNSV is transmitted naturally in soil by the zoospores of the chytrid fungus Olpidium bornovanus (Lange & Insunza, 1977; Campbell & Sim, 1994), when it attaches itself to the spore outer covering (Furuki, 1981). Once the virus invades the plant, it can be propagated mechanically during pruning or harvesting. Alternatively, this virus may be seed-propagated, although, in this case, efficient transmission requires the assistance of the fungal vector (Campbell et al., 1996).

The MNSV genome consists of a single-stranded, positive-sense RNA of approximately 4·3 kb (Riviere & Rochon, 1990) and is thought not to be 5′-capped (Rochon & Tremaine, 1989; Hearne et al., 1990; Huang et al., 2000). Several MNSV isolates have been cloned and sequenced [MNSV-Dutch, GenBank accession no. NC_001504 (Riviere & Rochon, 1990); MNSV-NH and NK, GenBank accession nos AB044291 and AB044292 (Ohshima et al., 2000); MNSV-YS and KS, GenBank accession nos AB189944 and AB189943 (Kubo et al., 2005)] and infectious transcripts have been obtained [MNSV-Mz5, GenBank accession no. AY122286 (Diaz et al., 2003); MNSV-264, GenBank accession no. AY330700 (Diaz et al., 2004)]. Molecular analysis of the MNSV genomic sequence revealed the presence of at least five open reading frames (ORFs) flanked by a short 5′ untranslated region (UTR) and a non-polyadenylated 3′ UTR. Interestingly, an avirulence determinant has been characterized in the 3′ UTR of isolate MNSV-264, allowing the infection of non-cucurbit species, in addition to overcoming the resistance conferred by the recessive gene nsv in melon (Diaz et al., 2004; Morales et al., 2005). The 5′-proximal ORF has the potential to encode a protein of 29 kDa (p29) terminating with an amber codon, whose read-through would result in a larger gene product of 89 kDa (p89). Two small, centrally located ORFs encode two consecutive proteins of 7 kDa (p7A and p7B). Interestingly, if read-through of the amber codon located at the end of p7A occurred, both proteins would be joined in frame, resulting in a fusion protein of 14 kDa. Finally, the 3′-proximal ORF encodes a coat protein of 42 kDa (p42), which is related to those of the genus Tombusvirus (Riviere et al., 1989; Riviere & Rochon, 1990; Canizares et al., 2001). p29 and its read-through protein p89 are expressed from the genomic-length RNA (gRNA), whereas the small p7A and p7B (or p14) proteins and the coat protein are translated from 1·9 and 1·6 kb subgenomic RNAs (sgRNAs), respectively (Riviere & Rochon, 1990).

Function of each MNSV ORF can be deduced from amino acid sequence comparisons with homologue proteins of...
other well-studied members of the same genus or family. Thus, p29 and p89 are probably viral components of the replicase–transcriptase complex (Hacker et al., 1992; White et al., 1995; Panavičienė et al., 2003; Panavas et al., 2005). The two central ORFs, p7A and p7B, could be involved in cell-to-cell movement (Hacker et al., 1992; Li et al., 1998; Marcos et al., 1999; Cohen et al., 2000) and homologues of p42, independently of their structural function as coat protein, have been involved in systemic movement (Cohen et al., 2000), suppression of post-transcriptional gene silencing (PTGS) (Qu et al., 2003; Thomas et al., 2003; Ryabov et al., 2004; Meng et al., 2006) and attachment to the surface of fungus-vector zoospores in natural transmission (McLean et al., 1994; Robbins et al., 1997; Kakani et al., 2001). However, the association between sequence information and biological function of viral proteins has not been demonstrated in the case of MNSV. Therefore, this study reports the synthesis of infectious transcripts corresponding to the MNSV genome of a Spanish isolate (MNSV-Al; Gosalvez et al., 2003) and subsequent mutational analysis by reverse genetics to explore proposed functions of each potential genome-encoded protein during viral infections of melon plants, the natural MNSV host. Furthermore, recombinant virus delivering the green fluorescent protein (GFP) marker was used to monitor viral factors affecting the spread of infection. Additionally, the putative role of each gene product in suppression of PTGS was studied.

**METHODS**

**Virus isolation and construction of a full-length cDNA clone of MNSV-Al gRNA.** Nucleotide sequences of the 5' and 3' termini of MNSV-Al gRNA were obtained by 3'-RACE of the corresponding double-stranded RNA (dsRNA), which was isolated from leaves of *Cucumis melo* L. subsp. *melo* 'Galía' plants infected with the MNSV-Al isolate (Gosalvez et al., 2003) as described previously (Astruc et al., 1996; Covelli et al., 2004) and purified after electrophoretic separation on polyacrylamide gels. Briefly, the dsRNAs were denatured, polyadenylated and reverse-transcribed with a primer containing a 3'-dT<sub>25</sub> tail with the last base degenerated. These modified RNAs were PCR-amplified by using an oligo(dT) primer combined with others derived from internal regions of the isolate MNSV-Dutch. Five clones from each terminus were sequenced and used to design MNSV-Al-specific primers corresponding to the 5' and 3' UTR ends. T7 promoter sequence was included in the 5' UTR-specific primer. Full-length cDNA construction was performed by RT-PCR amplification of three overlapping regions covering the entire gRNA of MNSV-Al, using primers corresponding to both MNSV-Al termini combined with others based on the MNSV-Dutch sequence. The three fragments were assembled by using single endonuclease-restriction sites located in the MNSV-Al sequence and ligated into the pUC18 vector (MBI Fermentas) by standard subcloning strategies, giving rise to the pMNSV(Al) clone. Therefore, any MNSV-Dutch specific sequences predetermined by the used primers were removed. All reverse transcriptase and amplification reactions were carried out by using the RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (MBI Fermentas) and Vent DNA polymerase (New England Biolabs), respectively, following the manufacturers’ instructions. PCR-amplification conditions included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 60 °C for 40 s and extension at 72 °C for 2 min.

**Construction of a recombinant GFP-MNSV(Al) clone.** The p42 ORF from the full-length clone pMNSV(Al) was replaced by the GFP ORF to generate the recombinant pMNSV(Al)-ΔcGFP-GFP clone. Nucleotides located between positions 2845 and 4000, corresponding to the almost-complete p42 gene, were removed by reverse-PCR strategies using primers VP598 and VP599 (see Supplementary Table S1, available in JGV Online), leading to the pMNSV-Al-ΔcP clone. The GFP ORF was PCR-amplified by using the pEGFP-C3 vector (Clontech) as a template and primer pair VP543 (5'-GGCGGCGCCGGTGGCGCAACCATTGGTGAG-3'); underlined endonuclease-restriction site, NotI) and VP551 (5'-CACAGGGCCGCTACTTGATACACGTGTCGCA-3'; underlined endonuclease-restriction site, Apol). Subsequently, the GFP cDNA was ligated to pMNSV-Al-ΔcP by the previously introduced NotI and Apol restriction sites and T4 DNA ligase (Promega), resulting in the translational fusion of the first 9 aa of p42 to the GFP ORF.

**Protein-deficient expression constructs.** MNSV-Al protein-deficient expression constructs used in this study are represented in Fig. 1. Mutants were obtained from both the full-length pMNSV-Al and recombinant pMNSV(Al)-ΔcP-GFP clones by oligonucleotide-directed mutagenesis using a QuickChangeR XL site-directed mutagenesis kit (Stratagene).

**Fig. 1.** Schematic representation of (a) pMNSV(Al) and (b) pMNSV(Al)-ΔcP-GFP constructs and the corresponding protein-deficient expression mutants. Names of putatively encoded proteins are shown at the bottom. The GFP ORF is indicated in white letters. Non-modified coding regions are shown as open boxes, whilst frame-shift mutations are represented by discontinuous lines within diagrams or (FS) in construct name (left). Symbols (+) and (−) represent presence/absence of the protein indicated in the construct name, respectively.
mutagenesis kit (Stratagene) according to the manufacturer’s protocol and primer pairs listed in Supplementary Table S1 (available in JGV Online). The plasmid series pMNSV(Al)-89(FS) and pMNSV(Al)-Δcp-GFP-89(FS), pMNSV(Al)-7A(FS) and pMNSV(Al)-Δcp-GFP-7A(FS), pMNSV(Al)-7B(FS) and pMNSV(Al)-Δcp-GFP-7B(FS), in addition to pMNSV(Al)-42(FS), were obtained by introducing a frame-shift mutation into the p89, p7A, p7B and p42 ORFs, respectively. A virus mutant allowing for p89 read-through-product expression, but not the p29 protein, was generated by introducing an amber stop codon into a tyrosine codon mutation at the end of the p29 ORF in the pMNSV(Al)-29(−) and pMNSV(Al)-Δcp-GFP-29(−) plasmids. Fusing p7A and p7B ORFs yielded p14 expression by changing an amber stop codon into an alanine codon mutation at the end of p7A in the pMNSV(Al)-14(+) and pMNSV(Al)-Δcp-GFP-14(+) plasmids.

In vitro transcription and inoculation of plants. Plasmids were linearized by restriction with endonuclease PstI. Non-viral nucleotides located at the 3′ end were removed by T4 DNA polymerase treatment and in vitro transcripts were synthesized by T7 RNA polymerase (Roche Diagnostics) standard reactions. Melon plants of 6–10 days old were inoculated mechanically by rubbing fully expanded cotyledons with the uncapped transcription products (approx. 5 μg RNA per cotyledon) in the presence of inoculation buffer (potassium phosphate buffer: 0.03 M, pH 8) and carbordum. Inoculated plants were kept in growth chambers with a 16 h day length, a daytime temperature of 25–26°C and a night-time temperature of 22°C. Total RNA from cotyledons was analysed by Northern blot and GFP expression was visualized with a TCS SL confocal laser-scanning microscope (Leica, using filter BP/450-490 LP515) with excitation at 488 nm and emission at 500–535 nm.

Total RNA extraction, Northern blot and tissue-blotting assay. Total nuclear acid extraction from either inoculated melon-plant cotyledons or agroinfiltrated Nicotiana benthamiana leaves was performed as described previously (Navarro et al., 2004). RNAs were electrophoresed through formaldehyde-denatured gel and transferred to positively charged nylon membranes (Roche). Alternatively, symptomatic cotyledons were crushed in the nylon membranes (Roche) for a tissue-blotting assay, as described previously (Más & Pallás, 1995). Northern and tissue-blot membranes were air-dried and nucleic acids were bound by using a UV cross-linker (approx. 5 μg RNA per cotyledon) in the presence of a UV cross-linker (0.03 M, pH 8) and carbordum. Inoculated plants were kept in growth chambers with a 16 h day length, a daytime temperature of 25°C and a night-time temperature of 22°C. Total RNA from cotyledons was analysed by Northern blot and GFP expression was visualized with a TCS SL confocal laser-scanning microscope (Leica, using filter BP/450-490 LP515) with excitation at 488 nm and emission at 500–535 nm.

RESULTS

Infectivity of MNSV-Al gRNAs produced in vitro

The complete nucleotide sequence of isolate MNSV-Al (GenBank accession no. DQ339157) revealed high nucleotide and amino acid identity with previously described MNSV strains [MNSV-Dutch (Riviere & Rochon, 1990); MNSV-NH and NK (Ohshima et al., 2000); Mz5 (Díaz et al., 2003); MNSV-264 (Díaz et al., 2004); MNSV-YS and KS (Kubo et al., 2005)]. A full-length cDNA clone of MNSV gRNA, including a T7 RNA promoter at the 5′ end (pMNSV-Al), was constructed to enable MNSV-Al gRNA synthesis without foreign sequences at the 3′ end. These viral transcripts produced a local, and occasionally systemic, infection in susceptible melon plants independently of the presence of a cap analogue at the 5′ terminus, as occurred with biologically active clones of isolates MNSV-Mz5 and MNSV-264 (Díaz et al., 2003, 2004). Symptoms produced by inoculation of in vitro MNSV-Al transcripts and the systemic spread rate were identical to those observed when viral RNA isolated from virions or purified virions was inoculated (data not shown). Local chlorotic spots became necrotic lesions in the cotyledons at approximately 5–6 days post-inoculation (d.p.i.). When systemic infection had developed, new young leaves emerged, filled almost completely with chlorotic spots.

p42 is a pathogenicity determinant necessary for systemic infection

Frame-shift or non-stop codon mutations of the five viral ORFs were introduced into the pMNSV-Al infectious clone. Run-off transcripts produced from these mutants and from the original pMNSV-Al clone (wild type) were inoculated onto melon-plant cotyledons. Neither local nor systemic symptoms were observed, even at 10 d.p.i., in melon plants inoculated with MNSV(Al)-29(−), MNSV(Al)-89(FS), MNSV(Al)-7A(FS) and MNSV(Al)-7B(FS) RNAs, which had impaired expression of p29, p89, p7A and p7B, respectively. Similar results were obtained with RNAs from pMNSV(Al)-14(+) variants, which lacked individual p7A and p7B, but expressed both proteins fused as p14 (data not shown). However, MNSV(Al)-42(FS) RNAs, containing a frame-shift mutation at p42, induced the appearance of local

Functional analysis of MNSV-encoded proteins
symptoms on inoculated cotyledons, but never in emerging leaves, unlike the situation observed for wild-type transcripts. Local symptoms included chlorotic spots, unlike the more severe necrotic lesions generated by infection with wild-type RNAs (Fig. 2a). Thus, the viral RNA distribution in both types of local symptoms was analysed by tissue-blotting assay. Hybridization signals of at least five different experiments consistently revealed that, in the absence of p42, infection foci were smaller than those observed in wild-type infections (Fig. 2a). No hybridization signal was detected when mock-inoculated plant cotyledons were also assayed (data not shown). These data suggest that p42 is an important factor controlling symptoms, which is required for systemic transport and also enhances cell-to-cell movement.

Replication of mutants was analysed by Northern blot of equivalent amounts of total RNA purified from all inoculated cotyledons. MNSV RNAs were detected only when MNSV(Al)-42(FS) RNA was inoculated (Fig. 2b, lane 8). No significant effect was observed with this mutation on either the accumulation level of viral RNAs or the synthesis of both sgRNAs when compared with those observed for the wild-type RNA or even for viral particles, indicating that replication/accumulation are not affected detectably by the absence of p42 (Fig. 2b, compare lane 8 with lanes 1 and 2). To study p42 function further, a deletion mutant was constructed (pMNSV-Al-Dcp) and tested for its replication ability. Inoculation of these truncated RNAs resulted in an asymptomatic infection where one gRNA and two sgRNAs of approximately 1-2 kb smaller than the corresponding wild-type RNAs were detected by Northern blot hybridization (Fig. 2b, lane 9). Furthermore, the accumulation levels of these MNSV-Al-Dcp RNAs were comparable to what is found in wild-type infections. These results suggest strongly that symptoms depend on the presence of the p42 coding region and the protein itself.

**p29 and p89 are essential for MNSV replication, whereas cell-to-cell movement is controlled by the small p7A and p7B proteins**

A GFP-based approach commonly used to monitor virus infections (Baulcombe et al., 1995) was developed to differentiate between replication and cell-to-cell movement-deficient mutants. Thus, a GFP-MNSV recombinant [pMNSV(Al)-Acp-GFP], obtained by replacing the p42 ORF from the full-length clone pMNSV(Al) by the GFP ORF, was constructed. The GFP gene was fused in frame with the first 9 aa of the p42 ORF without affecting the p7B overlapping amino acids (Fig. 1b). Transcripts derived from this chimeric construct [MNSV(Al)-Acp-GFP RNAs] were inoculated mechanically onto melon ‘Galia’ cotyledons and GFP expression was monitored by confocal microscopy as green fluorescence. At 3 d.p.i., fluorescent infection foci were observed in inoculated cotyledons, indicating that chimeric RNAs were able not only to replicate, as GFP expression is only possible if the corresponding sgRNA 2 is synthesized, but also to move from cell to cell (Fig. 3a). Systemic spread of the fluorescence was not detected and no symptoms were observed, consistent with the results obtained before with MNSV(Al)-Dcp RNAs.

We then made a set of pMNSV(Al)-Dcp-GFP mutants, similar to those generated in pMNSV(Al) (Fig. 1b), to discriminate between mutants that cannot replicate from those that have their cell-to-cell movement impaired. Therefore, the modified MNSV(Al)-Dcp-GFP RNAs were inoculated

---

**Fig. 2.** (a) Photographs of melon cotyledons showing local lesions produced by inoculation of transcripts of either pMNSV(Al) (left) or pMNSV(Al)-42(FS) (right). Corresponding tissue blotting is also shown beside both images. Assays were performed by using a p42 MNSV-specific riboprobe. (b) Detection by Northern blot analysis of MNSV RNAs in melon-plant cotyledons inoculated with purified virions (lane 1) and in vitro transcripts of pMNSV(Al), pMNSV(Al)-29(−), pMNSV(Al)-89(FS), pMNSV(Al)-7A(FS), pMNSV(Al)-7B(FS), pMNSV(Al)-14(+), pMNSV(Al)-42(FS) and pMNSV(Al)-Dcp (lanes 2–9, respectively). Total RNA extracts obtained from mock-inoculated plants were used as healthy controls (lane 10). MNSV genomic and subgenomic RNA positions are indicated in the margins. Relative sample loading is inferred from ethidium bromide staining of plant rRNA (bottom panel).
onto melon cotyledons and, at 3, 6 and 8 d.p.i., a total of 100 cotyledons were monitored for green fluorescence expression. RNAs from \textit{pMNSV(Al)}-\textit{D}cGFP-89(FS) and \textit{pMNSV(Al)}-\textit{D}cGFP-29(−), which were defective in the putative replicase p29 and p89 proteins, respectively, were unable to induce fluorescence (data not shown). Those from \textit{pMNSV(Al)}-\textit{D}cGFP-7A(FS) and \textit{pMNSV(Al)}-\textit{D}cGFP-7B(FS), which were defective in putative cell-to-cell movement proteins p7A and p7B, respectively, led to GFP expression in individual cells (approx. 10 single cells per cotyledon) (Fig. 3b, panels 1 and 2, respectively) until 8 d.p.i. Afterwards, the green fluorescence disappeared. Transcripts from \textit{pMNSV(Al)}-\textit{D}cGFP-14(+) provided similar results, indicating that viral RNA may not reach adjacent cells by using the p14 fusion protein alone (Fig. 3b, panel 3). These data suggest strongly that p29 and p89 are essential for virus RNA replication, whilst p7A and p7B are involved in cell-to-cell movement, but probably do not function as a p14 fusion protein.

Different complementation assays were performed to gain insight into the mechanism of cell-to-cell movement involving both p7A and p7B. Unlike the results reported for \textit{Turnip crinkle virus} (TCV) in \textit{Arabidopsis thaliana} (Li \textit{et al.}, 1998; Cohen \textit{et al.}, 2000), movement was not restored when RNAs from \textit{pMNSV(Al)}-\textit{D}cGFP-7A(FS) and \textit{pMNSV(Al)}-\textit{D}cGFP-7B(FS) were inoculated together. Transgenic melon plants overexpressing MNSV proteins remain unavailable and experimental plants like \textit{A. thaliana} or \textit{N. benthamiana} are not MNSV host plants (except for isolate MNSV-264; Diaz \textit{et al.}, 2003). Thus, we developed a different complementation approach using individual inoculation onto melon cotyledons of either \textit{MNSV(Al)}-\textit{D}cGFP-7A(FS) or \textit{MNSV(Al)}-\textit{D}cGFP-7B(FS) RNAs and providing each of the functional proteins by transient expression using \textit{A. tumefaciens} infiltration. Transient expression in melon-plant cotyledons was assessed previously by agroinfiltration of binary vectors carrying GFP or p42 ORFs. Major expression of either GFP or p42, assessed as green fluorescence appearance or by Western blot analysis, respectively, was observed at 5 d.p.i. Unlike in other experimental systems, such as \textit{N. benthamiana}, a non-uniform green fluorescence distribution among melon-cotyledon cells was observed (see Supplementary Fig. S1, available in JGV Online). Therefore, 10-day-old cotyledons were agroinfiltrated with binary vectors carrying non-mutated p7A or p7B ORFs (pMOG7A and pMOG7B) and, 5 days later, coincident with the peak of transient expression, they were inoculated with \textit{MNSV(Al)}-\textit{D}cGFP-7A(FS) or \textit{MNSV(Al)}-\textit{D}cGFP-7B(FS) RNAs, respectively. A putative model involving p7A/p14 or p7B/p14 was evaluated by inoculating \textit{MNSV(Al)}-\textit{D}cGFP-14(+) RNAs onto either pMOG7A- or pMOG7B-agroinfiltrated cotyledons. Fifty cotyledons from each complementation assay were monitored by confocal microscopy. Individual cells and small fluorescent groups of cells were observed in similar proportions when \textit{MNSV(Al)}-\textit{D}cGFP-7A(FS) and \textit{MNSV(Al)}-\textit{D}cGFP-7B(FS) RNAs were complemented with transient p7A expression (groups of no more than three to four cells) (Fig. 3c, panel 4) and p7B (groups of no

![Fig. 3. (a) Confocal imaging of infectious foci produced by inoculation of \textit{in vitro} transcripts of \textit{pMNSV(Al)}-\textit{D}cGFP onto melon-plant cotyledons. (b) Transcripts from \textit{pMNSV(Al)}-\textit{D}cGFP-7A(FS), \textit{pMNSV(Al)}-\textit{D}cGFP-7B(FS) and \textit{pMNSV(Al)}-\textit{D}cGFP-14(+) were inoculated onto melon-plant cotyledons, producing individual fluorescent cells (panels 1, 2 and 3, respectively). (c) Images from complementation assays of MNSV movement mutants by transient expression. Complementation of \textit{MNSV(Al)}-\textit{D}cGFP-7A(FS) or \textit{pMNSV(Al)}-\textit{D}cGFP-7B(FS) RNAs by transient expression of p7A or p7B resulted in fluorescent-cell groups (panels 4 and 5, respectively). Complementation assay of \textit{MNSV(Al)}-\textit{D}cGFP-14(+) RNA with p7A (panel 6) did not restore virus cell-to-cell-movement capacity. All photographs were taken at 6 d.p.i.](http://vir.sgmjournals.org)
more than six cells) (Fig. 3c, panel 5), respectively. By contrast, fluorescence was only detected in single cells in complementation assays performed with MNSV(Al)-Δcp-GFP-14(+ ) RNAs (Fig. 3c, panel 6). Although we cannot rule out the possibility that small foci originated by unconnected events of viral infection on adjacent cells, this seems very unlikely, as these results were never observed when movement mutants were inoculated onto pMOG800-agroinfiltrated cotyledons (data not shown).

Both transiently expressed proteins 7A and 7B were able to complement in trans the corresponding mutant transcripts, although the replicating mutant viruses never reached the levels of infection foci produced when both movement proteins were provided by MNSV(Al)-Δcp-GFP RNA (Fig. 3a). This was probably because the agroinfection was not distributed homogeneously into the cotyledon as described before (see Supplementary Fig. S1, available in JGV Online). Hence, the local spread of movement-deficient RNAs was completely dependent on the location of the initial virus-infected cells inside a region expressing the corresponding movement protein. In addition, a new difficulty must be circumvented, as proteins must be agro-expressed at levels able to support viral movement, coinciding in time with the presence of the viral RNA inside the cell.

**MNSV movement protein p7B and coat protein (p42) delayed RNA silencing in transient-expression experiments**

An *A. tumefaciens*-mediated transient-expression assay on transgenic *N. benthamiana* plants expressing GFP (lane 16c; Ruiz et al., 1998) was performed as reported previously (Voinnet et al., 2000; Qu et al., 2003) to study the capacity of all MNSV genome-encoded proteins to act as potential RNA-silencing suppressors (see Methods for detailed description of constructs). At 2 d.p.i., the transient GFP expression induced an evident increase of green fluorescence in all infiltrated leaves when compared with the fluorescence observed in non-agroinfiltrated (transgenically carrying the complete MNSV-Al genome. As *N. benthamiana* is a non-host of the MNSV-Al isolate, no sgRNAs are produced and p7A, p7B and p42 proteins are not expressed (Riviere & Rochon, 1990) (Fig. 4a). Therefore, expression of 7B as well as p42 contributed to the stabilization of GFP mRNA (Fig. 4b), which further led to elevated GFP fluorescence (Fig. 4a). The effect of p7B and p42 proteins on GFP silencing was approximately 10-fold weaker than that generated by HC-Pro as measured by the GFP mRNA accumulation levels at 5 d.p.i. (Fig. 4b).

**MNSV coat protein (p42) and potyviral helper component proteinase (HC-Pro) favour local spread of MNSV(Al)-Δcp-GFP RNAs**

As demonstrated above, p42 shows a RNA silencing-suppressor activity and it is involved in the final size to which infection foci develop during MNSV invasion. To study the effect of this protein on virus local spread and compare it with that of HC-Pro, we performed a complementation strategy based on a transient-expression assay. *A. tumefaciens* strains carrying pMOG800, pMOG42 or pMOG(HC-Pro) clones were agroinfiltrated into melon cotyledons. MNSV(Al)-Δcp-GFP RNAs were inoculated at day 5 post-infiltration. Local-spread progress was assessed at 3, 5, 8, 12 and 14 d.p.i. by monitoring green fluorescence. The differences observed from three independent experiments (eight cotyledons per assay) demonstrated clearly that the presence of p42 and HC-Pro produced an enhancing effect (higher in the case of HC-Pro) on infection-focus size, clearly obvious at 5 d.p.i. At this point, the mean diameter of infection foci in the presence of either p42 or HC-Pro was 750 ± 53 and 820 ± 45 μm, respectively (Fig. 5a, panels 1 and 2, respectively), whereas in the absence of both proteins it was 300 ± 13 μm (Fig. 5a, panel 3). Additionally, viral RNA accumulation/replication rates were maintained when p42 or HC-Pro was expressed, as all foci in infected cotyledons fused until the inoculated cotyledons were invaded completely at 14 d.p.i. (data not shown). Unlike these results, the smaller fluorescence foci produced in the absence of both proteins continued to spread slowly until 8–10 d.p.i., and then spreading stopped [and the fluorescence began to decay gradually (Fig. 5b)]. Green fluorescence was never observed in vascular tissues.

**DISCUSSION**

Plant virus infection involves intracellular replication, movement from an infected cell to adjacent healthy cells by crossing the cell wall through the plasmodesmata and, subsequently, long-distance spread to other plant parts via the vascular system. The accomplishment of this life cycle is also the consequence of antagonist balance between viral infection and plant host-defence mechanisms...
that specifically target virus replication or movement (e.g. PTGS and systemic acquired resistance). In this work, we revealed the putative function of every MNSV-encoded protein at each step of the infection, including replication, local and systemic movement and RNA silencing. To perform this study, we used mutational analysis by reverse genetics of both an infectious clone containing the whole genome of the isolate MNSV-Al and a chimeric construct carrying GFP instead of the p42 ORF. Firstly, MNSV RNA synthesis in inoculated melon plants was impaired when either the p29 or p89 ORF was inactivated in both series of mutants pMNSV(Al)-89(FS) and pMNSV(Al)-29(−) or pMNSV(Al)-Dcp-GFP-89(FS) and pMNSV(Al)-Dcp-GFP-29(−). Consequently, these overlapping proteins are essential for MNSV replication and are probably part of the replication complex, as has been described for related viruses such as TCV (Hacker et al., 1992; Rajendran et al., 2002) and Tomato bushy stunt virus (Rajendran & Nagy, 2003, 2004; Panavienė et al., 2003; Panavas et al., 2005).

Following intracellular replication, cell-to-cell movement of carmoviruses (Cohen et al., 2000; García-Castillo et al., 2003) seems to be controlled by two small proteins working in trans (Hacker et al., 1992; Li et al., 1998; Cohen et al., 2000), an RNA-binding protein (Marcos et al., 1999; Vilar et al., 2001) and a membrane protein (Vilar et al., 2002), referred to as double-gene-block proteins or DGBps (Hull, 2002). Interestingly, the homologous proteins from TCV have overlapping regions (Carrington et al., 1989), whilst MNSV-Al DGBps (p7A and p7B) are exceptional among sequenced carmoviruses, as both proteins are arranged in frame in such a way that a fusion protein consisting of the complete p7A–p7B ORFs (p14) could be synthesized by a read-through process (Riviere & Rochon, 1990). However,
The results reported here demonstrated that p7A and p7B operating in trans are sufficient to move viral RNAs among neighbouring cells of the natural host plant. Complementation in trans of homologous TCV proteins was demonstrated on experimental plants (Li et al., 1998; Cohen et al., 2000), although indirect evidence was reported in its natural host Brassica campestris (Hacker et al., 1992). p14 was, by contrast, unable to promote cell-to-cell movement, even in the presence of either p7A or p7B. This last observation was also suggested by the presence of a strong termination codon at the end of p7A ORF that avoids p14 synthesis in isolate MNSV-Mz5 (Díaz et al., 2003). Consequently, if this protein is still expressed in some isolates, it is unlikely to play a role in local spread.

On the other hand, the absence of p42 led to localized infections as well as a reduced infection-focus size, even though it has been reported that coat proteins are dispensable for carmovirus cell-to-cell movement (type I movement; Scholthof, 2005). However, it is possible that these coat protein effects on local and systemic spread are associated with their RNA silencing-suppression ability, as suggested for other plant viral systems (Qu & Morris, 2005). From data presented here and elsewhere, it is revealed that p42 has several functions throughout the MNSV life cycle. Primarily, this protein is responsible for the capsid structure (Riviere et al., 1989; Riviere & Rochon, 1990), but it is also a pathogenicity determinant (protein that increases viral symptoms) enhancing local spread and an essential factor for systemic infection. Moreover, p42 and p7B are weak silencing suppressors, as they delayed, but did not prevent, PTGS in transient-expression experiments on GFP-transgenic plants. Nevertheless, similar results were observed for other plant viral suppressors when they were analysed initially by using this transient-expression method (p25 of Potato virus X; p20 and CP encoded by Citrus tristeza virus; Lu et al., 2004). Analogous combinations of p42 functions have been reported for other viral proteins such as potyviral HC-Pro and cucumber mosaic virus 2b, including the related TCV coat protein (Brigneti et al., 1998; Qu et al., 2003; Thomas et al., 2003; Roth et al., 2004; Ryabov et al., 2004; Qu & Morris, 2005). In this sense, p42 can contribute to the development of lesions indirectly by facilitating virus replication and spreading. Interestingly, and in parallel to our observations with regard to the p7B component of the DGBp, a weak RNA-suppressor activity was initially described in agroinfiltration experiments for potato virus X p25, protein 1 from the triple-gene block of proteins that are required for cell-to-cell movement of potexviruses (Voinnet et al., 2000; Lough et al., 2001). These data suggest that PTGS could be controlled by a component of the cell-to-cell movement machinery as a common feature among a number of plant viruses whose silencing suppression might be linked to viral transport (Verchot-Lubicz, 2005). Although the PTGS mechanism stage (initiation, local or systemic spread of a silencing signal or maintenance) affected by both MNSV suppressors was not studied in this work, the transient expression of p42 was relevant at an early infection stage by stimulating cell-to-cell movement and maintaining RNA replication. However, further studies on transgenic melon plants or, alternatively, the use of MNSV isolates infecting experimental plants (MNSV-264; Díaz et al., 2003) are necessary.

**ACKNOWLEDGEMENTS**

We thank L. Corachán for her technical assistance and Dr D. C. Baulcombe for *N. benthamiana* 16c plants. This work was supported by grant BIO05-7331 from the granting agency DGICYT. J. A. N. and A. G. are recipients of a J3P contract and a PhD fellowship from the Consejo Superior de Investigaciones Científicas and the Spanish Ministerio de Educación y Ciencia, respectively.

**REFERENCES**


