The nine C-terminal residues of the grapevine fanleaf nepovirus movement protein are critical for systemic virus spread

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The grapevine fanleaf virus (GFLV) RNA2-encoded polyprotein P2 is proteolytically cleaved by the RNA1-encoded proteinase to yield protein 2A, 2Bmp movement protein and 2Ccp coat protein. To further investigate the role of the 2Bmp and 2Ccp proteins in virus movement, RNA2 was engineered by alternatively replacing the GFLV 2Bmp and 2Ccp genes with their counterparts from the closely related Arabis mosaic virus (ArMV). Transcripts of all chimeric RNA2s were able to replicate in Chenopodium quinoa protoplasts and form tubules in tobacco BY-2 protoplasts in the presence of the infectious transcript of GFLV RNA1. Virus particles were produced when the GFLV 2Ccp gene was replaced with its ArMV counterpart, but systemic virus spread did not occur in C. quinoa plants. In addition, chimeric RNA2 containing the complete ArMV 2Bmp gene was neither encapsidated nor infectious on plants, probably because polyprotein P2 was incompletely processed. However, chimeric RNA2 encoding ArMV 2Bmp, in which the nine C-terminal residues were those of GFLV 2Bmp, formed virus particles and were infectious in the presence of GFLV but not ArMV 2Ccp. These results suggest that the nine C-terminal residues of 2Bmp must be of the same virus origin as the proteinase for efficient proteolytic processing of polyprotein P2 and from the same virus origin as the 2Ccp for systemic virus spread.

Introduction

Grapevine fanleaf virus (GFLV) is a plant virus which belongs to the genus Nepovirus of the family Comoviridae (Ward, 1993). GFLV is widely distributed in most vineyards and causes serious economic damage. The genome of GFLV is composed of two plus-sense, single-stranded RNAs, called RNA1 and RNA2, which each carry a small covalently linked viral protein (VPg) at the 5′ extremity and a poly(A) stretch at the 3′ OH end (Pinck et al., 1988, 1991). Both RNAs code for a polyprotein which is cleaved by the viral proteinase encoded by RNA1. RNA1-encoded polyprotein P1 possesses all the information needed for RNA replication and protein maturation (Margis et al., 1991; Ritzenthaler et al., 1991). RNA2-encoded polyprotein P2 is processed into three final cleavage products. The N-proximal part corresponds to protein 2A (28 kDa), which is essential for RNA2 replication (Ritzenthaler, 1994), the central region produces the movement protein (MP) 2Bmp (38 kDa), which is cleaved out of the polyprotein P2 at the C/A (residues 257/258) and R/G (residues 605/686) sites (Margis et al., 1993) and the 56 kDa coat protein 2Ccp is located at the C terminus of polyprotein P2 (Serghini et al., 1990). The coat protein assembles into icosahedral particles which can accumulate as RNA-containing or empty capsids in infected plants, indicating that RNA is not required for capsid assembly. GFLV RNA1 and RNA2 have both been sequenced (Ritzenthaler et al., 1991; Serghini et al., 1990) and full-length cDNA clones are available for production of infectious in vitro run-off transcripts (Viry et al., 1993), referred to as Tr1 and Tr2, respectively.

Arabis mosaic virus (ArMV) is a Nepovirus closely related to GFLV, but with a wider host range, including Solanaceae and Cucurbitaceae as well as grapevine (Murant, 1981). The genome organization and expression of ArMV is analogous to that of GFLV (Loudes et al., 1995). These authors have shown that RNA2 of the ArMV (isolate Syrah) consists of a mixture of two distinct species, called RNA2-U and RNA2-L, which are
slightly different in size, but highly similar in 2BMP (95%) and 2CP (98%) sequences. P2 polyproteins of RNA2-U and -L have a high percentage of identity with P2 of GFLV, reaching 69% in the 2C domain and 88% and 86% in the 2BMP domain, respectively. An antiserum raised against the GFLV 2BMP protein allows detection of GFLV and ArMV 2BMP proteins (Ritzenthaler et al., 1995a). Despite these high percentages of identity, the GFLV polyprotease is inefficient in vitro at processing the R/G cleavage site between ArMV 2BMP and 2CP proteins (Loudes et al., 1995). This can probably be attributed to differences in the amino acid sequence upstream of the R/G site, since the identity between the GFLV and ArMV 2BMP proteins is only six out of ten residues within the C-terminal residues. Also, a sequence alignment between these two proteins detects two gaps in the ArMV 2BMP domains in positions 13 and 14 as numbered from the GFLV cleavage site R/G.

Ritzenthaler et al. (1995b) have detected tubular structures containing isometric virus-like particles in or expanding from the cell wall and/or plasmodesmata, proper encapsidation of the viral genome, and virions crossing the cell wall via the tubules.

In preliminary experiments, inoculations of C. quinoa plants with Tr1 and Tr2 transcripts devoid of 2C and 2BMP sequences have shown that GFLV could not spread within the plant in the absence of 2BMP and/or 2CP proteins (Ritzenthaler, 1994). To further investigate the role of GFLV 2BMP and 2CP in cell-to-cell movement, chimeric RNA2 molecules were constructed with different sequences of ArMV (isolate Syrah) substituted for their counterparts in a complete GFLV RNA2 cDNA clone. The resulting transcripts were co-inoculated with GFLV RNA1 transcript into protoplasts or plants and the chimeric molecules were tested for their capacity to replicate, to form tubules, to assemble virus particles and to establish a systemic infection in plants. Results are presented concerning complementation between GFLV and ArMV in virus spread as well as on the specificity of interaction between the 2BMP and 2CP proteins.

Methods

Construction of chimeric GFLV RNA2. Plasmid pVec is a pUC-derived plasmid containing the 5' and 3' non-coding sequences of GFLV RNA2 under the control of the T7 promoter. It also includes a stop codon and a SmalI site upstream of the 3' non-coding sequence (Ritzenthaler, 1994). It was used as a cloning vector to produce pVec2P and pVec2AB, which contain full-length GFLV RNA2 and GFLV RNA2 devoid of 2CP sequence, respectively. The cDNA sequences of ArMV RNA2-U and RNA2-L were derived from plasmids p60 and p81, respectively (Loudes et al., 1995).

NheI and Nael restriction sites were introduced into pVec2P for cloning purposes downstream of the 2BMP/2CP cleavage site to facilitate the exchange of the GFLV and ArMV 2CP genes (Fig. 1B). The HindIII–BamHI fragment (nt 194–2300) from pVec2P was cloned into pBSK+ plasmid (Stratagene) to produce single-stranded DNA for mutagenesis experiments. Mutagenesis of the GFLV sequence was performed according to the method of Kunkel (1985) with primer mutCP (5' AGAGGGCTACCCGGCAGAGG 3') complementary to the 5'-terminal sequence of the 2CP gene. In this primer, four nucleotides (underlined) were changed to introduce NheI and Nael sites without modifying the amino acid sequence. The BsmHI–BamHI fragment (nt 1535–2300) carrying the mutated sequence was then cloned back into pVec2P to give pVec2ABC (Fig. 1B).

The ArMV RNA2-U and -L full-length 2CP genes were amplified by PCR using cDNA clones of p60 and p81 as template, and the primers 5CP [5' CGGGGCTAGCCGGCGAGGATCT 3'], which corresponds to the 5'-terminal sequence of the ArMV 2CP gene and contains the NheI and Nael sites (underlined) and 3CP [5' GGGAATACGTACTATTAAACCTTAAAG 3'], which corresponds to the 3'-terminal sequence of the ArMV 2CP gene and contains two contiguous in-frame stop codons (bold) and a SmalI site (italics). The amplified ArMV-U and -L 2CP genes were then cloned into the NheI and SmalI sites of pVec2ABC to give pVec2ABC and pVec2ABC, respectively (Fig. 1A). The subscripts U or L identifies protein originating from ArMV RNA2-U or -L, respectively.

PCR fragments corresponding to full-length ArMV-U and -L 2BMP genes were produced from p60 and p81, respectively, using primers 5MPS (5' TGCGGTGTGGATGATTGACTACCCGGTGAGG 3') and primer 3CP (5' AACCTCTGCCGCGTAGCGCCTCAGG 3') which corresponds to the 5'-terminal sequence of the ArMV 2BMP gene and contains the NheI and Nael sites (underlined). The amplified fragments were digested with AgeI (bold) and Nael (underlined), and ligated into either AgeI- and Nael-digested pVec2ABC, or pVec2ABC, to generate pVec2AB, respectively, or AgeI- and Nael-digested pVec2ABC to give pVec2ABC, respectively (Fig. 1A).

Partial sequences of the ArMV-U and -L 2BMP genes were amplified by PCR using plasmids p60 and p81 as templates and primers 5MPS and either primer 3MPR (5' ATGTACTGATCCGAGGCGGCAATCCCGCTGACGTCGTTGAGG 3') or primer 3MRPL (5' TGAATTCGCTGATCCGAGGCGGCAATCCCGCTGACGTCGTTGAGG 3') in which an EspI site (bold) was introduced. Amplified fragments were digested with AgeI and EspI, and ligated into either AgeI- and EspI-digested pVec2ABC, or pVec2ABC, to give pVec2AB and pVec2ABC, respectively (Fig. 1A, B). All PCR amplifications were carried out using 1·5 U Tth DNA polymerase (Promega) and 0·1 U Vent (exo-) DNA polymerase (New England Biolabs) per 100 µl reaction. The integrity of the recombinant clones was checked by DNA sequencing of the complete PCR fragments introduced.

RNA transcription. Vector pMV13 (Viry et al., 1993) was used to produce full-length infectious transcripts of GFLV RNA1 after linearization with BglII. The cDNA clones corresponding to GFLV RNA2 or chimeric GFLV RNA2 were linearized with Sall prior to transcription. Capped full-length transcripts, called Tr1 for GFLV RNA1 transcripts and Tr2 for GFLV RNA2 or chimeric GFLV RNA2 transcripts, were
Fig. 1. Genetic map of the pVecP2-based GFLV RNA2 recombinants. (A) Schematic representation of the constructs. Boxes denote coding sequences and lines symbolize non-coding GFLV sequences. Open boxes represent GFLV sequences, hatched boxes represent ArMV RNA2-U sequences and filled boxes represent ArMV RNA2-L sequences. Arrows indicate the proteolytic cleavage sites C/A and R/G. The position of the restriction sites used for construction of the chimera are shown below. Nucleotide and amino acid sequence alignments at the R/G and C/A cleavage sites (arrow and dotted line) are presented in (B) and (C). Mutated nucleotides are underlined and ArMV protein sequences are in italics. Restriction enzyme sites used for cloning are in boxes. P2 ArMV-U corresponds to the p60 sequence. K* indicates the only modification introduced by the cloning strategy, leading to the replacement of R by K. Nucleotide numbering indicated to the left is taken from sequences deposited in the EMBL database under accession nos X16907 (GFLV RNA2), X81814 (ArMV RNA2-U) and X81815 (ArMV RNA2-L).

synthesized in vitro using the RiboMAX large-scale RNA production system according to the instructions provided by the manufacturer (Promega). Transcript size and integrity were checked by agarose–formaldehyde gel electrophoresis.

Inoculation of Chenopodium quinoa plants. C. quinoa plants were inoculated with purified transcripts according to Viry et al. (1993). Plants were monitored at regular time intervals for symptom development. Apical non-inoculated leaves were harvested 12 days post-
inoculation (p.i.) for RNA and protein analysis. Inoculation experiments were repeated ten times for each construct.

■ Transfection of C. quinoa and tobacco protoplasts. Mesophyll protoplasts of C. quinoa were isolated (Hans et al., 1992) and transfected according to Viry et al. (1993) using 5 µg Tr1 and 10 µg Tr2 or chimeric Tr2.

Tobacco BY-2 (T-BY2) protoplasts were prepared as described by Gaire (1998). Briefly, cells were collected from a 3–5-day-old culture and digested with 0.1% (w/v) pectolyase, 1% (w/v) cellulase RS (Onozuka) in 0.45 M mannitol, 3.6 mM MES, pH 5.5. After washing, 0.1 mM CaCl2 was added. Protoplasts were incubated for 1 h at 4 °C and transfected using 30 µg Tr1 and 30 µg Tr2 corresponding to GFLV RNA2 or chimeric GFLV RNA2. C. quinoa and T-BY2 protoplasts were harvested 3 days post-transfection (p.t.).

■ Western blot analysis. Proteins were extracted from 2 × 10⁶ C. quinoa protoplasts according to Ritzenhalter et al. (1995 a) or from C. quinoa leaves ground in liquid nitrogen. Total soluble proteins were denatured and clarified by centrifugation at 10000 g for 5 min prior to SDS–PAGE (Laemmli, 1970). Equivalent amounts of total proteins extracted from protoplasts or leaves were analysed by Western hybridization using 2BMP antisera according to Ritzenhalter et al. (1995 a).

■ RNAs encapsidation assays and Northern blot analysis. C. quinoa protoplasts (2 × 10⁶) were pelleted and resuspended either in 500 µl 100 mM Tris–HCl pH 8.0, 100 mM LiCl, 10 mM EDTA, 0.1% (w/v) SDS (TLES buffer) to obtain total RNA or in 500 µl 50 mM PIPES pH 6.5, 1% (v/v) Tween 20 and incubated for 20 min at 37 °C to degrade unencapsidated RNA. The RNA was then extracted by phenol–chloroform and further purified by precipitation with 0.3 M sodium acetate pH 5.2. For each sample, RNA corresponding to 2 × 10⁶ protoplasts was separated by electrophoresis on a 1% agarose–formaldehyde gel and transferred onto Hybond-N membrane (Amersham). GFLV RNA1 and -2 were detected using digoxigenin-labelled riboprobes prepared according to the manufacturer’s instructions (Boehringer). GFLV RNA1 was detected using a 375 nt riboprobe obtained after transcription with T3 polymerase of ScaI-linearized pVP7 (Margis et al., 1991), which corresponds to the proteinase-coding domain. GFLV RNA2 was detected using a 385 nt riboprobe obtained after transcription with the T3 polymerase of Rovr-linearized pKS-2A, previously called P28 (Margis et al., 1993).

■ Electron microscopy. C. quinoa protoplasts (5 × 10⁶) were pelleted and homogenized in phosphate buffer (18 mM KH₂PO₄, 42 mM Na₂HPO₄ pH 7.2) with small microtome pestles and several freeze–thaw cycles. Virus particles were separated from crude membranes by centrifugation for 15 min at 12000 g at 4 °C. Formvar/carbon-coated electron microscopy grids (Agar Scientific) were incubated with a mixture of GFLV 2C⁰ and ArMV 2C⁰ rabbit antiserum (each at 5 µg/ml in phosphate buffer) for 30 min at room temperature, washed and incubated with protoplast preparation for 4 h at 4 °C. Grids were then washed and incubated in saturation buffer [0.01%, (v/v) Triton X-100, 5% w/v, BSA in phosphate buffer containing goat anti-rabbit IgG at 12.5 µg/ml (Sigma)] for 1 h at room temperature. After washing, grids were floated on the same mixture of GFLV 2C⁰ and ArMV 2C⁰ antiserum used in the coating step. After 30 min incubation at room temperature, grids were washed and further incubated for 30 min with goat anti-rabbit IgG coupled to 10 nm gold particles (Amersham), diluted 40-fold in phosphate buffer. Finally, grids were rinsed with distilled water, negatively stained with 1% (w/v) ammonium molybdate, pH 7.0, and observed with a Philips EM208 electron microscope at 80 kV.

■ Detection of 2BMP by immunofluorescence. Immunostaining of 2BMP was performed with harvested T-BY2 protoplasts as described by Gaire (1998). Briefly, protoplasts were fixed in 5 mM PIPES pH 6.9, 0.5 mM EGTA, 0.2 mM MgCl₂, 0.4% (v/v) paraformaldehyde, and allowed to adhere to Superfrost/Plus glass slides. The protoplasts were permeabilized and blocked with PBS containing 0.5% Triton X-100, 25 mg/ml digitonin and a 20-fold dilution of blocking reagent (Boehringer). Then they were incubated with 2BMP antisera (Ritzenhalter et al., 1995 a) and 2BMP was labelled using CY3-conjugated anti-rabbit IgG (Jackson ImmunoResearch).

Results

Design of chimeric RNA2

To determine the role of GFLV RNA2 2BMP and 2C²⁰ proteins in the movement of virus and to characterize the interactions between these two proteins during virus movement, chimeric GFLV RNA2s were constructed in which segments of GFLV RNA2 were replaced with the homologous segments of ArMV RNA2. Since ArMV RNA2 comprises two distinct molecules, called RNA2-U and RNA2-L, with indistinguishable biological functions (Loudes et al., 1995), genomic exchanges were carried out using either ArMV RNA2-U or RNA2-L sequences.

pVec2ABC (Fig. 1A) was used as a starting point to produce chimeric plasmids. pVec2ABC is derived from the wild-type pVecP2 by the addition of two restriction sites, Nhel and Nael, to pVecP2 without altering the amino acid composition of polyprotein P2 (Fig. 1B). The functionality of pVec2ABC was verified by infectivity tests on C. quinoa plants and immunodetection of 2BMP protein. The results showed that C. quinoa displayed similar systemic symptoms and accumulated similar levels of 2BMP protein 10 days p.i. whether inoculated with Tr1 + Tr2 or Tr1 + Tr2ABC. These data indicate that the introduction of the two restriction sites, Nhel and Nael, into pVec2ABC did not affect the replication of Tr2ABC, virus multiplication or systemic movement.

Exchange of the complete 2C²⁰ genes between GFLV and ArMV was achieved using the novel Nael site and the SmalBI site to produce pVec2ABC_U and pVec2ABC_L (Fig. 1A). The exchange of 2BMP sequences using AgeI and Nael sites permitted us to transfer the 2BMP genes except for their six N-terminal residues. The resulting cDNA constructs, called pVec2AB₃₁₄_C₃₉, pVec2AB₁₅₇_C₇₇, pVec2AB₁₄₇ and pVec2AB₁₃, encode the complete 2BMP protein of either ArMV-U or ArMV-L, except for the six N-terminal residues, which were from GFLV 2BMP. The 2BMP proteins encoded by pVec2AB₃₁₄_C₃₉, pVec2AB₁₅₇_C₇₇, pVec2AB₁₄₇ and pVec2AB₁₃ have a Lys residue instead of an Arg residue in position 4 downstream of the C/A proteolytic cleavage site (K* in Fig. 1C).

In order to maintain the R/G proteolytic cleavage site in a GFLV environment, the AgeI–Espl fragment of GFLV-2BMP, which comprises residues 7–339 of 2BMP, was replaced by the corresponding fragment of ArMV-U or L 2BMP in pVec2ABC,
pVec2ABC_U and pVec2ABC_L, respectively. The resulting plasmids, called pVec2AB_U9C, pVec2AB_L9C, pVec2AB_U9C and pVec2AB_L9C (Fig. 1A), contain the complete ArMV 2BMP protein, except for the nine C-terminal amino acids, which were from GFLV (Fig. 1B).

Replication of chimeric GFLV RNA2

To test the influence of the genomic exchanges on RNA replication, protoplasts of C. quinoa were transfected with Tr1 and transcripts of chimeric GFLV RNA2. Total RNA was extracted 3 days p.t. (Fig. 2, lower panel) and analysed by Northern blotting using a mixture of riboprobes specific to GFLV RNA1 and RNA2 (Fig. 2, upper panel). The comparison of RNA progeny of Tr2ABC (Fig. 2, lane 1) and chimeric Tr2 (Fig. 2, lanes 2–11) revealed no major differences in RNA2 accumulation. We therefore conclude that the genomic exchanges of 2BMP and/or 2CP sequences had no influence on the efficiency of RNA replication.

In vivo processing of chimeric GFLV RNA2-encoded polyprotein

To study the influence of 2BMP and/or 2CP exchanges on the in vivo processing of GFLV RNA2-encoded polyproteins, C. quinoa protoplasts were transfected with Tr1 and chimeric Tr2. Analysis of total protein extracts 3 days p.t. with an antisemur specific to GFLV 2BMP protein (Fig. 3) showed similar patterns for Tr2ABC, Tr2ABC_U, and Tr2ABC_L (Fig. 3, lanes 1–3) and the absence of maturation intermediates at a detectable level. These data indicate that chimeric GFLV RNA2 constructs which included segments of ArMV RNA2-U and -L appeared

**Fig. 2.** Northern blot analysis of total RNA extracted from C. quinoa protoplasts transfected with 5 µg Tr1 plus 10 µg Tr2ABC (1), Tr2ABC_U (2), Tr2ABC_L (3), Tr2ABCU (4), Tr2ABCL (5), Tr2ABCU (6), Tr2ABCL (7), Tr2ABCU (8), Tr2ABCL (9), Tr2ABCU (10) or Tr2ABCL (11). Lane 12, mock-transfected protoplasts. Total RNA from 2×10^6 protoplasts were extracted 3 days p.t. with TLES buffer and electophoresed on a 1% agarose–formaldehyde gel. Upper panel, viral RNA was detected using two digoxigenin-labelled riboprobes complementary to RNA1-encoded proteinase and RNA2-encoded 2A sequences. The position of GFLV RNA1 and -2 is identified on the right. Lower panel, negative picture of ribosomal RNA stained by ethidium bromide added to the RNA samples before denaturation.

**Fig. 3.** Detection of 2BMP protein in C. quinoa protoplasts by Western blot analysis. Protoplasts were transfected with 5 µg Tr1 plus 10 µg Tr2ABC (1), Tr2ABC_U (2), Tr2ABC_L (3), Tr2ABCU (4), Tr2ABCL (5), Tr2ABCU (6), Tr2ABCL (7), Tr2ABCU (8), Tr2ABCL (9), Tr2ABCU (10) or Tr2ABCL (11). Lane 12, protoplasts were mock-transfected. At 3 days p.t., 2×10^6 protoplasts were pelleted, resuspended in electrophoresis loading buffer and electrophoresed on a 10% SDS–polyacrylamide gel. Proteins were transferred onto an Immobilon membrane and screened using antibodies raised against the GFLV 2BMP protein. Immunoreactive proteins were detected by chemiluminescence. The positions of the 2BMP proteins and maturation intermediates (2AB and 2BC) are indicated on the right. Standard molecular masses (kDa) are positioned on the left.

**Fig. 4.** Northern blot analysis of viral RNA from transfected C. quinoa protoplasts. Total RNA was extracted with TLES buffer (T), yielding both encapsidated and nonencapsidated RNAs (lanes with odd numbers) or with PIPES buffer (E), yielding only encapsidated RNAs (lanes with even numbers). Protoplasts were transfected with 5 µg Tr1 plus 10 µg Tr2ABC (1 and 2), Tr2AB (3 and 4), Tr2ABC_L (5 and 6), Tr2ABCU (7 and 8), Tr2ABCU (9 and 10), Tr2ABCL (11 and 12) or Tr2ABCL (13 and 14). Lane 15, RNA from mock-transfected protoplasts. The procedure for hybridization was as in Fig. 2. Positions of the different GFLV RNAs are indicated on the right.
Fig. 5. Immunoelectron micrographs of viral capsids after transfection of C. quinoa protoplasts with chimeric RNA2. Protoplasts were homogenized 3 days p.t. and lysates were incubated on grids pre-coated with a mixture of anti-ArMV-S 2CCP and anti-GFLV 2CCP antibodies. Labelling was carried out with the same mix of antisera used for coating and gold-conjugated secondary antibodies. Protoplasts were transfected with 5 µg Tr1 plus 10 µg Tr2ABC (A), Tr2ABCU (B), Tr2ABUCU (C), Tr2ABUC (D), Tr2ABUCu (E) or Tr2ABUCu (F). Protoplasts were also mock-transfected (G). Arrows indicate virus-like particles. Scale bar represents 100 nm.

To behave similarly regarding replication and polyprotein processing, we limited our further experiments to chimera between GFLV RNA2 and ArMV RNA2-U.

Effect of 2CCP produced from chimeric GFLV RNA2 on viral RNA protection

To evaluate the ability of chimera-encoded 2CCP proteins to protect viral RNA from nucleolytic attack, total cellular RNA and nuclease-resistant (presumably encapsidated) RNA was extracted from transfected protoplasts and analysed by Northern blotting using riboprobes specific to GFLV RNA1 and GFLV 2A sequences. Hybridization signals were obtained with total RNA regardless of the origin of the Tr2 (Fig. 4, lanes 1, 3, 5, 7, 9, 11 and 13). Nuclease-resistant, presumably encapsidated, viral RNA was detected only for protoplasts transfected with Tr2ABC, Tr2ABCu, Tr2ABUCU and Tr2ABUC (Fig. 4, lanes 2, 6, 12 and 14). These data show that the ArMV 2CCP protein protects GFLV RNA1 and chimeric RNA2 from endogenous ribonucleases. On the contrary, when protoplasts were transfected with Tr2ABUCu and Tr2ABUC, no viral RNA could be detected (Fig. 4, lanes 8 and 10). Taken together, these results indicate that RNA was protected when the nine C-terminal residues of 2B were from GFLV and suggest a correlation between the protection of RNA and the complete processing of polyprotein P2.

Effect of 2Bmp produced from chimeric GFLV RNA2 on virus particle formation

To determine whether RNA protection was indeed due to viral RNA encapsidation into virus particles or simply to 2CCP monomers covering the viral RNA, crude extracts of transfected protoplasts were examined by immunosorbent electron microscopy and gold labelling. Immunolabelled virus-like particles were observed only in protoplasts transfected with Tr2ABC, Tr2ABCu, Tr2ABUCU and Tr2ABUC (Fig. 5A, B, E, F). In contrast, when protoplasts were transfected with Tr2ABUCu and Tr2ABUC, no virus-like particles were visible. Together, these observations reveal a good correlation between viral RNA protection and virion formation and suggest that RNA protection is almost certainly due to the encapsidation of viral RNA in particles.

Effect of 2Bmp produced from chimeric GFLV RNA2 on tubule formation

The ability of chimeric GFLV RNA2 to induce tubules was investigated in T-BY2 protoplasts by immunofluorescence. T-BY2 protoplasts were preferred to C. quinoa protoplasts because they do not produce mature chloroplasts which can interfere with the fluorescence labelling. Tubules were observed emerging from the surface of protoplasts transfected with all chimeric GFLV RNA2 (Fig. 6A–E). These data
indicated that the exchange of 2C<sup>CP</sup> genes had no influence on tubule formation and that ArMV-2B<sup>MP</sup> protein was also able to form tubules that immuno-reacted with the GFLV 2B<sup>MP</sup> antiserum. An example is illustrated for Tr2AB<sub>T</sub>C<sub>U</sub> (Fig. 6C) and has also been noticed for Tr2AB<sub>T</sub>C (not shown). In protoplasts transfected with Tr2AB<sub>T</sub>C<sub>U</sub> and Tr2AB<sub>T</sub>C<sub>U</sub>, tubules were also observed (Fig. 6D, E). These results suggest that the nine C-terminal residues of 2B<sup>MP</sup> are not essential for tubule formation and/or that folding of 2B<sup>MP</sup> protein is not modified by exchange of sequence at the C terminus of 2B<sup>MP</sup>.

### Whole plant infectivity tests

The ability of the chimeric GFLV RNA2 to establish a systemic infection was investigated in <i>C. quinoa</i> plants. Typical GFLV-like symptoms appeared 10 days p.i. on apical leaves of <i>C. quinoa</i> plants co-inoculated with Tr1 and either Tr2ABC, Tr2AB<sub>T</sub>U<sub>C</sub> or Tr2AB<sub>T</sub>U<sub>C</sub>. Apical leaves of <i>C. quinoa</i> plants were tested by Western blotting using GFLV 2B<sup>MP</sup> antiserum. No 2B<sup>MP</sup> protein was detected when plants were inoculated with Tr1 and either Tr2AB<sub>T</sub>C<sub>U</sub> or Tr2AB<sub>T</sub>C. Apcial leaves of <i>C. quinoa</i> plants were tested by Western blotting using GFLV 2B<sup>MP</sup> antiserum. No 2B<sup>MP</sup> protein was detected when plants were inoculated with Tr1 and either Tr2AB<sub>T</sub>C<sub>U</sub> or Tr2AB<sub>T</sub>C. These results were expected since the virus progeny produced by these chimeric GFLV RNA2 do not produce particles (Fig. 4, lanes 8, 10; and Fig. 5C, D), confirming that a 2C<sup>CP</sup> protein able to form virus particles is essential for virus spread in plants. Considering those constructs which produce tubules, virus particles and which are able to protect progeny viral RNA, 2B<sup>MP</sup> could be detected in the protein extracts of plants inoculated with Tr1 and Tr2AB<sub>T</sub>U<sub>C</sub> (Fig. 7, lane 9), but not in plants inoculated with Tr1 and either Tr2ABC<sub>T</sub> or Tr2AB<sub>T</sub>C<sub>U</sub> (Fig. 7, lanes 2, 8). Similar results were obtained with the chimeric ArMV-L counterparts (Fig. 7, lanes 3, 10, 11). These results indicate that chimeric Tr2 with 2B<sup>MP</sup> from ArMV and the nine C-terminal residues of GFLV origin are competent for the spread of the virus and the establishment of a systemic infection. Since hybrid 2B<sup>MP</sup> from both Tr2AB<sub>T</sub>C<sub>U</sub> and Tr2AB<sub>T</sub>U<sub>C</sub> is functional and ArMV 2C<sup>CP</sup> produced from either Tr2ABC<sub>T</sub> or...
Tr2AB<sub>U</sub>C<sub>U</sub> is competent for particle assembly and for protection of RNA progenies, the inability of Tr2ABC<sub>U</sub> or Tr2AB<sub>U</sub>C<sub>T</sub>, Tr2ABC<sub>T</sub> and Tr2AB<sub>U</sub>C<sub>L</sub> to produce systemic infection cannot be attributed to incapacity of either individual 2B<sup>MP</sup> or 2C<sup>CP</sup>. Only chimeric GFLV RNA2 transcripts which have the nine C-terminal residues of 2B<sup>MP</sup> and the complete 2C<sup>CP</sup> of GFLV origin (i.e. Tr2AB<sub>U</sub>C and Tr2AB<sub>L</sub>C) were infectious on <i>C. quinoa</i> plants, suggesting the existence of virus-specific interactions between 2C<sup>CP</sup> and the C terminus of 2B<sup>MP</sup>.

**Discussion**

Our comparisons of the biological activity of various GFLV/ArMV chimeric RNA2s (Table 1) provide an insight into the role of the GFLV 2B<sup>MP</sup> and 2C<sup>CP</sup> proteins in the establishment of a systemic infection. Exchanges of the sequences encoding 2B<sup>MP</sup> and 2C<sup>CP</sup> have no influence on RNA replication, but highlight the importance of the nine C-terminal residues of the 2B<sup>MP</sup> protein in efficient processing of polyprotein P2 and in cell-to-cell movement of the virus.

The contrast between the infectivity of Tr2AB<sub>U</sub>C<sub>U</sub> and Tr2AB<sub>L</sub>C and the lack of infectivity of Tr2ABC<sub>L</sub>, Tr2ABC<sub>I</sub>, Tr2AB<sub>U</sub>C<sub>I</sub> and Tr2AB<sub>U</sub>C<sub>L</sub> on <i>C. quinoa</i> plants indicates that 2C<sup>CP</sup> and the nine C-terminal residues of 2B<sup>MP</sup> must be of the same virus origin for systemic infection. Thus, whereas RNA protection, tubule formation and assembly of virus particles occur for all the above constructs, only Tr2AB<sub>U</sub>C and Tr2AB<sub>L</sub>C can initiate a systemic infection, indicating that virus movement requires more than virion assembly and formation of tubules. We propose that the requirement that the nine C-terminal residues of the 2B<sup>MP</sup> domain and the 2C<sup>CP</sup> sequence derive from the same virus reflects a requirement for specific interactions between 2B<sup>MP</sup> and 2C<sup>CP</sup>. Such interactions between viral proteins have been hypothesized for *Alfalfa mosaic virus* (AIMV) (Reusken et al., 1995), which has a tubule-mediated movement strategy (Kasteel et al., 1997), as well as for *Cucurbita chlorotic mottle virus* (Sanchez-Navarro et al. (1997) have already illustrated that the MP and CP genes must be of the same virus origin to allow efficient spread of recombinants. For *Cucumber mosaic virus* and *Tomato aspermy virus*, Salanki et al. (1997) also hypothesized the existence of virus-specific interactions between the MP and CP and 2A protein to allow cell-to-cell movement of recombinant virus. The lack of virus spread when proteins are exchanged between GFLV and ArMV cannot be attributed to improper virus–host protein interactions, since <i>C. quinoa</i> is a common host for both viruses.

As empty virus-like particles were observed in tubules of *Cucurbita mosaic virus* (CPMV) (van Lent et al., 1991), the presence of particles in tubules probably depends only on specific recognition between MP and coat proteins, not on viral RNA–protein interactions. However, we cannot conclude whether the GFLV nine C-terminal residues of 2B<sup>MP</sup> directly interact with GFLV 2C<sup>CP</sup> or whether they are involved in conformational modifications of the ArMV 2B<sup>MP</sup> which allow the interaction with GFLV 2C<sup>CP</sup>. Our results can be compared with those of Lekkerkerker et al. (1996), who showed that a deletion in the C-terminal domain of CPMV MP resulted in empty tubules unable to take up virus particles. Similarly, the deletion of the three C-terminal amino acids of the MP rendered AIMV non-functional for cell-to-cell movement (van der Vossen et al., 1995). Lekkerkerker et al. (1996) hypothesized that the C-terminally deleted MP might produce tubules with a slightly smaller diameter than the native MP. In our conditions, however, it is

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<th>Chimeric transcripts</th>
<th>Replication</th>
<th>Cleavage at the R/G site</th>
<th>Capsid formation/RNA protection</th>
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*+, incomplete maturation at the R/G site; ND, not determined.*
unlikely that chimeric 2B*MP formed tubules with a reduced diameter since the hybrid and GFLV 2B*MP proteins are identical in size.

The ability of variants Tr2AB*U*CP and and Tr2AB*C to form tubules showed that the C terminus of 2B*MP can be exchanged without impairing tubule assembly. This finding is similar to the observations of Lekkerkerker et al. (1996) and Thomas & Maule (1995), who indicated that a C-terminal proximal domain of the CPMV and Cauliflower mosaic virus MP can be manipulated without affecting tubule formation. Taken together, these findings support the model of Thomas & Maule (1995), who suggested that there are structural similarities among the MP's of viruses having tubule-mediated movement.

Regarding proteolytic processing of the recombinant polyprotein P2 in protoplasts, we have shown that the presence of the nine C-terminal residues of GFLV 2B*MP allow optimal cleavage at the R/G site regardless of the parental origin of 2B*MP and 2C*CP. In contrast, for variants in which the complete 2B*MP originated from ArMV (Tr2AB*U*CP, Tr2AB*U*C, Tr2AB*C and Tr2AB*CP), abundant amounts of 2BC intermediate were detected and no virus particles were visualized, indicating the importance of a GFLV environment for the efficient cleavage at the R/G site by the GFLV RNA1-encoded protease. Our in vitro results are in agreement with previous in vitro maturation experiments (Loudes et al., 1995) which showed that the GFLV protease efficiently processes the C/A site between the GFLV 2A and the ArMV 2B*MP but is ineffective in cleaving the R/G site downstream of the ArMV 2B*MP. Similarly, Demangeat et al. (1991) have shown that GFLV polyprotein P2 was not cleaved in vitro by the translation products of tomato black ring virus RNA1. Such virus-specificity has also been described for proteases of the related CPMV and Red clover mosaic virus using recombinants (Shanks et al., 1996).

Our incompletely processed GFLV RNA2 variants (Tr2AB*U*CP and Tr2AB*CP) confirm that 2C*CP assembly into particles is essential for virus spread. Capsid formation and RNA protection were not ensured for these GFLV RNA2 variants since the chimeric polyproteins were apparently not sufficiently processed to produce enough free 2C*CP monomers for virion assembly. For Brome mosaic virus (BMV), which also undergoes tubule-mediated movement, Schmitz & Rao (1996) have shown that an encapsidation-competent CP is required for virus spread. Similarly, Dolja et al. (1995) have shown that an encapsidation-defective tobacco etch potyvirus mutant was restricted to the initially infected cell. The necessity of proper capsid formation is consistent with the presence of virions within tubules as shown for GFLV (Ritzenthaler et al., 1995 b), CPMV (van Lent et al., 1991) and for ALMV and BMV (Kasteel et al., 1997). Considering that the 2B*MP produced by Tr2AB*U*CP can form tubules, we can hypothesize that either (i) 2B*MP turnover is slower than that of 2C*CP, (ii) 2B*MP protein is needed at lower concentrations for tubule assembly, or (iii) the 2BC intermediate can participate in tubule formation.

Regarding viral RNA encapsidation, our results suggest that transcapsidation of GFLV RNA in ArMV coat protein is possible, since Tr2AB*UC and Tr2ABC*CP RNA survived in RNA protection assays and capsids were detected by immuno-sorbent electron microscopy experiments. Hence, the signal acting in cis for encapsidation is either of weak specificity or this signal is encompassed within the RNA sequence encoding 2C*CP. Assuming that the encapsidation signal is conserved among RNA1 and RNA2, or is at least very similar, the fact that GFLV RNA1 can be encapsidated by ArMV 2C*CP favours a weak specificity towards the virus.

Finally, it is to be noted that the similar behaviour of our ArMV-U- and ArMV-L-based chimeric Tr2 suggests that the two species of RNA2 are independent and self-sufficient for virus spread. This is consistent with the results of Liu et al. (1991), who showed that the doublet of RNA2 of ArMV isolate Lilac could be separated by successive cloning of local lesions to obtain an infectious ArMV-SF isolate.

In conclusion, we have shown that the C terminus of 2B*MP is critical for processing of GFLV polyprotein P2 by the GFLV protease and that the same virus origin between 2C*CP and the C-terminal part of 2B*MP is essential for movement. Work is in progress to restore recognition between the hybrid 2B*MP and the ArMV 2C*CP and then to determine which of the C-terminal residues of GFLV 2B*MP are implicated in processing at the R/G site and which are responsible for 2B*MP/2C*CP interactions.

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References


Protoplasts