Identification and characterization of RNA-binding activity in the ORF1-encoded replicase protein of *Pelargonium* flower break virus

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*Pelargonium flower break virus* (PFBV) belongs to the genus *Carmovirus* (family *Tombusviridae*) and, as with the remaining members of the group, possesses a monopartite genome of single-stranded, positive-sense RNA that contains five ORFs. The two 5′-proximal ORFs (ORFs 1 and 2) encode two polypeptides of 27 and 86 kDa (p27 and p86), respectively, that show homology with replication proteins. The p27 does not present any motif to explain its presumed involvement in replication, while p86 has the motifs conserved in RNA-dependent RNA polymerases. In this work, we have confirmed the necessity of p27 and p86 for PFBV replication. To gain insights into the function(s) of p27, we have expressed and purified the protein from *Escherichia coli* and tested its ability to bind RNA in vitro. The results have shown that p27 is able to bind ssRNA with high affinity and in a cooperative fashion and that it is also capable of binding other types of nucleic acids, though to a lesser extent. Additionally, competition experiments suggest that p27 has a preference for PFBV-derived ssRNAs. Using truncated forms of p27, it can be concluded that several regions of the protein contribute to its RNA-binding properties and that this contribution is additive. This study is the first to show nucleic acid-binding ability of the ORF1 product of a carmovirus and the data obtained suggest that this product plays an essential role in selection and recruitment of viral RNA replication templates.

**INTRODUCTION**

Multiplication of viral RNA genomes requires specific interaction between the viral RNA-dependent RNA polymerase (RdRp) and its cognate RNAs. Despite this, only minimal detailed information is available for this type of interaction (reviewed by Kim & Kao, 2008). Studies focused on analysing RNA-binding activity of auxiliary (virus- or host-encoded) replication proteins are yet scarcer, even though such proteins may be crucial in the selection and recruitment of viral RNA replication templates, the promotion of the hallmark asymmetrical (+) and (−) RNA synthesis or the switch from viral RNA translation to replication (Ahlquist et al., 2003; Wang & Nagy, 2008).

*Pelargonium flower break virus* (PFBV) is a member of the genus *Carmovirus* and, as do most members of the family *Tombusviridae* (Lommel et al., 2005), possesses a monopartite genome composed of a single-stranded (ss), positive-sense genomic RNA that harbours five ORFs (Rico & Hernández, 2004). The translation products of the two 5′-proximal ORFs, ORFs 1 and 2, are proteins of 27 and 86 kDa (p27 and p86), respectively, and reverse genetics experiments with the homologous proteins of other carmoviruses have shown that both are indispensable for viral replication (Genoveş et al., 2006; Hacker et al., 1992). Two internal ORFs encode small proteins that are probably involved in viral movement, and the 3′-proximal ORF encodes a polypeptide which plays a dual role as capsid protein and as suppressor of RNA silencing (Martínez-Turíño & Hernández, 2009). As occurs in most members of the *Tombusviridae*, ORF2 must be translated from the genomic RNA by readthrough of the leaky stop codon of ORF1 and, thus, p86 contains the sequence of p27 at the N terminus while its C-terminal portion presents the signature motifs of the RdRps (Koonin & Dolja, 1993). Based on the presence of these motifs, the predicted function of p86 is to synthesize viral RNA progeny and subgenomic RNAs for expression of the internal and 3′-terminal genes. Conversely, p27 does not contain obvious motifs that explain its presumed involvement in viral replication (Habili & Symons, 1989).

As a consequence of the translation strategy that gives rise to p86, p27 is produced in much larger amounts than p86. This suggests that p27, as proposed for equivalent proteins of members of the genus *Tombusvirus* (White & Nagy, 2008),
2004), may be a pivotal protein with diverse functions during the replication process. Our recent work indicates that p27 specifically associates with mitochondrial membranes (S. Martínez-Turín and C. Hernández, unpublished results), paralleling the results obtained with the ORF1 products of other members of the Tombusviridae that contain sorting signals for subcellular localization (Mochizuki et al., 2009; Navarro et al., 2004; Panavas et al., 2005; Turner et al., 2004; Weber-Lotfi et al., 2002). Besides its potential to target the replicative complex to specific cellular membranes, it could recruit viral RNA templates and other protein (viral- or host-coded) components required for RNA synthesis. To gain insights into the function(s) of p27, we report experiments aimed at confirming its requirement for viral replication and at assessing its RNA-binding properties. We present evidence showing that p27 is indeed essential for replication and interacts in vitro with RNA, which has not been previously reported for any member of the genus Carmovirus. In addition, we have analysed the contribution of different protein regions to RNA binding. The results indicate the presence of redundant information for RNA binding suggesting that the virus has evolved to ensure that a p27 key activity is preserved.

RESULTS

Both p27 and p86 are involved in PFBV replication

The requirement for the translation products of ORFs 1 and 2 for viral replication has so far been verified for two species of the genus Carmovirus, Turnip crinkle virus (TCV) and Melon necrotic spot virus (Genovés et al., 2006; Hacker et al., 1992), as well as for members of other genera of the family Tombusviridae (Castaño et al., 2009; Molnár et al., 1997; White & Nagy, 2004). In order to confirm that p27 and p86 of PFBV play a similar role, three different mutants were generated using an infectious viral cDNA clone (pSP18-IC; Rico & Hernández, 2006) as the template: (i) p27aug, which lacks the initiation codon of the first two ORFs (p27/p86); (ii) p27stop, bearing two stop codons in tandem that terminate ORF1; and (iii) p27tyr, in which the leaky stop codon or ORF1 has been replaced by a Tyr-encoding triplet (Fig. 1a). Inoculation of Chenopodium quinoa plants with transcripts derived from the mutant constructs did not induce local lesions on the inoculated leaves, in contrast to inoculation with RNAs synthesized from the wild-type (wt) pSP18-IC clone (not shown). A Northern blot analysis confirmed the absence of PFBV in leaves inoculated with p27aug, p27stop and p27tyr (Fig. 1b, left panel). Transfection of protoplasts with the latter mutants revealed that none of them accumulated at detectable levels, indicating that, as expected, replication is the step of the infectious cycle affected by the corresponding mutations (Fig. 1b, right panel) and thus confirming the involvement of p27 and p86 in replication. Remarkably, coinoculation of protoplasts with p27stop and p27tyr did not restore the defect of each individual mutant, as no viral RNAs could be detected in this type of assay (lane 5 in the right panel of Fig. 1b).

In vitro RNA binding by recombinant p27

To obtain suitable amounts of p27 for in vitro studies, the p27 gene was PCR-amplified from the plasmid pSP18-IC and cloned into the Escherichia coli expression vector pET-23d (+), which allowed fusion of a hexa-His tag at the C terminus of p27. Chromatography on nickel-nitrilotriacetic acid (Ni-NTA) columns of proteins from induced cultures expressing His-tagged p27 resulted in highly purified preparations of the recombinant protein, with an electrophoretic mobility consistent with that expected for its

Fig. 1. Bioassay of PFBV mutants. (a) Schematic representation of wt PFBV genomic RNA and derived mutants p27aug, p27stop and p27tyr. Open boxes correspond to predicted ORFs. Numbers above ORFs 1 and 2, encoding proteins p27 and p86, respectively, indicate nucleotide positions of the codons that define each ORF. Nucleotide substitutions were introduced into the full-length cDNA clone pSP18-IC leading to triplet changes that are depicted for each mutant. In mutants, dashed boxes correspond to ORFs that are not expected to be translated. RT, Stop codon readthrough. (b) Northern blot analysis of total RNA from C. quinoa leaves (left panel) or protoplasts (right panel) inoculated with transcripts derived from the wt construct pSP18-IC (lane 1) and from the mutant constructs p27aug (lanes 2–4, respectively); a mixture of p27stop and p27tyr (lanes 2–4, respectively); a mixture of p27stop and p27tyr was also used for protoplast inoculation (lane 5 in the right panel). The band corresponding to the PFBV genomic RNA (gRNA) is indicated by an arrow; lower bands correspond to subgenomic RNAs. A loading control of cellular RNA is included at the bottom.
predicted molecular mass (28.8 kDa, considering the size of the wt protein plus six additional amino acids resulting from the cloning procedure and the hexa-His tag at the C terminus) (Fig. 2). Western-blot analysis of a parallel gel confirmed that the prominent band in the purified preparations indeed corresponded to the full-length p27, because it reacted specifically with an anti-His6 antibody (not shown).

Possible nucleic acid-binding capability of p27 was studied by electrophoretic mobility shift assays (EMSAs) using a 32P-labelled ssRNA probe (3'-PFBV, encompassing the 3' terminal region of the PFBV genome) and increasing amounts of the recombinant His-tagged protein. This approach showed that p27 could bind RNA efficiently in vitro. The protein started to retard the probe at a concentration of 3 nM and retarded it completely at and above 93 nM (Fig. 3a). The RNA bound to the recombinant p27 essentially stayed in the well, probably due to the large size of the complex. Addition of BSA instead of p27 in control binding reactions did not lead to a change in RNA mobility (Supplementary Fig. S1, available in JGV Online), indicating that the RNA shift observed with p27 was not the result of non-specific protein–RNA interactions. The binding of distinct amounts of His-tagged p27 with a constant amount of the probe exhibited an 'all-or-none' behaviour, as no intermediately shifted bands (resulting from limited binding of the probe by p27) between the completely bound and free probe were detected in the EMSA. This observation is consistent with a cooperative binding of p27, in which most of the RNAs are either covered by p27 or are not bound to it at all (Citovsky et al., 1990; Lohman et al., 1986). To analyse the binding data further, non-linear regression was applied (Fig. 3b).

Fig. 2. Expression and purification of PFBV p27 in E. coli. Proteins were analysed by 15% SDS-PAGE and subjected to Coomassie blue staining. Lanes: 1, protein markers with their molecular masses (in kDa) indicated on the left; 2 and 3, total proteins from E. coli non-induced (NI) and induced (I) cultures transformed with construct pET-p27 which allowed expression of p27 fused to a His tag; 4, purified preparation (P) of His-tagged p27. The position of recombinant p27 is indicated by an arrow.

Fig. 3. Analysis of RNA-binding properties of PFBV p27 in vitro. (a) Representative EMSA showing interactions between the recombinant p27 and ssRNA. The 32P-labelled 3'-PFBV ssRNA probe (at 33 pM) was incubated with no protein (lane 1) or with the His-tagged p27 at 3, 7, 17, 24, 93 or 139 nM (lanes 2–7, respectively). The unbound, free RNA probe and the shifted (bound) RNA complexes are marked on the right. The two bands observed for the free probe correspond to two different conformations of the RNA, as they migrated as a single band after heat treatment; moreover, their relative ratio did not change significantly after denaturing and snap-cooling assays with dilutions of the RNA, indicating that the upper band does not result from dimerization of the probe but rather from intramolecular folding (not shown). (b) Best-fit curve of the binding data determined by non-linear regression. The curve depicts the single experiment shown in (a), but the analysis was repeated at least three times with similar results. (c) Influence of NaCl concentration on the RNA-binding activity of PFBV p27. The fraction of bound ssRNA was plotted against log[NaCl] (NaCl concentration in mM) to calculate the IC50.
RNA–protein complex formation was measured as the disappearance of the band corresponding to the unbound RNA from the EMSA (Carey, 1991; Daro’s & Carrington, 1997; Marcos et al., 1999). From the non-linear regression adjustment, an apparent $K_d$ of 11 nM could be determined.

In addition, the slope of the best-fit curve ($h$ or Hill slope) was found to be 1.4, supporting the notion of p27 ssRNA binding being cooperative ($h=1$ is indicative of no cooperativity, whereas values above 1 point to positive cooperativity).

EMSAs were also conducted using different salt concentrations in the presence of an amount of p27 that was sufficient to bind all ssRNA molecules. The appearance of free RNA was quantified to evaluate complex dissociation and the NaCl concentration at which binding was reduced to 50% of maximal levels (IC$_{50}$) was 390 mM (Fig. 3c). The stability of the p27–ssRNA complex at relatively high salt concentrations resembled that observed with other RNA-binding proteins from plant viruses (Herranz & Pallás, 2004; Li & Palukaitis, 1996; López et al., 2000; Navarro et al., 2006; Richmond et al., 1998; Wobbe et al., 1998) and suggested that p27–RNA complex formation is not due solely to the electrostatic interactions between the nucleic acid and the protein.

**Differential affinities of p27 for distinct types of nucleic acids**

To assess the ability of p27 to discriminate among different types of nucleic acids, formation of the protein complex with the ssRNA substrate was challenged by titrating the binding reactions with unlabelled competitors. The mixtures included 0.1 ng $^{32}$P-labelled 3’-PFBV ssRNA, the recombinant p27 at a concentration (93 nM) that resulted in complete retardation of the ssRNA probe in the absence of competitors and increasing amounts of unlabelled nucleic acids (1-, 10- and 100-fold molar excess over the radiolabelled probe) corresponding to either PFBV-derived ssRNAs (3’-PFBV, same as the probe, and 5’-PFBV, encompassing the 5’ region of the viral genome) or ssRNA, dsRNA, ssDNA and dsDNA molecules of heterologous origin. The effect of the inclusion of competitors was evaluated by EMSA (Fig. 4a). The competition data were analysed by a plot of the probe fraction bound to the protein in the presence of competitor vs the [competitor]:[probe] ratio (Fig. 4b). These experiments showed that the ssRNAs were the best competitors, whereas dsRNA, ssDNA and dsDNA templates were relatively poor competitors under the experimental conditions used. Comparison of results with ssRNA competitors of similar sizes corresponding to the 5’-region of the PFBV genome, to the 3’-region or to a non-specific sequence suggested that p27 preferably binds PFBV-derived ssRNAs. Collectively, the data suggested that p27 is an ssRNA-binding protein, although it is also capable of binding other nucleic acids.

**Fig. 4.** Effect of competitors on the ssRNA binding of p27 determined by EMSA. (a) Standard binding assay, containing p27 (at 93 nM) and $^{32}$P-labelled 3’-PFBV ssRNA probe (at 33 pM), was performed in the absence (lane 2) or presence (lanes 3–11) of increasing amounts (1, 10- and 100-fold molar excess) of unlabelled competitors as shown above the autoradiograms. Lanes 1, no protein. (b) Graphical representation of the EMSA results. The percentage of the RNA probe fraction bound to the protein in the presence of different competitors has been plotted against the competitor/probe molar ratio. The experiments were repeated twice with essentially identical results (data from one set of experiments are presented here).
Identification of the RNA-binding domain(s) of p27

In order to recognize the domain(s) of p27 responsible for its RNA-binding ability, we generated five truncated forms of the protein, p27(1–100), p27(1–144), p27(100–243), p27(140–243) and p27(171–243), by removing fragments of different sizes at its N- or C-terminal region (Fig. 5a). These p27 derivatives were fused to a hexa-His tag and purified in the same way as the wt p27. EMSAs with increasing amounts of the proteins were performed. Plots of the fraction of retarded probe at different protein concentrations showed that the truncated form p27(1–100) does not bind to RNA under the in vitro conditions, while p27(100–243) bound to the probe to an extent equivalent to that of the entire wt protein (Fig. 5a, b). Deletion of additional residues from the N terminus in the derivative p27(140–243) diminished RNA-binding affinity sixfold when compared with p27(100–243) (K_d = 69 nM vs 10 nM; Fig. 5). Further elimination of N-terminal residues in p27(171–243) did not abolish RNA-binding capability but this was reduced by about half with respect to the previous truncated p27(140–243) (K_d = 124 nM vs 69 nM). Additional assessment of the RNA-binding capacity of p27(171–243) was performed by replacing three arginines (positions 195–197 in the entire wt protein) by alanines, which yielded a protein, p27(171–243)*, that was virtually inactive for RNA binding (Fig. 5b and Supplementary Fig. S2). Finally, the derivative p27(1–144) exhibited RNA-binding activity with an apparent K_d close to, though lower than, that estimated for p27(140–243) (K_d = 42 nM vs 69 nM), despite the fact that they do not share a significant common region (Fig. 5). Collectively, the results indicated that the central (residues 100–139) and C-terminal (residues 140–170 and 171–243) portions of p27 contribute to its RNA-binding activity and that this contribution seems to be additive as, according to the estimated K_d values, increasing RNA-binding affinities were found as segments of greater length were included [compare results for p27(100–243), p27(140–243) and p27(171–243) in Fig. 5].

To confirm that the retardation of the probe was actually the result of its interaction with the p27 derivatives and not with any contaminant E. coli protein(s) accompanying the purified preparations, a Northwestern assay was conducted. To this aim, the full-length p27 and deletion forms were resolved by SDS-PAGE and subsequently blotted on to a nitrocellulose membrane. Hybridization of the membrane with the 32P-labelled 3'-PFBV probe revealed signals in positions perfectly coincident with those corresponding to the full-length p27, p27(100–243), p27(140–243) and p27(1–144) (Fig. 5c). A weak signal was also observed in a position coincident with p27(171–243) with longer exposures of the autoradiogram (not shown), whereas no signal could be detected in the portion of the membrane that contained p27(1–100) or p27(171–243)*. Hence, the Northwestern results were consistent with those obtained by EMSA regarding the formation of complexes with RNA of p27 derivatives and confirmed that various regions of p27 are endowed with RNA binding properties.
DISCUSSION

Through mutational analysis, in this work we have corroborated the involvement of the proteins p27 and p86, encoded by ORFs 1 and 2 of PFBV, in viral replication. These results are in agreement with those reported for other members of the family Tombusviridae which show that, besides the RdRp, the corresponding virus produces an additional, relatively small polypeptide that is indispensable for effective pathogen multiplication. The lack of complementation among p27stop and p27tyr mutants, which are impaired in p86 and p27 synthesis, respectively, suggests either a cis-preferential requirement for both viral replicase proteins and/or a coupling between translation and replication, as proposed for several members of the Tombusviridae (Okamoto et al., 2008; Oster et al., 1998; White et al., 1995) and also other viral families (e.g. Lewandowski & Dawson, 2000; Neeleman & Bol, 1999). It should nevertheless be noted that, within the family Tombusviridae, only the RdRp functioned in a cis-preferential manner in the case of Red clover necrotic mosaic virus (genus Dianthovirus) (Okamoto et al., 2008), whereas the opposite situation (i.e. cis-preferential functioning of ORF1 product) was reported for TCV and Tomato bushy stunt virus (TBSV; genus Tombusvirus) (Oster et al., 1998; White et al., 1995). Besides the probability that PFBV p27 and p86 do not function well in trans, the failure of p27- and p86-deficient PFBV mutants to accumulate in complementation assays might also be because (i) the tyrosine substitution introduced in p27tyr is not the best replacement for p86, (ii) the stoichiometry of p27 and p86 is suboptimal and/or (iii) the nucleotide changes introduced in each case lead to disruption of cis-acting elements. Concerning the last point, it is pertinent to mention that an RNA segment encompassing the readthrough portion of the RdRp coding region has been proposed to be involved in TBSV template recruitment into replication (Pogany et al., 2005). We cannot exclude that a similar internal recognition element was altered in p27stop and p27tyr mutants, which could account for the replication-incompetence of these viral RNAs.

As the virus-encoded RdRp is the catalytic subunit of the viral replicase, its interaction with RNA should exert a primary influence upon the different steps of viral RNA synthesis. However, the specific role that other essential, non-RdRp proteins (such as PFBV p27 and related products) play in replication is often uncertain. Our recent work shows that PFBV p27 contains signals for targeting to mitochondrial membranes (S. Martínez-Turiño and C. Hernández, unpublished results), which suggests that it might determine the subcellular localization of the replication complex, in agreement with that reported for ORF1 products of other members of the Tombusviridae (Mochizuki et al., 2009; Navarro et al., 2004; Panavas et al., 2005; Turner et al., 2004; Weber-Lotfi et al., 2002). It could also have additional functions during formation of the replication complex, such as recruitment of templates and other protein components. Consistent with this view, in this work we have shown that p27 is a nucleic acid-binding protein, with higher affinity for ssRNA than for dsRNA, ssDNA or dsDNA. In addition, the data suggest that p27 preferably binds to PFBV-derived ssRNAs, though more work is needed to confirm this point. Strikingly, p27 RNA binding seems to be sensitive not only to RNA sequence but also to RNA structure, as the conformation of the 3′-PFBV probe that migrated more slowly was bound preferentially in all assays (e.g. in Figs 3 and 4). The structural features that distinguish the two conformations of the ssRNA probe are not currently known, but it is probable that they are the result of the adoption of distinct higher-order structures similar to those described in the 3′-terminal region of other members of the Tombusviridae (McCormack et al., 2008; Na & White, 2006; Pogany et al., 2003; Wang & Wong, 2004) and also predicted in PFBV (Rico et al., 2006).

Though RNA-binding properties have not been described so far for p27 homologues of other carmoviruses, the p27-binding capability parallels the results obtained with the equivalent replicase protein of TBSV, p33, the only related protein for which this question has been investigated (Rajendran & Nagy, 2003). Moreover, preferences of p33 for different types of nucleic acids were reported to be similar to those found for PFBV p27, and both molecules exhibited cooperative ssRNA-binding behaviour. The lack of intermediate binding complexes in EMSAs performed with either p27 (this work) or p33 (Rajendran & Nagy, 2003) supports a cooperative view of the interaction, in which the first protein molecule binds and boosts subsequent protein binding, probably as a result of protein–protein interactions. Supporting this model, p33 has been shown to self-interact both in vitro and in vivo (Rajendran & Nagy, 2004). The cooperative nature of RNA binding, probably mediated by protein–protein interactions, has been reported for other viral proteins involved in replication, for example the poliovirus and hepatitis C virus RdRps (Pata et al., 1995; Wang et al., 2002). The functional significance of such cooperative binding is not clear, but it could increase the stability of the RNA–protein complex, resulting in protection of both viral template and replicase (Rajendran & Nagy, 2003). Other advantages of cooperative binding to the viral templates, such as enhancement of RdRp activity, have also been proposed (Hobson et al., 2001).

The apparent $K_d$ for the p27–ssRNA complex was estimated to be $11\ \text{nM}$, which is relatively low but in the range of those calculated for other viral RNA-binding proteins such as human immunodeficiency virus Rev and Tat (Burd & Dreyfuss, 1994), poliovirus polymerase (Oberste & Flanagan, 1988), vaccinia virus NPHI protein (Gross & Shuman, 1996) and citrus tristeza virus p23 (López et al., 2000). Most of these proteins, though not all, bind RNA in a sequence-specific fashion. As indicated above, the results of our competition assays suggest that PFBV p27 preferentially binds ssRNAs of viral origin, though non-viral ssRNAs also behaved as relatively good
RNA-binding properties of PFBV p27 protein

The assays of truncated forms of p27 revealed that neither N- nor C-terminal truncations abolish RNA-binding capability, though the affinity for RNA is affected by the removal of distinct protein segments (Fig. 5). As some of the truncated forms able to bind RNA did not share common regions, the results indicated that p27 contains various RNA-binding domains. Considering the negligible participation in RNA binding of the portion encompassing residues 1–100 and the high binding affinity of the truncated p27(1–144) (as witnessed by a $K_d$ of 42 nM), the central region embracing residues 101–144 contains an important determinant for RNA binding, a conclusion also supported by comparison of $K_d$ values of N-terminal truncated forms (Fig. 5). From the latter comparison, the region between residues 145 and 170 was also highlighted as relevant for RNA binding, and a contribution of the most C-terminal part (residues 171–243) could also be inferred. Despite being relatively low, the contribution of the C terminus was confirmed by mutation of three basic amino acids, which led to a protein unable to bind RNA. These results are consistent with the important role that positively charged amino acids play in nucleic acid-binding properties of numerous proteins (Genovés et al., 2009; Jones et al., 2001; Weiss & Narayana, 1998; Wobbe et al., 1998). Alignment of p27 with homologues from distinct carmoviruses did not reveal a clear common sequence signature in any of the delineated regions. However, in silico analysis of these proteins with the program RNABindR (Terribilini et al., 2006, 2007) allowed prediction of a central domain that was potentially involved in RNA binding in all of them, which would be positionally equivalent to that determined experimentally in PFBV p27 at residues 101–144 (Supplementary Fig. S3). This prediction suggests that RNA binding is a general trait of carmoviral ORF1 products. The finding of several regions in PFBV p27 endowed with RNA-binding ability contrasts with TBSV p33, for which an 8 aa segment, corresponding to an arginine- and proline-rich motif (called the RPR motif), has been identified as the primary RNA-binding site (Rajendran & Nagy, 2003).

It is reasonable to assume that, as the N-terminal portion of PFBV p86 overlaps with p27, p86 may also bind RNA, as expected for an RdRp. This point has been confirmed for p92, the RdRp of TBSV, which, besides the RPR motif that it shares with the pre-readthrough protein p33, has additional RNA-binding domains within its non-overlapping C-terminal region (Rajendran & Nagy, 2003). Whether PFBV p86 contains more RNA-binding regions than those present in p27 remains to be explored.

In conclusion, the results presented here show that PFBV p27 binds ssRNA with high affinity and that it has several regions that contribute to its binding ability. This observation suggests that such capacity is an essential function of p27 and that the protein has evolved to potentiate and preserve it. In addition, p27 seems to have a preference for PFBV-derived ssRNAs. This may favour the selection of templates, preventing amplification of ssRNAs of heterologous origin, though the participation of other viral/host proteins in template recognition cannot be discounted. Despite the low apparent $K_d$ of p27 binding, dissociation of the templates and/or products from the replication complex must occur to allow viral RNAs to move forward towards other steps of the infectious cycle (e.g. translation, intra- and intercellular movement, encapsidation). Such dissociation could be achieved by post-translational modifications of the protein that might reduce or abolish the RNA-binding properties. In line with this view, phosphorylation has been reported to diminish the RNA binding of TBSV p33 (Stork et al., 2005) and of other RNA-binding proteins (e.g. Ohndorf et al., 2001). Other post-translational modifications such as methylation of arginines have been proposed to regulate the RNA-binding activity of some proteins (Bedford & Richard, 2005; Liu & Dreyfuss, 1995; Siebel & Guthrie, 1996). Computer analysis of PFBV p27 predicts the existence of both phosphorylation and methylation sites in those regions found to be particularly relevant for RNA binding (not shown). Further work is needed to assess whether these potential modifications play any role in modulating p27–nucleic acid interaction.

METHODS

Recombinant plasmids. Plasmid pSP18-IC, which contains a wt full-length PFBV cDNA inserted into pUC18 downstream from a T7 RNA polymerase promoter, has been described previously (Rico & Hernández, 2006). This plasmid was used as a template to generate mutant constructs p27auG, p27stop and p27tyr (Fig. 1). Mutations were introduced with the QuickChange site-directed mutagenesis kit (Stratagene) and appropriate primers (CH296/CH297 for p27auG, CH298/CH299 for p27stop and CH117/CH118 for p27tyr).

For gene expression, the PFBV p27 gene was amplified from pSP18-IC with primers CH67/Ch68 and the Expand High Fidelity PCR system (Roche). The amplification product, bearing appropriate restriction sites at the ends, was cloned into E. coli expression vector pET-23d (+) (Novagen). The resulting construct, named pET-p27, harboured the p27 gene fused to a sequence coding for a hexa-His tag. A similar approach was used to generate constructs corresponding to p27 deletion-mutant derivatives pET-p27(1–100) (primers CH67/CH306), pET-p27(1–144) (CH67/CH264), pET-p27(100–243) (CH261/CH68), pET-p27(140–243) (CH262/CH68) and pET-p27(171–243) (CH307/CH68). An additional construct, pET-p27(171–243)*, identical to pET-p27(171–243) but with nucleotide substitutions that led to the replacement of three arginines by alanines (positions 195–197 in the entire protein), was generated by PCR with
the QuikChange site-directed mutagenesis kit (Stratagene) and CH321/CH322. Primers are listed in Supplementary Table S1. Each construct was verified by DNA sequencing with an ABI PRISM DNA sequencer 377 (Perkin-Elmer).

**Purification of p27 and derivative proteins from E. coli.** The pET23d(+) recombinant plasmids were used to transform E. coli Rosetta BL21(DE3)pLysS (Novagen). The His-tagged p27 protein and derivatives, whose expression was induced in the transformed bacteria with isopropyl β-D-thiogalactopyranoside at 0.4 mM for 4 h at 37 °C, were purified using Ni-NTA columns according to the supplier’s instructions (Sigma). The purified recombinant proteins were analysed and quantified by 15% SDS-PAGE after Coomassie brilliant blue staining. Quantification was confirmed by using the Bio-Rad protein assay, which is based on the Bradford method.

**Preparation of labelled and unlabelled nucleic acids.** Both labelled and unlabelled ssRNAs for gel-mobility shift and competition experiments were generated by *in vitro* transcription with T7 RNA polymerase following the manufacturer’s recommendations (Fermentas). Two distinct constructs were used as templates to synthesize PFBV-derived ssRNAs: (i) plasmid pSP18-IC, which, after digestion with EcoRI, allowed the production of an ssRNA probe (5’-PFBV) encompassing nt 1–141 of the PFBV genome; and (ii) plasmid pBS-3R, a pBluescript KS (+)D-thiogalactopyranoside at 0.4 mM for 4 h and incubated at 25°C for 30 min. In competition experiments, different amounts of unlabelled nucleic acids were added simultaneously with the labelled ssRNA probe to the binding mixtures. When indicated, the NaCl concentration was modified. After the binding reaction, samples were analysed by non-denaturing PAGE (5%) in TAE buffer (40 mM Tris/ HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.2). The gel was vacuum-dried and exposed for autoradiography or phosphorimaging analysis. Non-linear regression curves for the binding data and apparent Kd values were calculated using GraphPad Prism software version 5.03. This software was also used to estimate the IC50.

**Northwestern assay.** Purified proteins (about 0.2 μg) were separated by 15% SDS-PAGE and then transferred to nitrocellulose membranes (Scheicher & Schuell). The membranes were incubated overnight at room temperature in renaturation buffer (10 mM Tris/ HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.1% Triton X-100 and 1x Denhardt’s reagent) supplemented with 10% BSA, with three changes of buffer during the incubation. The membranes were probed with 32P-labelled 3’-PFBV ssRNA for 1 h, washed three times (15 min each) with the renaturation buffer, air dried and subjected to autoradiography.

**Inoculation of plants and protoplasts and analysis of viral infection.** *In vitro* synthesis of genomic PFBV transcripts, inoculation of plants and protoplasts and Northern blot analysis of the inoculated material were performed as described previously (Rico & Hernández, 2009).

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