Beet necrotic yellow vein virus subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement

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Beet necrotic yellow vein virus (BNYVV) is a multipartite RNA virus. BNYVV RNA3 does not accumulate in non-host transgenic Arabidopsis thaliana plants when expressed using a 35S promoter. However, a 3’-derivative species has been detected in transgenic plants and in transient expression assays conducted in Nicotiana benthamiana and Beta macrocarpa. The 3’-derivative species is similar to the previously reported subgenomic RNA3 produced during virus infection. 5’ RACE revealed that the truncated forms had identical 5’ ends. The 5’ termini carried the coremin motif also present on BNYVV RNA5, beet soil-borne mosaic virus RNA3 and 4, and cucumber mosaic virus group 2 RNAs. This RNA3 species lacks a m7Gppp at the 5’ end of the cleavage products, whether expressed transiently or virally. Mutagenesis revealed the importance of the coremin sequence for both long-distance movement and stabilization of the cleavage product in vivo and in vitro. The isolation of various RNA3 5’-end products suggests the existence of a cleavage between nt 212 and 1234 and subsequent exonucleolytic degradation, leading to the accumulation of a non-coding RNA. When RNA3 was incubated in wheatgerm extracts, truncated forms appeared rapidly and their appearance was protein- and divalent ion-dependent.

INTRODUCTION

Many positive-stranded RNA viruses possess polycistronic genomes that are supported by single or multiple RNAs. Viruses utilize different strategies for the expression of structural and non-structural proteins. Such mechanisms include cleavage of polyproteins, internal initiation of translation, leaky scanning, frameshift and read-through, as well as the expression of subgenomic RNAs (sgRNAs) collinear to one extremity (usually the 3’ end) of the viral genomic RNAs. This latter mechanism usually requires the synthesis of a viral cRNA that serves during the infection as a template for transcription of shortened forms of positive-sense viral RNAs from internal promoters recognized by the viral RNA-dependent RNA polymerase (RdRp). Such RNAs position the gene to be expressed at the 5’-proximal end of the molecule. Therefore, the prerequisite for sgRNA production is the presence of both a minus-strand template and a functional RdRp that recognizes structural motifs to initiate the internal transcription of sgRNAs (Miller & Koev, 2000). Recent studies have illustrated the accumulation of a novel class of sgRNAs that are produced independently of virus replication (de Wispelaere & Rao, 2009) and can be considered as non-coding RNAs (ncRNAs). One example of such a species is the red clover necrotic mosaic virus (RCNMV) SR1f RNA, which corresponds to a degradation product of genomic RCNMV RNA-1 (Iwakawa et al., 2008). Flaviviruses also generate a subgenomic flavivirus RNA (sfRNA) from incomplete degradation of genomic RNA and it plays an essential role in pathogenicity (Pijlman et al., 2008). Virus-derived ncRNA function varies from as-yet-unknown roles to involvement in recombination (Cheng et al., 2006; de Wispelaere et al., 2005; de Wispelaere & Rao, 2009), symptom attenuation or exacerbation (Esteban et al., 2008; Pijlman et al., 2008; Shi et al., 2008), as well as the regulation of translation and replication processes (Iwakawa et al., 2008).

Beet necrotic yellow vein virus (BNYVV) is the type member of the genus Benyivirus. Its genome is divided into four
RNA molecules (Gilmer & Ratti, 2012). RNA1 and 2 encode the essential functions for replication, encapsidation, cell-to-cell movement and silencing suppression, whereas several small genomic RNAs are essential to complete the natural infection cycle in the field (reviewed by Peltier et al., 2008). RNA3 is involved in the pathogenicity of the virus. The p25 protein affects leaf and root symptoms (Jupin et al., 1992; Peltier et al., 2011; Tamada et al., 1999) and the RNA3 core sequence is responsible for the long-distance movement of the virus in Beta spp. (Lauber et al., 1998). BNYVV RNA3-based long-distance movement can be complemented with the beet soil-borne mosaic virus RNA3 species (Ratti et al., 2009) that contains a short conserved sequence named coremin, suspected to complement the systemic spread, but its effect has not yet been demonstrated. During virus infection, RNA3 leads to the production of the species RNA3sub (Balmori et al., 1993) that presumably encodes a p4.6 ORF, expression of which has never been found (D. Gilmer, unpublished results).

Attempts to obtain transgenic lines producing full-length RNA3 led to the identification of a shortened RNA3 species referred to as RNA3sub. As no viral replicase was present in such plants, which are not hosts for BNYVV infection, we suspected this RNA to be a cleavage product. In this paper, we revisited the mechanism by which RNA3sub (Balmori et al., 1993) is generated. Our findings indicate that RNA3sub corresponds to a stable RNA3 decay product. Stabilization of the RNA3 decay product depends on the 20 nt long coremin sequence identified previously (Ratti et al., 2009). Using in vitro mutagenesis, we studied the in vivo and in vitro stability of RNA3sub and its role in long-distance movement of the virus in Beta macrocarpa.

RESULTS AND DISCUSSION

Expression of BNYVV RNA3 out of the viral context leads to its cleavage

Binary vectors expressing full-length (R3) and UTR-deleted (NR3) forms of RNA3 (Fig. 1a) were used both to produce Arabidopsis thaliana transgenic lines and to infiltrate N. benthamiana leaves with or without inclusion of the p19 silencing-suppressor protein (SSP). No RNA3s of the expected sizes were detected either in transgenic lines expressing 35S-driven RNA3 (data not shown), or when the same constructs were agroexpressed in leaves in the absence of p19 SSP. Northern blot analyses using a probe complementary to the 3' UTR did not reveal bands around 2100 and 1800 nt, expected from the predicted sizes for R3 and NR3 species, respectively. However, when p19 SSP was added to increase the transcript levels, Northern blotting allowed the detection of shortened forms of R3 and NR3 of about 870 and 820 nt, respectively, and a weak signal corresponding to the expected 1800 nt long NR3 RNA (Fig. 1b). Aside from the effect of the P19 SSP in increasing the levels of R3 and NR3 transcripts, our data suggest that processing from full-length RNA3 is more efficient to generate a shortened form, as it is no longer detected and the intensity of the corresponding shortened RNA is stronger than that of NR3. To test for a putative splicing event for the agroexpressed BNYVV RNA3 (expected to be expressed in the nucleus, whereas natural viral RNAs are known to be cytoplasmic), RNAs were Northern-blotted with a probe that hybridized specifically to the 5'-proximal RNA3 sequence (Fig. 1a). No signal of about 870/820 nt was detected, indicating that splicing did not produce such RNA species. A weak signal of about 200–300 nt was detected in leaves expressing R3 in the presence of the p19 SSP (Fig. 1c, arrow). Such a signal did not clearly appear in the T + control lane (Fig. 1c), but similar RNAs were revealed by RACE experiments (see below). NR3-derived sequences were not detected, as the probe is not expected to hybridize to such RNA (Fig. 1a), but the detection of the p25 protein from this NR3 construct supports the presence of the corresponding RNA. The p25 protein was not detected in R3-infiltrated leaves (Fig. 1d), corroborating a rapid degradation of the R3 RNA or suggesting the necessity of trans-acting viral factors for its expression. Taken together, these results suggest that constitutive expression of R3 and NR3 occurred in planta, but was followed by rapid degradation of the RNAs, leading to the accumulation of 3' RNA species. Size estimation of the 3'-end species suggests that the observed RNA3sub species extended into the terminator and polyadenylated sequences. Thus, we hypothesize that the RNA3sub species detected in the Northern blot loading control (T+, Fig. 1b) was not a bona fide sgRNA, but the result of processing of RNA3.

RNA3sub is a cleavage product

Viral sgRNAs are polymerization products generated by the action of the viral RdRp using the viral complementary genomic strand as a template. BNYVV RNAs and sgRNAs possess a cap structure (Gilmer et al., 1992a), whereas degradation or cleavage products should have a 5'-monophosphate residue. We determined the 5' terminus of the RNA species derived from R3 and NR3 and reinvestigated the nature of the BNYVV sgRNA termini. To do this, we performed 5' RACE experiments using a method that allowed us to distinguish between capped, mono- or triphosphorylated 5' ends. As BNYVV sgRNAs are not encapsidated, we used total RNAs collected from infected as well as from agroinfiltrated leaves.

RNAs were subjected to the four alternative treatments depicted in Fig. 2(a), which did or did not include a dephosphorylation step (calf intestinal phosphatase; CIP) to remove 5'-monophosphate ends, followed or not by tobacco acid pyrophosphatase (TAP) treatment to remove cap or pyrophosphate structures. Treated RNAs were ligated to the rRACE-5 oligoribonucleotide using T4 RNA ligase, reverse-transcribed using oligoT12 primer and PCR-amplified using specific primers complementary to viral BNYVV RNA3. As controls, we used total RNAs...
from plants infected with BNYVV to perform the same experiments on capped RNA2 as well as the RNA2 subgenomic species sub-a, -b and -c using specific primers. 5′ RACE of a neosynthesized RNA (with 5′ ppp or m7Gppp) is only possible on TAP-treated RNAs. However, 5′ RACE of a cleavage product is obtained only when a monophosphate remains, and is therefore possible only in the absence of CIP treatment. When these treatments were applied to control genomic (Fig. 2b) and subgenomic capped (Fig. 2c, d and e) RNAs, amplifications were detected in samples treated with TAP, validating the method. For the RNA3 samples (Fig. 2f and h), amplicons of identical sizes were visualized only when the RNA was not treated with CIP, whatever the TAP treatment. The RNA2-derived amplicons were gel-purified, cloned and sequenced. We thus confirmed the presence of a cap structure on BNYVV genomic and sgRNA2 species. The RNA2sub-a 5′ extremity corresponded to nt 2012, that of RNA2sub-b to nt 3215 and the 5′ extremity of RNA2sub-c was confirmed to map to nt 3992 of RNA2, as reported previously (Gilmer et al., 1992a). Our results demonstrate further that the RNA3sub expressed in the viral context was not capped (Fig. 2f), as thought previously (Balmori et al., 1993); nor was the shortened form of RNA3 detected when expressed ectopically (Fig. 2h). Moreover, this experiment permitted us to conclude that such species were not produced from internal initiation of transcription, as they do not have a 5′ triphosphate. Interestingly, sequence analysis indicated that a large majority (18 of 26 and five of eight in viral and transient expression, respectively) of the 5′ termini of ncRNA3 species were 2 nt upstream of the previously described coremin sequence, suspected to mediate benyvirus long-distance movement (Ratti et al., 2009). The coremin sequence is present on BNYVV RNA3,
BNYVV RNA5, beet soil-borne mosaic virus (BSBMV) RNA3 and 4, as well as on cucumovirus RNAs (Fig. 3a), and is also found at the 3' terminus of Scaevola virus A isolate RNA (GenBank accession no. JN127346.1). We conducted a similar approach to analysis of BNYVV RNA5 (Fig. 2g) and BSBMV RNA3 (not shown). BSBMV RNA4 was not included in our study. Here again, we identified eight of 10 uncapped ncRNA5 species with similar 5' termini, consisting of the coremin sequence with two extra nucleotides at the 5' end (Fig. 3a, lower panel). We concluded that RNA3sub is not an sgRNA, but rather corresponds to an RNA3 cleavage product.

Next, we characterized the 3' extremities of the 5' fragments weakly visible on the Northern blot (Fig. 1c). We self-ligated CIP- and TAP-treated RNAs to circularize them before reverse transcription and nested inverse PCR amplification with specific primers. Amplicons were obtained, cloned and characterized by sequencing. Sequences allowed the isolation of fragments ranging in size from 89 to 303 nt when RNA3 was transcribed in planta from the R3 construct. The same experiment conducted on infected tissues led to the identification of fragments from 89 to 212 nt long. However, in this latter case, extra nucleotides of unknown origin were found at the 3' ends (data not shown).

Taken together, our results indicate that RNA3 cleavage probably occurs between nt 303 and 1234 and is accompanied by a 5'→3' decay of full-length RNA3, involving
The presence of the smear below the ncRNA3 position has the stabilization of the ncRNA. RNA3sub (now ncRNA3) is the important role of the coremin sequence or structure for occur in infiltrated leaves. Such results demonstrate clearly detection of ncRNA3 in the viral context is explained by the implication of an RNA-induced silencing complex activity presence and in the absence of SSP, indicating the non-sequence was present. Hence, RNA3 cleavage occurs in the presence of wt RNA3, but not when the K, E or C mutants were used (Fig. 3c, Total). As p19 expression influenced the stability of ncRNA3 in agroinfiltrated leaves, we tested whether deletion of the p14 SSP could affect the production and the stability of ncRNA. We reproduced the experiment with RNA2 in vitro transcripts lacking the p14 ORF. Tiny local lesions appeared 6 days p.i. and the RNA contents of single local lesions were analysed as before. In the absence of p14 SSP, an identical pattern was observed (Fig. 3c, TotalASSP), indicating that RNA3 cleavage still occurred and that the ncRNA3 was stable only when the wt coremin sequence was present. Hence, RNA3 cleavage occurs in the presence and in the absence of SSP, indicating the non-implication of an RNA-induced silencing complex activity and the role of the coremin sequence in ncRNA stabilization. When compared with the transient expression assay, the detection of ncRNA3 in the viral context is explained by the amplification of the wt and mutated RNA3, which does not occur in infiltrated leaves. Such results demonstrate clearly the important role of the coremin sequence or structure for the stabilization of the ncRNA. RNA3sub (now ncRNA3) is not encapsidated and therefore cannot be visualized using the TM protocol (Gilmer et al., 1992b; Jupin et al., 1990). The presence of the smear below the ncRNA3 position has been investigated using 5’ RACE as described before (Fig. 3d). A parallel experiment using circular (c)RT-PCR fragment sequencing permitted sequence determination for the RNA produced with the K mutant (majority of the extremities corresponding to nt 1308), as well as the E and C mutants (nt 1281). None of the mutants was able to produce ncRNAs with a 5’ extremity corresponding to nt 1234. This experiment suggests that the coremin sequence is essential for the stabilization of the ncRNA3. This conclusion was corroborated by experiments in which RNA3 was expressed ectopically in agroinfiltrated leaves. Only wild-type ncRNA was detected. None of the mutated forms was able to accumulate, suggesting their likely constitutive degradation (Fig. 3c, Transient). Here again, neither wt nor mutated RNA3 was detected, suggesting that a viral product or function, such as replication and encapsidation of the genomic RNA3, is required for their stabilization.

Cleavage is a conserved mechanism that leads to the accumulation of viral ncRNAs

The presence of the coremin sequence, referred to as the Box1 sequence for cucumber mosaic virus (CMV) (Thompson et al., 2008) at the 5’ termini of benyvirus and cucumovirus RNA 3’ fragments (Fig. 3a), led us to focus on the possible role of this sequence in the virus life cycle and in the stabilization of the cleavage products leading to the ncRNAs. The known secondary structure of cucumovirus Box1 was used as a basis for site-directed mutagenesis to introduce base substitutions into the loop (Fig. 3b, K mutant), in both the stem and the loop (Fig. 3b, C mutant) and to substitute the sequence by the one present on the negative strand (Fig. 3b, E mutant). RNA3 and K, C, E mutants were inoculated with BNYVV RNA1 and 2 onto Chenopodium quinoa. Northern blot analyses were performed on total RNA extracted 7 days post-infection (p.i.) from local lesions using TRizol (Fig. 3c, Total) and encapsidated RNA extracted in the presence of Tris/MgCl2 following a 30 min incubation at 37 °C [TM protocol (Gilmer et al., 1992b)] (Fig. 3c, Encapsidated).

Wild-type (wt) and mutated RNA3 were replicated efficiently (Fig. 3c, Total) and packaged into virions (Fig. 3c, Encapsidated), indicating that the coremin sequence and subsequently ncRNA3 does not influence RNA3 replication. On the other hand, ncRNA3 was found only in lesions containing wt RNA3, but not when the K, E or C mutants were used (Fig. 3c, Total). As p19 expression influenced the stability of ncRNA3 in agroinfiltrated leaves, we tested whether deletion of the p14 SSP could affect the production and the stability of ncRNA. We reproduced the experiment with RNA2 in vitro transcripts lacking the p14 ORF. Tiny local lesions appeared 6 days p.i. and the RNA contents of single local lesions were analysed as before. In the absence of p14 SSP, an identical pattern was observed (Fig. 3c, TotalASSP), indicating that RNA3 cleavage still occurred and that the ncRNA3 was stable only when the wt coremin sequence was present. Hence, RNA3 cleavage occurs in the presence and in the absence of SSP, indicating the non-implication of an RNA-induced silencing complex activity and the role of the coremin sequence in ncRNA stabilization. When compared with the transient expression assay, the detection of ncRNA3 in the viral context is explained by the amplification of the wt and mutated RNA3, which does not occur in infiltrated leaves. Such results demonstrate clearly the important role of the coremin sequence or structure for the stabilization of the ncRNA. RNA3sub (now ncRNA3) is not encapsidated and therefore cannot be visualized using the TM protocol (Gilmer et al., 1992b; Jupin et al., 1990). The presence of the smear below the ncRNA3 position has been investigated using 5’ RACE as described before (Fig. 3d). A parallel experiment using circular (c)RT-PCR fragment sequencing permitted sequence determination for the RNA produced with the K mutant (majority of the extremities corresponding to nt 1308), as well as the E and C mutants (nt 1281). None of the mutants was able to produce ncRNAs with a 5’ extremity corresponding to nt 1234. This experiment suggests that the coremin sequence is essential for the stabilization of the ncRNA3. This conclusion was corroborated by experiments in which RNA3 was expressed ectopically in agroinfiltrated leaves. Only wild-type ncRNA was detected. None of the mutated forms was able to accumulate, suggesting their likely constitutive degradation (Fig. 3c, Transient). Here again, neither wt nor mutated RNA3 was detected, suggesting that a viral product or function, such as replication and encapsidation of the genomic RNA3, is required for their stabilization.

Coremin sequence is an RNA pathogenicity determinant that influences systemic spread

Next, we focused on the viral function of the coremin sequence. When the mutated forms of RNA3 were inoculated onto B. macrocarpa leaves together with BNYVV RNA1 and 2, no systemic spread was observed, in contrast to the efficient systemic spread of the virus when wt RNA3 was used (Fig. 3c, Systemic). This result illustrates the importance of the coremin sequence for long-distance movement of the virus in B. macrocarpa. However, it does not allow us to assign this role to its presence on full-length RNA3, ncRNA3 or both. In the case of CMV, deletion of RNA3 Box1 led to a 25% decrease in virus accumulation (de Wispelaere & Rao, 2009) and a reduction in long-distance spread. As ‘subgenomic RNA5s’ were still produced from CMV RNA1 and 2 (Thompson et al., 2008), long-distance movement was not affected. A similar situation may occur for BNYVV, as P-type RNA5 can promote long-distance movement in ≤5% of the inoculated plants with a BNYVV artificial isolate lacking RNA3 (C. Peltier & D. Gilmer, unpublished data). In contrast, however, no effect of the SR1f ncRNA is observed for systemic spread of RCNMV. Such ncRNA affects the translation of genomic RNA1 (Iwakawa et al., 2008). On this basis, we tested the effect of coremin mutations on p25 accumulation. We conducted Western blot analyses on individual local lesions and observed a decrease in p25 accumulation when the coremin sequence was mutated. This effect was only moderate for the K mutant, but was strong for the C and E mutants (Fig. 3e). The molecular bases of such a regulatory effect are not yet known and further studies will be needed to understand RNA3 translational regulation.

RNA3 cleavage occurs in vitro and depends on a protein activity

wt and mutated RNA3 were incubated in wheatgerm translation extracts (WG) for 2 h and RNA3 was detected by Northern blot hybridization (Fig. 4a). As in the in vivo experiments, RNA3-derived ncRNA was detected when the
wt coremin sequence was present (Fig. 4a, wt), but not when RNA3 contained a mutated coremin sequence (Fig. 4a, K, E, C). In a parallel experiment, wt RNA3 was incubated in WG buffer alone for up to 24 h without any degradation, suggesting that the cleavage was not due to an RNA3 cis-acting ribozyme activity (Fig. 4b). As ribozymes could be trans-acting (Been, 1994; Ohkawa et al., 1995), we phenol-purified total RNAs from WG extracts and incubated in vitro RNA3 transcripts with purified WG RNAs and buffer. Again, no visible degradation was
noticed after 6 h, supporting the absence of trans-acting ribozyme activity in WG extracts (data not shown).

A similar result was obtained when WG extracts were heat-denatured, suggesting the involvement of a protein (data not shown). Therefore, we carried out a phenol/chloroform treatment of WG extract to purify nucleic acids only partially, leaving some proteins. The extract was added to purified transcripts and, surprisingly, RNA3 was destabilized, suggesting the presence of the degradation activity. Conversely, the 600 nt long ncRNA3 appeared, with an approximately 800 nt intermediate mainly observed after 2 h of treatment (Fig. 4c, −EDTA, Probe 3'). The activity was found to be divalent ion-dependent, as the addition of EDTA prevented the 600 nt long ncRNA3 appeared, with an approximately estimated size of 500 nt, present 2 and 6 h after incubation. Signal intensity was comparable to the amount of RNA3 initially loaded, suggesting a complete cleavage of the RNA between position 500 and 1234. This 500 nt 5' cleavage product with an approximately estimated size of 500 nt, present 2 and 6 h after incubation. The presence of RNA3 and revealed a 5' cleavage product with an estimated size of 500 nt, present 2 and 6 h after incubation. A similar result was obtained when total RNAs from C. quinoa infected with BNYVV RNAs were incubated with WG extracts. Such experiments allowed us to rule out a protective effect of other viral RNA in vivo (data not shown).

Protein electrophoresis (Fig. 4e) and mass spectrometry analyses (MS-MS) of the phenol/chloroform-treated WG extract revealed the presence of the most conserved ribosomal protein L3-B2 (Brodersen & Nissen, 2005), together with ribosomal proteins S8, L2, L13a, L17-1, S11, L34 and (with lower confidence) L23. To our knowledge, no endoribonucleotide activity has been described for ribosomal proteins, although it was suspected previously to exist as part of a no-go decay surveillance mechanism, in which ribosomes have stalled during translation (Belasco, 2010; Hayes & Sauer, 2003; Passos et al., 2009; Sunohara et al., 2004), and could explain a non-site-specific cleavage of RNA3 in the vicinity of position 500. Interestingly, the p25 initiation codon is located at position 435 and ribosomal stall may occur and induce RNA cleavage. The existence of a conserved mechanism between plant and animal viruses favours a role for a conserved functional element, such as a ribosomal protein, but we cannot rule out the presence of other unidentified proteins undetected by our method. Hence, further work is needed to identify the exact nature of the nucleases involved in BNYVV RNA3 degradation.

Concluding remarks

Production of ncRNA from viral genomic RNAs seems to be a shared mechanism by animal and plant viruses and, in the case of BNYVV, appears non-host-specific. However, as far as our knowledge extends, the fate of such ncRNAs does not appear to be identical for different virus species. For BNYVV, functional effects have been found in long-distance movement on B. macrocarpa, but one should point out the non-requirement of BNYVV RNA3 for long-distance movement on N. benthamiana. Such a situation could indicate that these ncRNAs might act as tissue-specific regulatory elements, as RNA3 and p25 expression are essential prerequisites for the rhizomania syndrome. As A. thaliana does not constitute a host for BNYVV, the effect of ncRNA3 expression on the virus life cycle could not be addressed in this plant. The basis of the mode of action of viral ncRNA is yet to be discovered, but may involve RNA-silencing components together with viral proteins involved in the suppression of post-transcriptional gene silencing. The overproduction of ncRNAs could be a way for the virus to saturate the silencing machinery of the cell, as proposed for the adenovirus VA-RNA (Andersson et al., 2005; Lu & Cullen, 2004).

METHODS

Binary vectors. The B-type full-length RNA3 (R3) (Quillet et al., 1989) or a form (NR3) carrying nt 294–1725 of the cDNA sequence (Fig. 1a) flanked by XbaI and XmnI restriction sites was cloned into XbaI- and XmnI-digested pBin61 (Voinnet et al., 1998) to obtain the pBin-R3 and pBin-NR3 constructs, respectively. Vectors were introduced into Agrobacterium tumefaciens strain GV3101 (Holsters et al., 1980) using electroporation.

Transient expression of viral sequences in plant leaves. A. tumefaciens strains carrying pBin-R3, -NR3 or derivative constructs and empty binary vector pBin or pBin-p19 vector expressing the tomato bushy stunt virus silencing suppressor P19 (Voinnet et al., 2003) were infiltrated into leaf tissues as described by Voinnet et al. (1998). For co-infiltration of two Agrobacterium cultures carrying distinct vectors, equal volumes of both cultures (OD600 = 1) were mixed just before infiltration. Mature leaves of 4-week-old N. benthamiana plants were infiltrated and observed after 4, 6 and 9 days. Transgenic lines were produced as described previously (Peltier et al., 2011).

In vitro RNA synthesis, infection, plant RNA extractions and analyses. Full-length in vitro transcripts were produced and rub inoculated to plants as described previously (Quillet et al., 1989). Total RNAs from infiltrated N. benthamiana or infected C. quinoa and B. macrocarpa were extracted using TRizol reagent (Invitrogen) according to the manufacturer’s recommendations. Northern and Western blots were performed as described previously (Peltier et al., 1992a, b; Klein et al., 2007).

5’ RACE and cRT-PCR. Total RNA (20 μg) was used to perform four different 5’ RACE experiments. RNA (10 μg) was treated (+CIP) or not (−CIP) with 1 U CIP (Promega) for 30 min at 37 °C. CIP-treated and untreated RNAs (5 μg) were then incubated (+TAP) or not (−TAP) with 10 U TAP (Epicentre Biotechnologies) to remove any cap or triphosphate structures, following the manufacturer’s recommendations. RNA was purified and oligoribonucleotide rRACE-5 (5’-GAACACUGCGUUUGCUGGCUUUGAUGAAA-3’) was linked to 5’-phosphorylated RNA extremities using T4 RNA ligase before a reverse-transcription step using oligodT12 primer and avian myeloblastosis virus reverse transcriptase. Viral 5’ RNA extremities were then characterized by PCR amplification using RACE-5 primer
KOAc, 4.3 mM Mg(OAc), 10 mM DTT, 1 mM CaCl2, 1 mM spermidine, 24 mM HEPES pH 7.6. WG total RNA was phenol-and phenol/chloroform-extracted from 62.5 µl supplier WG before ethanol precipitation and was resuspended in 40 µl nuclease-free distilled water, whereas partially purified extracts were obtained similarly but omitting the phenol-extraction step.

**Protein preparation and identification by mass spectrometry.** A liquid-handler robot (Quadz215; Gilson International) was used to prepare gel pieces before trypsin digestion as described by Rabilloud et al. (2001). Briefly, gel bands were washed alternately with 100 µl 25 mM NH4HCO3 and then 100 µl 50% acetonitrile (ACN) (3 min wash with shaking, with the liquid being discarded before addition of the next solvent). This hydrating/dehydrating cycle was repeated twice and the pieces of gel were dried for 20 min before reduction (10 mM DTT, 25 mM NH4HCO3 buffer at 56°C for 45 min) and alkylation (25 mM iodoacetamide, 25 mM NH4HCO3 buffer for 45 min, room temperature). Afterwards, gel pieces were again washed with three cycles of 25 mM NH4HCO3/ACN alternately. Following a 20 min drying step, the gel pieces were rehydrated with 3 vols 25 mM NH4HCO3 buffer containing freshly prepared 12.5 ng trypsin (V5111, Promega) and incubated overnight at room temperature. Tryptic peptides were extracted from the gel by vigorous shaking for 30 min in 35% H2O2/60% ACN/5% HCOOH, followed by a 15 min sonication step.

**Matrix-assisted laser desorption/ionization–time of flight [MALDI-TOF/(TOF)] mass spectrometry and database search.** MALDI mass measurement was carried out on an Autoflex III Smartbeam (Bruker-Daltonik GmbH) MALDI-TOF TOF spectrometer used in reflector positive mode. A prepspotted anchorchip target (PAC system from Bruker-Daltonik, technical note TN011) with HCCA matrix was used to analyse tryptic digests. The resulting peptide mass fingerprinting data (PMF) and peptide fragment fingerprinting data (PFF) were combined by Biotools 3.2 software (Bruker-Daltonik) and transferred to an intranet version of the search engine Mascot (Matrix Science). Variable modifications (N-terminal protein acetylation, methionine oxidation and cysteine carbamidomethylation) and one tryptic missed cleavage were taken into account for protein identification by mass spectrometry and database search.

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