P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity

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Post-transcriptional gene silencing (PTGS), or RNA silencing, is one of the key mechanisms of antiviral defence used by plants. To counter this defence response, viruses produce suppressor proteins that are able to inhibit the PTGS pathway or to interfere with some of its function. The aim of this study was to evaluate the RNA silencing suppressor (RSS) activity of P0 proteins from selected European isolates of the beet-infecting poleroviruses beet chlorosis virus (BChV) and beet mild yellowing virus (BMYV) using two different experimental systems: (i) agro-infiltration of Nicotiana benthamiana green fluorescent protein-positive plants and (ii) mechanical inoculation of Chenopodium quinoa using a beet necrotic yellow vein virus (BNYVV, genus Benyvirus) RNA3-based replicon. The results demonstrated that P0 of most BMYV isolates exhibited RSS activity, although at various efficiencies among isolates. Conversely, P0 of BChV isolates displayed no RSS activity in either of the two systems under the experimental conditions used. These results are the first reported evidence that P0 proteins of two closely related beet poleroviruses show strain-specific differences in their effects on RNA silencing.

INTRODUCTION

Post-transcriptional gene silencing (PTGS), or RNA silencing, acts as a basic antiviral mechanism in plants and invertebrates (Ding & Voinnet, 2007) in a sequence-specific way. It is activated by dsRNA, produced during replication of viral RNA genomes, which is then sliced by an RNase III Dicer-like (DCL) enzyme and unwound by RNA helicase into single-stranded small interfering RNA molecules (ss-siRNA) (Zamore & Haley, 2005). Being involved in different steps of the silencing pathway (Vaucheret, 2006), ss-siRNAs are thought to guide annealing and endonucleolytic cleavage of complementary viral RNA molecules, following their incorporation into the RNA-induced silencing complex (RISC). The key component of RISC is the ARGONAUTE1 (AGO1) protein, which carries the RNA slicer activity (Baumberger & Baulcombe, 2005; Brodersen & Voinnet, 2006). It has been shown that many viral proteins can inhibit RNA silencing (Voinnet et al., 1999). Various viral RNA silencing suppressor (RSS) proteins appear to act at different steps in the PTGS pathway (Alvarado & Scholthof, 2009). The mechanism of action has begun to be understood for some well-studied RSSs (Li & Ding, 2006). However, the mode of action of the majority is still unclear and many unanswered questions remain. It has been suggested that dsRNA binding is a general silencing suppression strategy of plant viruses as many diverse RSSs show affinity to ds-siRNA (Lakatos et al., 2006). Among the best characterized RSSs with RNA-binding activity is the P19 protein of tombusviruses (Lakatos et al., 2006). It has also been shown that the 2b protein of cucumber mosaic virus (CMV, genus Cucumovirus), which interferes with the microRNA (miRNA) pathway (Zhang et al., 2006; Goto et al., 2007; Lewsey et al., 2007). However, recent findings have shown that several viruses have developed other modes of action. This is the case for P38 of turnip crinkle virus (genus Carmovirus), which blocks DCL4 and DCL2 activities in Arabidopsis thaliana (Deleris et al., 2006), and the 2b protein of CMV, which inhibits the cleavage function of AGO1 (Zhang et al., 2006).

The RSSs characterized for sugar beet viruses are: L2 of beet curly top virus (genus Curtovirus; Yang et al., 2007), P14 of beet necrotic yellow vein virus (BNYVV, genus Benyvirus;
Dunoyer et al., 2002), P21 of beet yellows virus (genus Closterovirus; Reed et al., 2003) and P0 of turnip yellows virus (TuYV, genus Polerovirus; Pfeffer et al., 2002). TuYV [syn. beet western yellows virus (BWYV), FL1 strain; D’Arcy & Domier, 2005; Beuve et al., 2008] is closely related to the poleroviruses beet chlorosis virus (BChV) and beet mild yellowing virus (BMYV) used in this study. Recently, it was shown that the P0 protein of TuYV and of cucurbit aphid-borne yellows virus (CABYV, genus Polerovirus) interacts through its F-box domain with Arabidopsis S-phase kinase-related protein 1 (SKP1) orthologues ASK1 and ASK2, components of the SKP1–Cullin F box (SCF) family of E3 ubiquitin ligases (Pazhouhandeh et al., 2006). It has been postulated that P0 might target AGO1 for degradation by acting as an F-box protein in an SCF complex (Baumberger et al., 2007; Bortolamiol et al., 2007).

Three distinct viruses belonging to the genus Polerovirus were described in Europe in relation to sugar beet: BMYV, TuYV and BChV, of which only BMYV and BChV are able to infect sugar beet and to display similar symptoms of ‘mild yellowing’ (D’Arcy & Domier, 2005). A further beet-infecting species, BWYV-USA, has also been described (Beuve et al., 2008). All poleroviruses, like other members of the family Luteoviridae, are phloem-limited and are transmitted in a circulative–persistent manner by several aphid species (Stevens et al., 2005). They have a positive-sense ssRNA genome with a genome-linked protein (VPg) at their 5’ end. The beet polerovirus genome consists of six open reading frames (ORFs) (P0–P5). P1 and P2 are required for virus replication (Mayo & Ziegler-Graff, 1996). ORF3 encodes the major coat protein (CP) involved in particle assembly and aphid transmission (Brault et al., 1995; Stevens et al., 2005). The product of ORF4 is thought to be a movement protein. P5 is expressed together with P3 as a fusion protein, the readthrough protein, whose N-terminal region contains important domains for aphid transmission and virus accumulation in plants (Brault et al., 1995; Bruyère et al., 1997).

So far, polerovirus P0 has been shown to be the RSS of TuYV and CABYV (Pfeffer et al., 2002), acting as an F-box protein and targeting a key step in the RNA-dependent RNA degradation pathway (Pazhouhandeh et al., 2006). The aim of this work was to evaluate and compare P0 RSS activity from a range of BMYV and BChV isolates, using both agro-infiltration of fluorescent Nicotiana benthamiana plants and inoculation of Chenopodium quinoa via a BNYVV replicon.

RESULTS

RSS activity analysis using a patch test in green fluorescent protein-positive (GFP⁺) transgenic N. benthamiana

To determine whether the P0 proteins of the poleroviruses BChV and BMYV could suppress GFP silencing in GFP-expressing transgenic N. benthamiana line 16c, binary vectors expressing P0 of BChV (P0BC) or BMYV (P0BM) were constructed, and those expressing P0 of TuYV (pBin-P0Tu) and P14 of BNYVV (pBin-P14) were used as described previously (Pfeffer et al., 2002). Expression of GFP in the leaves and stems of N. benthamiana line 16c resulted in a green fluorescence that could be visualized under UV light. However, silencing of this GFP expression was

![Fig. 1. Response of transgenic GFP-expressing N. benthamiana line 16c after agro-infiltration using vectors harbouring the P0 gene of several polerovirus isolates. Leaves at 4 days (panels 2–5) or 1 week (panels 1 and 6) after agro-infiltration with bacteria mixtures harbouring pBin-GFP and one of the following: 1, empty vector pBin-Ø; 2, pBin-P0Tu; 3, pBin-P14; 4, pBin-P0BM-19K (similar results were obtained with pBin-P0BM-21B, pBin-P0BM-N9 and pBin-P0BM-N27); 5, pBin-P0BM-26 (a similar result was obtained with pBin-P0BM-N32); 6, pBinP0BC-N13 (similar results were obtained with pBin-P0BC-2a, pBin-P0BC-18K, pBin-P0BC-O36, pBin-P0BC-M27 and pBin-P0BC-M26).](http://vir.sgmjournals.org)
visualized by red fluorescence after agro-infiltration of the 16c line with a binary vector pBin-GFP carrying 35S-GFP and designed to transiently express a GFP transcript. The same red fluorescence appeared when pBin-GFP was co-agro-infiltrated with the empty vector (pBin-Ø) (Fig. 1, panel 1). The GFP silencing produced in line 16c by infiltration of pBin-GFP can be suppressed by co-infiltration of a pBin vector expressing an RSS protein (Voinnet et al., 1998).

As expected, when pBin-GFP was co-infiltrated with most pBin-P0BM, the infiltrated patches displayed an intense green fluorescence at 4 days post-inoculation (p.i.), indicating that P0 of BMYV, as already shown for P0 of TuYV and P14 of BNYVV, exhibited an RSS activity (Fig. 1, panels 2–4; Table 1). Patches that were co-infiltrated with pBin-GFP/pBin-P0BC did not inhibit GFP silencing and displayed the same phenotype as patches infiltrated with pBin-GFP/pBin-Ø (Fig. 1, panel 6). In contrast, no or weak suppression of GFP silencing was detected for P0 of two BMYV isolates (BMYV-N32 and -26) (Fig. 1, panel 5, Table 1), even though GFP mRNA transcripts were detected in the corresponding plants (Fig. 2). This weak GFP fluorescence probably corresponded to incomplete suppression by P0 of the two isolates.

The persistence of the intense GFP fluorescence was observed even after 21 days p.i. in patches that were co-infiltrated with either pBin-GFP/pBin-P0Tu or pBin-GFP/pBin-P0BM (data not shown). In contrast, in the case of BNYVV pBin-P14 agro-infiltration, the intense green fluorescence faded after 1 week under our experimental conditions. Indeed, the transcripts of P14 underwent RNA silencing in turn, as the accumulation of siRNA of these transcripts has been revealed (H. Guilley, unpublished results). This could be due to P14 instability in a non-viral context. This observation suggests that P14 certainly does not have the same operating mode as P0 of TuYV and BMYV. In the case of P0BM-N32 and P0BM-26, the weak fluorescence faded after 1 week, similar to BNYVV P14-infiltrated patches (data not shown).

Molecular hybridization using a GFP probe (Himber et al., 2003) of total RNA extracted from patches at 4 days p.i. confirmed the visual observations (Fig. 2a, b). Northern blot analyses showed that GFP mRNA was moderately to highly abundant in the green fluorescent patches of leaves that were co-infiltrated with pBin-GFP/pBin-P0Tu, pBin-GFP/pBin-P0Tu or pBin-GFP/pBin-P0BM (Fig. 2a, lanes 3, 4, 11, 12, 15 and 16). Conversely, the siRNA accumulation found in these patches was not intense (Fig. 2b, lanes 3, 4, 11, 12, 15 and 16). Both fluorescence and RNA analyses of GFP expression in the infiltrated N. benthamiana leaves clearly identified most of the P0BM as RSSs, as already shown for P0Tu (Pfeffer et al., 2002). In contrast, for all P0BC studied, as well as for two P0BM (P0BM-N32 and P0BM-26), no mRNA GFP overaccumulation was observed (Fig. 2a, lanes 5–10 and 13 and 14, respectively) and in correlation with this, siRNA molecules accumulated to high levels (Fig. 2b, lanes 5–10 and 13, 14 respectively), indicating that these P0s, if they are stable under our experimental conditions, have an undetectable or weak RSS activity.

### RSS activity analysis using BNYVV inoculation of C. quinoa

In order to confirm the results of the aforementioned agro-infiltrations, we used a viral vector complementation method developed recently by Guilley et al. (2009) and based on a BNYVV-derived recombinant vector to identify and characterize RSS activity of different heterologous viral

<table>
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<tr>
<th>P0</th>
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<th>P0 sequence GenBank accession number</th>
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<th>Local lesion phenotype</th>
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*–, Lack of green fluorescence or local lesion phenotype; +, presence of high-level green fluorescence or local lesion phenotype; +/-, presence of weak green fluorescence; NT, not tested.

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Table 1. Polerovirus isolates used in this study and summary of the response of their P0 tested for its ability to suppress RNA silencing, using two experimental systems: fluorescence in agro-infiltrated N. benthamiana and local lesion phenotype in inoculated C. quinoa
proteins. One advantage of this assay lies in the fact that the expression of the heterologous potential RSS by the recombinant virus reproduces the natural conditions of a cytoplasmic viral infection. In this study, we used a variant of this technique as described below.

BNYVV RNA1, -2, -3 and -4 are necessary for the natural infection and transmission processes for sugar beet (Koenig et al., 1986; Bouzoubaa et al., 1991). However, when BNYVV is mechanically inoculated into experimental hosts such as *C. quinoa*, RNA1 and -2 are sufficient to induce and maintain infection (Quillet et al., 1989). At 6 days p.i., the inoculated plants displayed chlorotic local lesions of medium size (Fig. 3, panel 1).

We used a BNYVV RNA3-based replicon (Jupin et al., 1992; Bleykasten-Grosshans et al., 1997; Guilley et al., 2009) as an amplification–translation vector to express P0 proteins of different poleroviruses. To assess the capacity of P0BC and P0BM to cross-complement the RSS activity of BNYVV P14, we used infectious transcripts comprising RNA1 +2D P14 of BNYVV strain F2 (Quillet et al., 1989), an artificial strain deficient in P14 synthesis (Hehn et al., 1995). *C. quinoa* leaves inoculated with this strain developed small necrotic local lesions at 5 days p.i. (Fig. 3, panel 2). These lesions were ten times smaller than those developed after inoculation by wild-type BNYVV RNA1 + 2 (Fig. 3, panel 1). This phenotype of local lesions was consistent with a low RNA accumulation (Fig. 4, lane 9). As a positive control and in order to show that it was possible to cross-complement the lack of P14, we used tomato bushy stunt virus (TBSV; genus *Tobamovirus*) P19 protein and TuYV P0 protein as heterologous RSSs (Guilley et al., 2009).

To check the expression ability of RNA3 replicon-based constructs harbouring polerovirus P0 ORFs, we co-inoculated wild-type RNA1 + 2 along with a replicon, either empty (rep0) or containing the ORF of BNYVV P14, TBSV P19 or P0 of various beet poleroviruses (TuYV-FL1, BChV-2a and BMYV-2ITB). All of these replicons showed the same accumulation as wild-type RNA1 and -2, as visualized on Northern blots (Fig. 4, lanes 3–8). The accumu-

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**Fig. 2.** Molecular hybridization analysis. (a, b) Northern blot analysis of GFP mRNA (a) and GFP siRNA (b) at 4 days p.i. extracted from infiltrated patches of *N. benthamiana* line 16c after agro-inoculation with pBin-GFP plus one of the following: vector pBin (lane 2); pBin-P0Tu (lane 3); pBin-P14 (lane 4); pBin-P0BC-2a (lane 5); pBin-P0BC-18K (lane 6); pBin-P0BC-O36 (lane 7); pBin-P0BC-M27 (lane 8); pBin-P0BC-N13 (lane 9); pBin-P0BC-M26 (lane 10); pBin-P0BM-2ITB (lane 11); pBin-P0BM-19K (lane 12); pBin-P0BM-26 (lane 13); pBin-P0BM-N32 (lane 14); pBin-P0BM-N9 (lane 15); pBin-P0BM-N27 (lane 16). The RNA loaded in lane 1 came from a non-infiltrated leaf. +, High silencing suppression activity; +/−, moderate silencing suppression activity; −, no silencing suppression activity. (c) Loading control for cellular rRNA.

**Fig. 3.** Experiment demonstrating BNYVV P14 complementation. Leaves of *C. quinoa* plants were observed at 6 days p.i. as after inoculation as follows: 1, RNA1 + 2 alone (similar symptoms were obtained with RNA1 +2 + rep0 or RNA1 + 2 + any of the replicons analysed: repP14, repP19 TBSV , repP0BM-2ITB , repP0BC-2a); 2, RNA1 + 2ΔP14; 3, RNA1 + 2ΔP14 with repP0Tu (similar symptoms were obtained with repP14 or with repP0BM-2ITB); 4, RNA1 + 2ΔP14 with repP19 TBSV ; 5, RNA1 + 2ΔP14 with repP0BM-19K; 6, RNA1 + 2ΔP14 with rep0 (a similar phenotype, i.e. no symptoms, was obtained with repP0BM-26, repP0BM-N32, repP0BC-2a, repP0BC-18K, repP0BC-O36, rep0BC-N13 or repP0BC-M27). Bar, 0.5 cm.
attachment rate of viral RNA was somewhat variable between experiments. Moreover, P0 sequence insertion did not interfere with RNA1 and -2 replication (Fig. 4, lanes 6–8). This finding allowed us to use these replicons in heterocomplementation experiments.

Complementation tests were carried out with P0BC and P0BM RNA3 replicons, which were inoculated onto C. quinoa in the presence of helper RNA1 + 2ΔP14 transcripts. At 6 days p.i., chlorotic local lesions appeared on inoculated leaves in the case of P0BM (Fig. 3, panel 3, Table 1), similar to those with wild-type phenotype obtained with RNA1 + 2 (Fig. 3, panel 1) or with an inoculum containing RNA1 + 2ΔP14 + repP14. However, no local lesions were observed in the case of the replicons harbouring the P0 ORF from all BChV isolates and two BMYV isolates (BMYV-N32 and -26) (Table 1). This phenotype was similar to that displayed with RNA1 + 2ΔP14 + rep0 (RNA3 empty replicon construct) (Fig. 3, panel 6) and may result from an additional effect to RNA silencing, the defective interfering effect of the RNA replicon described previously (Hehn et al., 1994). The presence or lack of RNA accumulation of these replicons was confirmed by Northern blot analysis (Fig. 4). CP is produced when P14 is expressed but no CP can be detected when P14 is absent (Hehn et al., 1995). CP accumulation correlated with viral RNA multiplication, except for P0Tu, for which the CP accumulation was weaker than for the other P0s (Fig. 5, lane 10). This was probably due to its necrotic effect on C. quinoa leaves: the chlorotic local lesions were necrotic in the centre at 5 days p.i. (Fig. 3, panel 4) and became totally necrotic at 7 days p.i. (data not shown).

Discussion

Analysis of different plant viruses indicates that the ability to suppress RNA silencing is a widespread property (Voinnet, 2005). More than 35 individual RSSs have been identified from almost all plant virus taxa (Li & Ding, 2006). These proteins are structurally and functionally very different. For TuYV (syn. BWYV-FL1), the RNA silencing suppression function has been assigned to the P0 protein (Pfeffer et al., 2002). ORF0 is the most variable genomic region within the genus Polerovirus. However, phylogenetic clustering based on the P0 sequence of CAYBV, BMV and BWYV-USA has confirmed their high identity for this part of the genome (Fig. 6; Beuve et al., 2008) and has revealed the potential ability of P0 of these species to suppress RNA silencing.

The aim of this study was to investigate whether P0 of the closely related BMYV and BChV displayed a similar function and whether this ability varied among European isolates. Using two experimental systems based on either nuclear (agro-infiltration) or cytoplasmic (viral context) systems of P0 transcript expression, we demonstrated that the ability to suppress RNA silencing was variable, even among closely related members of the same virus.
genus. We showed that most P0s of BMYV displayed RSS activity in both of the heterologous systems used. Moreover, the suppression induced by P0 of BMYV was long-lasting in agro-infiltration experiments, similar to P0 of TuYV (Pfeffer et al., 2002) but in contrast to that induced by BNYVV P14, which decreased over a period of 6 days p.i. This observation suggests that P0 of BMYV and P14 of BNYVV may have different modes of action in their RSS mechanism. Conversely, we demonstrated that P0 of six BChV isolates had no detectable RSS activity.

The ability of P0BC and P0BM to suppress RNA silencing was investigated using the second system involving a viral context expression, i.e. C. quinoa inoculation experiments using BNYVV replicons as we described recently (Guilley et al., 2009). Thus, using TBSV P19 or P0Tu, we confirmed that suppression activity analysis by this method is a reliable means of testing various proteins for their RSS ability. We showed that replicons carrying P0 of most BMYV isolates can complement BNYVV P14, but replicons carrying P0BC, P0BM-N32 or P0BM-26 cannot. Therefore, these results are consistent with our aforementioned observations using agro-infiltration of the N. benthamiana 16c line.

In addition, we noted that, after C. quinoa inoculation, P0Tu induced chlorotic local lesions, becoming necrotic in the centre at 5 days p.i. (Fig. 3, panel 4) and totally necrotic at 7 days p.i. This protein is already known as a pathogenicity and symptom determinant (Pfeffer et al., 2002). In contrast, C. quinoa infected leaves with the P0BM RNA3 replicon displayed chlorotic local lesions (Fig. 3, panel 3), as well as the phenotype observed in the case of BNYVV.

![Fig. 5. BNYVV P14 complementation.](image)

![Fig. 6. Neighbour-joining phylogenetic tree of aligned amino acid sequences of various polerovirus P0 proteins.](image)
infection (Fig. 3, panel 1). Therefore, P0BM protein was able to restore a BNYVV wild-type phenotype similar to that produced in the case of BNYVV P14.

It is noteworthy that the BChV host range appears to be narrower than that of BMYV, BWYV-USA and TuYV (Hauser et al., 2002; Beuve et al., 2008), suggesting that BChV has evolved separately and probably appeared by recombination between an unidentified polerovirus-like ancestor, which provided the 5' part of the genome encoding P0 and the polymerase, and BMYV or TuYV, which provided ORF3, -4 and -5 (Hauser et al., 2002). Exchanging ORF0 sequences between BChV and BMYV in order to investigate their biological activity would be informative.

Among the six BMYV isolates, two isolates (BMYV-N32 and -26) did not exhibit RSS activity in either assay. The P0 sequences of these two isolates seem to be closest to the BChV P0s within the BMYV group (Fig. 6). However, amino acid sequence analysis of the isolates BMYV-N32 and -26 did not reveal any changes, in comparison with the other isolates of BMYV, in their F-box-like domain (Table 2).

Moreover, the amino acid sequence comparison with other poleroviruses revealed the presence of the short motif of the leucine-rich repeat sequence LP(LL)L/I (residues 61–65 of BChV), which matches the start of the F-box sequence in P0 of all BChV isolates (Table 2). However, the amino acid composition of a C-terminal-proximal sequence (K/R)IYGEDGX3FWR, which could be a so far undescribed part of the genome, was the case for BMYV in this study. Such an extreme variation in RSS ability in closely related members of a single virus group has been shown for potexviruses (Voinnet et al., 2000; Senshu et al., 2009) and for some CMV strains (Zhang et al., 2006; Lewsey et al., 2007). As we tested P0 as an isolated protein for its potential RSS ability, we cannot exclude the existence of another viral RSS protein, as has been reported for citrus tristeza virus (genus Closterovirus), which possesses at least three RSSs, P20, P23 and CP (P25) (Lu et al., 2004). In the case of BChV, the RSS could correspond to another viral protein or to an interaction between P0 and another viral factor. It is also possible that P0 proteins that do not show suppression activity could be unstable under our experimental conditions. However, as no specific antibodies targeting these proteins are currently available, we are unable to draw conclusions about their stability in planta. Further studies of beet polerovirus P0s are required to understand their variability and mode of action as RSSs. Thus, it would be informative to investigate recognition of AGO1 by these P0s in transgenic A. thaliana plants, as has been performed for P0Tu (Bortolamiol et al., 2007).

### Methods

**Origin of virus isolates and RT-PCR constructs.** BChV and BMYV isolates were collected in France (BChV-M26, -M27, -N13 and -O36; BMYV-N9, -N27, -N32 and -2ITB), Poland (BChV-18K; BMYV-19K and -26) and the UK (BChV-2a) (Table 1; Kozlowska-Makulska et al., 2009). Isolates BMYV-2ITB and BChV-2a have been fully sequenced (Guilley et al., 1995; Hauser et al., 2002). Viral cDNAs from different isolates were used as templates for PCR amplification of their P0 genes using appropriate specific primers (Hauser et al., 2000). XbaI and BamHI restriction sites were added to the ORF0.

**Table 2.** Amino acid sequences of P0 of TuYV, BWYV-USA, CABYV, BMYV-2ITB, BMYV-19K, BMYV-N27, BMYV-N32, BMYV-26, BChV-2a, BChV-N13.

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<th>Virus</th>
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*Number of first amino acid in sequence.
†The sequence in this region of P0 of BMYV-N9 is identical to that of BMYV-19K.
‡The sequences in this region of P0 of BChV-18K, BChV-M26, BChV-M27 and BChV-036 are identical to that of BChV-N13.
the 5’ extremity of the BChV and BMYV primers, respectively. RT-PCR was performed essentially as described by Hauser et al. (2000). XbaI- and BamHI-digested PCR fragments were ligated into pBin61 (Voinnet et al., 2000) to produce pBin-P0. In order to confirm the P0 insert, three PCR amplicons per isolate were cloned and sequenced.

Agro-infiltration of transgenic N. benthamiana. The agroinfiltration experiments described here were repeated at least three times. Six isolates each of BChV and BMYV were used for this experiment (Table 1). The plant material (N. benthamiana line 16c, transgenically expressing GFP and the Agrobacterium tumefaciens (strain C58C1) infiltration method have been described previously (Voinnet et al., 1998). For co-infiltration, equal volumes of A. tumefaciens (with pBin-GFP or pBin-P0) cultures (OD600 = 1) were mixed before infiltration. One or two lower leaves of 5-week-old N. benthamiana plants were infiltrated. The plants were then shifted to a growth chamber (temperature 20 ± 2 °C, photoperiod 16 h). Plants were observed under 100 W hand-held long-wave UV lamps (Voinnet et al., 1998) and patches (100 mg) were collected for total RNA extraction and Northern blot analysis at 4 days to 1 week p.i.

Construction of BNYVV replicons. Clones of RNA1, RNA2 and RNA3-derived replicon (rep0) used in this study have been described previously (Quillet et al., 1989; Lupin et al., 1992; Erhardt et al., 2000). The RNA2AP14 mutant carrying a mutation in the P14 ORF (+ 2 frameshift mutation) has been described by Hehn et al. (1995). The BChV and BMYV P0 sequences for insertion into rep0 were amplified by PCR with two primers that contained, respectively, a BamHI or XbaI restriction site. BChV and BMYV PCR fragments were then digested with both BamHI and XbaI and inserted into BamHI/XbaI-cleaved rep0 plasmid. Clones containing the PCR fragment in the appropriate orientation were identified by restriction enzyme digestion and the insert was confirmed by sequence analysis.

In vitro transcription and C. quinoa infection. BNYVV constructs and different replicons were linearized with HindIII prior to transcription. The transcription reaction was performed with a T7 RNA polymerase RibomAX transcription kit (Promega) following the manufacturer’s instructions. Transcripts (5 μg in 20 μl) were mechanically inoculated onto celite-dusted C. quinoa leaves (two leaves per plant) as described by Gilmer et al. (1992). Infected plants were grown in a greenhouse at 25 ± 2 °C with a photoperiod of 16 h.

RNA analysis. Total RNA was extracted using a polysome extraction buffer and a phenol/chloroform method (Jackson & Larkins, 1976). Following ethanol precipitation, high-molecular-mass RNA was separated from low-molecular-mass RNA by precipitation with 5% PEG 8000 and 0.5 M NaCl. High-molecular-mass RNA was recovered after solubilization of cellular DNA with 3 M sodium acetate. High- and low-molecular-mass RNA fractions were separated by 1% agarose or 15% polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Hybond-NX; Amersham). RNA samples for electrophoresis were adjusted to the same concentration by for electrophoresis were adjusted to the same concentration by high- and low-molecular-mass RNA fractions. High-molecular-mass RNA was detected with specific polyclonal antiserum (Niesbach-Kloßgen et al., 1992). The polerovirus F box protein P0 shows variable PTGS suppression activity.

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