Rapid screening of RNA silencing suppressors by using a recombinant virus derived from beet necrotic yellow vein virus

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To counteract plant defence mechanisms, plant viruses have evolved to encode RNA silencing suppressor (RSS) proteins. These proteins can be identified by a range of silencing suppressor assays. Here, we describe a simple method using beet necrotic yellow vein virus (BNYVV) that allows a rapid screening of RSS activity. The viral inoculum consisted of BNYVV RNA1, which encodes proteins involved in viral replication, and two BNYVV-derived replicons: rep3–P30, which expresses the movement protein P30 of tobacco mosaic virus, and rep5–X, which allows the expression of a putative RSS (X). This approach has been validated through the use of several known RSSs. Two potential candidates have been tested and we show that, in our system, the P13 protein of burdock mottle virus displays RSS activity while the P0 protein of cereal yellow dwarf virus-RPV does not.

RNA silencing suppressor (RSS) proteins are pathogenicity determinants widely expressed by plant and animal viruses (Li & Ding, 2006; Moissiard & Voinnet, 2004) that have recently also been identified amongst bacterial effectors (Navarro et al., 2008). Although these proteins are often multifunctional (Diaz-Pendon & Ding, 2008), the activity that will concern us here is their ability to block or attenuate plant host defence mechanisms, particularly post-transcriptional gene silencing. A large number of RSSs have been identified using procedures described in recent reviews (Li & Ding, 2006; Moissiard & Voinnet, 2004; Qu & Morris, 2005). The most popular assay is the ‘patch’ technique developed by Voinnet et al. (1998) based on the infiltration of Agrobacterium tumefaciens cultures harbouring the putative RSS and a reporter gene [usually the gene for green fluorescent protein (GFP)] on Nicotiana benthamiana. Viral vectors such as potato virus X have also proven to be useful to express and characterize RSS in a silencing reversal assay (Voinnet et al., 1999). More recently, alternative technologies based on functional complementation of defective viral mutants have been developed (Chiba et al., 2006; Powers et al., 2008). Here, we describe another simple experimental approach to screen for RSS activity based on a viral system derived from beet necrotic yellow vein virus (BNYVV). This test was used to assess the RSS activity of two uncharacterized viral proteins.

For successful amplification on a plant host, most viruses need to fulfil three main functions: (i) replication, (ii) movement from the initial point of infection and (iii) suppression of the host RNA silencing mechanism. If infection is successful, symptoms can be observed and progeny RNA is detected. BNYVV has intrinsic properties that lend themselves to use in an assay for RNA silencing suppression activity. First, BNYVV RNA1, which encodes the viral RNA-dependent RNA polymerase, can replicate autonomously (Bouzoubaa & Scheidecker, 1990; Gilmer et al., 1992). Second, functional replicons have been constructed from genomic RNA3 and 5, viral RNAs which are not required for viral multiplication on leaves but are needed in the natural cycle of infection of roots (Jupin et al., 1990; Schmidlin et al., 2005). Replicons based on these RNA species, rep3 and rep5, respectively (Fig. 1a), can express distinct functional heterologous proteins and, importantly, this can occur in the same infected cell (Schmidlin et al., 2005).

In the assay described here, RNA1 is supplemented with rep3–P30 (which we will refer to as rep30) encoding the tobacco mosaic virus (TMV) movement protein (Lauber et al., 1998) and rep5–X encoding a putative RSS (X). Movement and silencing suppressor functions are normally encoded by RNA2, provided by the triple gene block (TGB) and the cysteine-rich protein P14, respectively (Dunoyer et al., 2002) (Fig. 1a). Note that the coat protein is dispensable for cell-to-cell movement for both TMV and BNYVV (Deom et al., 1992; Schmitt et al., 1992).

The following characterized RSS have been tested in this study: P14 of BNYVV and P15 of peanut clump virus...
(PCV), two cysteine-rich proteins (Dunoyer et al., 2002); P19 of tomato bushy stunt virus (TBSV), a short interfering RNA (siRNA) duplex-binding protein (Silhavy et al., 2002); P0 of beet western yellows virus (BWYV), a protein that targets ARGONAUTE1 (Bortolamiol et al., 2007; Pazhouhandeh et al., 2006); HcPro of turnip mosaic virus (TuMV; Kasschau et al., 2003); and Tas of the retrovirus primate foamy virus type 1 (PFV-1; Lecellier et al., 2005). We have also tested a mutant RSS protein that has lost its suppression capacity, P19mut of TBSV (Qiu et al., 2002), as well as two proteins that have not been characterized so far, P13 of burdock mottle virus (BdMV; Rush, 2003), a tentative member of the genus Benyvirus and P0 of cereal yellow dwarf virus-RPV (CYDV-RPV; Vincent et al., 1991), a monocot-infecting polerovirus.

In order to express known or putative RSS protein from rep5, we designed two sets of primers for each cDNA:

Fig. 1. (a) Schematic maps of BNYVV RNA1, RNA2 and the artificial replicons rep30 and rep5-X derived from RNA3 and RNA5, respectively. rep30 encodes the TMV P30 movement protein and rep5-X encodes a putative RSS (X). P14 encoded by RNA2 is the natural BNYVV silencing suppressor protein. (b, c) Northern blot analysis of progeny viral RNA in C. quinoa leaves inoculated with transcripts of BNYVV RNA1 plus BNYVV RNA2 (b) or rep30 (c) alone (lane 2) or supplemented with either empty rep5-0 (lane 3) or rep5 expressing a potential functional or mutated RSS, P14BNYVV (lane 4), P19TBSV (lanes 5 and 6), P0BWYVV (lane 7), P15PCV (lane 8), HcProTuMV (lane 9), TasPFV-1 (lanes 10), P0CYDV (lane 11) and P13BdMV (lane 12). Total RNA was extracted 6 days p.i. from equal weights of pooled local lesions or inoculated plant tissue. Lane 1 was loaded with RNA extracted from the same weight of non-inoculated leaf tissue. rRNA corresponds to the loading control. 32P-Labelled anti-sense RNA probes used to detect the different viral RNA and replicons (identified to the right) have been described (Lauber et al., 1998, 2005). In (c), the position of rep30 RNA is indicated by an asterisk (*).
forward primer containing an NcoI site and a reverse primer containing a BglII site. Both sites are present in the polylinker of the empty replicon rep5. PCR products were prepared from cDNA of each suppressor, cut with NcoI and BglII and cloned into rep5 digested with the same enzymes and treated with calf intestinal alkaline phosphatase. In two of the constructions, the cDNA itself contained an NcoI or BglII site, requiring a two-step cloning procedure. The resulting recombinant clones were checked by sequencing and will be referred to as rep5–P14BNYVV, rep5–P19TBSV, rep5–P19mutTBSV, rep5–P0BWYV, rep5–P15PCV, rep5–HcProTuMV, rep5–TasPFV-1, rep5–P0CYDV and rep5–P13BdMV. The different recombinant replicons were linearized with HindIII or NheI prior to transcription (Quillet et al., 1989).

First, we tested the capacity of each replicon transcript to replicate in a BNYVV context, i.e. in the presence of BNYVV RNA1 and RNA2 transcripts. For this purpose, plants of Chenopodium quinoa, a local lesion host of BNYVV, were inoculated with in vitro transcripts of BNYVV RNA1 and RNA2, supplemented or not supplemented with transcripts of the various replicon constructs. As a control, we used an empty replicon (rep5–0). Fig. 1(b) shows that all replicons (rep5–X) were able to replicate. We also verified that both P14BNYVV and P19TBSV suppressor proteins for which antibodies were available were expressed. The level of expression of the other proteins is unknown.

We next inoculated C. quinoa with BNYVV RNA1 and rep30 transcripts supplemented with a transcript of rep5 encoding one of the various RSSs. The control inocula consisting of BNYVV RNA1 and rep30 (providing the movement function), alone or with rep5–0, did not produce local lesions on C. quinoa (Fig. 2, ii and iii). This prompted us to verify the replication capacity of these samples by adding a transcript of rep5 encoding the GFP (rep5–GFP; Schmidlin et al., 2005). Inoculated leaves were observed by confocal microscopy. At 2 days post-inoculation (p.i.), the infection foci showed an average of 10–15 fluorescent cells, increasing to more than 40 epidermal and mesophyll cells at 6 days p.i. (Supplementary Fig. S1, available in JGV Online). After that, no further spread could be detected and the fluorescence intensity started to diminish. This observation clearly indicated that RNA1 supplemented with the movement function of P30 of TMV is able to replicate and spread over many cells, but these foci were too small to be visible by eye (Fig. 2, ii and iii). In contrast, when RNA1 was supplemented with rep5–GFP

![Fig. 2. Symptoms (local lesions) observed on leaves of C. quinoa mock-inoculated (i) or inoculated with transcripts of BNYVV RNA1 plus rep30 alone (ii) or rep30 supplemented with empty rep5 (iii) or rep5 expressing a potential functional or mutated RSS: P14BNYVV (iv), P19TBSV (v and vi), P0BWYV (vii), P15PCV (viii), HcProTuMV (ix), TasPFV-1 (x), P0CYDV (xii) or P13BdMV (xiii). Pictures were taken 4 days p.i. for plants whose inoculum contained the RSS of TBSV, PCV and TuMV and 6 days p.i. for the other plants. Symptoms produced by inoculation of transcripts of BNYVV RNA1 plus RNA2 are also shown (xiii).](image-url)
alone, only single fluorescent cells were observed (Supplementary Fig. S1). We hypothesized that the lack of efficient spread, even in the presence of P30, was due to the absence of a silencing suppressor (normally P14 encoded by RNA2) and that this allowed the plant defence mechanism to restrict expansion of the infection foci. We reasoned that coexpression of an RSS will counteract this local defence and allow the viral RNA to move from cell to cell and produce a local lesion.

Among the plants inoculated with BNYVV RNA1, rep30 and a recombinant rep5 construct, two types of observations could be made by visual inspection of leaves (Fig. 2). Plants expressing a characterized RSS exhibited local lesions: this was the case for P14BNYVV, P19TBSV, P0BWYV, P15PCV, HeProTaMV and TasPFEV-1 (Fig. 2, panels iv, v, vii, vii, ix and x, respectively). On the other hand, plants for which the inoculum contained an empty replicon (rep5–0) or a replicon expressing a nonfunctional suppressor (rep5–P14mutTBSV; Fig. 2, iv and vi, respectively) did not show symptoms. Necrotic lesions were observed early (4 days p.i.) for P14TBSV, P15PCV and HeProTaMV. Small necrotic symptoms were also observed early for TasPFEV-1 but curiously were more readily visible from the back side of the inoculated leaf. Chlorotic and necrotic symptoms appeared 6 to 7 days p.i. on plants expressing P14BNYVV and P0BWYV, respectively. Note that local lesions are much smaller for infections in which the function of movement is driven by the TMV-P30 movement protein than for an infection where the movement is dependent on the TGB proteins and the coat protein encoded by BNYVV RNA2 (Fig. 2, xiii; see also Lauber et al., 1998).

Total RNA extracted from the infected leaves was analysed by Northern blot hybridization. Little, if any, viral RNA could be detected in leaves inoculated with BNYVV RNA1 and rep30 alone (Fig. 1c, lane 2) or supplemented with empty replicon rep5–0 (Fig. 1c, lane 3). Similar results were obtained with the replicon expressing P19mutTBSV which does not show suppressor activity (Qiu et al., 2002) (Fig. 1c, lane 6). In contrast, viral RNAs were well amplified in leaves inoculated with RNA1 plus rep30 supplemented with rep5 expressing known RSS (Fig. 1c, lanes 4, 5, 7, 8 and 9) with the exception of the TasPFEV-1 suppressor. Surprisingly, expression of TasPFEV-1 in our viral system led to the appearance of necrotic local lesions, although no viral progeny RNA could be detected in the Northern blot presented (Fig. 2, x, and Fig. 1c, lane 10). However, low levels of viral RNA could be detected in other experiments for the samples containing no functional RSS or TasPFEV-1, confirming their replication ability (data not shown), and previously shown using the reporter rep5–GFP (Supplementary Fig. S1). TasPFEV-1 could be an RSS specifically targeting a step in the microRNA pathway (Lecellier et al., 2005) which is not relevant in plant viral infections. However, the fact that necrotic local lesions were obtained indicates that TasPFEV-1 is able to induce a pathogenic response in C. quinoa.

We also tested the potential RSS activity of two uncharacterized proteins: P13BDMV, which is phylogenetically related to P14BNYVV (Rush, 2003), and P0CYDV, which is related to P0BWYV (Mayo & Ziegler-Graff, 1996). P13BDMV protein behaved as an authentic RSS, since local lesions were observed, although they were smaller than those obtained with P14BNYVV (Fig. 2, compare xii and iv), and amplification of the RNA progeny was comparable to that obtained with protein P14BNYVV (Fig. 1c, lanes 12 and 4). In contrast, P0CYDV did not lead to the appearance of symptoms on C. quinoa and no progeny RNA could be detected (Fig. 2, xi, and Fig. 1c, lane 11). Thus, unlike P0 of BWYV and P0 of cucurbit aphid borne yellow virus (CABYV; Pfeffer et al., 2002), P0 of CYDV-RPV did not exhibit RSS activity in this assay. The absence of silencing suppression activity was confirmed in transient agro-infiltration patch experiments (data not shown). CYDV-RPV evidently differs from BWYV and CABYV by its host range [monocots versus dicots (D‘Arcy & Domier, 2005)], which might explain the lack of RSS activity of P0CYDV in N. benthamiana and C. quinoa leaves. However, the P0 protein of the monocot-infecting virus sugar cane yellow leaf virus (SCYLV) was shown to inhibit RNA silencing in patch assays, although the virus does not infect Nicotiana species (Mangwende et al., 2009). Another plausible explanation for the absence of RSS activity of P0CYDV might be the limitations of our assay, which addresses only the potential effect on local silencing, and not on movement of the systemic RNA silencing signal. Further experiments will be required to resolve this subject.

More generally, our results highlight a remarkable diversity in the activity of RSS among viruses from a single virus genus. Such observations have already been made for silencing suppressor proteins from other virus families and are potentially important for gaining an understanding of the link with pathogenicity (Vanitharani et al., 2004; Zhang et al., 2006).

We do not yet understand the variable level of RNA progeny obtained in our assay for the different RSS proteins. Thus, viral RNA accumulation was high for P14BNYVV, P19TBSV, P15PCV and P13BDMV, but lower for P0BWYV and HeProTaMV (Fig. 1c). This variation might reflect different modes of action of the RSS and/or the necrotic effect linked to their pathogenicity.

In this work, we have developed a new assay based on viral multiplication complementation to identify the potential RSS activity of proteins. It is a rapid and straightforward method as it relies only on inoculation of in vitro transcripts followed by observation of symptoms on inoculated leaves, without the need for any reporter gene. RNA amplification is confirmed by a simple Northern blot analysis. Powers et al. (2008) published a similar method based on a mixed expression technique in which an RSS-deficient turnip crinkle virus transcript was heterocomplemented by a potential RSS protein transiently expressed by infiltration of recombinant A. tumefaciens.
The size of the infection foci was monitored by the expression of the GFP reporter (inserted in place of the RSS gene). In our system, the heterologous proteins are entirely expressed by the virus, thereby reproducing the natural conditions of a cytoplasmic viral infection. Moreover, using an uncommon host for RSS assays (C. quinoa) might reveal some unexpected features. Finally, our replication-based assay provides an alternative and easy-to-use tool to identify and characterize the RSS activity of multifunctional viral proteins.

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References


