Many phytoviruses have been used for the production of functional proteins in plants, based on viral genome amplification within infected cells (for reviews, see Pogue et al., 1998, 2002; Awram et al., 2002; Porta & Lomonossoff, 2002; Gleba et al., 2004). Using a plant virus as a vector allows rapid expression of post-translationally modified functional proteins.

We have shown previously that Beet necrotic yellow vein virus (BNYVV) can be used as such a vector. BNYVV has five genomic RNAs, but only RNA-1 and -2 are required for virus propagation in leaves of Chenopodium quinoa (Quillet et al., 1989). Thus, in principle, it should be possible to use one of the other three RNAs as a ‘replicon’, i.e. a BNYVV replication-dependent vehicle for expression of a foreign gene inserted in place of the open reading frame (ORF) for the normally encoded protein. Indeed, BNYVV RNA-3 has already been used in this way to express green fluorescence protein (GFP) (Erhardt et al., 2000; Vetter et al., 2004) or proteins encoded by BNYVV RNA-2 (Hehn et al., 1995; Bleykasten-Grosshans et al., 1997; Lauber et al., 1998a). However, it has not proved possible to routinely express two foreign proteins from different RNA-3-based replicons; the replicons appear to compete with one another during replication, so that one or the other is eliminated quickly from the infection site (Lauber et al., 1999). This prompted us to examine the possibility of using one of the other small BNYVV RNAs as a replicon in the hope that, as RNA-3, -4 and -5 multiply together during normal virus replication, replicons derived from different molecules might also co-exist.

Preliminary experiments with RNA-4 revealed that it has drawbacks for use as a replicon, as it has cis-essential replication sequences that extend more than 393 nt from the 5′ terminus (Gilmer et al., 1992; unpublished observations). We therefore concentrated on RNA-5, for which a full-length cDNA clone has recently become available (D. Link, L. Schmidlin, A. Schirmer, E. Klein, M. Erhardt, O. Lemaire & D. Gilmer, unpublished data). In this study, we inserted the ORFs for several proteins, such as GFP, the BNYVV RNA-2 proteins P13 and P15 (expressed from a dicistronic construct; Bleykasten-Grosshans et al., 1997; Lauber et al., 1998a) and the RNA-5 protein p26 modified by the addition of a haemagglutinin (HA) tag (p26HA), into an RNA-5-based replicon (RNA-5-hA) and compared their expression levels with those obtained by using the well-characterized RNA-3 replicon (Fig. 1a). The effect on the behaviour of the replicons of the addition to the inoculum of various combinations of the other small BNYVV RNAs was also analysed.

Construction of the RNA-5 replicon was performed by replacing the p26-encoded sequence with a linker sequence (containing restriction sites for NcoI, EcoRV, BglII and XbaI), whilst retaining the 5′-proximal 438 nt of RNA-5 and the 243 nt preceding the RNA-5 poly(A) tail. The GFP sequence was inserted between the NcoI and BglII

Use of a *Beet necrotic yellow vein virus* RNA-5-derived replicon as a new tool for gene expression

Laure Schmidlin, Didier Link, Jérôme Mutterer, Hubert Guilley and David Gilmer

Institut de Biologie Moléculaire des Plantes, 12 rue du Général Zimmer, 67084 Strasbourg cedex, France

A new gene-expression system based on RNA-5 of *Beet necrotic yellow vein virus* (BNYVV) was constructed to allow the expression of recombinant proteins in virally infected cells. Replication and expression levels of the RNA-5-based replicon containing the green fluorescence protein (GFP) gene were compared with those obtained with the well-characterized RNA-3-derived replicon (Rep-3). When RNA-3 and/or RNA-4 BNYVV RNAs were added to the inoculum, the expression levels of RNA-5-encoded GFP were considerably reduced. To a lesser extent, RNA-3-derived GFP expression was also affected by the presence of RNA-4 and -5. Both RNA-3- and RNA-5-derived molecules were able to express proteins within the same infected cells. Together with Rep-3, the RNA-5-derived replicon thus provides a new tool for the co-expression of different recombinant proteins. In Beta macrocarpa, Rep-5-GFP was able to move in systemic tissues in the presence of RNA-3 and thus provides a new expression system that is not restricted to the inoculated leaves.
C. quinoa

RNAs within local lesions of genes. Drawings are not to scale. (b) Accumulation levels of lines. Black boxes refer, respectively, to the p6.8 and p4.8 indicated by labelled rectangles and non-coding regions by RNA-5HA molecules and their derivative replicons. ORFs are nations. (a) Diagram of the coding capacities of RNA-3 and capacities of BNYVV replicons using different inoculum combi-

Analysis of RNA (b and d) and protein (c) expression

Fig. 1. Analysis of RNA (b and d) and protein (c) expression capacities of BNYVV replicons using different inoculum combinations. (a) Diagram of the coding capacities of RNA-3 and RNA-5HA molecules and their derivative replicons. ORFs are indicated by labelled rectangles and non-coding regions by lines. Black boxes refer, respectively, to the p6.8 and p4.8 genes. Drawings are not to scale. (b) Accumulation levels of BNYVV RNAs within local lesions of C. quinoa leaves 6 days p.i. with Stras12 alone (1) or supplemented with RNA-3 (2), RNA-5HA (3), RNA-3 and RNA-4 plus RNA-5HA (4), Rep-3-GFP (5), Rep-5-GFP (6), Rep-3-GFP plus RNA-5HA (7), Rep-5-GFP plus RNA-3 (8), Rep-3-GFP plus RNA-4 and RNA-5HA (9), Rep-5-GFP plus RNA-3 and RNA-4 (10), and Rep-3-GFP plus Rep-5-GFP (11). Membranes were probed with specific riboprobes complementary to sequences of RNA-1, RNA-2 and GFP (i), RNA-3 (ii), RNA-4 (iii) or RNA-5HA (iv). Note that Rep-3-GFP, Rep-5-GFP and RNA-5HA co-migrate. (c) Immunodetection of BNYVV-expressed proteins within local lesions of C. quinoa leaves 6 days p.i. with the above constructs. MS is an image of the membrane stained with Serva blue G. (d) Analysis of the accumulation levels of RNA-3- and -5-derived replicons expressing the dicistronic P13-P15 sequence. Accumulation level of BNYVV RNAs within local lesions of C. quinoa leaves 6 days p.i. with Stras12 alone (12), or supplemented with Rep-3-P13-P15 (13) or Rep-5-P13-P15 (14). Membranes were probed with riboprobes complementary to RNA-1 and -2. The RNA-2-specific riboprobe was chosen so as to detect the P13-P15 sequence expressed in both replicons. Similar amounts of total RNA were loaded. Identities of RNAs and specifically immunodetected proteins are indicated on the right.

Extended analysis of lesion phenotypes induced by different inocula was performed and is summarized in Table 1. When Stras12 was used alone or supplemented with Rep-3- and/or Rep-5-derived molecules, ~2 mm green chlorotic local lesions that are typical of Stras12 were obtained. The presence of Rep-3-GFP induced the expression of GFP in infected tissues, which produced a strong fluorescent ring under UV illumination. In the case of Rep-5-GFP, fluorescence within local lesions was high in the initially infected tissues (i.e. the centre of the lesion) and lower in the periphery of the infected tissues.

The effects of the other small replicable RNAs on symptom formation and GFP expression levels were tested. When RNA-3 was added to the inoculum, ~2 mm yellow chlorotic spots were observed (Table 1), which displayed a faint fluorescence when Rep-5-GFP was added to the inoculum. Lesion fluorescence was lowered when RNA-4 was also added to the inoculum. An identical approach was performed by using full-length RNA-5HA. Expression of p26HA induced the necrosis of infected C. quinoa tissues and resulted in the production of ~1 mm necrotic lesions. Such necrosis was increased dramatically in the presence of RNA-4, leading to ~0.5 mm necrotic spots (Table 1). Rep-3-GFP expression in the presence of RNA-5HA was restricted to the periphery of the lesion and was not affected by the presence of RNA-4 (Table 1). Taken together, these findings suggested that, in C. quinoa, RNA-5-driven protein expression occurs later during the infection process and probably with lower efficiency than that driven by RNA-3.

Combined tissues from three local lesions from the above inoculations were analysed by Northern blotting. After TRIzol extraction (Invitrogen), similar amounts of total RNA (not shown) were separated on a denaturing gel, transferred onto a nitrocellulose membrane and probed with riboprobes specific for each viral RNA (Lemaire et al., 1988) or for GFP, as described previously (Lauber et al., 1998b; Erhardt et al., 2000). Accumulation of the RNA was quantified with a Fuji MAS 1000 BioAnalyser. The results are summarized in Fig. 1(b). In each case, viral RNA-1 and -2 were detected in similar amounts [Fig. 1b(i)],

restriction sites to produce Rep-5-GFP. Dicistronic Rep-5-P13-P15 was obtained by inserting an Ncol/BglII-digested PCR product, obtained from the full-length RNA-2 cDNA clone, between the Ncol and BglII restriction sites of Rep-5. Rep-3-GFP was produced as described previously (Erhardt et al., 2000). The GFP sequence was replaced by the monomeric red fluorescent protein (mRFP) sequence to produce Rep-3-RFP by using the Ncol and BamHI restriction sites. C. quinoa leaves were inoculated with the Stras12 helper strain (i.e. RNA-1 and -2) together with different combinations of the small BNYVV RNAs. Local lesions appeared 6 days post-infection (p.i.) and were analysed for their phenotype, for GFP fluorescence (where appropriate) and for RNA and protein contents by Northern and Western blotting.

Downloaded from www.microbiologyresearch.org by
IP:  54.191.40.80
On: Thu, 31 Aug 2017 23:07:33

Journal of General Virology 86

464

Downloaded from www.microbiologyresearch.org by
IP:  54.191.40.80
On: Thu, 31 Aug 2017 23:07:33
as were RNA-3 and Rep-3-GFP [Fig. 1b(ii), compare lanes 2 and 5]. Rep-3-GFP accumulated similarly when inoculated alone or together with RNA-5HA or Rep-5-GFP [Fig. 1b(i) and (ii), lanes 5, 7 and 11], but its accumulation was six times lower when RNA-4 was added to the inoculum [Fig. 1b(i) and (ii), lane 9]. In contrast, RNA-4 replication was not affected significantly by the presence of other small viral RNAs [Fig. 1b(iii), lanes 4, 9 and 10]. The replication of RNA-5HA was affected when RNA-3 was added to the inoculum [Fig. 1b(iv), compare lanes 3 and 4] and it should be noted that replacement of p26HA by GFP did not modify the size of the RNA, but did diminish accumulation of the RNA-5-derived viral vector [Fig. 1b(iv), compare lanes 3 and 6]. Such a result may be due to a possible cis-acting effect of the p26HA protein or the presence of replication regulatory elements within the p26 ORF, as described previously for RNA-4 (Gilmer et al., 1992). Interestingly, the addition of RNA-4 together with Rep-3-GFP did not alter the accumulation of RNA-5HA [Fig. 1b(iv), compare lanes 4 and 9]. Quantification of the detected signals revealed that Rep-5-GFP accumulated at a level four- to sixfold lower than Rep-3-GFP. Moreover, Rep-5-GFP replication was affected dramatically by the presence of other small BNYVV RNAs [Fig. 1b(i), compare lanes 5 and 6, 7 and 8, and 9 and 10, and Fig. 1b(iv), lanes 6, 8 and 10]. These lower replication levels explained the low accumulation of GFP fluorescence observed within infected tissues (Table 1).

To confirm that the GFP expression patterns correlated with the observed RNA replication levels, immunodetection of virally expressed proteins was performed on single local lesions. The lesions were ground in 50 μl Laemmli buffer, centrifuged and heat-denatured and equivalent amounts of protein were separated by SDS-PAGE before transfer on to Immobilon membrane (Niesbach-Klösgen et al., 1990; Lauber et al., 1998b; Erhardt et al., 2000). Total protein content was estimated by staining the membrane with Serva blue G and appeared to be equal in each sample (Fig. 1c, MS). After each immunodetection, the membrane was stripped by a 5 min incubation in 0·2 M NaOH and extensive washes in water. Signals were detected by using a Chemidoc analyser and quantified with Quantity One software (Bio-Rad). In each case, similar amounts of BNYVV p21 coat protein were detected (Fig. 1c, CP), permitting comparison of the accumulation levels of the other virally expressed proteins. These comparisons revealed that the relative expression levels of RNA-3-encoded p25 (Fig. 1c, p25, lanes 2, 4, 8 and 10) paralleled the accumulation levels of full-length RNA-3 described above. However, the accumulation of RNA-5-encoded p26HA did not correlate exactly with the lowered replication of RNA-5HA that was observed in the presence of RNA-3 and -4 (compare Fig. 1c, p26HA, lanes 3 and 4), suggesting that the p26HA protein possesses a high stability in vivo.

The spatial and temporal patterns of GFP expression from Rep-3 and Rep-5 were distinct, as different accumulation patterns of fluorescence were observed within infected tissues (Table 1). This observation was confirmed by the immunodetection of GFP within local lesions. Thus, inoculation with Rep-3-GFP produced GFP fluorescence in infected tissues (Table 1) and strong signals were observed following Western blotting using a GFP-specific antibody (Fig. 1c, GFP, lanes 5, 7, 9 and 11). However, only low amounts of GFP protein were immunodetected when Rep-5-GFP was inoculated (Fig. 1c, GFP, lanes 6, 8 and 10), reflecting the low fluorescence that was observed within local lesions obtained under these conditions (Table 1). For similar RNA accumulation levels [Fig. 1b(i), lanes 6

### Table 1. Summary of C. quinoa local lesion sizes, phenotypes and their GFP expression patterns (where applicable) 6 days p.i. with Stras12 supplemented with different inoculum compositions, carrying the GFP sequence on RNA-3- and/or RNA-5-derived replicants

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Lesion diameter (mm)</th>
<th>Symptoms</th>
<th>GFP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intensity</td>
</tr>
<tr>
<td>Stras12 supplemented with:</td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>–</td>
<td>~2</td>
<td>CS</td>
<td>NA</td>
</tr>
<tr>
<td>RNA-3</td>
<td>~2</td>
<td>YS</td>
<td>NA</td>
</tr>
<tr>
<td>RNA-5HA</td>
<td>~1</td>
<td>Nec</td>
<td>Strong</td>
</tr>
<tr>
<td>Rep-3-GFP</td>
<td>~2</td>
<td>CS</td>
<td>Faint</td>
</tr>
<tr>
<td>Rep-5-GFP</td>
<td>~2</td>
<td>CS</td>
<td>Strong</td>
</tr>
<tr>
<td>Rep-3-GFP + RNA-5HA</td>
<td>~2</td>
<td>CS</td>
<td>Strong</td>
</tr>
<tr>
<td>RNA-3 + Rep-5-GFP</td>
<td>~1</td>
<td>Nec</td>
<td>NA</td>
</tr>
<tr>
<td>RNA-4 + RNA-4 + RNA-5HA</td>
<td>~0·5</td>
<td>Nec</td>
<td>Strong</td>
</tr>
<tr>
<td>RNA-5HA + RNA-4 + RNA-5HA</td>
<td>~1</td>
<td>Nec</td>
<td>NA</td>
</tr>
<tr>
<td>RNA-3 + RNA-4 + Rep-5-GFP</td>
<td>~2</td>
<td>YS</td>
<td>Faint</td>
</tr>
<tr>
<td>RNA-3 + Rep-5-GFP</td>
<td>~2</td>
<td>CS</td>
<td>Faint</td>
</tr>
<tr>
<td>RNA-5HA</td>
<td>~2</td>
<td>CS</td>
<td>Strong</td>
</tr>
</tbody>
</table>

CS, Chlorotic spots; YS, yellow spots; Nec, necrotic spots; NA, not applicable.
and 9), quantitative comparison of GFP protein levels (Fig. 1c, GFP, lanes 6 and 9) indicated that protein expression from Rep-5 was four to five times lower than that from Rep-3. Importantly, when both replicons were co-inoculated together with Stras12, both were able to replicate [Fig. 1b(ii) and (iv), lane 11] and to express GFP protein (Table 1; Fig. 1c, GFP, lane 11; see also Fig. 2e).

The lower accumulation level of Rep-5 was further confirmed by the use of replicons expressing the dicistronic P13-P15 sequence from RNA-2 (Rep-3-P13-P15 and Rep-5-P13-P15). When inoculated with Stras12, both replicons were detected, but Rep-5-P13-P15 accumulated at a level about sixfold lower than Rep-3-P13-P15 (Fig. 1d, compare lanes 13 and 14).

We have reported previously that RNA-3 is required for long-distance movement of the virus in systemic hosts and that Rep-3 derivatives are not able to invade the plant systemically, restricting their utilization to the inoculated tissues (Lauber et al., 1998b; unpublished observations). Beta macrocarpa inoculated mechanically with Stras12 supplemented with RNA-3 and Rep-5-GFP permitted the detection of fluorescence in infected leaves (Fig. 2a), but also in upper leaves (Fig. 2b and c) and in rootlets (Fig. 2d), indicating that Rep-5 can produce recombinant protein in systemically infected tissues.

To demonstrate further that both replicons were able to replicate and express two proteins in the same infected cell, we used C. quinoa leaves inoculated with Stras12 supplemented with Rep-3-RFP and Rep-5-GFP. Fluorescence monitoring using a specific band-pass filter permitted the detection of both GFP [Fig. 2e, row (i)] and RFP [Fig. 2e, row (ii)] in the same infected cells [Fig. 2e, row (iii)]. Such results allowed us to conclude that Rep-3 and Rep-5 RNAs replicate together within infected cells and thus allow co-expression of recombinant proteins for interaction studies in planta [e.g. fluorescence resonance-energy transfer (FRET)].

Fig. 2. Rep-5-GFP moves systemically within B. macrocarpa in the presence of Stras12 supplemented with RNA-3 (a–d) and is co-expressed with mRFP in the same C. quinoa infected cells (e). (a) Leaf of B. macrocarpa inoculated with Stras12 supplemented with RNA-3 and Rep-5-GFP. (b–d) Detection of RNA-5-driven GFP expression within systemically infected leaves (b and c) and in rootlets (d). Photographs were taken under UV illumination with a 4× magnification binocular equipped with a Nikon Coolpix 5000 digital camera. The red fluorescence in (a–c) is due to chlorophyll. (e) Co-expression of mRFP and GFP. Visualization of the fluorescence patterns within C. quinoa local lesions produced by Stras12 supplemented with Rep-3-RFP and Rep-5-GFP (1), or confocal laser-scanning microscopy (CLSM) observations of cellular fluorescence pattern within the aforesaid lesions (2). (i) Expression pattern of GFP; (ii) expression pattern of mRFP; (iii) merged images. Pictures of GFP and mRFP expression patterns were acquired with a Nikon E800 microscope equipped with a Nikon DXM1200 camera and processed by using Nikon ACT1 software. CLSM images were obtained with a LSM510 Meta Zeiss confocal microscope using a 63 ×/1.2 water-immersion objective. Bars, 0·5 mm for the first column; 5 μm for the CLSM images.
In this study, we have shown that Rep-5 represents a useful new tool for the expression of recombinant proteins, in particular together with RNA-3 when using a BNYVV systemic host. On local host crops, both replicons allow the co-expression of proteins. Whilst Rep-3 derivatives permit high levels of protein synthesis in infected tissues, the use of Rep-5 may be preferable in situations where overexpression leads to undesired side effects in infected cells.

Acknowledgements

We thank Malek Alioua for sequence analysis, Daniele Scheidecker and Audrey Schirmer for technical support, Pierre Pfeiffer for providing the mRFP-containing vector and Ken Richards for critical reading of this manuscript. L. S. was supported by SES-Advanta under the CIFRE programme. D. L. was supported by ITB and Region Alsace under a Bourse Régionale convention. The Inter-Institut confocal microscopy plate-form was co-financed by CNRS, Université Louis Pasteur, Region Alsace and ARC.

References


