DOI 10.1099/vir.0.81477-0

Short Communication

Zygocactus virus X-based expression vectors and formation of rod-shaped virus-like particles in plants by the expressed coat proteins of *Beet necrotic yellow vein virus* and *Soil-borne cereal mosaic virus*

R. Koenig,[1] D.-E. Lesemann,[1] S. Loss,[1] J. Engelmann,[1] U. Commandeur,[2] G. Deml,[1] J. Schiemann,[1] H. Aust[3] and W. Burgermeister[1]

[1]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Messeweg 11, D-38104 Braunschweig, Germany

[2]Rheinisch-Westfälische Technische Hochschule Aachen, Institut für Biologie VII, Molekulare Biotechnologie, Worringerweg 1, D-52074 Aachen, Germany

[3]Technical University Braunschweig, Institut für Mikrobiologie, Spielmann-Str. 7, D-38106 Braunschweig, Germany

Correspondence
R. Koenig
r.koenig@bba.de

Received 31 August 2005
Accepted 24 October 2005

Expression vectors were constructed from 35S promoter-containing full-length cDNA clones of Zygocactus virus X (ZVX). The expression of foreign genes was driven by the ZVX coat protein (cp) subgenomic promoter. It was successful only when the variable region downstream of the conserved putative promoter region GSTTAAGTT(X_{12-13})GAA was retained. Most of the ZVX cp gene, except for a short 3′ part, was replaced by the corresponding sequence of the related Schlumbergera virus X (SVX) and its cp subgenomic promoter to enable encapsidation of the transcribed RNA by an SVX/ZVX hybrid cp. Vector-expressed cp of *Beet necrotic yellow vein virus* (BNYVV) assembled in *Chenopodium quinoa*, *Tetragonia expansa* and *Beta vulgaris* leaves into particles resembling true BNYVV particles. The virus produced from these constructs retained its ability to express BNYVV cp in local infections during successive passages on *C. quinoa*. This ability was lost, however, in the rarely occurring systemic infections.

Full-length cDNA clones of plant viruses can be modified in such a way that they allow the expression of inserted foreign genes in plants. Such plant virus-based expression vectors are powerful tools for various applications (Awram *et al.*, 2002; Pogue *et al.*, 2002; Porta & Lomonossoff, 2002; Gleba *et al.*, 2004). Diagnostic or therapeutic proteins or enzymes – if need be optimized for specific applications by PCR-introduced mutations – can be produced in plants in a cheaper and safer way than in other eukaryotic systems. Genome portions of plant viruses can be checked for their functions (Gilardi *et al.*, 1998) and their influence on virus multiplication (Culver, 1996) without the need for producing transgenic plants. Functions of plant genes, including resistance genes, may be assessed, because vector-expressed gene portions are multiplied via a double-stranded RNA step, making the genes susceptible to virus-induced gene silencing (Lu *et al.*, 2003; Brigneti *et al.*, 2004). Most plant virus expression vectors used so far are based on *Tobacco mosaic virus* or *Potato virus X*, which mainly infect solanaceous hosts. For studying various aspects of the *Beet necrotic yellow vein virus* (BNYVV)-induced rhizomania disease, we were interested in expression vectors that would be able to initiate infections in experimental and natural hosts of BNYVV, such as *Chenopodium quinoa*, *Tetragonia expansa* and *Beta vulgaris*. Viruses on which such vectors would be based should have a number of properties favouring the practical application of the anticipated expression systems: they should cause mild systemic infections in the above-mentioned hosts of BNYVV; for safety reasons, they should not be dangerous pathogens for any important crops; they should have elongated particles to avoid packaging problems due to enlarged RNAs; for convenient handling, they should preferably have monopartite genomes; and related viruses should be available for exchanging promoters and other genome elements. Potexvirus isolates from various genera in the Cactaceae, originally grouped together under the name *Cactus virus X* (Milicic *et al.*, 1966), seemed to fulfil all of these criteria. Three isolates originating from *Zygocactus* sp., *Schlumbergera* sp. and *Opuntia* sp., respectively, are related only rather distantly, both serologically and at the molecular level, and it has been proposed that they represent distinct virus species, Zygocactus virus X (ZVX), Schlumbergera virus X (SVX) and Opuntia virus X (OVX), respectively (Koenig *et al.*, 2004). Biologically

0008-1477 © 2006 SGM Printed in Great Britain

active, 35S promoter-driven full-length cDNA clones of ZVX (Koenig *et al.*, 2004) have served as a basis for the vector constructs described here.

The design of our vector constructs resembles that of certain tobamovirus-based vectors in which the coat protein (cp) subgenomic promoter is used to drive expression of foreign genes and the original cp gene is replaced by the cp subgenomic promoter and the cp gene of a related virus (Donson *et al.*, 1991; Shivprasad *et al.*, 1999). In our constructs (Fig. 1), the putative ZVX cp subgenomic promoter is used to drive expression of foreign genes inserted via newly created unique *Asc*I and *Spe*I sites. Most of the ZVX cp gene up to a *Stu*I site close to its 3′ end is replaced by the corresponding portion of the cp gene of the related SVX, together with the putative SVX cp subgenomic promoter. It has been suggested that the highly conserved sequence GSTTAAGTT(X_{12-13})GAA (written as DNA) upstream of potexviral cp genes (Fig. 2, top) represents the cp subgenomic promoter (Memelink *et al.*, 1990; Chen *et al.*, 2005), but its exact size has not yet been proven experimentally. To increase the likelihood that the inserted SVX sequence (Fig. 1) contained the complete SVX cp subgenomic promoter, 16 additional nucleotides upstream of the conserved sequence were included in the insert. The SVX sequence upstream of the cp gene thus consisted of 45 nt. It shared 58 % identity with the corresponding ZVX sequence.

Between the putative subgenomic promoters and the cp genes, there is a stretch of nucleotides in potexviral genomes that is highly variable in size and composition (examples in Fig. 2, top). Three constructs were prepared to introduce the *Asc*I and *Spe*I sites needed for the insertion of foreign genes into our vector system (Fig. 2, bottom). In pA, the 14 nt of the two restriction sites replaced the variable region of the ZVX sequence, except for the 3′-terminal 'G'. In pB, the variable region was retained and the two sites were inserted immediately downstream of it. In pC, the two sites were inserted 18 nt further downstream to include the 5′ end of the ZVX cp gene in which the ATG start codon was inactivated by replacing the 'G' by a 'T'.

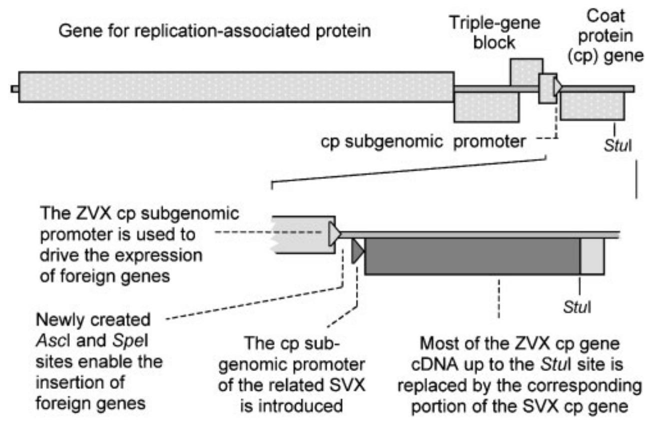
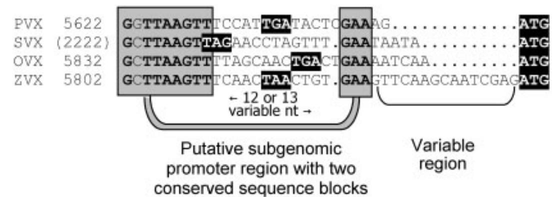


Fig. 1. Genome organization of wild-type ZVX (top) and enlarged 3′ end of its cDNA showing the modifications introduced in order to allow the expression of foreign genes (bottom).



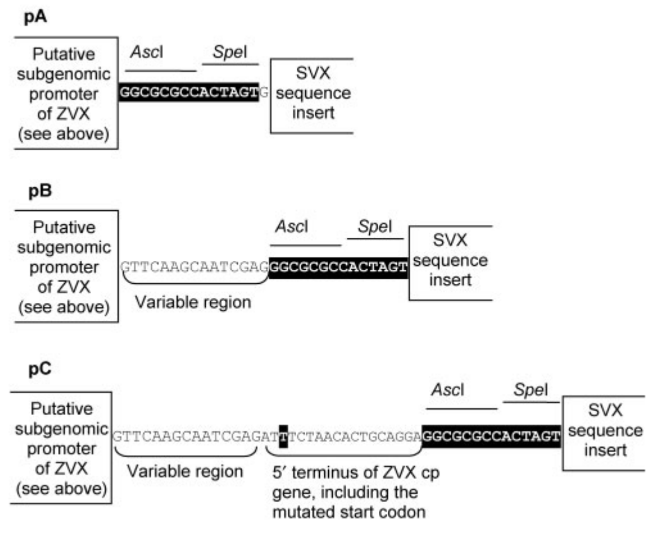


Fig. 2. Top: location of the putative cp subgenomic promoters in the genomes of various potexviruses with respect to the TGBP3 stop and the cp start codons. Bottom: sequence portions of constructs pA, pB and pC in which the *Asc*I and *Spe*I sites were introduced in three different ways into the vector system outlined in Fig. 1. Stop and start codons, the *Asc*I and *Spe*I sites and the 'T' replacing the original 'G' in the ZVX cp start codon in pC are highlighted by white letters on a black background.

Via the newly created *Asc*I/*Spe*I sites, the BNYVV cp gene was inserted into pA, pB and pC, yielding pA/BNYVVcpg, pB/BNYVVcpg and pC/BNYVVcpg, respectively. These plasmids (10 μg in 10 μl) were rubbed on carborundum-dusted leaves of *C. quinoa*. Infections were detected readily in the inoculated leaves 3 weeks post-infection (p.i.) by means of ELISA using antiserum to the ZVX-related OVX. Immunoelectron microscopy (Milne & Lesemann, 1984; Milne, 1984) using this antiserum revealed the presence of numerous potexvirus particles (Fig. 3a), indicating that the SVX cp subgenomic promoter is present in the 45 nt upstream of the SVX cp gene start codon and is able to drive expression of a hybrid cp encapsidating the transcribed RNA. ZVX and SVX antisera failed to react in ELISA and immunoelectron microscopy. Whereas the failure of the ZVX antisera may be due to the replacement of most of the ZVX cp gene by the corresponding SVX sequence, the failure

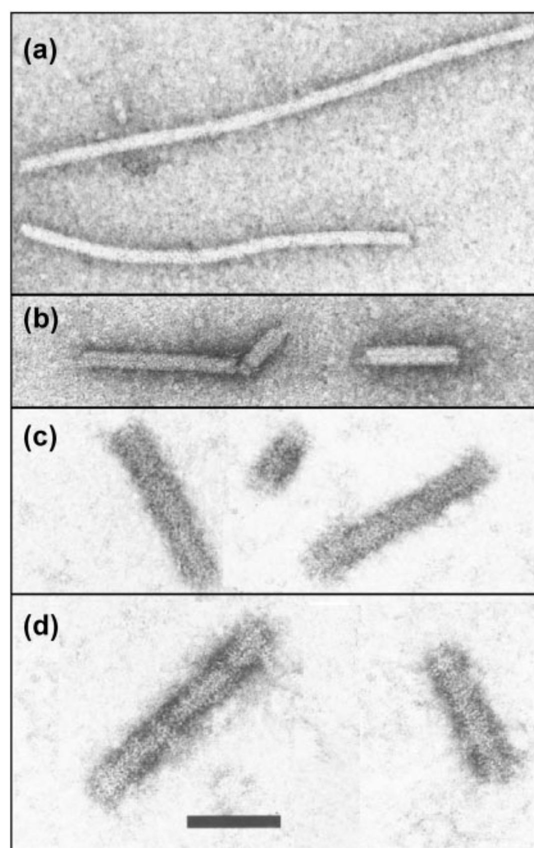


Fig. 3. Virus and virus-like particles trapped by means of antibodies from sap of *C. quinoa* leaves in which infections had been initiated by construct pB carrying the cp gene of either BNYVV (a–c) or SBCMV (d). (a) Potexvirus particles trapped by means of antibodies to OVX. (b, c) Rod-shaped particles trapped by means of antibodies to BNYVV. (d) Rod-shaped particles trapped by means of antibodies to SBCMV. In (c) and (d), the particles were decorated by antibodies to the respective viruses. An axial channel is clearly visible, particularly in the undecorated rod-shaped particles in (b). Bar, 100 nm.

of the SVX antisera was unexpected. Possibly the hybrid cp folds in a slightly different manner, which exposes epitopes that are recognized more readily by OVX rather than SVX or ZVX antisera. Alternatively, there were indications that our original OVX and SVX preparations had actually contained mixtures of various potexviruses. The viruses that were immunodominant in these preparations may not have been the same ones as those that have been sequenced.

ELISA readily detected BNYVV cp in leaves inoculated with pB/BNYVVcpg or pC/BNYVVcpg, but not in leaves inoculated with pA/BNYVVcpg, which lacks the variable region downstream of the conserved GSTTAAGTT(X_{12-13})GAA block (Fig. 2). This suggests that this variable region is, at least in the ZVX sequence, either an essential part of the cp subgenomic promoter or that the palindromic *Asc*I site sequence, which is retained upstream of inserted foreign genes, interferes with the functionality of the ZVX cp subgenomic promoter when it is located directly downstream of the GSTTAAGTT(X_{12-13})GAA block.

The infections initiated by our constructs in *C. quinoa* – like those initiated by the original full-length ZVX cDNA clones – differed from those produced by wild-type ZVX in that they were symptomless and rarely became systemic. ELISA with inoculated leaves dissected into squares of 7×7 mm^2 indicated that the infections remained confined to a few small areas. In the rarely occurring systemic infections, no BNYVV cp was detected, suggesting loss of the inserted gene during systemic spread. BNYVV cp was, however, detected readily in leaves of *C. quinoa* rubbed with sap from plasmid-inoculated *C. quinoa* leaves and also after at least three further passages in *C. quinoa*. Further studies with pB/BNYVVcpg revealed that the BNYVV cp gene was also expressed in leaves of *T. expansa* inoculated by rubbing and in leaves of *B. vulgaris* inoculated by means of vortexing whole seedlings (Koenig & Stein, 1990). Expression was most efficient in *T. expansa*, where ELISA readings for BNYVV were sometimes more than 10 times higher than those for OVX, whereas in *C. quinoa* they were usually lower than those for OVX. In sugar beet, positive BNYVV ELISA readings sometimes showed up only after several hours of incubation.

Immunosorbent electron microscopy with BNYVV-specific antibodies revealed that the expressed BNYVV cp in all three plant species assembled into rod-shaped virus-like particles that, like true BNYVV particles, had a diameter of approximately 20 nm and a clearly visible axial canal (Fig. 3b). Measurement of 200 particles formed from vector-expressed cp revealed a main population with lengths ranging between 40 and 150 nm, with some particles being up to 450 nm long. The particles were decorated readily by BNYVV antibodies (Fig. 3c). The counts for BNYVV-like particles in *C. quinoa* were up to 10 times higher than those for potexvirus particles. Although these particle counts are not absolutely comparable, because the trapping capacities of the two antisera may be different, they suggest that the formation of BNYVV-like particles may be quite efficient. Formation of rod-shaped virus-like particles was also observed when the *Soil-borne cereal mosaic virus* (SBCMV) cp gene was expressed in *C. quinoa* by means of construct pB. These particles also contained the typical axial canal and were decorated strongly by SBCMV antibodies (Fig. 3d).

The formation of numerous BNYVV-like particles in our experiments was unexpected because of the absence of the 75 kDa BNYVV cp readthrough protein. It has been suggested that this readthrough protein plays an important role in efficient particle formation in true BNYVV infections (Schmitt *et al.*, 1992; Haeberlé *et al.*, 1994). For *Soil-borne wheat mosaic virus*, which is related to SBCMV, it has previously been shown that the cp readthrough protein is not required for particle formation (Yamamiya & Shirako, 2000).

The sedimentation behaviour of the BNYVV-like particles formed from vector-expressed cp was checked in

isopycnic caesium chloride gradients. Sap from infected plants (50–200 μl depending on ELISA readings) was diluted with 50 μM phosphate buffer (pH 7·2) to a final volume of 4·7 ml and 2·3 g caesium chloride was added. The mixture was centrifuged in a swingout rotor at 110 000 ***g*** for 2 or 3 days. Fractions of 160 μl, diluted with 160 μl 2 × ELISA sample buffer, were tested for BNYVV cp by ELISA. True BNYVV particles and those formed from vector-expressed BNYVV cp were found in the same fractions, i.e. in fractions 16–20 out of 27. This suggested that the BNYVV-like particles formed from expressed cp contained nucleic acid.

Our vector constructs pB and pC seem to be promising tools for studying various aspects of rhizomania disease. So far only one other vector system – based on *Tobacco rattle virus* – has briefly been described to be able to initiate infections in sugar beet (MacFarlane & Popovich, 2000). The weakened aggressiveness of the virus derived from our constructs – possibly due to changes introduced into the primary transcripts by the nuclear RNA-processing machinery (Gleba *et al.*, 2004) – and the apparent loss of the inserted gene in the rarely occurring systemic infections may be advantageous for biosafety reasons, because virus expression vectors 'need to be sufficiently stable to express foreign genes in plants, but sufficiently unstable that the foreign sequence will not remain in the environment after production' (Shivprasad *et al.*, 1999). A lack of symptoms is also considered to be a desirable feature in virus-based gene silencing systems, as virus-induced symptoms would not mask phenotypes associated with plant gene silencing (Naylor *et al.*, 2005). With other virus-based expression systems, it has been shown that the building of an effective vector is not a 'trivial exercise' (Shivprasad *et al.*, 1999). The efficiency of tobamovirus-based vectors has been improved considerably by combining genome portions from several tobamoviruses (Shivprasad *et al.*, 1999) and by DNA shuffling of the movement protein genes (Toth *et al.*, 2002). Another factor influencing vector efficiency is the method used for introducing it into plants. With our constructs, mechanical leaf inoculation and vortexing in the case of sugar beet have been used. Mechanical inoculation is the easiest, but obviously also the least-efficient method for introducing viral cDNA clones and their derivatives into the nuclei of plant cells. Thus, cDNA clones of *Potato virus M* seemed to be non-infectious in mechanical-inoculation tests, although agrodelivery revealed that they were highly infectious (Flatken & Maiss, 2005). Liu & Lomonossoff (2002) and Lu *et al.* (2003) consider agroinfection to be the method of choice for introducing *Cowpea mosaic virus*- and *Potato virus X*-based vector constructs into plants. Further work involving these techniques is planned to evaluate the potential of our constructs for practical applications.

Acknowledgements

We are greatly indebted to Professor Dr Maiss, University of Hannover, Germany, for kindly supplying the plasmid pe35Stu_pa, to Professor Dr H. Jeske and Dr A. Kadri for their advice concerning sedimentation analyses, and to the Deutsche Forschungsgemeinschaft (grant Ko518/14-1), the Arbeitsgemeinschaft industrieller Forschungsvereinigungen (AiF grant 14163 N72, GFP BR42/04) and the Niedersächsisches Ministerium für Wissenschaft und Kultur (grant ZN1401) for financially supporting different aspects of this work.

References

Awram, P., Gardner, R. C., Forster, R. L. & Bellamy, A. R. (2002). The potential of plant viral vectors and transgenic plants for subunit vaccine production. *Adv Virus Res* **58**, 81–124.

Brigneti, G., Martín-Hernández, A. M., Jin, H., Chen, J., Baulcombe, D. C., Baker, B. & Jones, J. D. G. (2004). Virus-induced gene silencing in *Solanum* species. *Plant J* **39**, 264–272.

Chen, J., Shi, Y.-H., Adams, M. J. & Chen, J.-P. (2005). The complete sequence of the genomic RNA of an isolate of *Lily virus X* (genus *Potexvirus*). *Arch Virol* **150**, 825–832.

Culver, J. N. (1996). Tobamovirus cross protection using a potexvirus vector. *Virology* **226**, 228–235.

Donson, J., Kearney, C. M., Hilf, M. E. & Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc Natl Acad Sci U S A* **88**, 7204–7208.

Flatken, S. & Maiss, E. (2005). Construction and agroinfection of a *Potato virus M* full-length clone. In *Fourth Joint Meeting of Dutch and German Plant Virologists*, Wageningen, 10–11 March 2005, p. 22.

Gilardi, P., García-Luque, I. & Serra, M. T. (1998). Pepper mild mottle virus coat protein alone can elicit the *Capsicum* spp. L^3 gene-mediated resistance. *Mol Plant Microbe Interact* **11**, 1253–1257.

Gleba, Y., Marillonnet, S. & Klimyuk, V. (2004). Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr Opin Plant Biol* **7**, 182–188.

Haeberlé, A.-M., Stussi-Garaud, C., Schmitt, C., Garaud, J.-C., Richards, K. E., Guilley, H. & Jonard, G. (1994). Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Arch Virol* **134**, 195–203.

Koenig, R. & Stein, B. (1990). Distribution of beet necrotic yellow vein virus in mechanically inoculated sugarbeet plantlets of cultivars with different degrees of rhizomania resistance. In *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, Braunschweig, Germany, 21–24 August 1990, pp. 87–90.

Koenig, R., Pleij, C. W. A., Loss, S., Burgermeister, W., Aust, H. & Schiemann, J. (2004). Molecular characterisation of potexviruses isolated from three different genera in the family *Cactaceae*. *Arch Virol* **149**, 903–914.

Liu, L. & Lomonossoff, G. P. (2002). Agroinfection as a rapid method for propagating *Cowpea mosaic virus*-based constructs. *J Virol Methods* **105**, 343–348.

Lu, R., Martín-Hernández, A. M., Peart, J. R., Malcuit, I. & Baulcombe, D. C. (2003). Virus-induced gene silencing in plants. *Methods* **30**, 296–303.

MacFarlane, S. A. & Popovich, A. H. (2000). Efficient expression of foreign proteins in roots from tobravirus vectors. *Virology* **267**, 29–35.

Memelink, J., van der Vlugt, C. I. M., Linthorst, H. J. M., Derks, A. F. L. M., Asjes, C. J. & Bol, J. F. (1990). Homologies between the genomes of a carlavirus (lily symptomless virus) and a potexvirus (lily virus X) from lily plants. *J Gen Virol* **71**, 917–924.

Milicic, D., Plese, N., Bercks, R., Brandes, J., Casper, R. & Chessin, M. (1966). Vergleichende serologische und elektronenmikroskopische Untersuchungen an Isolaten des Kakteen-X-Virus. *Phytopathol Z* **55**, 211–217 (in German).

Milne, R. G. (1984). Electron microscopy for the identification of plant viruses in in vitro preparations. *Methods Virol* **7**, 87–120.

Milne, R. G. & Lesemann, D.-E. (1984). Immunosorbent electron microscopy in plant virus studies. *Methods Virol* **8**, 85–101.

Naylor, M., Reeves, J., Cooper, J. I., Edwards, M.-L. & Wang, H. (2005). Construction and properties of a gene-silencing vector based on Poplar mosaic virus (genus *Carlavirus*). *J Virol Methods* **124**, 27–36.

Pogue, G. P., Lindbo, J. A., Garger, S. J. & Fitzmaurice, W. P. (2002). Making an ally from an enemy: plant virology and the new agriculture. *Annu Rev Phytopathol* **40**, 45–74.

Porta, C. & Lomonossoff, G. P. (2002). Viruses as vectors for the expression of foreign sequences in plants. *Biotechnol Genet Eng Rev* **19**, 245–291.

Schmitt, C., Balmori, E., Jonard, G., Richards, K. E. & Guilley, H. (1992). *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proc Natl Acad Sci U S A* **89**, 5715–5719.

Shivprasad, S., Pogue, G. P., Lewandowski, D. J., Hidalgo, J., Donson, J., Grill, L. K. & Dawson, W. O. (1999). Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* **255**, 312–323.

Toth, R. L., Pogue, G. P. & Chapman, S. (2002). Improvement of the movement and host range properties of a plant virus vector through DNA shuffling. *Plant J* **30**, 593–600.

Yamamiya, A. & Shirako, Y. (2000). Construction of full-length cDNA clones to *Soil-borne wheat mosaic virus* RNA1 and RNA2 from which infectious cDNAs are transcribed *in vitro*: virion formation and systemic infection without expression of the N-terminal and C-terminal extensions to the capsid protein. *Virology* **277**, 66–75.