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

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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(X12–13)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 (Gilaridi 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
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 AscI and SpeI sites. Most of the ZVX cp gene up to a Stul 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(X12–13)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 AscI and SpeI 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'. Via the newly created AscI/SpeI 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. 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).](image-url)
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(X12–13)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 Asd 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(X12–13)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 mm² 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 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.

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; Haebler 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 extremity of beet necrotic yellow vein virus particles. (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.

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