Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus

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An infectious full-length cDNA clone of the RNA genome of the potyvirus zucchini yellow mosaic virus (ZYMV) was constructed under the control of the cauliflower mosaic virus 35S promoter. All squash, cucumber, melon and watermelon plants inoculated with the cloned cDNA of ZYMV by particle bombardment become infected. Bombardment technology is 106-fold more effective than mechanical inoculation. Due to the great increase in efficiency, ineffective constructs now became infective (i.e. cDNA under the control of the 35S promoter without the NOS terminator; with an addition of 127 nucleotides at the 5' end of the viral cDNA; uncapped transcripts), and the infectivity of capped-transcripts was maximized. Inoculation by particle bombardment produced visual symptoms rapidly (3-4 days), allowing the detection of viral coat protein and virions after 2 and 3 days in systemically infected leaves and inoculated cotyledons respectively.

The use of infectious clones of RNA viruses has permitted investigations of viral gene function. Infectious clones of several potyviruses have been constructed, under the control of either a bacterial promoter (in vitro transcript production) or a plant expression promoter [e.g. the cauliflower mosaic virus (CaMV) 35S promoter] (see Boyer & Haenni, 1994). The use of infectious transcripts is the less efficient process, as in vitro transcription is more complicated and requires 5'-capping, and RNA transcripts can be degraded during the inoculation process (see Boyer & Haenni, 1994).

We have previously constructed a full-length clone (FLC) of zucchini yellow mosaic virus (ZYMV), from which infectious transcripts were produced (Gal-On et al., 1991). Increased efficiency of a full-length viral clone under the control of a promoter effective in planta (Benfey & Chua, 1990) would enable the study of mutations which reduce, but do not abolish, gene function. Particle bombardment is a well known technique to introduce foreign genes into plants (Sanford, 1988). Particle bombardment has also been used for infection with RNA (Klein et al., 1987) and DNA viruses (Garzon-Tiznado et al., 1993; Hagen et al., 1994).

Four full-length ZYMV cDNA constructs were made and cloned in pBluescript KS(+) (Stratagene) (Fig. 1a-d). The 35S promoter was excised from the plasmid pBI221 (Clontech) and cloned into pKS (Stratagene). The generated clone (pKS35SXB) contained unique XbaI and BamHI sites downstream of the transcription initiation of the 35S promoter. The 5' end of ZYMV cDNA (2-3 kb) (Gal-On et al., 1991) was cleaved with BamHI and XbaI, and cloned into the appropriate sites of pKS35SXB, and the new clone designated pKS35S+16B. The NOS terminator was excised from pBI221 by EcoRI/SacI and cloned, after blunt ending, to pKS35S+16B, and the generated construct was designated pKS35S+16BNOS. Sequencing confirmed an extra 21 nucleotides between the poly(A) tail and the NOS terminator. Finally, the 3' two-thirds cDNA fragment (7.4 kb BamHI/Asp718 double-digested) from the full-length clone pKSM16322M (Gal-On et al., 1991) was introduced into clone pKS35S+16BNOS, producing pKS35S+ZYMVNOS (Fig. 1a). In order to link the transcription initiation of the 35S promoter to the viral cDNA we used a PCR [with the Expand Long Template PCR system (Boehringer Mannheim)] of clone pKS35S+16BNOS...
from the 3′ end of the 35S promoter to the 5′ end of the viral cDNA. The PCR fragment (6.4 kb) was closed by ligation forming pKS35S16BNOS. The 3′ two-thirds BamHI-Asp718 fragment from the FLC was cloned into the same sites of clone pKS35S16BNOS, synthesizing pKS35SZYMVNOS (Fig. 1b). The third construct, lacking the NOS termination region, was created by subcloning the FLC linked to the 35S promoter (double-digested with Asp718 and NotI) to the appropriate sites of pKS, producing pKS35SZYMV (Fig. 1c). The four clones, a–d (Fig. 1), were tested for infectivity by mechanical and bombardment inoculation of cucurbit plants.

**ZYMV** causes severe epidemics in all important cultivated species of Cucurbitaceae (Lisa et al., 1981), producing a high rate of infection in squash, melon and cucumber, but is less efficient in watermelon. Symptom appearance (vein clearing, followed by a yellow mosaic on the infected systemic leaf) requires 7–10 days after sap inoculation of squash, cucumber and melon. Using the full-length cDNA clone pKS35SZYMVNOS (1 µg dose/plant at 0.5 µg/µl; n = 20–35 plants) we obtained 100% systemic infection of ZYMV in all four cucurbit species by particle bombardment. A particle inflow gun was constructed (Gray et al., 1994), modified only by the use of locally available components. Preparation of the DNA was basically according to Perl et al. (1992) using calcium nitrate at pH 10.5 for plasmid DNA. However, transcripts were precipitated onto the M20 tungsten particles using 1.25 M-calcium nitrate, pH 8. The target cotyledon was less than 10 cm from the discharge point.

Symptoms appeared 3–4 days after inoculation in squash (Cucurbita pepo L. cv. Ma’ayan), cucumber (Cucumis sativus L. cv. Bet Alpha) and melon (Cucumis melo L. cv. Arava). ZYMV was detected in watermelon (Citrullus lanatus Schad cv. Malali) by ELISA 7 days post-inoculation (p.i.) as the early stages of ZYMV infection are symptomless in this species. However, mechanical inoculation using about the same concentration and dose per cotyledon caused a low efficiency of infection in squash (19%), cucumber (7%) and melon (4%), and none in watermelon (n = 27–47 plants). Symptoms appeared between 10 to 14 days p.i. The other published potyviral infectious clone under the control of the 35S promoter (plum pox virus: Maiss et al., 1992) was efficient on Nicotiana benthamiana, but less than the 100% obtained here.

The constructs pKS35SZYMVNOS and pKS35SZYMV (with and without the NOS terminator respectively) (Fig. 1b, c) infected 100% (n = 35 and 18) of
squash plants by particle bombardment inoculation. The NOS terminator is therefore not essential for infection, as shown by Yamaya et al. (1988). However, mechanical inoculation with the construct lacking the NOS terminator is non-infective with 1 μg (Fig. 1 c) or even 5 μg DNA/plant. This difference in infectivity is probably due to the great increase in efficiency due to particle bombardment inoculation.

The construct pKS35S+ZYMVNOS with an extra 127 nt between the 35S transcription initiation and the 5' end of the ZYMV genome (Fig. 1 a) shows a low level of infectivity by particle bombardment, and no infectivity by mechanical inoculation at 1 μg (Fig. 1 a) or even 10 μg/plant. The infection resulting from inoculation with this cDNA clone (pKS35S+ZYMVNOS) showed symptoms similar to the wild-type ZYMV, although progeny virions were not tested for the possibility of extra nucleotides at the 5' end of the ZYMV genome. Previous reports (see Boyer & Haenni, 1994) showed that 1–15 extra nucleotides at the 5' end abolish or greatly reduce infection by in vitro transcripts. With an extra 40 nonviral nucleotides before the 5' end of the cDNA of beet necrotic yellow vein virus the construct was still infectious when driven by the 35S promoter, whereas in vitro transcripts are inactive (Commandeur et al., 1991).

The pKSM16322M construct which contains the T7 promoter was non-infective (Fig. 1 d), probably because the bacterial T7 promoter is not recognized by the plant DNA-dependent RNA-polymerase(s).

Control experiments were necessary because of the high infectivity of our constructs. In each experiment plants were mock-bombarded (without DNA) after treatments with experimental constructs. ZYMV infections were not observed in any of these experiments (n = 42).

Squash seedlings were bombarded with different quantities of plasmid DNA of the clone pKS35S-ZYMVNOS (Fig. 2). Here we have converted the data to amount of DNA bombarded/plant. There may be errors in the dose received by the plant due to variations in the binding of the DNA to the tungsten particles. However, almost all of the DNA in the mixture was found to be associated with the tungsten particles (A. Gal-On, unpublished data). Not all of the tungsten particles may strike the plant and many do not penetrate the epidermis. However, the values given (Fig. 2) are the maximum possible dose of DNA received by the plant, and these calculations allow a direct comparison with mechanical inoculation using the same clone (pKS35SZYMNOS). Infection by particle bombardment by a dose as small as 1 ng/plant was maximal, and then decreased linearly with log dose to 20% infection at 1 pg cDNA/plant (Fig. 2). The low rate of infection by mechanical inoculation with 1 μg/plant was abolished by

![Fig. 2. Dose response of squash plants to cloned ZYMV (pKS35SZYMVNOS) inoculated by particle bombardment (○) or mechanically (■). The x-axis has a logarithmic scale. All points are the sum of five separate experiments with five plants each, with the exception of the results for bombardment with 100 ng, 20 ng and 0.2 ng/plant, which represent the results of two such experiments.](image-url)
to prove systemic infection), we detached the inoculated cotyledon immediately after bombardment. The plants from which the cotyledons had been removed did not show infection, while the intact plants developed symptoms 3–4 days p.i.

A notable facet of the work reported here is the great efficiency of bombardment inoculation, and the rapidity of symptom development. The earlier appearance of ZYMV symptoms following bombardment inoculation (3–4 days), compared to mechanical inoculation (10–14 days) with cDNA or RNA transcripts could be due to many more cells being infected by bombardment than by mechanical inoculation. Particle bombardment is known to be able to deliver foreign DNA directly to the host nucleus (Yamashita et al., 1991), thereby also increasing the overall efficiency of infection. The particle inflow gun (Finer et al., 1992; Gray et al., 1994) used here is capable of projecting DNA into xylem cells in melon cotyledons (V. Gaba, unpublished data). It is also possible that bombardment results in immediate infection of plasmid companion cells, or cells near the plasmid, permitting rapid systemic movement of the virus (Leisner & Howell, 1993). Direct infection of plasmid cells is also a possible explanation of the effectiveness of bombardment with DNA viruses (Garzon-Tiznado et al., 1990; Hagen et al., 1994).

To the best of our knowledge this is the first report of the use of particle bombardment with a cloned plant RNA virus, and the combination of the two technologies (strong plant promoter and particle bombardment) produced rapid infection at high efficiency. The high efficiency enabled us to observe infections where previously constructs had not been infectious. This technology is expected to allow us to determine viral gene function in future studies of recombination and complementation events.

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