Inoculation of Viral RNA and cDNA to Potato and Tobacco Plants Using the Helios™ Gene Gun

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Introduction

Particle bombardment is a physical method widely used for gene transfer into plants, mammals, fungi and bacteria (Armaleo et al. 1990, Klein et al. 1992, Shark et al. 1991, Williams et al. 1991, Zelenin et al. 1989). The hand-held Helios gene gun provides wider possibilities for gene transfer to plant tissue both in vivo and in vitro, because it is not limited by the large size of the target, and bombardment does not need to be carried out under vacuum.

The particle bombardment technique has been used to infect plants with DNA viruses, RNA viruses and cloned RNA viruses (Gal-On et al. 1995, Gilbertson et al. 1991, Klein et al. 1992). The PDS-1000/He system is efficient for inoculation of detached leaves, but the Helios gene gun enables inoculation of an intact plant. In this study we have used the Helios gene gun to inoculate whole tobacco and potato plants with the cDNA of an RNA virus, potato virus A (PVA; Puurand et al. 1996). We wanted to optimize bombardment parameters to achieve 100% efficiency of PVA infection in susceptible tobacco plants. In the case of potato plants, we used potato clone 'A6' (Solanum demissum Lindl. x S. tuberosum cv. Aquila), which develops necrotic lesions at the site of PVA infection (Valkonen et al. 1995),and thus enables counting of the number of initial infection sites. The number of lesions can be used as a readily observed measure for optimal inoculation conditions. The bombardment parameters examined in this study were helium pressure, distance of target tissue from the spacer, polyvinylpyrrolidone (PVP) concentration, viral DNA and RNA amounts, and the size and number of gold particles (microcarriers).

PVA belongs to the family Potyviridae, the largest and economically the most important group of plant viruses (Bartels 1971). PVA is widely distributed in potato-growing areas and decreases the yield of infected potato plants by up to 40% (Brunt 1992). A full-length cDNA clone of the RNA genome of PVA has been constructed under the control of a T7 promoter (PVA-T7), from which infectious transcripts can be produced in vitro for inoculation (Puurand et al. 1996). The same construct is also available under the control of a cauliflower mosaic virus (CMV) 35S promoter (PVA-35S) that can be used directly for inoculation (Ü. Puurand pers. comm., Rajamaki and Valkonen 1999).

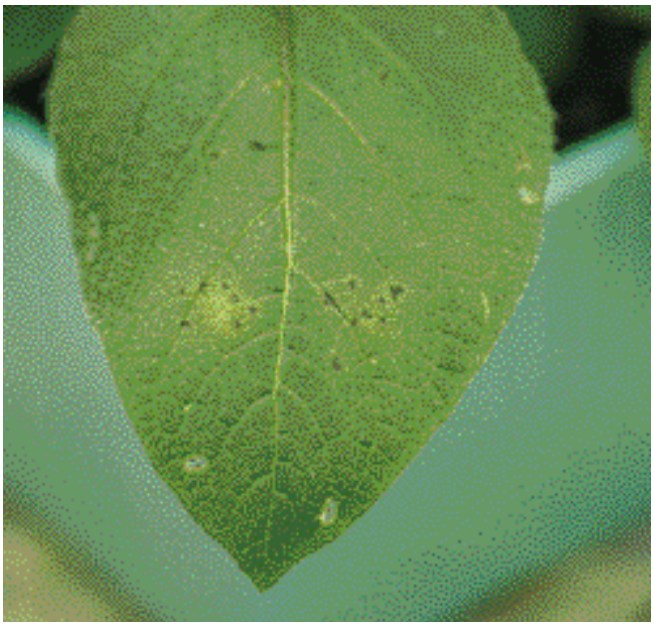

Fig. 1. Bombardment of potato clone 'A6' with viral cDNA at 80 (right side) and 100 psi (left side), at 0 (lower half) and 2 cm distance (upper half of the leaf).

Methods

Plant Material. Tobacco (Nicotiana tabacum L. cv. Samsun) is susceptible to PVA, whereas the test potato clone 'A6' reacts with the development of necrotic lesions (hypersensitive response) at the sites of infection (Valkonen et al. 1995). Tobacco and potato plants were grown in a growth chamber under an 18 hr photoperiod (250 µE s^{-1} m^{-2}) at 19/17 °C (day/night) and a relative humidity of 40%. Tobacco plants were inoculated by particle bombardment when the first true leaf was fully expanded. Potato plants propagated by taking cuttings were grown in the growth chamber for four weeks before bombardment.

Preparation of DNA or RNA for Bombardment. The PVA-T7 construct was linearized with AgeI prior to in vitro synthesis of the 5'-capped transcripts according to the manufacturer's instructions (RiboMax, Promega). Transcript quality and concentration were determined by electrophoresis in an agarose gel. RNA was precipitated on gold particles by addition of 1/10 volume of 3 M NaAc (pH 7.4) and 3 volumes of 99.5% ethanol. The suspension was mixed by tapping the

Fig. 2. Extensive death of the cells in the bombarded area of tobacco leaf (pressure 150 psi, distance 0 cm).

tube and placing it at -20 °C for 30 min. After a quick centrifugation (~5 sec), the supernatant was discarded and the gold pellet washed three times with 1 ml 99.5% ethanol. The gold particles were then resuspended in 200 µl of ethanol containing the appropriate concentration of PVP. The suspension was transferred to a 15 ml polypropylene centrifuge tube, and 2.8 ml ethanol/PVP solution was added. The suspension was immediately used for preparation of cartridges.

PVA-35S plasmid was isolated from an E. coli XL1-Blue culture using the QIAGEN® Plasmid Maxi Kit (QIAGEN Ltd., UK) according to the manufacturer's instructions. PVA-35S was linearized prior to precipitation of the DNA on gold particles, as described above for RNA. Cartridges were prepared as described in the Helios gene gun instruction manuals (catalog # 165-2431 and 165-2432, Bio-Rad).

Inoculation. For each tobacco plant, the first full-grown true leaf was inoculated with one bombardment. For the potato clone 'A6', one or two full-grown leaves were bombarded either one or multiple times on different sides of the midvein. The latter arrangement permitted comparison of two or more different bombardment parameters on the same 'A6' leaf (Figure 1). Before bombardment, plants were kept in the dark for at least five hr. After bombardment, plants were sprayed with water and placed in a transparent box in dim light overnight. The next day, the plants were returned to the growth chamber, and the extent of mechanical damage on bombarded areas of the leaves was observed. Symptoms of PVA infection in 'A6' (necrotic lesions) were observed four days after bombardment. In tobacco, PVA infection was detected twelve days after bombardment of upper uninoculated leaves. The assay was a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using monoclonal antibodies and alkaline phosphatase-conjugated monoclonal antibodies to PVA (MAb 58/0; Rajamaki et al. 1998) obtained from Adgen, UK.

Optimization of Bombardment Parameters. We tested various bombardment conditions in order to identify those that resulted in the largest number of necrotic lesions on 'A6' leaves and in infection of all inoculated tobacco plants. First, different helium pressures (80, 100, 150, 200, 250 and 300 psi) were tested using 2 µg PVA-35S or PVA-T7 per mg gold (1.0 µm), 0.05 mg PVP per ml 99.5% ethanol, and 0.5 mg gold per bombardment as recommended for starting conditions in the Helios gene gun instruction manual. Bombardments were done with 0 or 2 cm distance between the spacer and the target. The first experiments showed that bombardment at 250 and 300 psi damaged leaves extensively, so these pressures were not included in further experiments.

Next, PVP concentrations were varied (0, 0.05 and 0.1 mg per ml 99.5% ethanol). Third, using the previously determined optimal helium pressure and PVP concentration, different sizes of microcarriers (0.6, 1.0 and 1.6 µm) were tested. In the last experiment, various amounts of microcarriers per shot (the microcarrier loading quantity, or MLQ) and various amounts of PVA cDNA coated on microcarriers (the DNA loading ratio, or DLR) were tested.

In each optimization step, three cartridges of PVA-T7 transcript (RNA) or PVA-35S (cDNA) were bombarded onto tobacco and potato plants. Each test was carried out at least three times. After each optimization step, the optimal parameter was used in further experiments, as explained above. Significance of observed differences was tested using analysis of variance and calculation of the least significant differences (Steel and Torrie 1981).

Results

Pressure and Distance. In tobacco, 100% infection was achieved using a helium pressure of 150 or 200 psi at a distance of 0 cm, but extensive death of the cells in the middle of the bombarded area was observed (Figure 2). At the higher pressure of 250 or 300 psi, leaves tended to be ripped. No infection was detected in tobacco plants bombarded at the low pressures of 80 and 100 psi, and at these pressures the bombarded areas usually remained undamaged. Results were similar for PVA-T7 (RNA) and PVA-35S (cDNA).

Table 1. Ratios of Microcarriers and PVA cDNA Tested

mg microcarrier/shot	µg DNA/shot	DNA/microcarrier (µg/mg)
0.5	0.1	0.2
0.75	1	1.33
0.5	1	2
0.125	1	8
0.5	5	10

In the potato clone ‘A6’, infection initiation sites could be visually identified by the development of necrotic lesions as a result of the hypersensitive response to PVA. A few necrotic lesions characteristic of PVA infection (Valkonen et al. 1995) were observed at 80 psi, and their numbers increased with increased pressure (Figure 3). At lower pressures (80 and 100 psi), the bombarded area of leaf was usually undamaged and just a few necrotic lesions were observed. Bombardment with higher pressures (150 and 200 psi) led to cell death in the middle part of the bombarded tissue, but numerous necrotic lesions were still observed in the surrounding surviving tissue (Figure 4).

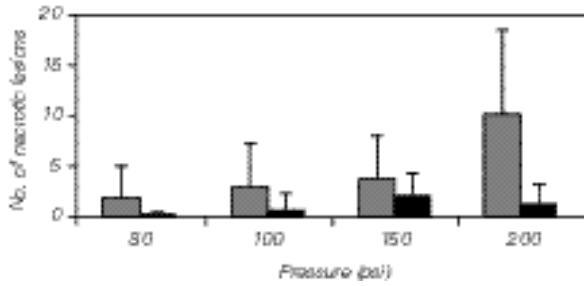

Fig. 3. Effect of helium pressure on the number of necrotic lesions in potato clone ‘A6’ using PVA cDNA. Gray bars, bombardment at 0 cm from the spacer; black bars, bombardment at 2 cm. Standard deviation is indicated on each bar.

The pressure of 200 psi and the distance of 0 cm were optimal for ‘A6’, differing significantly ($p < 0.01$) from the other pressures used. The higher pressures of 250 and 300 psi ripped the leaves and are thus not recommended for use. Although bombardment at a 2 cm distance from the target tissue resulted in spreading of the microcarriers (and subsequently the necrotic lesions) over a larger area on the leaf, the total number of necrotic lesions was no greater than after bombardment at a distance of 0 cm. No differences were observed between bombardment using PVA RNA or cDNA (data not shown).

Fig. 4. Bombardment of potato clone ‘A6’ with viral cDNA at 80 (left, upper part of leaves), 100 (right, upper part of leaves) 150 (left, lower part of leaves) and 200 psi (right, lower part of leaves) at 0 cm.

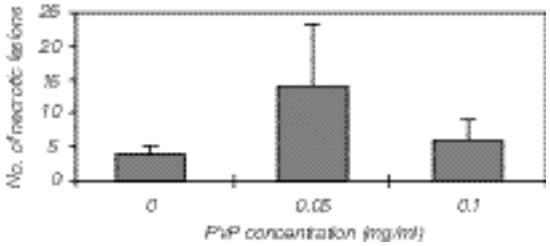

Fig. 5. Effect of different PVP concentrations on the number of necrotic lesions in potato clone ‘A6’ using PVA cDNA. Standard deviation is indicated on each bar.

PVP Concentration and Microcarrier Size. PVP serves as an adhesive during the coating of DNA/(or RNA)/microcarrier suspension to the walls of the Goldcoat tubing. With both DNA and RNA of PVA, the optimal concentration was shown to be 0.05 mg PVP/ml 99.5% ethanol. This resulted in significantly more lesions ($p < 0.01$) in ‘A6’ leaves than at PVP concentrations of 0 or 0.1 mg/ml (Figure 5).

Three sizes of microcarriers were used: 0.6, 1.0 and 1.6 µm. The 0.6 µm carriers were significantly more efficient in initiation of infection ($p < 0.01$) than the other two sizes used (Figure 6).

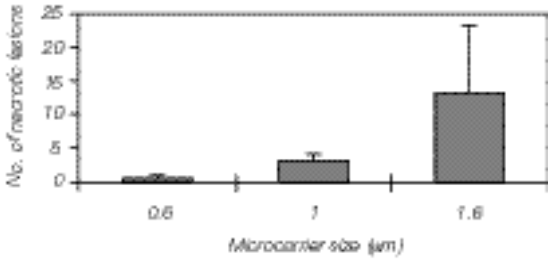

Fig. 6. Effect of the size of microcarriers on the number of necrotic lesions in potato clone ‘A6’ using PVA cDNA (values normalized to compare equal numbers of particles).

Ratios of Microcarrier and DNA Amounts. Various ratios of microcarriers and viral cDNA (Table 1) were tested by bombarding ‘A6’ leaves. The numbers of infection sites observed with different ratios of microcarriers and DNA were not significantly different statistically. As little as 0.1 µg viral cDNA per bombardment was sufficient to trigger development of necrotic lesions in ‘A6’.

Discussion

The Helios gene gun is being used in several laboratories for plant transformation and also virus inoculation. We have optimized bombardment parameters for a cloned RNA virus (PVA) using an experimental host widely used for many plant viruses (N. tabacum L. cv. Samsun) and also using a host that reacts with a hypersensitive response to virus infection (potato clone ‘A6’). The latter type of host is useful for an optimization experiment because the sites of infection can easily be identified visually after only a few days. Because the aim of our study was to inoculate a cloned virus (RNA transcripts or linearized viral cDNA), it was inferred that the optimal bombardment parameters could be different from those used for plant transformation. For this reason, optimization of parameters was needed.

For successful infection, it is important to optimize the condition of plants before and after inoculation. We noticed that shading the plants overnight before bombardment and keeping them under humid conditions in dim light after bombardment improved initiation of infection considerably. Such treatments are known to increase susceptibility of plants to mechanical inoculation (Matthews 1995).

This study showed that it is important to determine the optimal pressure, PVP concentration and microcarrier size, because differences in these parameters provided varying success in infection. These parameters may also vary for different host plants. In our study, the low pressure of 80 psi caused a few necrotic lesions in 'A6' leaves, whereas in tobacco no PVA infection was detected following inoculation at a pressure of 80 or 100 psi. In contrast, at a pressure of 150 or 200 psi, all tobacco plants became infected. PVP concentration of 0.05 mg/ml and microcarrier size of 1.6 µm were the best for both plants. The number of microcarriers and amount of viral cDNA tested did not affect infection efficiency.

We suggest the starting conditions indicated in Table 2 to optimize viral RNA or cDNA inoculation of tobacco and potato plants. They have been used successfully for many more recent studies in our laboratory (Hamalainen et al. in press, Rajamaki and Valkonen 1999).

Table 2. Suggested Starting Parameters for Inoculation of Potato and Tobacco Plants by RNA Transcripts or Linearized Viral cDNA

Plant	Pressure (psi)	PVP (mg/ml)	Microcarrier (mg/target)	cDNA or RNA (µg/target)
Tobacco	150–200	0.05	0.125–0.5	1
Potato	200	0.05	0.125–0.5	1

References

Armaleo, D., et al., Biolistic nuclear transformation of Saccharomyces cerevisiae and other fungi, Curr. Genet., **17**, 97–103 (1990)

Bartels, R., Potato virus A, CMI/AAB Desc. Pl. Viruses, **54** (1971)

Brunt, A. A., The general properties of potyviruses, Arch. Virol., **5**, 3-16 (1992)

Gal-On, A., et al., Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus, J. Gen. Virol., **76**, 3223–3227 (1995)

Gilbertson, R. L., et al., Cloning of the complete DNA genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration, Phytopathology, **81**, 980–985 (1991)

Hamalainen, J. H., et al., Recessive and dominant genes interfere with the vascular transport of potato virus A in diploid potatoes, Mol. Plant Microbe Interact. (in press).

Klein R. M., et al., High-velocity microprojectiles for delivering nucleic acids into living cells, BioTechnology, **24**, 384-386 (1992)

Matthews, R. E. F., Plant Virology (3rd ed.), Academic Press, London (1995)

Puurand, Ü, et al., Infectious in vitro transcripts from cloned cDNA of the potato A potyvirus, Virus Res., **40**, 135–140 (1996)

Rajamaki, M. L., et al., Biological, serological and molecular differences among isolates of potato A potyvirus, Phytopathology, **88**, 311–321 (1998)

Rajamaki, M. L. and Valkonen, J. P. T., The 6K2 protein and the VPg of potato virus A are determinants of systemic infection in Nicandra physaloides, Mol. Plant Microbe Interact., **12**, 1074-1081 (1999)

Shark, K. B., et al., Biolistic transformation of a procaryote, Bacillus megaterium, Appl. Environ. Microbiol., **57**, 480–485 (1991)

Steel, R. G. D. and Torrie, J. H., Principles and Procedures of Statistics (2nd ed.), McGraw-Hill Book Company, London (1981)

Valkonen, J. P. T., et al., Three strain groups of potato A potyvirus based on hypersensitive response in potato, serological properties and coat protein sequences, Plant Dis., **79**, 748–753 (1995)

Williams, R. S., et al., Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles, Proc. Natl. Acad. Sci. USA, **88**, 2726–2730 (1991)

Zelenin A. V., et al., Genetic transformation of mouse cultured cells with the help of high-velocity mechanical DNA injection, FEBS Lett., **244**, 65–67 (1989)

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