

# Expression of PVX coat protein gene under the control of extensin-gene promoter confers virus resistance on transgenic potato plants

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## Abstract

Tuber discs of potato (*Solanum tuberosum* L.) cultivars Desirée and Gracia were infected by *Agrobacterium tumefaciens* carrying a binary vector with the coat protein gene of potato virus X controlled by the carrot extensin gene long-transcript promoter. Several transgenic potato plants have been obtained by direct regeneration of shoots on culture medium with kanamycin used for selection. The presence of the coat protein gene was proved by Southern hybridization in several transformants. Its low but detectable expression level was shown by Northern and Western analysis. Ethepon treatment resulted in a five-fold increase in the amounts of the coat protein mRNA. The majority of transformants exhibited reduced accumulation of virus RNA in inoculated leaves. Potentials in the use of an ethylene-inducible promoter in the production of virus-resistant transgenic plants will be discussed.

**Abbreviations:** CP: coat protein, PVX: potato virus X, HRGP: hydroxyproline-rich glycoprotein, ELISA: enzyme-linked immunosorbent assay

## Introduction

Genetically engineered resistance of different plant species to virus infection by expression of viral coat proteins (CPs) has been demonstrated for several plant viruses (as reviewed by Beachy et al. 1990, Nelson et al. 1990, Nejidat et al. 1990). In each case, expression of the coat protein gene from the target virus conferred resistance against the homologous virus or different strains of the same virus, and in some cases even a broader spectrum of resistance could be observed (for review see Nejidat et al. 1990).

In most of these studies, the introduced virus genes were linked to the cauliflower mosaic virus (CaMV) 35S promoter providing high level of expression in the cells of transgenic plants. Resistance of plants to diseases, however, involves inducible defense mechanisms (Sequeira 1983, Collinge and Slusarenko 1987, Bol et al. 1990). These responses can be induced artificially by wounding and/or treatment of plant tissues or cultured cells with elicitors or ethylene (e.g. Lawton and Lamb 1987,

Ecker and Davis 1987).

Based on these observations, we describe here the use of the ethylene-inducible promoter of carrot extensin gene (Chen and Varner 1985) in the production of virus-resistant potato plants expressing a chimeric PVX coat protein gene.

## Materials and Methods

**Plant Material and Culture Conditions.** *Solanum tuberosum* L. cv. Gracia and Desirée plants were maintained as *in vitro* shoot cultures on hormone free MS (Murashige and Skoog 1962) medium. Tissue culture steps, including the shoot regeneration from tuber tissues were performed in a phytotron chamber at 12 hours fluorescent light ( $52\text{--}120 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 20/15 °C day/night temperature.

For *Agrobacterium* infection, spontaneously formed young (2–3 cm in size) tubers of *in vitro* or greenhouse grown plants (derived also from *in vitro* cultures) were used. Sterilization of thoroughly washed greenhouse tubers was carried out in 0.1% HgCl<sub>2</sub> solution (2 min.) and 70% ethanol (30 sec.) followed by washing with a large volume of sterile water.

Shoot regeneration was induced on agar-solidified MS medium supplemented with 2 mg l<sup>-1</sup> zeatin-riboside, 0.02 mg l<sup>-1</sup> naphthalene-1-acetic acid and 0.02 mg l<sup>-1</sup> gibberellic acid as hormones, 100 mg l<sup>-1</sup> kanamycin for selection and 300 mg l<sup>-1</sup> carbenicillin to prevent bacterial growth. Tissues were subcultured every four weeks on the same medium. Regenerated shoots were excised and transferred to hormone free MS medium containing 100–100 mg l<sup>-1</sup> kanamycin and carbenicillin.

**Ethepon treatment.** Control and transgenic potato plants were sprayed (10 ml per square meter) with a 0.03 M solution of the ethylene-releasing agent ethepon (2-chloroethylphosphonic acid) six weeks after transferring the *in vitro* plantlets into soil.

**Cloning.** All of the cloning steps were carried out using standard protocols (Maniatis et al. 1982) as described elsewhere in details (Zakharyev et al. 1989).

**Agrobacterium-infection.** The *Agrobacterium tumefaciens* strain LBA 4404 carrying the recombinant pBin 19 vector (Bevan 1984) was grown overnight at 28 °C in 10 ml of LB medium containing 50 mg l<sup>-1</sup> kanamycin.

The outer part (2–3 mm) of sterile tubers was cut off with a scalpel thereby removing the periderm and the buds. The interior tissue was cut into slices 2–3 mm thick which were

floated for 20 minutes in 20 ml of MS medium inoculated with 2 ml of an overnight culture of the *Agrobacterium* strain. After removing excess fluid from tuber slices with sterile filter paper, they were plated onto agar solidified regeneration medium (see above) without antibiotics for two days. Regeneration and selection of transgenic shoots were carried out as described above.

**Activity tests of neomycin phosphotransferase.** Kanamycin resistance of putative transformants was investigated through induction of rooting as well as callus formation (MS medium with 5 mg l<sup>-1</sup> naphthalene-1-acetic acid and 0.1 mg l<sup>-1</sup> benzyladenine) in the presence of 100 mg l<sup>-1</sup> kanamycin. Some of the regenerants were tested for neomycin phosphotransferase enzyme activity (Reiss et al. 1984).

**DNA and RNA analysis.** DNA was purified from leaves of *in vitro* potato plants according to Rogers and Bendich (1988) and was double digested by PstI and XhoI (BRL) and 7 µg DNA was loaded per lane in 0.8% agarose gels. After transfer to Hybond-N nylon filter (Amersham), the restricted DNA samples were hybridized with the 587 bp internal PstI/XhoI fragment of the PVX coat protein gene. Labelling of the probe with digoxigenine-UTP and hybridization were performed by using the non-radioactive DNA labelling and detection kit from Boehringer, according to the manufacturer's instructions.

Total RNA isolation from leaves of greenhouse grown plants, poly-A<sup>+</sup> RNA purification via oligo(dT)-cellulose chromatography, electrophoretic separation in formaldehyde containing agarose gels as well as the Northern analysis were carried out using standard procedures (Maniatis et al. 1982). As probes, the same fragment of the CP gene as for Southern hybridization, the 920 bp PstI fragment of the *neo* gene of the pNeo plasmid (Pharmacia) and a randomly chosen clone (CD 26) from a tomato leaf cDNA bank originally isolated for restriction fragment length polymorphism studies (Tanksley et al. 1987) were used. Labelling was performed by the random priming method resulting in around 10<sup>9</sup> cpm/µg specific activities.

**Immunoblotting.** Immunoblotting of 10 µg soluble extracted protein of control and transgenic plants was done according to Hockema et al. (1989) by using rabbit antibodies to PVX coat protein (Zakharyev et al. 1989) and horseradish peroxidase-linked donkey anti-rabbit antibody (Amersham).

**Coat protein mediated cross protection test.** Greenhouse grown potato plants were inoculated with purified PVX (G strain), 0.5 µg/ml in 150 mM phosphate buffer pH 7.0. Samples were cut out from inoculated leaves (8 mm in diameter) and pressed in Eppendorf tubes. The supernatant of the centrifuged crude extract was used (1-5 µl) in hybridization experiments. Oligonucleotide probes (two 40 nucleotides long complementary to the 3' end of the PVX genomic RNA sequences (Morozov et al. 1987) were prepared and labelled with <sup>32</sup>P-ATP using T4 polynucleotide kinase (Boehringer Mannheim). Specific activities were about 10<sup>9</sup> cpm/µg. Solution hybridization and hybrid collection were made essentially after Rouhiainen et al. (1991). The bound radioactivity was measured in a beta counter (Beckmann LS 7000).

## Results and Discussion

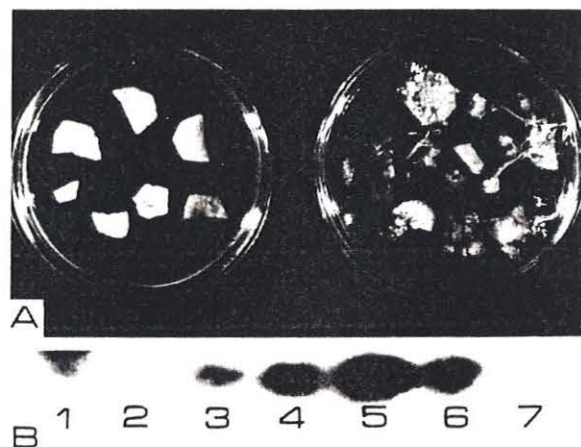
Expression of viral genes in transgenic plants has been found to be useful in several instances in the development of virus-resistant plants (for review see: Beachy et al. 1990). Especially, the phenomenon of coat-protein mediated resistance has been thoroughly investigated. Protection could be correlated in some cases with CP levels in different transgenic plants (see the review of Nelson et al. (1990) and it could be concluded that a threshold level of CP-expression is

apparently required for protection. To provide a high CP concentration in the cells of transgenic plants, the 35S promoter of cauliflower mosaic virus, which is an especially strong promoter in plants, was used to drive the expression of CP genes in most of the experiments (Beachy et al. 1990). However, besides the high expression level, the properly regulated expression of the introduced virus genes would also be of great importance in transgenic plants produced for practical purposes. Plant-virus interaction is accompanied by numerous metabolic changes (Collinge and Slusarenko 1987, Bol et al. 1990). One general effect is the induction of enzymes that are involved in the synthesis of ethylene, a plant hormone able to induce many stress responses (e.g. Yang and Hoffman 1984). The induction of enzymes of the phenylpropanoid pathway, oxidative enzymes and hydroxyproline-rich glycoproteins (HRGPs, extensins) is partially related to cell wall modifications that lead to the formation of a physical barrier to further spread of the virus from the site of infection (Cassab and Varner 1988). In carrots, different extensin mRNA transcripts have been found to accumulate in response to wounding and ethylene treatment, respectively (Ecker and Davis 1987). Both mRNAs have been mapped to a single carrot HRGP gene and differed only in the length of their 5' untranslated region (Chen and Varner 1985). It has been proposed that each transcript has its own promoter (Chen and Varner 1985), and that the upstream (long-transcript) HRGP promoter responds to the ethylene, while the downstream (short-transcript) promoter responds to the wound induction (Ecker and Davis 1987).

### Vector construction and transformation.

Based on the fact that the response of plant cells to infection by viruses (as well as by other plant pathogens) is accompanied by an increase in the amount of endogenous ethylene, we constructed a chimeric PVX CP gene under the control of the above mentioned ethylene inducible promoter of the carrot extensin gene.

The long-transcript promoter of the carrot extensin gene was isolated as a Sau3A/EcoRI fragment of the plasmid pDC5A<sub>1</sub> (Chen and Varner 1985). The structural gene of PVX coat protein was obtained by BamHI/HindIII restriction of the plasmid pVX-72 (see: Zakharyev et al. 1989). This fragment contained 11 bp from pUC 19 polylinker (BamHI-SalGI), 5' upstream region (54 bp), coat protein gene, 3' downstream region (75 bp) and poly-(A)-tail. The chimeric gene was supplied with the Nos terminator sequence. Cloning was performed in pUC 19 and finally the chimeric gene was recloned into pBin 19 (Bevan 1984) as an EcoRI/HindIII fragment. This disarmed binary vector contained also the neomycin phosphotransferase (*neo*) gene conferring kanamycin resistance to plant cells. This gene construct was expected to provide synthesis of a considerable amount of PVX CP in the transgenic plants under stress conditions only (e.g. after virus infection). The gene construct was introduced into the genome of two potato cultivars (Desirée, Gracia) via *Agrobacterium*-mediated transformation of tuber cells. Slices of minitubers of *in vitro* or young greenhouse grown plants exhibited high morphogenetic capability similarly as has been reported by Ishida et al. (1989).



**Figure 1** Regeneration of kanamycin-resistant shoots after *Agrobacterium*-mediated transformation. A: Most of the *Agrobacterium*-infected tuber slices regenerated several shoots on 100 mg l<sup>-1</sup> kanamycin containing medium (right), while this antibiotic concentration completely inhibited regeneration of control, uninfected tissues (left). B: Neomycin phosphotransferase activity in crude leaf extracts of primary transgenic regenerants (lane 1 - positive control; lanes 2-6 - transgenic potato clones; lane 7 - untransformed control Gracia plant).

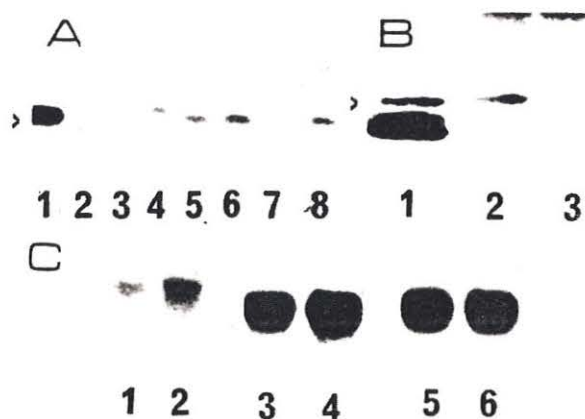
Almost all *Agrobacterium* infected tuber discs have regenerated several (3-10) shoots (Fig. 1/A). There was no significant difference between the two cultivars in shoot regeneration under selective conditions. Kanamycin resistance of the regenerated shoots was retested by the analysis of rooting and callus formation of leaf segments in the presence of kanamycin. On the basis of these tests, 25-50 % escapees were observed. However, control, uninfected tuber discs did not form any shoots in the presence of kanamycin (Fig. 1/A). In later experiments, we separated the shoot regeneration and elongation phases. First, shoot regeneration was induced on a medium with a high concentration of zeatin-riboside (4 mg l<sup>-1</sup> for 3-4 weeks) followed by the transfer of tissues with preformed shoot primordia to shoot elongation medium with only gibberellic acid (0.1 mg l<sup>-1</sup>). By this way, kanamycin selection was more efficient.

In ten randomly chosen kanamycin resistant clones, the expression of the bacterial *neo* gene was proved by neomycin phosphotransferase enzyme activity test (Fig. 1/B). All of the investigated greenhouse grown plants possessed the active enzyme.

#### *Presence and expression of the PVX coat protein gene in the transformants.*

So far, twenty kanamycin resistant plants have been tested for the presence of the viral gene sequence by Southern hybridization. Using the internal PstI/XhoI fragment of the coat protein gene as probe, and the double digestion of plant DNA by the same enzymes, the success of the gene transfer was unambiguously proved (Fig. 2/A) in 18 out of the 20 investigated plants.

In leaf extracts of transgenic Desirée plants (DR17/1) with high resistance in artificial virus infection experiments (Fig. 3), the virus coat



**Figure 2** Presence and expression of the introduced chimeric PVX CP-gene in transgenic potato plants. A: Southern hybridization revealed the presence of the interior PstI/XhoI fragment of the PVX CP-gene in transgenic potato plants (lane 1 - the fragment itself used as probe; lane 2 - empty; lane 3 - untransformed Gracia control; lanes 4,5,6 - transgenic Gracia plants; lane 7 - empty; lane 8 - transgenic Desirée plant). B: Detection of the PVX CP in the leaves of DR17/1 transgenic clone by Western analysis (lane 1 - 500 ng purified PVX CP (26 kD) and its degraded (22 kD) form; lane 2 - 10 µg total protein of DR17/1 clone; lane 3 - 10 µg total protein of Desirée control). C: mRNA levels in leaves of Ethephon treated (lanes 2,4,6) and untreated (lanes 1,3,5) Desirée transgenic clone (DR17/1). 8-8 µg of poly-A<sup>+</sup> RNA was loaded per lane and probed with PVX CP-gene fragment (lanes 1,2), *neo*-gene fragment (lanes 3,4) and tomato leaf cDNA clone CD 26 as inside control (lanes 4,5).

protein could be detected by Western blotting (Fig. 2/B).

The expression level seemed to be at least one order of magnitude lower than in transgenic potato (cv. Zarevo) plants carrying the same PVX CP-gene under the control of 35S promoter (O. A. Shulga, unpublished results).

The long-transcript promoter of the carrot extensin gene has been shown to be inducible by ethylene (Chen and Varner 1985, Ecker and Davis 1987). Therefore, we have analyzed the level of expression controlled by this promoter in control and Ethephon treated plants. Transcripts of the CP and *neo* genes could not be detected in the control Desirée plants (data not shown). The mRNA levels in the transgenic potato plants (DR17/1) with or without Ethephon treatment are shown in Fig. 2/C. In the untreated leaves, the expression of the extensin promoter controlled CP-gene was found at least one order of magnitude weaker than the expression of the Nos promoter driven *neo* gene (the specific activity of the used probes was in the same range, 0.8-1.0x10<sup>9</sup>cpm/µg). Similar expression levels have been observed in a transformed Gracia clone (data not shown). Since the Nos promoter is not considered a strong promoter in plants, this comparison might indicate a weak expression for the introduced virus gene in these plants. One possible reason is that the expression of HRGPs is tissue specific (Cassab and Varner 1988, Tierney and Varner 1987, Ye and Varner 1991) with a very low level in leaf tissues (Tierney and Varner 1987). The detectable expression of the chimeric CP-gene in leaf tissues could result from some stresses that influenced

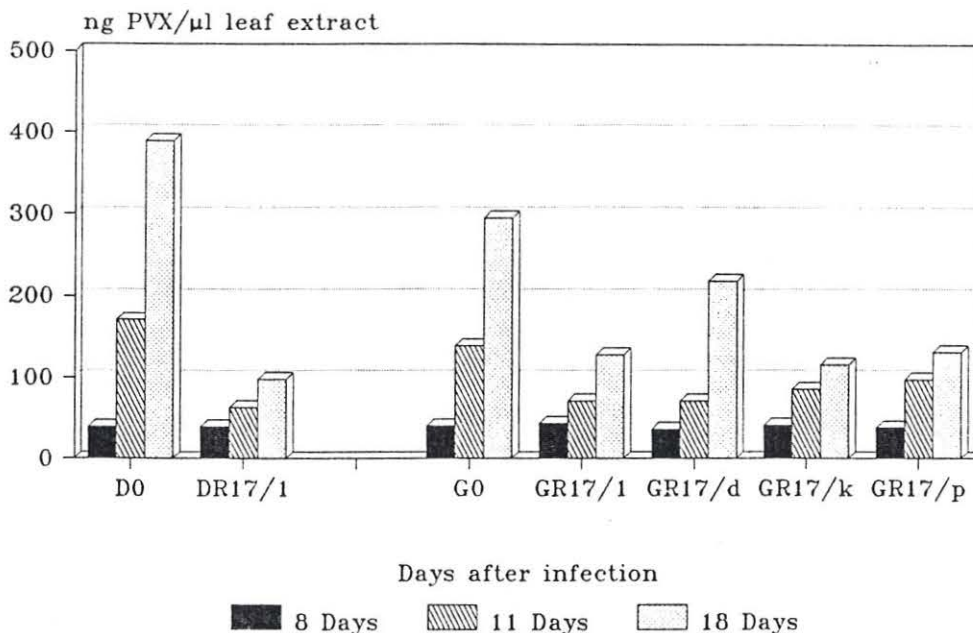


Figure 3 PVX virus RNA accumulation in infected leaves of control and transgenic potato plants after artificial infection (D0 - Desirée control; G0 - Gracia control; DR17/1 - Desirée transformant; GR17/1,d,k,p - Gracia transformants).

the greenhouse grown plants. The gene expression was enhanced about five times at the mRNA level 24 hours after spraying whole plants with Ethephon. This treatment, however, did not increase the amount of transcripts of the *neo* and CD 26 genes used as controls (Fig. 2/C), that indicates a specificity of the treatment in changing of gene expression pattern, for the extensin promoter.

Since the complexity of leaf tissue, the type and number of cells responding to ethylene generated from Ethephon are unknown. If only a small percentage of cells in the entire organ responds to the treatment, at the level of individual cells this ethylene induced increase in CP-mRNA amounts could be much higher than it is indicated by an analysis of the whole leaf. A cell type specific increase in the amount of extensin has been reported by Cassab et al. (1988) in the epidermal and cortical cells as well as for some degree in the vascular system upon ethylene treatment of pea epicotyls.

#### *Virus accumulation in PVX inoculated control and transgenic plants.*

On the basis of at least two independent experiments, the nine investigated clones could be divided into four groups according to the accumulation of the virus RNA. At the end of the investigated period (14 or 18 days after virus inoculation), the amounts of virus RNA exceeded those of control plants in the case of one transgenic clone. In further four clones, the virus RNA level ranged between 55 and 74 % of the controls, while in three transformants these values were 40 and 44 %, and in one case 10 and 25 %. The result of one of these experiments involving one Desirée (DR17/1 clone used for gene expression studies as well) and four Gracia transformants is shown in Fig. 3.

Artificial PVX infection of the transgenic potato

plants revealed that the introduction and expression of the chimeric CP-gene construct resulted in significantly decreased PVX propagation in most of the transgenic clones. The differences in the degree of virus RNA accumulation and the appearance of a clone with similar virus RNA levels as the control potato plants most probably can be ascribed to differences in the CP-gene expression as a result of position effect or structural changes in the transforming DNA during transformation and integration.

In the case of one of the transgenic clones (DR17/1) as compared to control plants, 4 to 10 fold reduction could be observed in the virus RNA levels 18 days after infection. A similar decline in virus accumulation was detected by Hemenway et al. (1988) in inoculated leaves of transgenic tobacco plants expressing the PVX CP-gene under the control of the 35S promoter. Hoekema et al. (1989) and Van den Elzen et al. (1989) have reported, however, 20 to 30 fold and even 100 fold differences in the virus level of infected control and transgenic potato plants expressing the PVX CP-gene at a very high level (up to 3 % CP of total soluble protein). In these experiments, the ELISA method was used to follow virus propagation, while we measured directly the amounts of virus RNA via oligonucleotide hybridization, that makes the direct comparison of the data to be difficult.

The detection system used in our study is simple, accurate and rapid, allowing detection of the viral RNA in plant sap in four hours. In infected plants coat proteins are translated from subgenomic RNA, and in the replication cycle of virus all viral RNAs have definitive roles. Detection of the total viral related RNAs in diseased plants gives a more comprehensive picture on the disease than detecting

only virions. The sensitivity of this test is higher than the ELISA technique commonly used. This sensitivity was further increased by using oligonucleotide probes hybridizing close to the poly-A<sup>+</sup> region of viral genomic DNA sequences, as oligo(dT) chromatography was used to collect the hybrid molecules. These oligonucleotides, however, can not discriminate between viral CP-RNA and the mRNA synthesized by the plant. This means that the effectiveness of the cross protection might have been underestimated. The background CP-mRNA level due to the expression of the chimeric CP-gene in the transgenic plants, can however be very low on the basis of the Northern analysis (see above).

The low level of expression of PVX CP-gene detected in the untreated transgenic plants can not possibly be responsible for the observed decline in virus RNA accumulation after inoculation of these plants with PVX. It could be that virus infection itself induces PVX CP production through the induction of ethylene synthesis in the plant cells similarly to Ethepon treatment. This hypothesis could be further augmented by analyzing PVX CP-gene expression in these transgenic potato plants following infection with other non-related potato viruses (e.g. PVY). Experiments are also in progress to investigate the transformants under field conditions in respect to their virus resistance.

As there are observations that HRGP mRNAs are mostly abundant in outer and inner phloem cells in petunia, tobacco, tomato and potato stems (Ye and Varner 1991) the usefulness of similar gene constructs in the evaluation of CP-mediated resistance against leaf roll virus (PLRV) in potato is apparent, since this virus occurs predominantly in the phloem (Van den Elzen et al. 1989).

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