Nicotiana benthamiana Plants Transgenic for PPV-SWC Coat Protein are Resistant to PPV Infection

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Abstract

Two constructs containing the coat protein gene (CP) of PPV-SwC – in (+) and (–) sense – were obtained and used to transform *Nicotiana benthamiana* plants via *Agrobacterium tumefaciens*. Transformed lines were analysed for the integration of the *CP* gene by PCR and Southern blot. Sixty transgenic lines were selected. Expression of *CP* gene was investigated by immuno-western blot using a monoclonal antibody specific for PPV-SwC. Various levels of CP expression were detected in transgenic plants. R1 lines were challenged with the homologous virus and with isolates belonging to D, M and EA PPV groups. A variable degree of resistance was obtained, going from complete susceptibility to immunity. Susceptible plants showed a slight delay in symptoms expression when compared to non transformed control. No correlation was established by CP expression level and observed resistance.

Keywords: *Plum pox virus*; cherry; transgenic plants; induced resistance

INTRODUCTION

Plum pox virus (PPV), the causal agent of *Sharka* disease of stone fruits, belongs to *Potyvirus* genus, *Potyviridae* family. It is considered the most important pathogen in plum, apricot and peach. Cherry plants have been regarded as resistant to PPV (DOSBA *et al*. 1987). However, reports from Italy (CRESCENZI *et al*. 1994) and Moldova (KALASHYAN *et al*. 1994) have shown that sweet (*Prunus avium*) and sour cherry (*P. cerasus*) can be naturally infected by PPV.

PPV-SwC isolate recovered from *P. avium* is very different from conventional strains of PPV and it is closely related to the sour cherry isolate PPV-SoC (CRESCENZI *et al*. 1996, 1997; MYRTA *et al*. 2000).

The characterisation of different PPV isolates recovered in cherry plants in different countries (CRESCENZI *et al*. 1994; KALASHYAN *et al*. 1994; NEMCHINOV, HADIDI 1998; MAXIM *et al*. 2002) and their distribution suggest the adaptation of a natural PPV population to the host cherry, not influenced by particular ecological conditions and highlights the high incidence of virus presence.

A study performed on fifty different cultivars of *P. avium* and *P. cerasus* showed that the great part of tested cultivars is susceptible to PPV-SwC (CRESCENZI unpubl. data).

A construct for the induction of non conventional resistance has been obtained using PPV-D derived sequences, and has been used for producing resistant plum (RAVELONANDRO *et al*. 1997). It has been hypothesised that the induced resistance is based on post-transcriptional gene silencing (SCORZA *et al*. 2001).

The remarkable divergence of sequence existing between PPV infecting cherry (PPV-C) and PPV isolates belonging to D, M and EA groups gives rise to the need to have available a construct based on PPV-SwC sequences in order to confer cherry resistance against PPV-C.

The aim of the present work is to obtain such a construct useful for the transformation of *P. avium* and *P. cerasus*.

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MATERIALS AND METHODS

PPV strains used in this study were PPV-SwC (CRESCENZI *et al*. 1997), ISPAVE 31 (an Italian isolate of PPV-D), a Greek isolate of PPV-M and PPV-El Amar.

They were mantained in *Nicotiana benthamiana* and were used as inoculum sources. Infection and strain identity were confirmed by TAS-ELISA using monoclonal antibodies specific to M, D, EA and C groups (BOSCIA *et al*. 1998).

General cloning steps for construction of plant expression vector followed standard protocols (SAMBROOK *et al*. 1989). Plasmid pCR-PPV-21 containing the 3' terminal part of PPV-SwC RNA (3'-terminal region of the *NIb* gene, *CP* gene and part of 3'-NCR) was sequenced and used for sub-cloning the whole *CP* gene. Two primers were designed to amplify *CP* gene by PCR and to introduce the leader sequence of TMV CP mRNA having an ATG start codon in phase with the sequence of PPV-SwC *CP* gene at its 5' end: 5'-GGATCCGTTTTAAAT*ATGGCCAAGGAGGGAAATGATGACGACG*-3' and 5'-GGATCC*CTACACTCCCCTCACACCGAGGAGGT*-3'. PCR product was cloned in both directions into pRT103 vector (TOPFER *et al*. 1987).

Two recombinant plasmids were obtained, pRT-PPV-cp11, (–) sense, and pRT-PPV-cp38, (+) sense.

(+) and (–) sense sequences of *CP* gene from these plasmids were digested and introduced into pGA482 vector. The resulting binary plasmids, termed pGA-PPV-cp11, (–) sense, and pGA-PPV-cp38, (+) sense, were mobilised into *Agrobacterium tumefaciens* LBA 4404 strain by triparental mating.

Leaf discs of *N. benthamiana* were transformed, shoots regenerated and plants selected using standard techniques (HORSCH *et al*. 1985). Seeds of first generation plants were screened for Kanamycin resistance.

About 10 plants of each of the 30 R1 transgenic lines obtained for each construct were challenged with PPV-SwC (infected plant sap, purified virus and genomic viral RNA). Inoculated plants were observed daily for symptoms development. Leaf tissue from these plants was also used to back-inoculate non-transgenic *N. benthamiana*.

PCR amplification was performed on total DNA isolated from leaves of putative transformed *N. benthamiana* as described by DELLAPORTA *et al*. (1983), using primers specific for the 3' end of PPV CP (WETZEL *et al*. 1991) and the same primers used for preparing the constructs.

PAGE fractionated PCR products were denatured and electroblotted onto nylon Hybond-N membranes (*Amersham*) and hybridised with a digoxigenin-labeled RNA probe corresponding to PPV-SwC *CP* gene, produced using DIG RNA labelling and detection kits (*Roche*).

Soluble proteins were extracted from leaf tissue of transgenic plants, electrophoresed and electroblotted onto a nitrocellulose membrane as described by CRESCENZI *et al*. (1997). PPV-SwC *CP* was finally detected by MabAC (MYRTA *et al*. 2000).

RESULTS AND DISCUSSION

The (+) and (–) sense constructs of PPV-SwC *CP* gene contained the whole coding region with no flanking viral sequences, preceded by an ATG initiation codon and the leader sequence of TMV CP mRNA.

For each construct, about 60 transgenic lines of *N. benthamiana* plants were regenerated by *A. tumefaciens* co-cultivation of leaf discs. Transgenic plants were normal in appearance and grew and developed like non-transgenic plants.

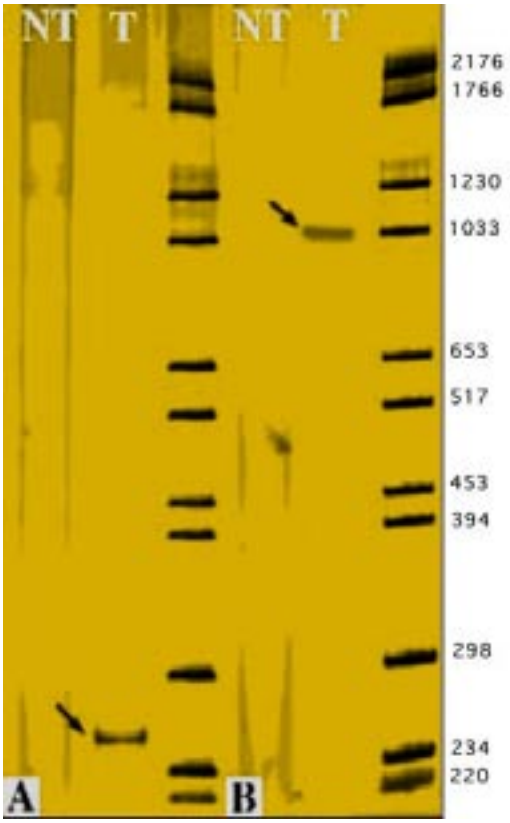

Figure 1. PAGE of PCR products. NT – not transformed; T – transformed. A – products obtained with primers specific for the 3' end of PPV *CP* gene. B – products obtained with primers specific for PPV-SwC *CP* gene

Figure 2. Different transformed lines of *N. benthamiana* plants 12 days post inoculation with PPV-SwC

Figure 3. Transformed lines of *N. benthamiana* plants 21 days post inoculation with PPV-SwC

Self-fertilised seedlings (R1 generation) population of 30 lines segregated with a ratio of 3:1 for kanamycin resistants: non-resistants, indicating a single insertion of the T-DNA.

PCR amplification with primers for a part of its 3' terminal region and for the whole PPV-SwC *CP* produced fragments of the expected size (Figure 1), confirming the identity of transgene as the *CP* gene. All transgenic lines selected on kanamycin-containing media were shown to bear the expected transgene.

All PCR amplification products hybridised with a PPV-SwC specific riboprobe (data not shown).

R1 transgenic plants tested by challenge inoculation with PPV-SwC showed 3 different behaviours: resistant (R), with no visible symptoms; intermediate (I), developing symptoms with a delay of 5–7 days; susceptible (S), showing a slight delay of symptom appearance (1–2 days) in comparison to non transformed plants (Figures 2 and 3). The developed symptoms did not depend on the source of inoculum.

No challenger virus was re-isolated from newly formed leaves of R plants, indicating the absence of systemic infection. On the contrary, all transgenic R, I and S plants became infected, like not transgenic ones, when they where inoculated with isolates belonging to PPV D, M and EA groups.

According to immuno-western blot analysis performed on transgenic plants transformed with pGA-PPV-cp38 construct, it was possible to establish that no correlation exists between the accumulation of CP expressed by transgene and observed resistance level. It was possible to observe CP accumulation both in immune and completely susceptible transgenic plants (Figure 4).

These results indicate that resistance induced by PPV-SwC *CP* gene is strain specific and depends on the sequence homology between the transgene and sequences of challenger virus. The high divergence existing between different PPV strains used in this study could explain the results obtained.

In this work we report the transformation of *N. benthamiana* with PPV-SwC coat protein gene in (+) and (–) sense direction via *A. tumefaciens*, and the resistance induced in these plants against homologous virus. Further efforts are needed for the final transformation of cherry plants.

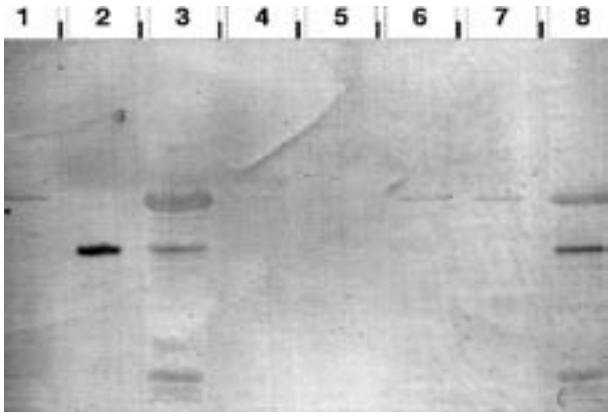

Figure 4. Immuno western blot of different lines of *N. benthamiana* plants transformed with pGA-PPV-cp38. It was performed with a monoclonal antiserum specific to PPV-SwC, MabAC. 1 – non transformed control; 2 – proteins extracted from purified virus; 3, 4 – resistant lines (R); 5, 6 – less resistant lines (I); 7, 8 – susceptible lines (S)

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