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PVY Resistant Transgenic Potato Plants (cv Claustar) Expressing the Viral Coat Protein

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Abstract

The coat protein mediated resistance to potato virus Y is assessed here in transgenic potato plants (*Solanum tuberosum* L., cv Claustar). Therefore, the corresponding cDNA from tunisian isolate of the virus was cloned into *Agrobacterium tumefaciens* binary vector. The transgenic lines were subsequently analysed for the presence and expression of the transgene. The *CP* cDNA copy number was determined for kanamycin resistant plants. Three selected transgenic lines and their S1 progeny resulting from tuber germination showed a high protection level against the virus. These data appear to support the hypothesis that the virus resistance is mediated by the translated viral coat protein expressed in transgenic potato lines.

Key words: Coat protein mediated resistance, Potato virus Y, *Solanum tuberosum* L., Transgenic plants

Introduction

Potato virus Y (PVY) is the type member of potyvirus group, the largest known one among plant viruses, and the economically most important group. This virus causes considerable yield losses in a number of economically important Solanaceous crops. The single stranded RNA genome of PVY encodes a polyprotein precursor with the CP at its C-terminus (review in Riechmann et al., 1992). Three viral proteases are responsible for autocatalytic cleavage of this polyprotein.

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The coat protein (CP) mediated resistance is now considered as a general method to achieve virus protection in plants. This strategy was applied for a great number of economically important plant viruses. Indeed, such pathogen mediated resistance of transgenic plants was described against a variety of virus groups (review in Beachy et al., 1990; Gadani et al., 1990). In the particular case of potyviruses, the expression of *CP* genes in transgenic plants was also reported (Stark and Beachy, 1989; Lawson et al., 1990; Kaniewski et al., 1990; Hassaïri et al., 1998). Different protection levels against the challenge virus were described. They varied from a reduced rate of systemic infection associated to a delayed appearance and low virus titres to a complete immunity of transgenic plants. The CP mediated approach was also investigated in transgenic potato lines (Farinelli et al., 1992; Lawson et al., 1990; Kaniewski et al., 1990). All these studies reported a decrease of infection rate or a complete resistance to PVY.

The mechanism underlying the resistance seems to differ with the virus involved (Beachy, 1997). It can be principally based on the presence of PVY CP RNA (Van der Vlugt et al., 1992; Masmoudi et al., 2002) or on the accumulation of viral coat protein (Powell et al., 1990). The RNA mediated resistance seems to be the consequence of RNA interference rather than the presence of translated coat protein itself. The so called post transcriptional gene silencing phenomena (Baulcombe, 1996) is due to the formation of double stranded RNA that direct the specific degradation of the target RNA in the plant cell. However, the translated CP-mediated resistance may be most likely due to an inhibition of uncoating early step in virus infection cycle.

We report here, the transformation of a commercial potato cultivar (Claustar) with the *CP* cDNA of a Tunisian isolate of PVY^{O}. Transgenic plants expressing the PVY-CP showed a high level of resistance to the challenge virus accumulation in the mechanically inoculated plants. This resistance was observed in lines containing 1-3 copies of the transgene whereas in previous results in our laboratory (Masmoudi et al., 2002) a high transgene copy number was required to ensure a complete resistance in transgenic tobacco plants expressing the untranslated form of the same *CP* gene.

Materials and methods

Virus and viral RNA purification

The Tunisian isolate of PVY^{O} was a gift from Mrs M. Mnari-Hattab (Tunisian National Institute for Agronomic Research, Tunis, INR AT). The virus was propagated in *Nicotiana tabacum* L., cv Xanthi. Viral particles and RNA were purified as described by Robaglia et al. (1989)

Construction of pBSCP320 and pPKYCP20 plasmids

Complementary DNA (cDNA) of the 3' end of PVY^{O} genomic RNA was synthesized using an oligo(dT) primer and the cDNA synthesis kit (Amersham) as recommended by the supplier. The *CP* double stranded cDNA was then synthesized by PCR using a specific primer derived from *CP* gene sequence of the French isolate (Robaglia et al., 1989). This CP primer used has the following sequence: 5' CGGATCCAGATCTGAACCATGGCAAATGAC3' and it leads to a construction containing a Kozak (1981) consensus sequence upstream of the ATG. The cDNA was cloned into the pBluescript SK+ (GIBCO-BRL, Life Science) treated by SmaI. After sequencing of the cDNA, a subcloning in the binary vector pKYLX35S2 (J. Albouy, INRA Versailles, France) was performed. Therefore, the pBSCP320 was treated by HindIII and SacI and the PVY *CP* cDNA was purified and ligated into the pKYLX35S2 binary vector treated by the same restriction enzymes. Under these conditions, the cDNA was inserted downstream of the enhanced Cauliflower mosaic virus (CaMV) 35S promoter and upstream of the 3' termination signal of the small subunit of the pea ribulose biphosphate-carboxylase synthetase gene (3'-RbcS). The neomycin phosphotransferase (*nptII*) gene driven by the nopaline synthetase promoter (*pnos*) is also inserted in the binary vector. The resulting recombinant plasmid was introduced into *Agrobacterium tumefaciens* C58 strain containing the disarmed Ti plasmid (pGV2260) harbouring the vir region (Deblaere et al., 1985). *Agrobacterium* transformation was performed by electroporation (Mersereau et al., 1990).

Plant transformation

The Claustar cultivar of potato plant (*Solanum tuberosum* L.) was used in leaf disc transformation. These plants were cultivated *in vitro* in a solid MS basal medium (Murashige and Skoog, 1962). Plants were *in vitro* cultured in a growth chamber at 21℃ with a day length of 16 h under 250 $\mu Em^{-2} S^{-1}$ light intensity. Agrobacterium tumefaciens recombinant strain was grown at 30℃ overnight in YEP medium supplemented with rifampicin (100 mg/L), streptomycin (100 mg/L) and kanamycin (50 mg/L). Bacteria were then collected by centrifugation at 4000 rpm for 5 min. The pellet was resuspended in 20 ml MS medium and transferred in a Petri dish containing the leaf discs. The mixture was incubated at room temperature for 20 min. Leaf discs were then transferred in a new MS medium and incubated for 3 days at 21℃.

Plant tissues were then transferred into regeneration medium (MS medium supplemented with ANA 0.2 mg/L, Zeatin 2 mg/L and GA_3 0.02 mg/L) supplemented with cefotaxime 250 mg/L and kanamycin 50 mg/L and incubated at 21℃ in the growth chamber. Leaf discs were subcultured every 15 days. The first seedlings were observed on calli 4 to 5 weeks later.

DNA analysis

DNA isolation was performed as described by Dellaporta et al., (1983). The purified DNA (20 µg) was digested with restriction enzymes as recommended by Sambrook et al., (1989). The DNA fragments were separated overnight on a 0.8% agarose gel and transferred to Hybond N+ filters (Amersham). The probe corresponding to the purified *CP* cDNA was randomly ^{32}P- labelled using rediprime kit (Amersham). DNA hybridization was performed overnight at 65℃ as described by Sambrook et al. (1989). The filters were exposed for autoradiography at -70℃ for few hours to few days.

Protein analysis

Proteins were extracted from fresh leaves of transgenic and control plants (1g) in a phosphate-buffered saline (PBS) solution (5ml) containing 137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4 and 2 mM PMSF (phenylmethylsulphonyl fluoride). For ELISA test microtiter wells were coated with crude protein extracts (20 µg), then

100 µl of anti- PVY CP specific monoclonal antibody (Rouis et al., 2001) were loaded and finally 100 µl of peroxidase conjugated anti-mouse antibodies were added. After each step, plates were washed three times with PBS containing 0.05% Tween 20. The absorbance at 490nm was measured 0.5 to 1h after addition of the 3,3' diaminobenzidine tetra-hydrochloride (DAB) substrate.

Greenhouse planting and inoculation of transgenic plants with PVY

The transgenic lines (T1 generation), the untransformed plants cultivated in vitro for 2 to 3 weeks and the S1 generation from tuber germination were transferred in a greenhouse. Fifteen plants from each line were tested under these conditions. Three weeks after transplantation, ten plants from transgenic and control lines were inoculated with 1:10 dilution of extract from tobacco leaf infected with PVY. Inoculation was performed by dusting of two lower leaves using carborundum. A second inoculation was performed one week later. Plants were tested for the presence of the virus every week by ELISA on 20 µg of crude protein extract, using the anti-PVY CP monoclonal antibody.

Results

Transformation, regeneration and selection of plants

The PVY coat protein cDNA was subcloned in a binary vector, PKYLX35S2-P5CS, downstream of the enhanced CaMV35S constitutive promoter (Figure 1). This plasmid was then transferred in *Agrobacterium tumefaciens* C58 (pGV2260) line for transformation of potato tissues. The transformed potato plants were selected on kanamycin containing MS medium. Transformation efficiency was of about 50%. Three callus derived plant lines were stably established.

In order to detect the PVY *CP* cDNA in the regenerated kanamycin resistant plants, Southern blot hybridization of genomic DNA digested by BglII was performed using the *CP* cDNA as probe (Figure 2). The hybridization profile obtained showed that the $Cl20_5$ and $Cl20_{11}$ lines possess two copies of the transgene each; whereas the $Cl20_9$ one seems to have only one copy. However, the intensity of the upper band for $Cl20_5$ may be the result of two comigrating fragment containing the *CP* transgene.

The expression of the *CP* transgene in the selected transformed lines was investigated by ELISA test using a monoclonal antibody directed against the viral CP (Figure 3). These data suggest that the expression level of transgene is different depending on the transgenic line.

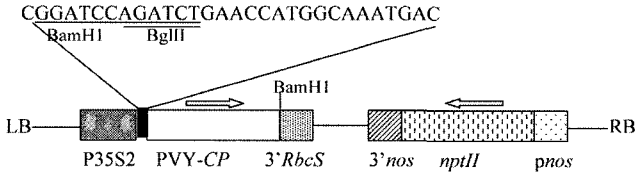


Figure 1. Structure of the plasmid pKYLX35S2-CPVY containing the PVY *CP*-cDNA used in transformation of potato lines. RB: right border, left border; *nptII*: neomycin phosphotransferase II; p35S: cauliflower mosaic virus 35S RNA promoter; p*nos*: nopaline synthase gene promoter; 3' nos: the 3' terminator of nopaline synthase gene. The Kozak consensus sequence is mentioned upstream of the added ATG. Restriction enzyme sites (BamHI and BglII) were underlined.

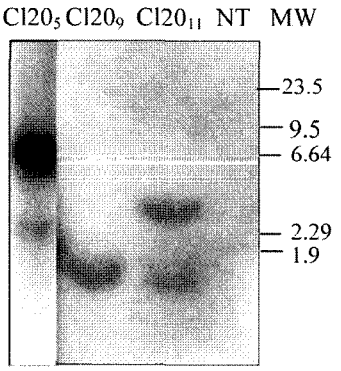


Figure 2. Southern blot analysis of BglII digested total DNA isolated from kanamycin resistant potato lines ($Cl20_5$, $Cl20_9$ and $Cl20_{11}$) and a non transformed plant (NT), probed with α^{32}P-labelled *CP* cDNA sequence. MW: λ-DNA marker digested with HindIII

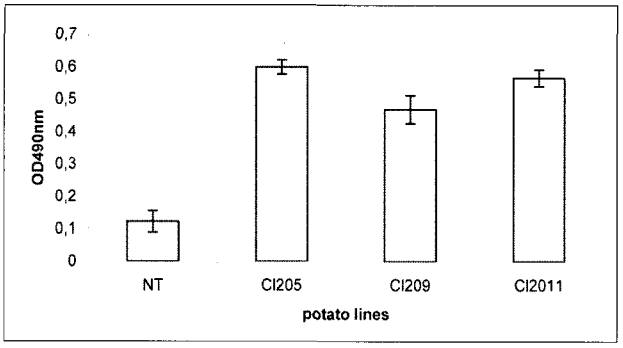


Figure 3. Detection of PVY CP protein in transgenic lines (Cl205, Cl209 and Cl2011) and non transferred plant (NT) by ELISA test using a specific monoclonal antibody diluted to 1/2000.

Investigation of virus resistance

Fifteen plants from each selected transgenic line ($CI20_5$, $CI20_9$ and $CI20_{11}$) of the T1 generation were transferred in the greenhouse for investigation of their resistance to PVY infection. Mechanical inoculation with PVY was performed on ten plants and the others were used as controls. Untransformed plants were also treated under the same conditions and were used as negative control. Virus infection and propagation were measured weekly by ELISA test (Figure 4) using the monoclonal antibody directed against the PVY CP. The data obtained showed that all transgenic lines tested were able to overcome virus infection. Indeed, no symptoms were observed on leaves, while the control untransformed plants were dramatically altered. The latter plants showed severe stunting, a discoloration and a deformation of the leaves in addition to an important reduction in plant growth. Virus accumulation measured by ELISA test was important in control untransformed plants. However, no virus content increase was observed in inoculated transgenic lines. The initial OD in transgenic lines resulting from the expressed PVY CP was subtracted from values presented in Figure 4

In order to confirm these results, the protection assays were performed on tuber grown plants from transgenic lines. The integration of *CP*-cDNA was performed by Southern blot analysis of genomic DNA treated by BamH1 (Figure 5a) while the expression was confirmed by ELISA (Figure 5b). The resulting plants were inoculated mechanically by the PVY^O strain as described previously. Infection symptoms and virus accumulation were also measured (Figure 6). The resulting data, confirmed the complete resistance of the transgenic lines expressing the translated PVY CP to the challenge virus infection.

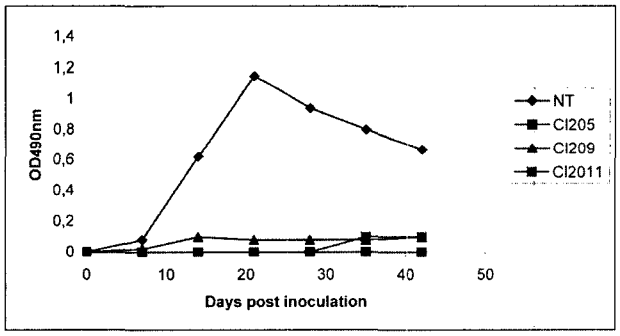
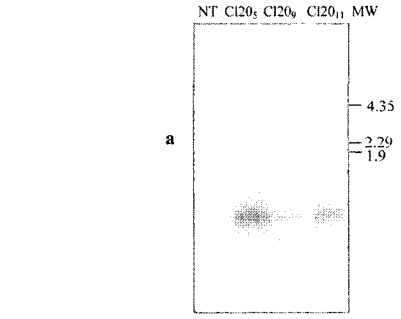


Figure 4. Accumulation of PVY in potato plants ($CI20_5$, $CI20_9$ and $CI20_{11}$) and non transferred plant (NT) inoculated with PVY^O (1/10 dilution of inoculum from tobacco fresh leaves infected) at various days post-inoculation. The accumulation of the virus was detected by ELISA and expressed as the OD_{490}nm values. Initial OD of transgenic plants was subtracted from indicated values.



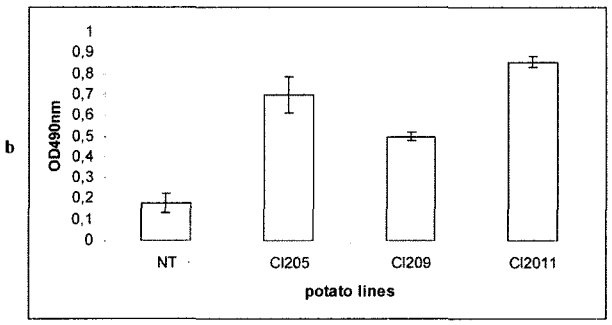


Figure 5. (a) Southern blot analysis of BamH1 digested total DNA isolated from S1 tuber grown potato plants ($CI20_5$, $CI20_9$ and $CI20_{11}$) and a non transformed plant (NT), probed with α ^{32}P-labelled *CP* cDNA sequence. MW: λ-DNA marker digested with *HindIII* (b) Detection of PVY CP protein in these S1 lines by ELISA test using a specific monoclonal antibody.

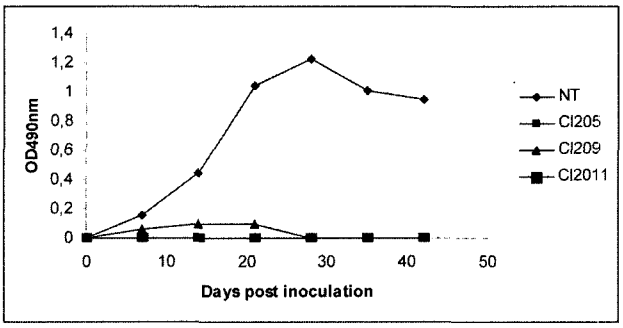


Figure 6. Accumulation of PVY in S1 tuber grown potato plants ($CI20_5$, $CI20_9$ and $CI20_{11}$) and non transferred plant (NT) inoculated with PVY^O (1/10 dilution of inoculum from tobacco fresh leaves infected) at various days post-inoculation. The accumulation of the virus was detected by ELISA and expressed as the OD_{490}nm values. Initial OD of selected transgenic plants was subtracted from indicated values.

Discussion

In this paper, we describe the data confirming that the coat protein mediated resistance is an efficient strategy

against PVY propagation in transgenic potato plants of the commercial variety Claustar. Indeed, immunity against PVY^0 was observed in all the transgenic plants expressing the translated PVY CP. This high PVY resistance is also transmitted to the S1 tuber germination derived progeny.

In previous reports (Lawson et al., 1990 and Kaniewski et al., 1990), three transgenic potato plants (cv. Bintje) containing the genes encoding the CP of PVX and PVY^0 and expressing detectable levels of transgenic PVY CP were described. These data showed that the complete resistance against the challenge virus was observed for only one line whereas the two other were partially resistant.

On the other hand, Malnoë et al. (1994) described two transgenic potato plants (cv. Russet Burbank) expressing the PVY^N CP and containing 2-3 copies of the *CP* gene. They showed that one line was resistant to the virus while the other was sensitive and in both lines it was impossible to detect the transgenic CP. It seems that the efficiency of CP mediated resistance is dependant on potato plant cultivar.

PVY immunity was also described in transgenic potato plants expressing a heterologous potyviral CP (Hassairi et al., 1998). Our results as all those described previously suggest that resistance may be due to inhibition or interference with the early virion uncoating step (Register and Beachy, 1988). They are also in agreement with data described by Ferreira et al. (2002) on Papaya ringspot virus resistant papaya transgenic plants. However, pathogen derived resistance was frequently explained by a post transcriptional gene silencing based on specific degradation of invading RNA (Wassenegger, 2002). In a previous work obtained in our laboratory (Masmoudi et al., 2002), transgenic tobacco plants expressing the untranslatable PVY *CP* gene from the same virus isolate were produced. They showed that resistance against the challenge virus was directly related to the transgene copy number. Indeed, plants possessing more than four transgene copies were resistant whereas those possessing one or two gene copies were susceptible to the virus. These results support our hypothesis that the translated CP is responsible for PVY resistance. Indeed, the transgenic potato plants described here, have inserted one to three copies of the transgene leading to detectable levels of the corresponding CP and a strong resistance against the virus. In a recent study, Missiou et al. (2004) showed that a protection of transgenic potato plants can be observed by expression of double stranded RNA corresponding to the 3' terminal part of the PVY *CP* gene.

All these data confirmed that the resistance in CP transgenic plants can be divided into two categories: protein-mediated and RNA-mediated mechanisms (Tepfer 2002). This is most probably due to the diversity of roles of the CP in the plant-virus interaction (Callaway et al., 2001).

Acknowledgments

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