

BEHAVIOUR OF TRANSGENIC *PLUM POX VIRUS*-RESISTANT *PRUNUS DOMESTICA* L. CLONE C5 GROWN IN THE OPEN FIELD UNDER A HIGH AND PERMANENT INFECTION PRESSURE OF THE PPV-REC STRAIN

J. Polák¹, J. Pívalová¹, J.K. Kundu¹, M. Jokeš¹, R. Scorza² and M. Ravelonandro³

¹ Crop Research Institute, Division of Plant Health, Prague, Czech Republic

² USDA-ARS Appalachian Fruit Research Station, Kearneysville, USA

³ INRA, UMR Génomique Diversité Pouvoir Pathogène 1090, 33883 Villenave d'Ornon, France

SUMMARY

Transgenic plum (*Prunus domestica* L.) clone C5 was grafted on virus-free St. Julien rootstocks and inoculated by bud grafting with the recombinant strain (PPV-Rec) of the *Plum pox virus* (PPV). Non inoculated C5 plums were used as controls. Transgenic plums infected with PPV-Rec and control plants were evaluated for six years in the open field. PPV symptoms, mild diffuse spots and rings, appeared on the basal leaves the second year after inoculation. The presence of PPV was confirmed by DAS-ELISA, ISEM, and RT-PCR. Severe PPV symptoms appeared in the leaves of shoots grown from non-transgenic inoculum buds. A reduction of symptoms and a decrease of the relative PPV concentration, as determined by DAS-ELISA in transgenic C5 plants, were observed from the third to the fifth year after virus inoculation. Very mild symptoms in older leaves of the basal branches of trees subsided by the end of the vegetative period, and were difficult to observe. The level of PPV detection by DAS-ELISA dropped from June to August together with a reduction of symptoms. PPV was undetectable by DAS-ELISA in more than half of the trees by the end of August. PPV-Rec was detected by RT-PCR in the basal and middle parts of the C5 scions. The upper leaves did not contain a detectable level of the virus. These results show the high level and stability of PPV resistance in graft-inoculated C5 trees.

Key words: Transgenic resistance, *P. domestica*, resistance, PPV-Rec.

INTRODUCTION

Several clones of plum (*Prunus domestica* L.) transformed with the *Plum pox virus* (PPV) coat protein (CP) gene were obtained by Scorza *et al.* (1994). One clone named C5 proved to be highly resistant to PPV under

glasshouse conditions (Ravelonandro *et al.*, 1997). Malinowski *et al.* (1998) showed that trees of clone C5 are not immune to PPV-D, but are highly resistant under field conditions. The resistance to PPV based on gene silencing (Scorza *et al.*, 2001) is stable under field conditions (Hily *et al.*, 2004). In field tests conducted in Poland and Spain (Malinowski *et al.*, 1998; 2006) and in Romania (Ravelonandro *et al.*, 2002), with PPV susceptible control trees providing a ready source of inoculum, no C5 trees were found to be infected by aphids over a seven-year period. Bud-graft inoculated C5 trees showed mild and transient infection close to the inoculum. A high and permanent infection pressure of PPV was provided by bud grafting of inoculum in the field trial of clone C5 conducted in the Czech Republic in 2002, in which PPV-infected and healthy control trees were used. Moreover, trees with combined inoculations by PPV, ACLSV and PDV were also used in the trial. Preliminary results of two-year observation were presented at the European Meeting '04 on plum pox in Rogów-Skierniewice, Poland, and published (Polák *et al.*, 2005). Further results of the behaviour of transgenic plants of clone C5 infected with PPV-Rec and of healthy controls are presented in this contribution.

MATERIALS AND METHODS

Transgenic plums, virus inoculation, field trial. Buds of plum *P. domestica* clone C5 transformed with the CP gene of PPV (Scorza *et al.*, 1994) were grafted onto virus-free St. Julien rootstocks in March 2002. Eleven transgenic plum trees were inoculated in August 2002 by grafting directly onto the transgenic C5 scion peach GF-305 buds infected with PPV (Polák *et al.*, 2005). The isolate used for inoculation was originally classified as strain PPV-M. Later it was re-classified by P. Komínek as PPV-Rec (see Glasa *et al.* 2004) and was detected by RT-PCR as described by Šubr *et al.* (2004). Transgenic plum trees inoculated with PPV-Rec and eleven non-inoculated control trees were transferred to an open field and evaluated for five years in the period 2003-2007. PPV-Rec infected buds (IB) were allowed to grow throughout the period of evaluation (2003-2007).

Shoots from IB were shortened to about 40 cm in length by pruning every spring. Transgenic trees were grown five years under a high and permanent infection pressure of PPV-Rec. The field trial, approved by Ministry of Environment of the Czech Republic (GM planting No. 881/OER/GMO/01) was established in isolation, being surrounded by grass and cereals in a diameter of one kilometer.

Visual, serological, electron microscopic, and RT-PCR evaluation. Symptoms caused by PPV-Rec in the leaves of transgenic plum trees were evaluated monthly

every year during the vegetative period. DAS-ELISA and ISEM detection of PPV using polyclonal antisera (Clark and Adams, 1977) were done every year (2003-2007) in June. A second DAS-ELISA assay was made at the end of August. Commercial ELISA kits (Bioreba, Switzerland) were used for all tests.

The relative concentration of PPV-Rec was determined by semiquantitative DAS-ELISA in samples prepared from symptomatic leaves in June 2005 and 2007. The relative concentration of PPV protein was established by determining the lowest dilution of leaf sample with positive reaction in DAS-ELISA (Albrechtová *et*

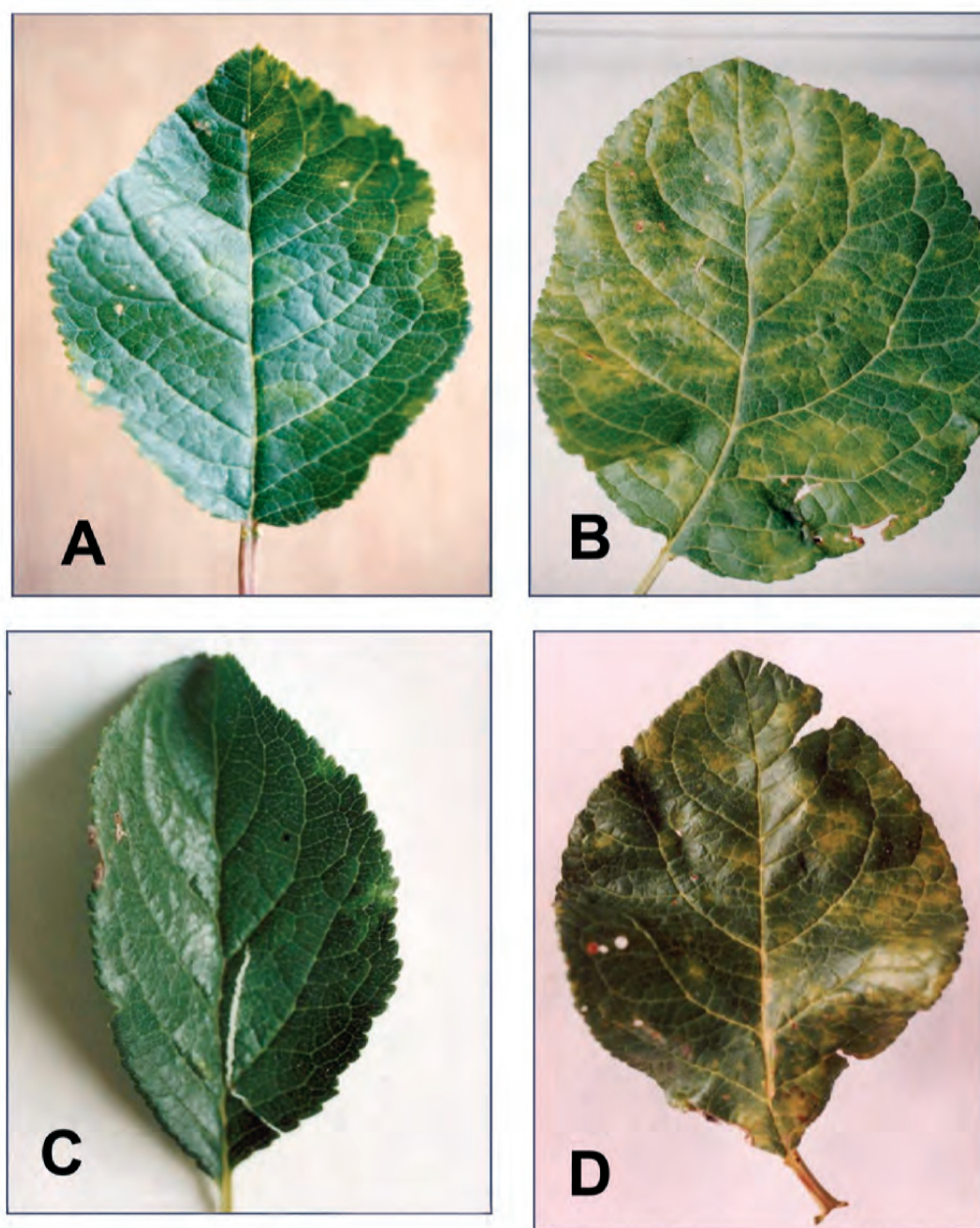


Fig. 1. A. Mild diffuse spots in a leaf of C5 inoculated with PPV-Rec (second year after inoculation, June 2004). B. Severe yellow spots and rings in a leaf emerged from a non-transgenic bud (IB) infected with PPV-Rec (second year after inoculation, June 2004). C. Very mild diffuse spots in a leaf of C5 inoculated with PPV-Rec (fifth year after inoculation, July 2007). D. Severe yellow spots and rings in a leaf g emerged from a non-transgenic bud (IB) infected with PPV-Rec (fifth year after inoculation, July 2007).



Fig. 2. C5 transgenic plum trees infected with PPV-Rec (A) and healthy controls (B).

al., 1986). The PPV titre in a sample of sap was expressed as the reciprocal value of the sample dilution. Leaf samples were extracted in a 1:10 ratio of plant material to phosphate-buffered saline, pH 7.2 with 0.05% Tween 20, 0.2% polyvinyl pyrrolidone and 0.2% egg albumine. Further dilutions of samples 1:2, 1:4, 1:8... were in phosphate-buffered saline. Samples extracted from IB leaves were used as a second positive control.

PPV immunoglobulins diluted 1:2000 were used for immunosorbent electron microscopy. Decorated PPV particles present in homogenates obtained from leaves of transgenic C5 plum trees were observed in a Philips 208 S electron microscope.

PPV-Rec was detected by RT-PCR using primer pair mD5/mM3 as described by Šubr *et al.* (2004). One hundred mg of ground C5 leaf tissues were used for total RNA extracted by RNAeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's procedure.

RESULTS AND DISCUSSION

No PPV symptoms appeared in the leaves of C5 transgenic plums in the first year after inoculation with PPV-Rec by chip budding. PPV symptoms appeared only in the leaves that emerged from IB. Mild diffuse spots (Fig. 1A) or rings appeared in the second year after inoculation in some leaves of all C5 trees inoculated

with PPV-Rec. Symptoms developed in June 2004 and virus presence was confirmed by DAS-ELISA and ISEM in inoculated trees in the middle of June. Very severe PPV symptoms appeared in all leaves grown from untransformed control buds infected with PPV-Rec (Fig. 1B).

Mild PPV symptoms appeared in some leaves of C5 in 2005-2007. A reduction of symptoms in C5 plants was observed from the third to the fifth year after virus inoculation. PPV symptoms were observed in the basal leaves and symptoms were milder in each year. Very mild PPV symptoms appeared only in a few (two to seven) basal C5 leaves in 2007. One to three mild diffuse spots per leaf (Fig. 1C) developed on the first to fourth oldest leaves on a branch. PPV presence in symptomatic leaves of C5 was confirmed by DAS-ELISA and ISEM. Only very mild PPV symptoms were observed by the end of May 2007 and a further reduction of symptoms was observed during June and July. Severe PPV symptoms were observed in the leaves emerged from untransformed control buds infected with PPV-Rec in the course of 2004-2007 by the end of August (Fig. 1D).

The relative concentration of PPV-Rec in symptomatic C5 leaves determined by DAS-ELISA fluctuated from 1.56×10^{-2} to 9.76×10^{-4} in 2005, and from 5.0×10^{-1} to 7.81×10^{-3} in 2007 in individual trees. The relative concentration PPV determined by semiquantitative DAS-ELISA dropped ten to thirty times over a three-

year period in accord with the reduction of symptoms. PPV was not detected by DAS-ELISA in more than half of the trees by the end of August 2007. No symptoms appeared in the leaves of non-inoculated control trees of C5 throughout the experiment. PPV-Rec was not detected in control trees by DAS-ELISA, ISEM, and RT-PCR. The growth of C5 control trees (Fig. 2B) was more vigorous in comparison with trees inoculated with PPV-Rec (Fig. 2A). The results of interactions of PPV-Rec with PDV and ACLSV in C5 plants were presented at the 5th International Conference on Bioresources and Viruses in Kyiv, Ukraine, 2007 and are now in press (Polák *et al.*, 2008).

In summary, the results of the present trial have confirmed both the high level and stability of PPV resistance in graft-inoculated C5 trees and have shown that transgenic plants have a high level of resistance to PPV-Rec.

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