Interactions of *Plum Pox Virus* Strain Rec with *Apple Chlorotic Leafspot Virus* and *Prune Dwarf Virus* in Field-Grown Transgenic Plum *Prunus domestica* L., Clone C5

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Abstract

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Transgenic plums, *Prunus domestica* L. clone C5, were inoculated by bud grafting with *Plum pox virus* (PPV-Rec, recombinant strain originated from plum), PPV-Rec + *Apple chlorotic leafspot virus* (ACLSV), PPV-Rec + *Prune dwarf virus* (PDV), and PPV-Rec + ACLSV + PDV. Non-inoculated transgenic plums served as controls. Plants were grown in an open field for 5 years. They were evaluated by visible symptoms, by DAS-ELISA and RT-PCR. Mild PPV symptoms, diffuse spots or rings appeared two years after inoculation in some leaves of plants artificially inoculated with PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, and PPV-Rec + ACLSV + PDV. Severe PPV symptoms appeared in leaves of shoots growing from infected buds used for inoculation. During the following three years, further weakening of PPV symptoms was observed in transgenic plants. In 2007, very mild PPV symptoms were found in only a few leaves, and over 60%, resp. 70% of the C5 trees showed no PPV symptoms. The presence of PPV was confirmed by ELISA, ISEM and RT-PCR. No difference in PPV symptoms was observed between PPV-Rec and combinations PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV. No symptoms of ACLSV appeared in combinations of ACLSV with PPV-Rec and PPV-Rec + PDV during 2004–2007, but the presence of ACLSV in leaves of transgenic plants clone C5 was proved by ELISA and RT-PCR. Neither synergistic nor antagonistic effects of ACLSV on PPV-Rec were observed. No symptoms of PDV appeared in combinations of viruses with PDV during 2004–2007. PDV was not detected by ELISA, and the presence of PDV was uncertain by RT-PCR in most of inoculated trees in 2006 and 2007. The results of RT-PCR will be further confirmed by sequence analysis and discussed. These results suggest a possible antagonistic interaction between PPV-Rec and PDV in plum clone C5.

Keywords: transgenic plum; resistance; sharka; interactions; PPV; PDV; ACLSV

Transgenic clone C5 of plum, *Prunus domestica* L., resistant to PPV (Ravelonandro *et al*. 1997; Scorza *et al.* 2001), was used for a study of mixed infection in the field. *Plum pox virus* (PPV) and two other common pathogens infecting stone-fruit trees, *Trichovirus – Apple chlorotic leafspot*

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virus (ACLSV) and *Ilarvirus – Prune dwarf virus* (PDV), were used to inoculate the PPV-resistant plum trees, clone C5, by bud grafting in 2002. The PPV-M strain originally used for inoculation in 2002 (Polák *et al.* 2005) was requalified in 2006 as PPV recombinant strain (PPV-Rec) after discovering natural recombinant isolates of PPV (Glasa *et al.* 2004). The four inocula were: PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV and PPV-Rec + ACLSV + PDV.

The aim of research with *P. domestica* clone C5 in the Czech Republic is to evaluate the interactions of the *Plum pox virus* recombinant strain and its combinations with ACLSV and PDV with transgenic plants of clone C5 grown in the field. Results of five years (2003–2007) of this field trial are presented.

MATERIALS AND METHODS

Transgenic plum trees, inoculation of viruses, field trial. Budwood of plum *P. domestica* clone C5 transformed with the coat protein gene of PPV (Scorza *et al*. 1994) were used. C5 buds were grafted onto virus-free rootstocks of St. Julien in 2002, and 55 trees of *P. domestica* clone C5/St. Julien were obtained. The procedure of inoculation of trees by bud grafting with PPV-Rec and with combinations of PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV and establishing a plantation were described in published preliminary results (Polák *et al*. 2005). The transgenic plum trees were evaluated during the years 2003–2007 (Figure 1).

Symptom evaluation, serological and electron microscopical detection of viruses. Plants inoculated with individual combinations (PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV, and the control = non-inoculated trees) were monitored monthly by symptom evaluation during the vegetation period of every year. ELISA and ISEM testing were performed every year in June. Polyclonal antibodies raised against PPV, ACLSV and PDV (Bioreba, Switzerland) were used in DAS-ELISA (Clark & Adams 1977). Leaf samples were extracted in phosphate-buffered saline. PPV immunoglobulins in the dilution 1:2000 were used for immunosorbent electron microscopy of PPV. Decorated PPV particles present in homogenates obtained from leaves of transgenic C5 plum trees were observed in an electron microscope Philips 208 S.

Figure 1. Orchard of transgenic plums, *Prunus domestica*, clone C5, in summer 2007

Detection by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). 100 mg of ground leaf tissues of C5 were used for total RNA extraction by using RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the procedure recommended by the manufacturer. PPV-Rec was detected by RT-PCR using primer pair mD5/mM3 as described by Šubr *et al*. (2004). PDV and ACLSV were also detected by RT-PCR. For PDV the primer pair PDVcpR/PDVcpF, amplifying partial coat protein (CP) gene (Ravelonandro *et al.* 2006) was used. For ACLSV detection a primer pair ACLSVutrR/ACFrIII, at 3-terminal of the ACLSV genome, spanning CP and UTR (un-translated region) gene (Ravelonandro *et al.* 2006) was used.

RESULTS AND DISCUSSION

PPV symptoms, mild diffuse spots (Figure 2) and rings, appeared two years after inoculation in leaves of C5 plants inoculated with PPV-Rec and in those inoculated with virus combinations PPV-Rec + ACLSV, PPV-Rec + PDV, and PPV-Rec + ACLSV + PDV. Symptoms of PPV were evident in June 2004, and virus presence was confirmed by ELISA and ISEM in most inoculated trees. PPV intact particles were found in leaves with PPV symptoms also in the years 2005–2007. Yet the PPV symptoms in the C5 parts of trees were much milder than in the parts from infected buds (IB).

No symptoms of PDV appeared during the vegetation periods of 2003–2007. PDV was not detected in leaves of C5 trees inoculated with PPV-Rec + PDV and PPV-Rec + PDV + ACLSV by ELISA in these years. PDV was detected by ELISA only in leaves

Figure 2. Diffuse spots, symptoms of PPV in leaves of *Prunus domestica*, clone C5, infected with PPV-Rec

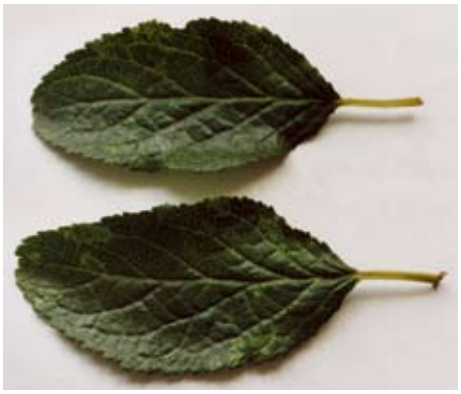

Figure 3. Very mild diffuse spots in leaves of the lower parts of transgenic plum, clone C5, inoculated with PPV-Rec + ACLSV

growing from the IB part. The presence of PDV was uncertain by RT-PCR in most of the inoculated C5 trees in 2006 and 2007 because a non-specific PCR fragment, of approximately 200 bp, was always amplified by RT-PCR rather than the expected 688 bp. These results suggest a possible antagonistic interaction between PPV-Rec and PDV in C5 trees.

No symptoms of ACLSV appeared in C5 trees and the IB portions of trees inoculated with PPV-Rec + ACLSV and PPV-Rec + ACLSV + PDV. However, ACLSV was detected by ELISA and RT-PCR in leaves of all C5 trees inoculated with PPV-Rec + ACLSV and PPV-Rec + ACLSV + PDV. Neither synergistic nor antagonistic effects of ACLSV on PPV-Rec were observed. The summarised results of ELISA are presented in Table 1.

The PPV symptoms, mild diffuse spots and rings, which appeared two years after inoculation in leaves of C5 plants inoculated with PPV-Rec and with the three combinations of viruses, were of the same intensity; no differences were observed between the combinations PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV and PPV-Rec + ACLSV + PDV. The PPV symptoms got milder with every year. In 2007, very mild PPV symptoms (Figure 3) appeared in only a few (two to seven) leaves on the lower parts of C5 trees, while over 60% of the C5 trees had no PPV symptoms. The PPV symptoms that had been visible by the end of May 2007, had almost disappeared in July, when over 70% of the C5 trees showed no symptoms. The silencing of PPV symptoms is presented in Table 2. The effect of gene silencing in C5 trees was obvious even under the high and permanent infection pressure by PPV-Rec. Resistance to PPV is maintained in the presence of *trichovirus* and *ilarvirus* infection. This is in agreement with results obtained by Ra-

Figure 4. Very severe symptoms in leaves of non-transgenic part of tree derived from inoculum buds infected with PPV-Rec + PDV + ACLSV (in 2003)

Figure 5. Very severe symptoms in leaves of non-transgenic part of tree derived from inoculum buds infected with PPV-Rec + PDV + ACLSV (in 2007)

Table 1. Interactions of PPV-Rec with PDV and ACLSV in field grown plants of transgenic plum, clone C5 – summarised results of ELISA

	Virus combination				Control trees
	PPV-Rec	PPV-Rec + ACLSV	PPV-Rec + PDV	PPV-Rec + ACLSV + PDV	
Number of inoculated trees	9 (11)$^{+}$	11	10 (11)$^{++}$	11	11
PPV positive	9	11	10	11	0
ACLSV positive	nt	8	nt	10	0
PDV positive	nt	nt	0	0	0

$^{+}$2 trees died, $^{++}$1 tree died, nt = not tested

Table 2. Silencing of PPV symptoms

Trees with and without PPV symptoms	Virus combination				Control trees
	PPV-Rec	PPV-Rec + ACLSV	PPV-Rec + PDV	PPV-Rec + ACLSV + PDV	
Mild	9	11	10	11	0
No symptoms (June 2004)	0	0	0	0	11
Very mild	4	3	3	3	0
No symptoms (May 2007)	5	8	7	8	11
Very mild	1	3	2	3	0
No symptoms (July 2007)	8	8	8	8	11

velonandro *et al.* (2007). No atypical symptoms were observed in combinations of PPV-Rec with PDV and ACLSV, and thus as a result of mixed infections.

The very severe or severe PPV symptoms which appeared first in 2003 in IB leaves growing from buds infected with PPV-Rec (Figure 4) appeared again in the following years 2004–2007 (Figure 5). No differences in the intensity of PPV symptoms among different virus combinations were observed in the years 2005–2007. Virus symptoms did not appear in leaves of 11 non-inoculated control trees of C5 in the years 2003–2007. Similarly, PPV, ACLSV and PDV were not detected by ELISA in the control trees during these years. Growth of the non-inoculated control trees was stronger in comparison with trees inoculated with PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV. There were no differences in growth between groups of trees inoculated with these four inocula.

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References

Clark M.F., Adams A.N. (1977): Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. Journal of General Virology, **34**: 51–57.

Glasa M., Palkovics L., Komínek P., Labonne G., Pittnerová S., Kúdela O., Candresse T., Šubr Z. (2004): Geographically and temporally distant natural recombinant isolates of *Plum pox virus* (PPV) are genetically very similar and form a unique PPV subgroup. Journal of General Virology, **85**: 2671–2681.

Polák J., Pívalová J., Jokeš M., Svoboda J., Scorza R., Ravelonandro M. (2005): Preliminary results of interactions of *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), and *Apple chlorotic leafspot virus* (ACLSV) with transgenic plants of plum *Prunus domestica*,

clone C-5 grown in an open field. Phytopathologia Polonica, **36**: 115–122.

Ravelonandro M., Scorza R., Bachelier J.C., Labonne G., Levy L., Damsteegt V., Callahan A., Dunez J. (1997): Transgenic *Prunus domestica* resistant to plum pox virus infection. Plant Disease, **81**: 1231–1235.

Ravelonandro M., Briard P., Kundu J., Hily J.M., Monsion M., Scorza R. (2006): Silencing in *Prunus*: A natural defence developed by woody fruit-trees in response to virus infection. In: Book of Abstracts XXth International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops – XIth International Symposium on Small Fruit Virus Diseases, May 22–26, 2006, Antalya: 22.

Ravelonandro M., Kundu J., Briard P., Monsion M., Scorza R. (2007): The effect of co-existing *Prunus* viruses on transgenic *Plum pox virus* resistant plums. Acta Horticulturae, **738**: 653–656.

Scorza R., Ravelonandro M., Callahan A.M., Cordts J.M., Fuchs M., Dunez J., Gonsalves D. (1994): Transgenic plums *(Prunus domestica* L.) express the *Plum pox virus* coat protein gene. Plant Cell Reports, **14**: 18–22.

Scorza R., Callahan A.M., Levy L., Damsteegt V., Ravelonandro M. (2001): Resistance to *Plum pox potyvirus* in a transgenic woody perennial fruit tree, European plum (*Prunus domestica* L.) result from post-transcriptional gene silencing. Acta Horticulturae, **550**: 425–430.

Šubr Z., Pittnerová S., Glasa M. (2004): A simplified RT-PCR-based detection of recombinant Plum pox virus isolates. Acta Virologica, **48**: 173–176.

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