PLUM POX VIRUS SILENCING OF C5 TRANSGENIC PLUMS IS STABLE UNDER CHALLENGE INOCULATION WITH HETEROLOGOUS VIRUSES

I. Zagrai[1], N. Capote[2], M. Ravelonandro[3], M. Cambra[2], L. Zagrai[1] and R. Scorza[4]

[1] *Statiunea de Cercetare-Dezvoltare pentru Pomicultura Bistrita, 3, Drumul Dumitrei Nou Street, 420127 Bistrita, Romania*
[2] *Instituto Valenciano de Investigaciones Agrarias, 46113 Moncada, Valencia, Spain*
[3] *INRA, UMR Génomique Diversité Pouvoir Pathogène 1090, 33883 Villenave d'Ornon, France*
[4] *Appalachian Fruit Research Station-USDA ARS, Kearneysville, USA*

SUMMARY

Transgenic C5 cv. Honey Sweet is a clone of *Prunus domestica* L. transformed with the *Plum pox virus* coat protein gene (PPV-CP). This transgenic plum displays post-transcriptional gene silencing (PTGS) which makes it highly resistant to PPV infection. To test the effect of heterologous viruses on the efficacy and stability of PTGS against PPV, transgenic C5 trees were graft-inoculated with different combinations of *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leaf spot virus* (ACLSV), *Prune dwarf virus* (PDV) and PPV-D strain. The potential for suppression of the silencing mechanism mediated by these viruses was evaluated. Challenge experiments were performed under greenhouse, nursery and field conditions in Romania and Spain, comprising two different environments, continental and Mediterranean, respectively. Virus infections were evaluated by visual monitoring of symptom and by serological and molecular assays. The engineered resistance to PPV in C5 transgenic plums was stable and was not suppressed by the presence of the challenging heterologous viruses over a three-year experimental period across all trials.

Key words: Honey Sweet, engineered resistance, gene silencing, PPV-D, PDV, PNRSV, ACLSV, co-infection.

INTRODUCTION

Transgenic plants expressing viral genes have been shown to exhibit varying degrees of resistance to the virus that provides the viral transgene, and to closely related viruses (Beachy *et al.*, 1990). The viral genes most commonly used in this pathogen-derived resistance (PDR) strategy have been coat protein (CP) genes. Since the initial report of CP-mediated resistance against *Tobacco mosaic virus* (TMV) infection in transgenic tobacco (Abel *et al.*, 1986), this strategy has allowed the development of many virus-resistant crops. *Plum pox virus* (PPV) is the causal agent of one of the most devastating diseases of *Prunus* species, eliciting important agronomic and economic losses (Cambra *et al.*, 2006). PPV can be transmitted by grafting and by a number of aphid species in a nonpersistent manner (Labonne *et al.*, 1995; Cambra *et al.*, 2006a). Since its first description in Bulgaria (Atanassov, 1932), the virus has spread to a large part of the European continent, around the Mediterranean basin and Near and Middle East, South and North America (Argentina, Canada, Chile and USA) and Asia (China, Iran, Kazakhstan and Pakistan) (Capote *et al.*, 2006; García and Cambra, 2007). Nevertheless, there are control measures against PPV based on two strategies: the reduction or elimination of the viral inoculum by quarantine measures and eradication programs, and the development of PPV resistant plants by conventional breeding or through the use of genetically modified plants (Scorza and Ravelonandro, 2006).

Following the latter strategy, transgenic European plums (*Prunus domestica* L.) containing the CP gene of PPV were developed as an approach to obtain PPV-resistant plums. One transgenic line, C5, subsequently named 'Honey Sweet' was found to be highly resistant to graft- and aphid-mediated inoculation by PPV in greenhouse and field tests (Ravelonandro *et al.*, 1997, 2000; Hily *et al.*, 2004; Malinowski *et al.*, 2006). C5 viral resistance is based on RNA silencing (Scorza *et al.*, 2001), i.e. a sequence-specific RNA degradation mechanism widely observed in animals, fungi and plants. In plants, it is called post-transcriptional gene silencing (PTGS) (Baulcombe, 2004; Hannon, 2002; Kooter *et al.*, 1999; Matzke *et al.*, 2001; Vaucheret *et al.*, 2001; Wianny and Zernicka-Goetz, 2000). The roles of RNA silencing include the developmental regulation of gene expression and protection from transposable elements and viruses.

Virus infection in plants can trigger the PTGS pathway in which siRNAs are produced (Hamilton and Baulcome, 1999; Hily *et al.*, 2005). As a response to this defence mechanism, many viruses encode gene-silencing suppressor proteins acting at different points in the PT-

Corresponding author: I. Zagrai
Fax: + 40. 263.214752
E-mail: izagrai@yahoo.com

GS pathway (Anandalakshmi *et al*., 1998; Voinnet, 2001). It has been shown that the helper component proteinase (HC-Pro) of potyviruses is a suppressor of the PTGS pathway (Brigneti *et al*., 1998; Kasschau *et al*., 1998), which interferes with the maintenance of the silencing by inhibiting degradation of the target mRNA and, consequently, preventing siRNA accumulation (Anandalakshmi *et al*., 1998; Llave *et al*., 2000; Mallory *et al*., 2001). Suppressor protein 2b from cucumoviruses, is unable to reverse already established RNA silencing, but prevents its initiation at the growing points of the plant by inhibiting the long-range activity of the silencing signal produced during the silencing reaction (Béclin *et al*., 1998; Brigneti *et al*., 1998; Guo and Ding, 2002). The p25 suppressor protein of the potexviruses, also affects RNA silencing by preventing spreading of the silencing signal (Voinnet *et al*., 2000). Other reported suppressors such as 19 kDa protein (p19) of tombusviruses (Zamore, 2004), the p21 protein of closteroviruses (Simón-Mateo *et al*., 2003; Keqiong and Dinshaw 2005) and the p14 of aureoviruses (Mérai *et al*., 2005) can bind siRNA suggesting that these proteins might sequester doubled-stranded siRNA as a general plant RNA viral strategy to suppress RNA silencing (Mérai *et al*., 2006; Lakatos *et al*., 2006). Viral PTGS suppressors have also been shown to suppress PTGS of non-viral transgenes (Beclin *et al*., 1998). While viral suppression of gene silencing has been demonstrated in herbaceous species (García and Cambra, 2007), it has not been reported in most crops including tree species or under field conditions.

The objective of this work was to determine the stability of RNA silencing in transgenic plums by assessing if suppression of PTGS occurs under mixed infection of PPV and heterologous viruses that commonly infect *P. domestica* and other *Prunus* species.

MATERIALS AND METHODS

Plant material and inoculated viruses. Challenge experiments were performed under greenhouse conditions and in experimental fields, located in Romania and Spain which represent contrasting environments, continental and Mediterranean, respectively. Challenge viruses were: *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leafspot virus* (ACLSV) and *Prune dwarf virus* (PDV).

Romania

Studies were carried out at the Fruit Research and Development Station, Bistrita. Two experiments were initiated in the field, in an experimental orchard and in a nursery. In addition, another assay was performed under greenhouse conditions. The C5 transgenic clone was chip-bud graft inoculated with PPV (D strain) and with the combinations PPV+PDV or PPV+PNRSV. The isolates used were 4Kr P2 for PDV, and Renclod P1 Isip for PNRSV, both from Bistrita, Romania. Buds infected with individual viruses were used for chip-bud inoculation. Conventional non transgenic plums were similarly inoculated and used as controls.

Experimental orchard. Ten C5 transgenic clones grafted on myrobalan rootstock were planted in the field in 1998 in an orchard with a high PPV infection pressure. Clone C5 exhibited a high resistance to natural PPV infection. In September 2003 six C5 trees were subjected to chip-bud graft inoculation. Two C5 plum trees were used for each virus combination. On each plum tree, half of the branches of the canopy were graft-inoculated and the other half was not. On each inoculated branch 10 buds individually infected with the test viruses were alternately grafted.

Experimental nursery. Myrobalan rootstocks were grown in an experimental nursery in April 2003 and grafted with buds from C5 or conventional plums on August 2003. The inoculation with the viruses was made as follow: buds of transgenic or conventional plums were inserted at the top of the grafting area, buds with PPV were inserted in the middle and buds with heterologous viruses were inserted at the bottom. Ten plants were used for each virus combination.

Greenhouse assays. Graft inoculations under greenhouse conditions were done as in the nursery. The budding was made in March 2004. Five plants were inoculated with each virus combination.

Spain

Experimental orchard. Eight C5 transgenic plums grafted on *P. marianna* rootstock were established in an experimental orchard in Llíria, Valencia in 1997. The orchard contained other transgenic European plums that were susceptible to natural PPV infection, and conventional European and Japanese plums (Malinowski *et al*., 2006). A high pressure of PPV inoculum was present in the area. After corroboration of the high resistance of the C5 transgenic plums to natural PPV infection, C5 plums were chip-bud graft inoculated in June 2003 with *Prunus salicina* shoots infected with combinations of PPV+ PNRSV, PPV+ACLSV or PPV+PNRSV+ACLSV (two C5 trees for each variant). PPV-D was used in all virus combinations. The isolates used were PPV L13, ACLSV L17, and PNRSV L180 from Lluxent, Valencia. Half of the branches of the canopy of C5 trees were chip-bud inoculated (two chip-buds per branch) and the other half of the tree was not. As controls, one C5 tree was inoculated only with PPV and another remained non inoculated. Two trees were reinoculated with ACLSV in 2004 due to necrosis produced by the virus in the grafts performed in 2003.

Virus monitoring

Romania. Virus infection (PPV and heterologous viruses) was evaluated by visual monitoring of symptom development and by serological and molecular methods. For testing, leaf samples were collected from different parts of the plants as follow: (i) in the case of the experimental orchard, leaves were collected from graft-inoculated branches, non-graft inoculated branches, and shoots developed from the grafted chip buds; (ii) in the case of the greenhouse and nursery experiments, leaves were collected from the basal half of the plants, from the top half of the plants and shoots developed from the grafted buds (inoculum). Serological detection was by DAS-ELISA using polyclonal antisera to PPV, PDV and PNRSV according to the manufacturer (Bioreba, Switzerland). Molecular detection was by IC-RT-PCR using the pair of primers P1/P2 for PPV (Wetzel *et al.*, 1991), PNRSV-10F/PNRSV-10R for PNRSV (Marbot *et al.*, 2003) and PDV-17F/PDV-12R for PDV (Kummert *et al.*, 2001). Assays were made before grafting (to check the virus-free status of the rootstocks and C5 trees) and after inoculation in June 2004, July 2005 and June 2006. In June 2004 all plants were analyzed. In July 2005 and June 2006, in the case of nursery and greenhouse experiments only two plants for each treatment showing the best symptoms and the highest absorbance values were subjected to analysis. In the case of C5 inoculated with PPV and PPV+PNRSV only one greenhouse-grown plant was analyzed.

Spain. The presence of PPV and the heterologous viruses was monitored in different parts of the tree: (i) *P. salicina* inoculum (shoot developed from the grafted chip bud); (ii) grafted C5 branch; (iii) non-grafted C5 branch, and (iv) *P. marianna* rootstock. PPV was detected by visual monitoring of symptom development and by the OEPP recommended methods (OEPP, 2004): DASI-ELISA using 5B-IVIA monoclonal antibody (Durviz, Spain) and IC-RT-PCR using P1/P2 primers (Wetzel *et al.*, 1991). PNRSV was monitored by symptom observation, DAS-ELISA using a PNRSV-specific commercial polyclonal antiserum (Loewe Biochemica, Germany) according manufacturers, and IC-RT-PCR using PNRSV-10F/PNRSV-10R primers (Marbot *et al.*, 2003). ACLSV was detected by DAS-ELISA using a polyclonal antiserum (Serodiag, France) and RT-PCR using A52 and A53 primers (Candresse *et al.*, 1995) with a previous RNA extraction step using the RNeasy plant minikit (Qiagen, USA). Visual inspection and serological and molecular analyses were done prior to inoculations in May 2003 (for assessing the virus-free status of C5 trees), and one, two and three years post-inoculation (May 2004, 2005 and 2006).

RESULTS

Romania

Experimental orchard (Table 1). PPV, PDV and PNRSV could not be detected in inoculated C5 trees by symptom observation and DAS-ELISA one year post-inoculation. Obvious PPV symptoms appeared on shoots that developed from the grafted chip buds (inoculum). DAS-ELISA results confirmed the presence of all viruses in the shoots derived from the inoculum. Low concentrations of PPV and PDV were detected in the inoculated conventional plums.

Two years post-inoculation DAS-ELISA tests revealed that the heterologous viruses (PDV and PNRSV) were translocated from the inoculum to the C5 trees. PPV could be detected with a very low titer in C5 trees only on a few discretely symptomatic leaves from graft inoculated branches. Conversely, very severe PPV symptoms and high viral concentration were present on graft-inoculated branches from conventional plums. In addition, the PPV symptoms observed in the noninoculated branches indicated that the virus invaded a large part of the canopy of conventional plums.

The evaluation performed three years post-inoculation showed no spread of PPV infection in C5 trees. In all cases (both single and mixed infections on C5), PPV could be observed (discrete diffuse and sporadic spots) and detected by DAS-ELISA and IC-RT-PCR only near the inoculum points. No PPV symptoms were observed in the non-inoculated part of the C5 canopy. The absence of the virus was confirmed by IC-RT-PCR. Although PDV and PNRSV induced no clear-cut symptoms they were both detected in the inoculated and in the non-inoculated part of the canopy of the C5 trees.

No differences in symptoms expression or in PPV spread and detection were observed in the C5 trees when PPV was inoculated alone or in combination with heterologous viruses.

Experimental nursery (Table 2). PDV readily invaded C5 plants inoculated with the PPV+PDV combination. PPV also translocated from the inoculum bud to C5 but the virus could only be detected at the basal part of the inoculated plants. Although PPV was detected one year post-inoculation, the infection did not proceed in the following two years (2005 and 2006).

In C5 plants inoculated with PPV+PNRSV, diffuse spots due to PPV appeared sporadically on a few leaves at the basal part of the plants. DAS-ELISA (2004 and 2005) and IC-RT-PCR (2006) confirmed the presence of PPV only limitedly to this part of the plants. However, PPV could not be detected by ELISA and IC-RT-PCR in the top half part of the inoculated plants. Although PNRSV produced no symptoms, the virus was detected with high titer in the whole plant since the first year post-inoculation.

Table 1. Evaluation of *Plum pox virus* (PPV) *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) in different parts of the graft-inoculated transgenic C5 "Honey Sweet" and conventional European plums in the Romanian experimental orchard over a three-year experimental period. Inoculation September 2003.

Tree type	Inoculum	Analysed tree part	2004			2005						2006					
			DAS-ELISA			DAS-ELISA			Symptoms intensity			IC-RT-PCR			Symptoms intensity		
			PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV
C5	PPV	graft (inoculum)	++++			+++++			+++++			+			+++++		
		grafted branch	-			+			±			+			+		
		non-grafted branch	-			-			-			-			-		
C5	PPV	graft (inoculum)	+++++			+++++			+++++			+			+++++		
		grafted branch	-			+			±			+			+		
		non-grafted branch	-			-			-			-			-		
C5	PPV PDV	graft (inoculum)	++++	++++		+++++	+++++		+++++	+		+	+		+++++	+	
		grafted branch	-	-		+	+++++		±	+		+	+		+	+	
		non-grafted branch	-	-		-	+		-	±		-	+		-	±	
C5	PPV PDV	graft (inoculum)	++++	+++		+++++	+++++		+++++	+		+	+		+++++	+	
		grafted branch	-	-		+	+++++		±	+		+	+		+	±	
		non-grafted branch	-	-		-	++		-	±		-	+		-	±	
C5	PPV PNRSV	graft (inoculum)	+++		+++	+++++		++++	+++++		+	+		+	+++++		±
		grafted branch	-		-	±		++	+		±	+		+	+		±
		non-grafted branch	-		-	-		±	-		-	-		+	-		-
C5	PPV PNRSV	graft (inoculum)	+++++		++	+++++		++++	+++++		+	+		+	+++++		±
		grafted branch	-		-	+		+++	±		±	+		+	±		±
		non-grafted branch	-		-	-		+	-		-	-		+	-		-
Conventional	PPV	graft (inoculum)	++++			+++++			+++++			+			+++++		
		grafted branch	++			++++			+++			+			+++++		
		non-grafted branch	-			++			+			+			++		
Conventional	PPV PDV	graft (inoculum)	+++++	+++++		+++++	+++++		+++++	+		+	+		+++++	+	
		grafted branch	+	±		++++	+++++		+++	+		+	+		+++++	+	
		non-grafted branch	-	-		++	++		+	±		+	+		++	+	
Conventional	PPV PNRSV	graft (inoculum)	++++		++++	+++++		+++++	+++++		+	+		+	+++++		+
		grafted branch	+		-	++++		+++	+++		±	+		+	+++++		++
		non-grafted branch	-		-	++		+	+		-	+		+	++		±

Table 2. Evaluation of *Plum pox virus* (PPV) *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) in different parts of the graft-inoculated transgenic C5 "Honey Sweet" and conventional European plums in the nursery over a three-year experimental period. Inoculation August 2003.

Tree type	Inoculum	Analysed plant part	2004			2005						2006					
			DAS-ELISA			DAS-ELISA			Symptoms intensity			IC-RT-PCR			Symptoms intensity		
			PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV
C5	PPV	bottom half	++			+			+			+			+		
		top half	-			-			-			-			-		
C5	PPV	bottom half	+			+			±			+			+		
		top half	-			-			-			-			-		
C5	PPV PDV	bottom half	±	++++		+	+++++		+	+++		+	+		±	++++	
		top half	-	++++		-	+++++		-	+++		-	+		-	+++	
C5	PPV PDV	bottom half	++	++++		++	+++++		+	+++		+	+		±	++++	
		top half	-	++++		-	+++++		-	+++		-	+		-	++++	
C5	PPV PNRSV	bottom half	±		+++++	+		++++	+		-	+		+	±		-
		top half	-		+++	-		++++	-		-	-		+	-		-
C5	PPV PNRSV	bottom half	+++		+++++	++		++++	+		-	+		+	±		-
		top half	-		++++	-		+++	-		-	-		+	-		-
Conventional	PPV	bottom half	+++++			+++++			+++++			+			+++++		
		top half	+++			++++			++++			+			++++		
Conventional	PPV PDV	bottom half	+++++	++++		+++++	+++++		++	+++		+	+		+++	++++	
		top half	++	++++		++++	+++++		+	+++		+	+		++	++++	
Conventional	PPV PNRSV	bottom half	+++++		+++++	+++++		++++	+++++		-	+		+	+++++		-
		top half	+++		++	+++		++++	+++		-	+		+	++++		-

Table 3. Evaluation of *Plum pox virus* (PPV) *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) in different parts of the graft-inoculated transgenic C5 "Honey Sweet" and conventional European plums in the greenhouse over a three-year experimental period. Inoculation March 2004.

Tree type	Inoculum	Analysed plant part	2004			2005						2006					
			DAS-ELISA			DAS-ELISA			Symptoms intensity			IC-RT-PCR			Symptoms intensity		
			PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV	PPV	PDV	PNRSV
C5	PPV	bottom half	+++			++			+			+			+		
		top half	-			-			-			-			-		
C5	PPV PDV	bottom half	+++	+++++		++	+++++		±	+++++		+	+		±	+++++	
		top half	-	+++++		-	+++++		-	+++++		-	+		-	+++++	
C5	PPV PDV	bottom half	++	+++++		++	+++++		+	+++++		+	+		+	+++++	
		top half	-	+++++		-	+++++		-	+++++		-	+		-	+++++	
C5	PPV PNRSV	bottom half	++		+++++	+		++++	+		-	+		+	±		-
		top half	-		+++++	-		+++	-		-	-		+	-		-
Conventional	PPV	bottom half	+++++			+++++			++++			+			++++		
		top half	++			+++			++			+			+++		
Conventional	PPV PDV	bottom half	++++	++++		+++++	++++		++++	+++		+	+		++++	++++	
		top half	+++	++++		++++	++++		++	+++		+	+		++	++++	
Conventional	PPV PNRSV	bottom half	+++++		+++++	+++++		++++	+++++		-	+		+	+++++		-
		top half	+++		+++	+++		++++	+++		-	+		+	++++		-

A similar PPV behaviour were observed in C5 plants inoculated with PPV alone.

No differences in symptoms development or PPV spread was observed on C5 grown in the nursery when PPV was inoculated alone or in combination with the other viruses.

Greenhouse assays (Table 3). In C5 plants inoculated with the PPV+PDV combination, PPV was detected by DAS-ELISA at the basal part of C5 plants three months and one year (2005) after inoculation. In the following year (2006), IC-RT-PCR tests confirmed that the spread of PPV was blocked in C5. On the other hand, the plants showed very severe PDV symptoms throughout the vegetative period. In the case of C5 inoculated with PPV+ PNRSV, a very low concentration of PPV was detected by DAS-ELISA one year post inoculation, and only on a few symptomatic leaves from the basal part of the plants. Two years post-inoculation, PPV could be detected by IC-RT-PCR but only on a few very discretely symptomatic leaves from the basal part of the tree. PNRSV showed no symptoms but the presence of the virus was confirmed by DAS-ELISA and IC-RT-PCR, both in the basal and distal sampling points of the plants.

No differences in symptom expression or PPV spread were observed on C5 grown in the greenhouse when this virus was inoculated alone or in combination with heterologous viruses.

Spain

Experimental orchard (Table 4). PPV, ACLSV and PNRSV could not be detected in inoculated C5 trees by symptom observation and DAS-ELISA one year post-inoculation. Obvious PPV symptoms appeared on the shoots developed from the grafted chip buds. DAS-ELISA results confirmed the presence of all viruses in the shoots derived from the inoculum. Low concentrations of PPV and PDV were detected in the inoculated conventional plums.

In C5 trees inoculated with PPV+PNRSV, PPV could be detected only in the shoot that grew from the inoculum bud one year post-inoculation. In the years that followed, the virus was detected by IC-RT-PCR and DASI-ELISA in the C5-inoculated branch, near the inoculum point, with low titer (low ELISA values and a weak amplicon in a gel). No symptoms of PPV were observed. Conversely, PNRSV translocated from the inoculum to the C5 tree rapidly invading the whole plant in the first year post-inoculation. When PNSRV was co-inoculated with ACLSV and PPV its spreading was slower, only reaching the inoculated branch or the whole canopy of the tree, three years post-inoculation.

In C5 trees inoculated with PPV+ACLSV, PPV showed the same behavior as in the other virus combinations. ACLSV spread slowly and could only be detected in the infected shoot the first year post-inoculation and in the graft-inoculated C5 branch in the suc-

Table 4. Evaluation of *Plum pox virus* (PPV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple chlorotic leaf spot virus* (ACLSV) in different parts of the graft-inoculated transgenic C5 "Honey Sweet" in the Spanish experimental orchard over a three-year experimental period. Inoculation: June 2003.

Tree type	Inoculum	Analysed tree part	2004						2005						2006					
			DASI-ELISA			IC-RT-PCR or RT-PCR			DASI-ELISA			IC-RT-PCR or RT-PCR			DASI-ELISA			IC-RT-PCR or RT-PCR		
			PPV	PNRSV	ACLSV	PPV	PNRSV	ACLSV	PPV	PNRSV	ACLSV	PPV	PNRSV	ACLSV	PPV	PNRSV	ACLSV	PPV	PNRSV	ACLSV
C5	PPV PNRSV	rootstock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		graft (inoculum)	+++	+++	-	+	+	-	++++	+++	-	+	+	-	++++	+++	-	+	+	-
		grafted branch	-	+	-	-	+	-	-	++	-	+	+	-	±	++	-	+	+	-
		non-grafted branch	-	-	-	-	+	-	-	++	-	-	+	-	-	++	-	-	+	-
C5	PPV PNRSV	rootstock	+++	-	-	+	-	-	+++	-	-	+	-	-	+++	-	-	+	-	-
		graft (inoculum)	+++	+++	-	+	+	-	+++	+++	-	+	+	-	+++	+++	-	+	+	-
		grafted branch	-	+	-	-	+	-	-	++	-	-	+	-	-	++	-	-	+	-
		non-grafted branch	-	+	-	-	+	-	-	+	-	-	+	-	-	++	-	-	+	-
C5	PPV ACLSV	rootstock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		graft	+++	-	++	+	-	+	+++	-	+	+	-	+	+++	-	+	+	-	+
		grafted branch	-	-	-	-	-	-	±	-	+	+	-	+	±	-	+	+	-	+
		non-grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	PPV ACLSV	rootstock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		graft (inoculum)	+++	-	++	+	-	+	+++	-	+	+	-	+	+++	-	+	+	-	+
		grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		non-grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	PPV PNRSV ACLSV	rootstock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		graft (inoculum)	+++	+++	+	+	+	+	+++	+++	+	+	+	+	+++	+++	+	+	+	+
		grafted branch	-	-	-	-	-	-	+	-	±	+	-	+	+	+	±	+	+	+
		non-grafted branch	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	+
C5	PPV PNRSV ACLSV	rootstock	+++	-	-	+	-	-	+++	-	-	+	-	-	+++	-	-	+	-	-
		graft (inoculum)	+++	+++	-	+	+	-	+++	+++	+	+	+	+	+++	+++	+	+	+	+
		grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
		non-grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	PPV	rootstock	+++	-	-	+	-	-	+++	-	-	+	-	-	+++	-	-	+	-	-
		graft (inoculum)	+++	-	-	+	-	-	+++	-	-	+	-	-	+++	-	-	+	-	-
		grafted branch	-	-	-	-	-	-	±	-	-	+	-	-	±	-	-	+	-	-
		non-grafted branch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	non-inoculated	rootstock	+++	-	-	+	-	-	+++	-	-	+	-	-	+++	-	-	+	-	-
		non-grafted tree	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

cessive years, but with low titer. In some cases, as in one C5 tree inoculated with the three viruses, ACLSV was not detected one year post-inoculation due to necrosis of the graft caused by it. Re-grafting was necessary in these cases to ensure ACLSV infection.

The C5 tree inoculated only with PPV was slightly infected by the virus. Infection, however, was localized near the inoculum, the virus had a low titer and did not spread to the rest of the tree over the three-year experimental period. No symptoms of PPV were observed.

No PPV infection was detected in the non inoculated C5 control tree over the entire experimental period, in spite of the PPV infection of the *P. marianna* rootstock since the beginning of the 3-year experimental period.

Conventional European plums were naturally infected by PPV by aphid vectors. The virus spread throughout the trees over the 3-year experimental period reaching a high level of infection.

DISCUSSION

Regardless of single (PPV) or mixed (PPV+PDV, PPV+PNRSV, PPV+ACLSV or PPV+PNRSV+ ACLSV) infection, C5 transgenic plums showed a similar behaviour versus PPV infection: This virus was able to induce only a very mild and limited infection in C5 independently of the presence of heterologous viruses. Symptoms were extremely mild, sporadic or absent, indicating effective inhibition of virus multiplication. It should be stressed that C5 trees remained uninfected despite the fact that some *P. marianna* rootstocks were PPV infected. The infection of this very sensitive rootstock took place through the suckers. Along the whole duration of the experiments (orchard, nursery, greenhouse), and in the two different ecological areas assayed (continental and Mediterranean) PPV infection remained close to the inoculation site and did not spread. Malinowski *et al.* (2006) showed the same infection patters in C5 trees graft-inoculated with PPV in an experimental open-field trial in Poland.

Previous field and greenhouse results clearly showed the resistance of C5 to PPV infection through aphid vectors and by graft inoculation (Hily *et al.*, 2004; Malinowski *et al.*, 2006). The resistance can be transferred to seedlings through cross-hybridization (breeding) (Ravelonandro *et al.*, 1998; Scorza *et al.*, 1998). In addition, graft-inoculation of transgenic plums with PPV and ilarviruses (PNRSV and PDV), or trichoviruses (ACLSV) in the field and in the greenhouse did not affect the efficacy and stability of PTGS over a three-year period. As a consequence, resistance to PPV did not break down. Because of all these characteristics and for the high productivity and the excellent quality of the crop, the transgenic C5 named 'Honey Sweet' is being considered for deregulation in the USA (Scorza *et al.*, 2007).

ACKNOWLEDGEMENTS

The authors thank the European Commission for supporting the project entitled "Environmental impact assessment of transgenic grapevines and plums on the diversity and dynamics of virus populations" under the competitive grant program contract no. QLK 3-2002 -0240.

REFERENCES

Abel P.P., Nelson R.S., De B., Hoffmann N., Rogers S.G., Fraley RT., Beachy R.N., 1986. Delay of disease development in transgenic plants that express the Tobacco mosaic virus coat protein gene. *Science* **232**: 738-743.

Anandalakshmi R., Pruss G.J., Ge, X., Marathe R., Mallory A. C., Smith T. H., Vance V., 1998. A viral suppressor of gene silencing in plants. *Proceedings of the National Academy of Sciences USA* **95**: 13079-13084.

Atanassov D., 1932. Plum pox. A new virus disease. *Annals of the University of Sofia Faculty of Agriculture and Silviculture* **11**: 49-69.

Baulcombe D., 2004. RNA silencing in plants. *Nature* **431**: 356-363.

Beachy R.N., Loesch-Fries S., Tumer N.E., 1990. Coat protein mediated resistance against virus infection. *Annual Review of Phytopathology* **28**: 451-474.

Béclin C., Berthomé R., Palauqui J.-C., Tepfer M., Vaucheret H., 1998. Infection of tobacco or Arabidopsis plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. *Virology* **252**: 313-317.

Brigneti G., Voinnet O., Li W.X., Ji L.H., Ding S.W., Baulcombe D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO Journal* **17**: 6739-6746.

Cambra M., Capote N., Myrta A., Llácer G., 2006a. *Plum pox virus* and estimated costs associated to sharka disease. *Bulletin OEPP/EPPO Bulletin* **36**: 202-204.

Cambra M., Capote N., Cambra M.A., Llácer G., Botella P., López-Quílez A. 2006b. Epidemiology of sharka disease in Spain. *Bulletin OEPP/EPPO Bulletin* **36**: 271-275.

Candresse T., Lanneau M., Revers F., Grasseau N., Macquaire G., German S., Malinowsky T., Dunez J., 1995. An immunocapture PCR assay adapted to the detection and the analysis of the molecular variability of the Apple chlorotic leafspot virus. *Acta Horticulturae* **386**: 136-147.

Capote N., Cambra M., Llácer G., Petter F., Platts L.G., Roy A.S., Smith I.M., 2006. A review of *Plum pox virus*. *Bulletin OEPP/EPPO Bulletin* **36**: 201-349.

García J.A., Cambra M., 2007. *Plum pox virus* and sharka disease. *Plant Viruses* **1**: 69-79.

Guo H.S., Ding S.W., 2002. A viral protein inhibits the long range signalling activity of the gene silencing signal. *EMBO Journal* **21**: 398-407.

Hamilton A.J., Baulcombe D.C., 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**: 950-952.

Hannon G.J., 2002. RNA interference. *Nature* **418**: 244-251.

Hily J.M., Scorza R., Malinowski T., Zawadzka B., Ravelonandro M., 2004. Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Research* **13**: 427-436.

Hily J.M., Scorza R., Webb K., Ravelonandro M., 2005. Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in a transgenic woody perennial plum tree. *Molecular Plant Microbe Interactions* **18**: 794-799.

Kasschau K.D., Carrington J.C., 1998. A counterdefensive strategy of plant viruses: Suppression of postranscriptional gene silencing. *Cell* **95**: 461-470.

Keqiong Y., Dinshaw, J.P., 2005. RNA silencing suppressor p21 of *Beet yellows virus* forms an RNA binding octameric ring structure. *Structure* **13**: 1375-1384.

Kooter J.M., Matzke M.A., Meyer P., 1999. Listening to the silent genes: Transgene silencing, gene regulation and pathogen control. *Trends in Plant Science* **4**: 340-347.

Kummert J., Vendrame M., Lepoivre P., Steyer S., 2001. Development of routine RT-PCR and ELISA tests for fruit tree certification. *Acta Horticulturae* **550**: 45-52.

Labonne G., Yvon M., Quiot J.B., Avinent L., Llácer G., 1995. Aphids as potential vectors of *Plum pox virus*: comparison of methods of testing and epidemiological consequences. *Acta Horticulturae* **386**: 207-218.

Lakatos L., Csorba T., Pantaleo V., Chapman E.J., Carrington J.C., Liu Y.P., Dolja V.V., Calvino L.F., Lopez-Moya J.J., Burgyan J., 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO Journal* **25**: 2768-2780.

Llave C., Kassachau K.D., Carrington J.C., 2000. Virus-encoded suppressor of postranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences USA* **97**: 13401-13406.

Malinowski T., Cambra M., Capote N., Zawadzka B., Gorris M.T., Scorza R., Ravelonandro M., 2006. Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. *Plant Disease* **90**: 1012-1018.

Mallory A.C., Ely L., Smith T.H., Marathe R., Anandalakshmi R., Fagard M., Vaucheret H., Pruss,G., Bowman L., Vance V.B., 2001. HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *Plant Cell* **13**: 571-583.

Marbot S., Salmon M., Vendrame M., Huwaert A., Kummert J., Dutrecq O., Lepoivre P., 2003. Development of Real-Time RT-PCR assay for detection of *Prunus necrotic ringspot virus* in fruit trees. *Plant Disease* **87**: 1344-1348.

Matzke M.A., Matzke A.J., Pruss G.J., Vance V.B., 2001. RNA based silencing strategies in plants. *Current Opinions in Genetic Development* **11**: 221-227.

Mérai Z., Kerényi Z., Molnár A., Barta E., Válóczi A., Bisztray G., Hayelda Z., Burgyán J., Silhavy D., 2005. Aureusvirus P14 is an efficient RNA silencing suppressor that binds double-stranded RNAs without size specificity. *Journal of Virology* **79**: 7217-7226.

Mérai Z., Kerenyi Z., Kertesz S., Magna M., Lakatos L., Silhavy D., 2006. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *Journal of Virology* **80**: 5747-5756.

OEPP, 2004. Diagnostic protocols for regulated pests. *Plum pox potyvirus*, EPPO standards. *Bulletin OEPP/EPPO Bulletin* **34**: 247-256.

Ravelonandro M., Scorza R., Bachelier J.C., Labonne G., Levy L., Damsteegt V., Callahan A.M., Dunez J., 1997. Resistance of transgenic *Prunus domestica* to *Plum pox virus* infection. *Plant Disease* **81**: 1231-1235.

Ravelonandro M., Scorza R., Renaud R., Salesses G., 1998. Transgenic plums resistant to *Plum pox virus* infection and preliminary results of cross-hybridization. *Acta Horticulturae* **478**: 515-524.

Ravelonandro M., Scorza R., Callahan A., Levy L., Jacquet C., Monsion M., Damsteegt V., 2000. The use of transgenic fruit trees as a resistance strategy for virus epidemscs: the plum pox (sharka) model. *Virus Research* **71**: 63-69.

Scorza R., Callahan A., Levy L., Damsteegt V., Ravelonandro M., 1998. Transfering potyvirus coat protein genes through hybridization of transgenic plants to produce plum pox virus resistant plums (*Prunus domestica* L.). *Acta Horticulturae* **472**: 421-425.

Scorza R., Callahan A., Levy L., Damsteegt V., Webb K., Ravelonandro M., 2001. Post-transcriptional gene silencing in plum pox resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Research* **10**: 201-209.

Scorza R., Ravelonandro M., 2006. Control of *Plum pox virus* through the use of genetically modified plants. *Bulletin OEPP/EPPO Bulletin* **36**: 337-340.

Scorza R., Hily J.M., Callahan A., Malinowski T., Cambra M., Capote N., Zagrai I., Damsteegt V., Briard P., Ravelonandro M., 2007. Deregulation of plum pox resistant transgenic plum "Honey Sweet". *Acta Horticulturae* **738**: 669-674.

Simón-Mateo C., López-Moya J.J., Guo H.S., González E., García J.A., 2003. Suppressor activity of potyviral and cucumoviral infections in potyvirus-induced transgene silencing. *Journal of General Virology* **84**: 2877-2883.

Vaucheret H., Béclin C., Fagard M., 2001. Post-transcriptional gene silencing in plants. *Journal of Cell Science* **114**: 3083-3091.

Voinnet O., 2001. RNA silencing as a plant immune system against viruses. *Trends in Genetics* **17**: 449-459.

Voinnet O., Lederer C., Baulcombe D.C., 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**: 157-167.

Wianny F., Zernicka-Goetz M., 2000. Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biology* **2**: 70-75.

Wetzel T., Candresse T., Ravelonandro M., Dunez J., 1991. A polymerase chain reaction assay adapted to plum pox potyvirus detection. *Journal of Virological Methods* **33**: 355-365.

Zamore P.D., 2004. Plant RNAi: How a viral silencing suppressor inactivates siRNA. *Current Biology* **14**: 198-200.