

Development of Plum Pox Virus Resistance in Plum via Expression of an Intron-Spliced Hairpin HC-Pro Transgene

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ABSTRACT

Plum pox virus (PPV) is a serious viral disease of *Prunus* species. Few sources of natural resistance to PPV are available. In this light, biotechnology is a strong option to develop PPV resistance in plants. We have previously shown that a transgene consisting of a region of the PPV HC-Pro sequence in an intron-spliced hairpin conformation could provide high level of resistance to PPV in *Nicotiana benthamiana*, a herbaceous model host. We have now generated transgenic plum lines expressing the same transgene. Plants were inoculated with PPV virus using a chip bud grafting method and were subjected to three cycles of dormant/growth periods. Plants were analyzed for PPV resistance after each cold treatment (dormant period) using a real-time polymerase chain reaction (PCR) assay. After the first cold treatment, transgenic plum plants remained PPV-free whereas all the control plants were PPV positive. After three cycles of cold treatment, many transgenic plants still exhibited strong resistance to PPV. Presence of siRNA molecules corresponding to the HC-Pro transgene were confirmed in transgenic lines that were highly resistant to PPV. This study shows that induction of PPV-specific RNA silencing via expression of an intron-spliced hairpin HC-Pro transgene is an effective approach for high level and stable resistance to plum pox virus in plum.

Keywords: plum pox virus disease, post transcriptional gene silencing, *Prunus domestica*

Abbreviations: NPTII, neomycin phosphotransferase; PCR, polymerase chain reaction; PPV, Plum pox virus; PTGS, post transcription gene silencing; siRNA, small interfering RNA

INTRODUCTION

Plum pox virus (PPV) is a viral disease that affects stone fruit species (*Prunus* spp.) including peach, nectarine, plum, apricot, cherry as well as ornamental *Prunus* plants (Nemeth 1994). PPV is the casual agent of plum pox disease (known as sharka in Europe) and is considered to be the most economically-devastating disease of *Prunus* fruit plants. The infected trees produce poor quality fruits which include lower sugar content, deformation, and colour abnormality (Nemeth 1994; Kegler *et al.* 1998) Also, the fruit of infected trees can have various symptoms such as chlorotic spots and ring patterns, which affect fruit marketability. In addition, PPV infection can result in premature fruit drop or even premature tree death (Nemeth 1994; Kegler *et al.* 1998). The loss resulting from PPV disease can be up to 80-100% (Lopez-Moya *et al.* 2000). Since its discovery in Bulgaria in 1917 (reviewed by Nemeth 1994), PPV disease has progressively spread across many countries in the world, including most of the countries in Europe and the Mediterranean region (Nemeth 1994). The disease has also been found in the USA (Levy *et al.* 2000) and in Canada (Thompson *et al.* 2001; James and Varga 2005).

Since the discovery of PPV, natural resistance through screening varieties has been sought (Kegler *et al.* 1998). However, few natural resistance sources have been found which can be used to develop PPV resistance in *Prunus* plants (Nemeth 1994; Kegler *et al.* 1998; Lopez-Moya 2000). Consequently, other approaches have been tested and used for the control of PPV, including control of aphids which transmit the virus, removal of infected trees, and quarantine approaches. Due to various factors, these methods are ineffective in controlling PPV disease and preventing

PPV spread.

Alternatively, genetic resistance to PPV may be developed using the induction of PPV-specific RNA silencing (Baulcombe 1999; Waterhouse *et al.* 1999). The coding regions of various PPV proteins (P1, CI, NIa-NIb, CP) have been engineered in plants and PPV resistant lines have been generated (e.g. Ravelonandro *et al.* 1998; Scorza *et al.* 2001). However, induction of post transcription gene silencing (PTGS) via direct transfer of viral genome fragments is often unpredictable and screening a large number of transgenic lines is needed to identify the resistant lines. Smith *et al.* (2000) reported that a transgene design allowing the expression of intron-spliced hairpin RNAs could induce RNA silencing more efficiently in transgenic plants. With intron-spliced hairpins, PPV resistance could be achieved in a more predictable manner and a large number of plants generated showed PPV resistance as demonstrated in model herbaceous plants (Pandolfini *et al.* 2003; Nicola-Negri *et al.* 2005).

We previously reported that a transgene consisting of a highly conserved region of the PPV HC-Pro coding region in an intron-spliced hairpin conformation could lead to high levels of resistance to PPV in the model plant *Nicotiana benthamiana* (Zhang *et al.* 2006). In this study, we have explored the feasibility of transferring the PPV resistance in plum plants using the intron-spliced hairpin HC-Pro transgene. We demonstrated a high level of resistance to PPV in the transgenic lines. The resistance was stable for an extended period of time and over repeated growth and dormant periods.

MATERIALS AND METHODS

Construction of plasmids

A conserved region of the HC-Pro coding region of PPV with a nucleotide sequence identity of 98% among different PPV isolates was selected to engineer resistance to PPV in model plants (Zhang *et al.* 2006). The region was amplified from a cDNA clone from Canadian isolate 5 (Rochon *et al.* 2003) and inserted in the sense and antisense orientation in plasmid pBBI-S13 separated by a DNA fragment containing the intron 1 S13 of sweet cherry S-allele of the Schneiders Variety (Wiersma *et al.* 2001). A double 35S promoter was used to drive the expression of the transgene. The expression cassette was transferred to a binary vector (pBin19). The detailed procedure of transformation vector development and construction was described in a previous report (Zhang *et al.* 2006).

Plum transformation

Mature plum fruits were harvested from trees of European plum cv. 'Stanley' in Vineland Station, Ontario, Canada. Plant transformation and plant regeneration followed the procedures described previously (Tian *et al.* 2006, 2009). Briefly, *Agrobacterium tumefaciens* (LBA4404) cultures were grown overnight with appropriate antibiotics. Plum seed explants were infected with the *Agrobacterium* cultures resuspended to OD₆₀₀ = 0.2 just before inoculation. The explants were transferred to shoot induction MS medium containing 20 mg·L⁻¹ acetosyringone for 3 days at 24 ± 1°C. After co-cultivation, the explants were transferred to fresh shoot induction medium containing 300 mg·L⁻¹ timentin for one week then transferred onto shoot induction medium containing 75 mg·L⁻¹ kanamycin and 300 mg·L⁻¹ timentin. The explants were subcultured to fresh induction medium every three to four weeks. Putatively transformed shoots were excised from the explants and placed in Magenta boxes containing a rooting medium (Tian *et al.* 2006). After rooting, the plantlets were transferred to soil and grown for 3-4 months under greenhouse conditions.

PPV inoculation and resistance assay

PPV (strain D) source plants were established by grafting PPV positive buds obtained from infected peach trees found in the Niagara peninsula region of Ontario, Canada. Positive source materials were also obtained from peach seedlings that were infected by allowing aphids which contained the PPV virus to feed on the plants and transmit the virus. The PPV-infected plants were grown at 21 to 23°C, 16-hr light period for approximately four months and then cycled for a dormant period at 4°C for approximately 3 months. All positive materials were tested prior to use by real-time PCR to confirm presence of the virus.

Transgenic plants were inoculated in a containment room for disease control by grafting three positive PPV buds from different source plants onto the main stem of the plant. The graft was allowed to adhere and transmit the virus for the entire growing period. Two months after the first inoculation, the plants were infected by PPV again by grafting another PPV positive bud on the stem. The plants were grown for another month, at which time they were transferred to a cold room with a temperature at 4°C and no light to mimic a dormant period. Three months later, the plants were brought out of the cold treatment and allowed to grow for about a month at the same growth conditions as described previously. The plants were tested for PPV presence. The plants were grown for another two months and then once again subjected to cold treatment. In total, the plants went through three cycles of growth and dormancy. Plants that tested strongly positive for the presence of PPV for the entire line were discarded after each cycle except for the controls. A total of 18 transgenic line and five control plants were used in the research study (Table 1).

Real-time PCR test for PPV

Ten leaves were randomly sampled from each plant. Samples were taken to make sure that leaves from the tip and the base of the branches were equally sampled. Wherever possible, visibly infec-

Table 1 PPV resistance assay for plum plants transformed with a PPV HC-Pro transgene in an intron-spliced hairpin conformation after three different cycles of cold treatments.

Plant Line	No of plants	% of PPV-negative (using real-time PCR assay)			
		Cycle 1	Cycle 2	Cycle 3	siRNA**
KL5	5	100%	100%	80%	+++
KL21-2	3	100	100	100	+++
KL21-5	3	66.7	0	-*	
KL22-1	3	100	100	100	+++
KL22-2	3	66.7	0	-	NA
KL22-3	3	66.6	66.6	50	+++
KL22-5	3	66.6	66.6	0	+
KL22-6	3	66.6	33.3	0	-
KL22-7	3	33.3	0	-	+
KL22-8	3	66.6	0	-	NA
KL22-10	2	0	0	-	-
KL22-11	3	100	100	66.6	+++
KL22-12	3	33.3	0	-	+
KL22-16	3	100	100	66.6	NA
KL23-1	3	33.3	0	-	-
KL23-2	3	100	100	100	++
KL27-1	3	100	100	66.6	++
KL27-2	3	66.7	66.7	-	NA
Control	5	0	0	0	-

* Plants were either dead or discarded (due to PPV-positive).

**Concentration of HC-Pro specific siRNA detected in the lines prior to the PPV inoculations (NA: not available).

ted leaves were included in the sample. A 0.5 g sub-sample was taken from the sampled 10 leaves, placed in a Bioreba extraction bag (Bioreba AG, Switzerland) with 5 mL of DiPEP buffer (Agdia Inc., Elkhart IN, USA) and ground using a Homex tissue homogenizer (Bioreba AG, Switzerland). The presence of PPV was tested by real-time PCR assay using PPV D specific primers as described in Kim *et al.* (2008). Each sample was run once and a positive and blank was used as comparison each time. A sample was considered PPV positive if the cycle threshold (CT value) was 35 or less.

Detection of siRNAs in transgenic lines

Northern blot was used for siRNA detection. Total RNA was isolated from transgenic plum leaves using the PURESCRIPT kit (Gentra Systems). RNA (40 µg) was separated on a 15% acrylamide, 7 M urea gel in the presence of 0.5X TBE buffer (250 to 300 V for 3 hrs). The RNA was transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) using a semi-dry transfer cell (BIO-RAD) as described by the supplier. The RNAs were fixed to the membrane by UV-crosslinking and baking at 80°C for 1 hr. The blot was pre-hybridized for 4 hrs at 38°C in ULTRAhybridization buffer (Ambion). The HC-Pro probe was synthesized by RT-PCR using the specific primers used for the transgene design and was labelled by random-priming (Zhang *et al.* 2006). The probe (2.5 × 10⁶ dpm/ml) was hybridized to the RNA blot overnight at 38°C. The membrane was washed twice in 2X SSC, 0.5% SDS at 42°C and the blot was exposed to a phosphor-imager screen (Storm) for 48 hrs. Primers were used as molecular mass markers.

RESULTS AND DISCUSSION

Transgenic plum plants were selected *via* PCR using *NPTII* gene primers (not shown) and primers specific for the intron sequences (5'-ATGGTGAGTTGCGTTACAATC-3' and 5'-ATCCTGCCAAGTAAAAAGAGG-3'). Several plants were produced for each transgenic line following a previously described protocol (Tian *et al.* 2006, 2009).

Three to five plants from each line were inoculated with PPV virus. After cold treatment, plants were brought back to normal growth conditions. Leaves started to develop about one week after the cold treatment. Leaves were fully developed after about four weeks. Leaf samples were taken and PPV presence was analyzed via real-time PCR as described previously in research methods. Of the 18 transgenic lines analyzed, 17 lines (94.7%) showed certain degree of

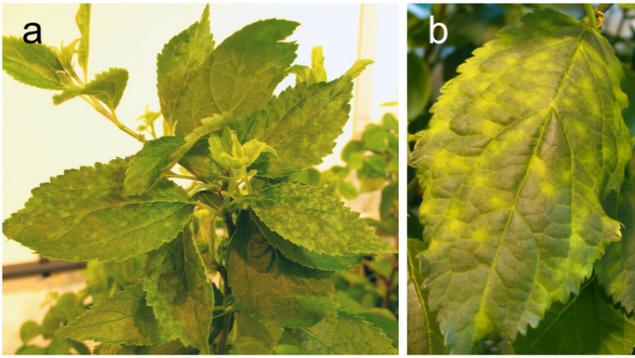


Fig. 1 Typical PPV symptoms in susceptible plum plants. (a) Chlorotic spots. (b) Close-up of leaf showing clearing of veins.

PPV resistance. On the other hand, five control plants which were generated *via* regular regeneration procedure were all PPV-positive (**Table 1**). Typical PPV symptoms, such as chlorotic spots on the leaves and clearing of the veins, could be observed in some plants that tested strongly positive for PPV (**Fig. 1**). However, none of the PPV-negative plants showed any viral symptoms. In some cases, one or two plants within one line were PPV-positive but the other plants were PPV negative (**Table 1**). Thus, after the first cold treatment the majority of transgenic lines appeared to be PPV-negative.

After a second cold treatment, PPV resistance testing showed that while some plants appeared to have lost the PPV resistance, the majority of the transgenic plants were still PPV-negative (**Table 1**). After the third cold treatment, a few plants did not survive probably due to the fact that the plants were stressed by cycling them more quickly than they would otherwise experience in a normal year of growth and dormancy (**Table 1**). Most plants that tested negative for PPV in cycle one and two remained PPV-negative in the third cycle. In three lines, namely, KL21-2, KL22-1, KL23-2, every single plant was PPV-negative after three cycles of cold treatment, suggesting a strong level of PPV resistance in these lines. Comparatively, all the control plants were

consistently positive for PPV virus over the three cycles of cold treatments (**Table 1**). Real-time PCR analysis of representative transgenic plum plants is shown in **Fig. 2**.

The results show that the intron-spliced hairpin design of the HC-Pro transgene which induced high levels of PPV resistance in *N. benthamiana* plants (Zhang *et al.* 2006) is also very effective in conferring PPV resistance in *Prunus* woody plants, namely plum. The use of hairpin design induced PTGS more effectively and therefore generated a higher efficiency of PPV resistance compared to that via direct transfer of PPV genome fragments into plum (Ravelonandro *et al.* 1998; Scorza *et al.* 2001). Other viral genome sequences (P1 and CP) have also been used to generate PPV resistance in plum using a hairpin design (Wang *et al.* 2008). However, the level of resistance varied with the specific viral region used. The results presented here indicate that by using a conserved region of the HC-Pro gene a high proportion of transgenic lines become highly resistant to PPV.

The presence of transgene-specific siRNAs was analyzed in transgenic lines *via* Northern blot assay using a probe specific for the HC-Pro transgene. To ensure that siRNAs were derived from the transgene and not from the infecting virus, these experiments were conducted prior to the PPV inoculations. Two species of siRNAs of approximately 21-22 nts and 25-27 nts in length were detected in most transgenic plants (**Table 1**, **Fig. 3**). The size of these RNAs corresponded to the two classes of siRNAs which are associated with the induction of RNA silencing in transgenic plants (Hamilton *et al.* 2002; Hily *et al.* 2005). The presence of siRNA molecules in plants appeared to be associated with PPV resistance. For instance, line KL5, KL22-3, KL21-2, KL22-1, KL22-11, KL23-2 KL27-1, showed high levels of PPV resistance over different cold treatments and siRNA molecules were clearly detected in these plants. On the other hand, siRNAs were not detected in line KL22-10 which was susceptible to PPV over different times (**Table 1**). As expected, siRNA molecules were also not detected in any of the control plants (**Fig. 3**).

In conclusion, we have shown that PPV resistance can be efficiently induced in transgenic plum plants with an RNA silencing-based strategy using an HC-Pro transgene in an intron-spliced hairpin conformation. The resistance was

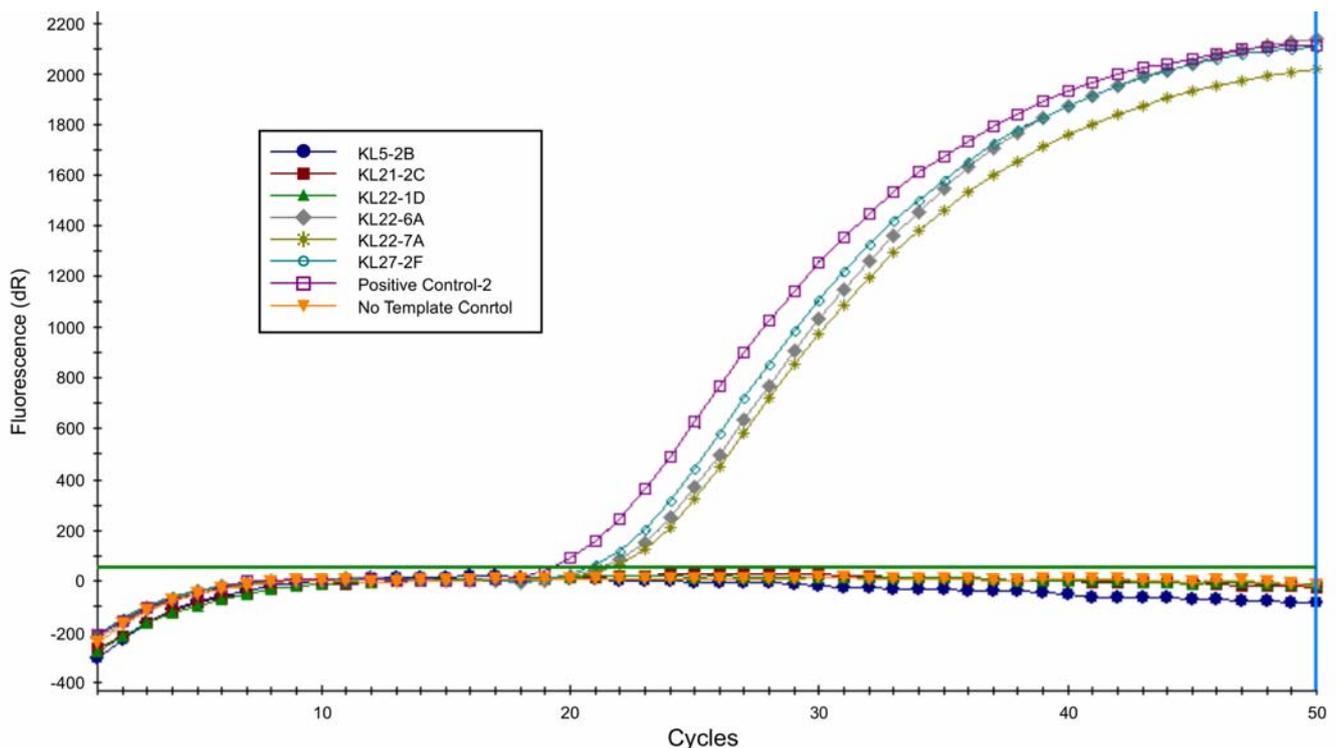


Fig. 2 Amplification curves of analysis of representative transgenic plum plants. The plants for PPV resistance were tested with real-time PCR using a D-primer for PPV and HEX probe. Samples were taken after the 3rd dormant period and plants were deemed susceptible if the C_T was less than 35.

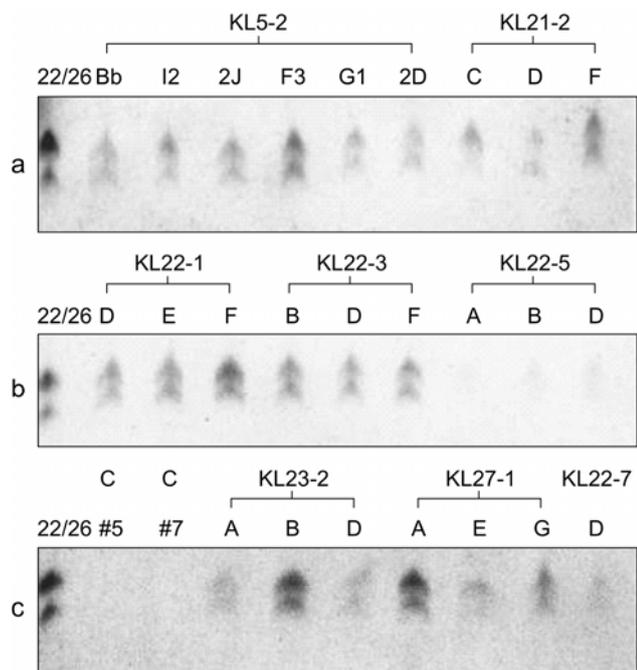


Fig. 3 siRNA analysis in selected plum transgenic plants and control plants. Total RNA was isolated from plants and was analyzed via Northern blot using PPV HC-Pro sequence as a probe. Sub-figures with a, b, c: different siRNA detection experiments. KL5-2, KL21-2, KL22-1, KL22-3, KL22-5, KL23-2, KL27-1, KL22-7: different transgenic plum lines. The number and letters under each line stand for individual plants within the line. C: control plant.

stable over an extended time period and over repeated growth and dormancy periods.

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REFERENCES

Baulcombe D (1999) Viruses and gene silencing in plants. *Archives of Virology* **15** (Suppl), 189-201
Hamilton AO, Voinnet L, Chappell, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. *The EMBO Journal* **21**, 4671-4679
Hily JM, 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

James D, Varga A (2005) Nucleotide sequence analysis of plum pox virus isolate W3174: evidence of a new strain. *Virus Research* **110**, 143-150
Kegler H, Fuchs E, Gruntzig M, Schwarz S (1998) Some results of 50 years of research on the resistance to plum pox virus. *Acta Virologica* **42**, 200-215
Kim WS, Stobbs LW, Lehman SM, James D, Svircev AM (2008) Direct real-time PCR detection of Plum pox virus in field surveys in Ontario. *Canadian Journal of Plant Pathology* **30**, 308-317
Levy L, Damsteegt V, Mavrodieva V, Goley E, Welliver R, Luster D (2000) Partial characterization of the Pennsylvania strain of plum pox virus (PPV-PA). *Phytopathology* **90**, S48 (Abstract)
Lopez-Moya JJ, Fernandez-Fernandez MR, Cambra M, Garcia JA (2000) Biotechnological aspects of plum pox virus. *Journal of Biotechnology* **76**, 121-136
Nemeth M (1994) History and importance of plum pox in stone fruit production. *EPPO Bulletin* **24**, 525-536
Nicola-Negri ED, Brunetti A, Tavazza M, Ilardi V (2005) Hairpin RNA-mediated silencing of plum pox virus P1 and HC-Pro genes for efficient and predictable resistance to the virus. *Transgenic Research* **14**, 989-994
Pandolfini T, Molesini B, Avesani L, Spina A, Polverari A (2003) Expression of self-complementary hairpin RNA under the control of the rolC promoter confers systemic disease resistance to plum pox virus without preventing local infection. *BMC Biotechnology* **3**, 7
Ravelonandro M, Scorza R, Dunez J (1998) Characterization of phenotype resistance to plum pox of transgenic plums expressing plum pox virus capsid gene. *Acta Virologica* **42**, 270-272
Rochon D, Theilmann J, James D, Reade R, Yang L, Upton C (2003) Partial molecular characterization of plum pox virus isolates occurring in Canada. *Canadian Journal of Plant Pathology* **25**, 198-208
Scorza R, Callahan A, Levy V, Damsteegt V, Webb K, Ravelonandro M (2001) Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Research* **10**, 201-209
Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320
Thompson D, McCann M, MacLeod M, Lye D, Green M, James D (2001) First report of plum pox potyvirus in Canada. *Plant Disease* **85**, 97
Tian L, Sibbald S, Subramanian J, Svircev A (2006) Characterization of *Prunus domestica* L. *in vitro* regeneration via hypocotyls. *Scientia Horticulturae* **112**, 462-466
Tian L, Fatih C, Wang X, Sibbald S (2009) Genetic transformation of *Prunus domestica* L. using the *hpt* gene coding for hygromycin resistance as the selectable marker. *Scientia Horticulturae* **119**, 339-343
Wang A, Tian L, Svircev A, Brown D, Stobbs L, Sanfaçon H, Miki B (2009) The development of genetic resistance to Plum pox virus in transgenic *Nicotiana benthamiana* and *Prunus domestica*. *Acta Horticulturae* **839**, 665-672
Waterhouse PM, Smith NA, Wang MB (1999) Virus resistance and gene silencing: killing the messenger. *Trends in Plant Science* **4**, 452-457
Wiersma PA, Wu Z, Zhou L, Hampson C, Kappel F (2001) Identification of new self-incompatibility alleles in sweet cherry (*Prunus avium* L.) and clarification of incompatibility groups by PCR and sequencing analysis. *Theoretical and Applied Genetics* **102**, 700-708
Zhang CS, Tian L, Svircev A, Sibbald S, Schneider KE, Barszcz ES, Wen R, Sanfaçon H (2006) Engineering resistance to Plum pox potyvirus (PPV) through the expression of PPV-specific hairpin RNAs in transgenic plants. *Canadian Journal of Plant Pathology* **28**, 263-270