

Hairpin Plum pox virus coat protein (hpPPV-CP) structure in ‘HoneySweet’ C5 plum seedlings provides PPV resistance when genetically engineered into plum (*Prunus domestica*)

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Abstract

The genetically engineered plum ‘HoneySweet’ (aka C5) has proven to be highly resistant to *Plum pox virus* (PPV) for over 10 years in field trials. The original vector used for transformation to develop ‘HoneySweet’ carried a single sense sequence of the full length PPV coat protein (*ppv-cp*) gene, yet DNA blot analyses indicated that there was an inserted copy of the *ppv-cp* that appeared to be an inverted repeat structure. Since the resistance mechanism of ‘HoneySweet’ was found to be based on post-transcriptional gene silencing (PTGS), it was hypothesized that the inverted repeat structure conferred the resistance to PPV in ‘HoneySweet’. Sequencing of the transgene insertions confirmed the presence of an inverted repeat of the PPV-CP sequence. We hypothesized that transcription from this structure produced a hairpin (hp) RNA that was responsible for PTGS of the transgene and the destruction of PPV viral RNA resulting in the high level of resistance to PPV infection. In order to confirm this hypothesis the hpPPV-CP insert was cloned from ‘HoneySweet’ and transferred into ‘Bluebyrd’ plum seedlings through *Agrobacterium tumefaciens* transformation of hypocotyl slices. The introduced DNA contained the CP inverted repeat flanked by 35S promoters on either end. Transgenic plum plants containing single or multiple copies of this hp insert were inoculated with PPV D isolated from Pennsylvania, USA. PPV infection was evaluated through three cycles of cold-induced dormancy (CID) by symptom expression and by two or more ELISA and PCR tests. Of the 18 plants evaluated, eight were always virus-free, five occasionally had weak or moderate infections, and five plants were clearly infected in multiple tests. While all plants of some clones were virus-free others had a mix of uninfected and mildly infected plants of the same clone. Most of the resistant plants contained a single copy of the hp construct. These data strongly support the hypothesis that the hp structure of the *PPV-CP* insert in ‘HoneySweet’ plum can confer PPV resistance.

Keywords: breeding, gene silencing, Rosaceae, sharka

Introduction

The earliest reports of gene silencing involved the transformation of petunia with the chalcone synthase gene for the purpose of increasing purple flower color. Unexpectedly, activity of the native chalcone synthase gene was shut down and white or partially white flowers resulted (Napoli et al., 1990). This homology-related gene silencing phenomenon appeared to be RNA-related and was shown to be triggered by double stranded (ds) RNA (Hannon 2002; Kooter et al., 1999; Matzke et al., 2001; Vaucheret et al., 2001; Waterhouse et al., 2001). RNA silencing participates in the regulation of endogenous gene expression in developmental processes and serves as a component of the protective mechanism against mobile genetic elements, such as transposons and viruses (Voinnet, 2005). A key component of gene silencing is short interfering RNA (siRNA). siRNAs are derived from the cleavage of dsRNA by the Dicer enzyme a member of the RNase II family that specifically cleaves dsRNA. ds RNAs are cleaved into RNA duplexes of 21 to 28 nucleotides (Hamilton and Balcombe, 1999; Bernstein et al., 2001; Elbashier et al., 2001; Baulcombe 2004). These siRNAs, corresponding to both sense and antisense strands, guide a multi-subunit ribonuclease, the RNA-induced silencing complex (RISC), and ensure that it specifically degrades RNAs that share sequence similarity with the dsRNA. Inverted repeats of the target gene have been shown to efficiently trigger silencing (Stam et al., 1997) presumably through the generation of dsRNAs. Self-complementary hairpin-RNA (hpRNA) constructs induce a high level of PTGS in transgenic plants (Wesley et al., 2001). The presence of an intron between the two complementary regions enhances silencing efficiency (Smith et al., 2000).

Specifically for resistance to PPV Pandolfini et al. (2003) showed that the expression of hpRNA containing a *PPV* sequence conferred systemic resistance to PPV but did not prevent local infection when introduced into *N. benthamiana* under the control of the *rolC* promoter. Di Nicola-Negri et al. (2005) reported that more than 90 % of transgenic *N. benthamiana* lines were resistant to the virus when engineered with hairpin constructs using *PPV-PI* and *PPV-Hc-Pro* gene sequences under 35S-Cauliflower mosaic virus (CaMV) promoter. Hily et al (2007) tested four ihpRNA gene constructs of the PPV-CP gene and showed that full-length (1 kb) and 213 bp ihpRNA PPV-CP sequences could induce siRNA production, gene silencing, and PPV resistance.

The clearest case of gene silencing based PPV resistance has been reported in plum in a clone that was originally transformed with a sense construct of the PPV-CP gene (Scorza et al, 1994; Ravelonandro et al, 1997). The high level of resistance of the C5 clone (cv. 'HoneySweet') has been demonstrated for over 10 years in field tests in 4 countries (Hily et al, 2004; Malinowski et al, 2006; Zagrai et al., 2008). The complex insertion of the sense PPV-CP gene (Scorza et al., 1994) appeared to include adjacent complementary copies of the PPV-CP gene which would produce hpRNA (Figure 1). In this report we confirm the presence of the predicted rearranged PPV-CP (hp) sequence through sequencing. We show the hp nature of the insert and through cloning of the insert and transformation into plum we demonstrate that the *PPV-CP* hp sequence from 'HoneySweet' plum provides PPV resistance.

Materials and methods

BAC library construction followed the procedures of Georgi et al. (2002). High-molecular-weight DNA was extracted in solution from leaves of transgenic plum C5.

Hybridization: Probes were made from PCR fragments representing PPV-CP and labeled with $\alpha^{32}\text{P}$ dCTP by random priming. Each BAC clone from the 'HoneySweet' library was double spotted on a membrane and hybridized with the probe. Hybridization signals were detected autoradiographically.

BAC DNA extraction: BAC DNA was extracted by a modified alkaline-lysis procedure. Larger-scale BAC DNA extractions (50–500 ml culture volume) were additionally purified on cesium chloride density gradients using a Beckman TL100 ultracentrifuge.

Confirmation of selected clones: BAC DNA prepared from positive clones was digested with *EcoRI*, *BamHI* or *HindIII*, electrophoresed on 0.8 % SeaKem LE agarose and stained with ethidium bromide. Southern transfer of the DNA to Hybond N+ membranes was performed using the manufacturer's Alkaline Transfer Protocol. Southern blots were hybridized with appropriate probes.

Subcloning and sequencing: For sequencing, BAC DNA was digested with *Sau3AI*, *EcoRI*, *BamHI* or *HindIII*, and the resulting fragments were ligated into pUC19 or pBluescript and transformed into *Escherichia coli* strain DH5 α by calcium/heat shock (Sambrook et al 1989). Clones were individually grown and plasmid DNA for sequencing was prepared using a protocol similar to that used for BAC DNA. Subclones were sequenced using ABI's Dye-deoxy terminator cycle sequencing kit and an ABI377 DNA sequencer. Sequences were assembled using Sequencher 4.2 software (GeneCodes Corp.).

Plasmid construction: A 3.1 kb *HindIII* fragment from a BAC subclone was ligated into pBINPLUS/ARS a pBIN19-based plant transformation plasmid (van Engelen et al., 1995). Sequence 1738 consisted of the sense and antisense complementary PPV-CP sequences and their respective 35S promoters (Figure 1). *Agrobacterium tumefaciens* EHA 105 was electrotransformed with this plasmid yielding the strain WV1738.



Fig. 1 The PPV-CP hairpin insert cloned out of C5 plum and used to produce new transgenic plum clones. (35S = cauliflower mosaic virus promoter; CP = the Plum pox virus coat protein gene; CP-3' = the 3' an incomplete untranslated end of CP; 3'-CP = a longer incomplete 3' untranslated end of CP.)

Plum transformation and transgenic plant production: Seed hypocotyls of 'Bluebyrd' (Scorza and Fogle, 1999), a PPV susceptible plum variety, were used as explants for transformation using the protocol of Petri et al. (2008). Plum plants that regenerated shoots and rooted in vitro under kanamycin selection were transferred to a greenhouse. DNA blotting following the procedures of Petri et al. (2008) was used to confirm integration of the insert into the plant genome and to estimate transgene copy number in each transgenic line.

Evaluation of PPV infection: The PPV virus source for aphid inoculation and PPV infection evaluation were as described in Hily et al. (2007). Briefly, aphids were starved for 30 min and then allowed to feed on detached, highly symptomatic leaves of GF305 peach seedlings for virus acquisition. Infected leaves with aphids were then placed onto plum test plants in cages which included transformed and non-transformed susceptible plum seedlings. Aphids were allowed to move from peach leaf pieces to plum leaves over a 24-hour inoculation access period (IAP) and inoculations were repeated up to 3 times if aphid numbers were low. Plants were sprayed with an appropriate insecticide.

Inoculated plants were placed under natural daylight conditions supplemented with 400-W lamps to provide a 15-h photoperiod. Temperature was maintained at 24 °C. All plants were assayed by ELISA and any plants testing negative for two consecutive assays were re-inoculated as above. All plants then were exposed to cold-induced dormancy (CID) at 4-5 °C for 60 days. Plants were removed from the CID treatment, allowed to re-flush in a warm greenhouse, and re-evaluated for infection.

PPV infection of test plants was evaluated by visual observation of symptoms and by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Leaf samples ranged from 0.25 to 0.5 g, and were ground in PBS (Phosphate Buffered Saline) -Tween ELISA buffer at a 1:10 ratio (w:v). Leaf extract samples were centrifuged for 5 min at 1200 xG (Beckman GPR centrifuge; Beckman Instruments, Palo Alto, CA). A one ml sample of the supernatant was collected and then processed according to the manufacturer's protocol (REAL[®] Kit, Durviz S.L.U., Paterna, Spain). Plant samples with ELISA values three times greater than the background were considered positive.

Results

Sequence analyses of 'HoneySweet': One BAC clone contained sequence that consisted of *ppv-cp* and 35S-35S promoter but did not have any of the other sequence from the vector including the marker *uidA* gene or NPTII. Sequence analysis determined that this sequence represented the postulated inverted repeat sequence. The 35S-35S promoter sequence was followed by the cp sequence and an incomplete 3' untranslated end, then the inverse of an incomplete but longer stretch of the 3' untranslated end, the coding sequence of the cp and the 35S-35S promoter sequence (Figure 1). This structure was inserted into plum DNA.

Transfer of *ppv-cp* inverted repeat: A 3.1 kb fragment was transferred to plum seedlings through *Agrobacterium* mediated transformation. Eight distinct clones containing the C5 insert (*C5IN*) were selected, and DNA blotting revealed that C5IN copy number ranged from 1 to 5 in regenerated clones.

PPV Resistance: Eight unique transgenic clones were evaluated. Five of the eight unique clones were vegetatively multiplied through self-rooting to produce a total of 19 plants that along with control plants (non-transgenic and C5) that were inoculated with PPV. Over the course of three consecutive cycles each cycle consisting of a growth period and a CID period the plants were monitored for symptoms and periodically tested during the growing period for the presence of the virus by ELISA, PCR and by quantitative real-time PCR to detect very low levels of infection. Of 18 plants evaluated, eight plants had no detectable levels of virus, five plants on occasion had weak or moderate levels of infection, and five plants were clearly infected in multiple tests (Table 1).

Tab. 1 Results of multiple evaluations of PPV infection of transgenic plum plants containing the PPV-CP hairpin insert from plum clone C5. Inoculations were aphid mediated with the PPV-D serotype from Pennsylvania USA. All clones contain the same construct but may have single or multiple copy inserts (ND = no data).

Clone/plant	# C5 inserts	Symptoms	ELISA 1	ELISA 2	PCR 1	PCR 2	PCR 3
D/1	1	-	-	-	-	-	-
D/2	1	-	-	-	-	-	-
F/1	1	-	-	-	-	-	ND
F/2	1	-	-	-	-	-	ND
F/3	1	-	-	-	-	ND	ND
G/1	1	-	-	-	-	+	+
G/2	1	-	-	-	-	ND	ND
G/3	1	-	-	+/-	-	ND	ND
G/4	1	-	+/-	+/-	-	ND	ND
K/1	2	-	-	-	-	-	ND
A/1	2	++	+	+	-	++	+
L/1	2-3	+	+	+	-	ND	ND
L/2	2-3	-	-	+	-	++	++
L/3	2-3	+	+	+	-	++	++
L/4	2-3	-	-	+	+	-	ND
E/1	4	-	+	+	-	++	++
H/1	>5	-	-	-	-	-	-
H/2	>5	+	-	+	-	ND	ND

Where multiple plants of a single clone were tested, all the plants for two clones, D and F were resistant while in G and H some plants tested completely negative and some gave a weak positive reaction. Several plants in clones A, E, and L gave strong positive reactions in some tests, generally PCR. There were few other strong positive reactions. Non-transformed control plants were clearly infected and strongly positive early in the inoculation tests. The original C5 clone was clearly resistant.

Discussion

C5 plum originated from transformation with a sense PPV-CP construct. This clone was the only highly resistant clone produced using that particular PPV-CP construct. All other clones were susceptible. C5 contained a duplicated and rearranged transgene insert (Scorza et al., 1994). It was suspected that one component of the insert in C5 was a tandem duplication of the CP gene and this component may have been responsible, at least in part, for resistance. Through the construction of BAC libraries of C5 plum, followed by gene cloning and sequencing we isolated the PPV-CP hairpin structure from C5. This structure was then engineered into a plant transformation vector and used to develop new C5 insert (C5IN) transgenic plants. Upon inoculation with PPV these plants were generally resistant to PPV indicating that the PPV-CP hp portion of the transgene insert in C5 is, if not solely, at least one of the components of the C5 transgene insert responsible for PPV resistance in this clone. Variability in resistance when using hp constructs is common and the basis for this variability is not known. Although all of the clones that were clearly susceptible to PPV in this trial were multicopy clones, other authors have found either no correlation between hp insert copy number and resistance (Hily et al., 2007) or a positive correlation between resistance and copy number (Kalantidis et al., 2002). Smith et al. (2000) found that many of the virus “immune” plants developed from hp transformants contained single copy inserts.

The confirmation of a PPV-CP hairpin insert in the PPV resistant plum clone C5 provides conclusive evidence of the mechanism for resistance. Further, almost 20 years of work with the C5 clone including over 10 years of field testing of resistance in four countries demonstrated the stability, efficacy and safety of hp gene silencing for PPV resistance in plum (Capote et al., 2008; Fuchs et al., 2007; Hily et al., 2004, 2005, 2007; Kundu et al., 2008; Malinowski et al., 2006; Polak et al., 2005, 2008; Ravelonandro et al., 1994, 1997, 1998a,b,c, 2001, 2002a,b, 2004; Scorza et al., 1994, 1998, 2001a,b, 2007; Scorza and Ravelonandro, 2006; Zagrai et al., 2007, 2008). Current efforts with C5 (‘HoneySweet’) are focused on the deregulation of this cultivar (Scorza et al., 2007) in order to make it available to breeders and growers who face the serious threat of PPV.

Literature

- Baulcombe, D.C.; 2004: RNA silencing in plants. *Nature* **431**, 356-363.
- Bernstein, E.; Caudy, A. A.; Hammond, S. M.; Hannon, G. J.; 2001: Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.
- Capote, N.; Perez-Panades, J.; Monzo, C.; Carbonell, E.; Urbaneja, A.; Scorza, R.; Ravelonandro, M.; Cambra, M.; 2008: Assessment of the diversity and dynamics of Plum pox virus and aphid populations in transgenic European plums under Mediterranean conditions. *Transgenic Res.* **17**, 367-377.
- Di Nicola-Negri, E.; 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 Res.* **14**, 989-994.
- Elbashier, S. M.; Lendeckel, W.; Tuschl, T.; 2001: RNA interference is mediated by 21 and 22 nt RNAs. *Genes and Dev.* **15**, 188-200.
- Fuchs, M.; Cambra, M.; Capote, N.; Jelkmann, W.; Kundu, J.; Laval, V.; Martelli, G.P.; Minafra, A.; Petrovic, N.; Pfeiffer, P.; Pompe-Novak, M.; Ravelonandro, M.; Saldarelli, P.; Stussi-Garaud, C.; Vigne, E.; Zagrai, I.; 2007: Safety assessment of transgenic plums and grapevines expressing viral coat protein genes: New insights into real environmental impact of perennial plants engineered for virus resistance. *J. Plant Path.* **89**, 5-12.
- Georgi, L. L.; Y. Wang, Y.; Yvergnaux, D.; Ormsbee, T.; Iñigo, M.; Reighard, G.; Abbott, A. G.; 2002: Construction of a BAC library and its application to the identification of simple sequence repeats in peach [*Prunus persica* (L.) Batsch.]. *Theor Appl Genet.* **105**, 1151-1158.
- 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.; Ravelonandro, M.; Damsteegt, V.; Bassett, C.; Petri, C.; Liu, Z.; Scorza, R.; 2007: Plum pox potyvirus coat protein gene intron-hairpin-RNA (ihpRNA) constructs provide resistance to Plum pox virus in *Nicotiana benthamiana* Domin. and plum (*Prunus domestica* L.). *J. Amer. Soc. Hort. Sci.* **132**, 850-858.
- 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 Res.* **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. *MPMI.* **18**, 794-799.

- Kooter, J. M.; Matzke, M. A.; Meyer, P.; 1999: Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* **4**, 340-347.
- Kundu, J.; Pascal, B.; Hily, J. M.; Ravelonandro, M.; Scorza, R.; 2008: Role of the 25-26 nt long siRNA in the resistance of transgenic *Prunus domestica* graft inoculate with Plum Pox Virus. *Virus Genes* **36**, 215-220.
- Liu, Z.; Scorza, R.; Hily, J. M.; Scott, S. W.; James, D.; 2007: Engineering resistance to multiple stone fruit viruses through expression of chimeric hairpins. *J. Amer. Soc. Hort. Sci.* **132**, 407-414.
- Malinowski, T.; Cambra, M.; Capote, N.; 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.
- Matzke, M. A.; Matzke, A. J.; Pruss, G. J.; Vance, V. B.; 2001: RNA-based silencing strategies in plants. *Curr. Opin. Genet. Dev.* **11**, 221-227.
- Pandolfini, T.; Molesini, B.; Avesani, L.; Spena, 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. *BioMed Central Biotechnology*. **3**, 7.
- Petri, C.; Webb, K.; Hily, J. M.; Dardick, C.; Scorza, R.; 2008: High transformation efficiency in plum (*Prunus domestica* L.): a new tool for functional genomic studies in *Prunus* spp. *Mol. Breed.* **22**, 581-591.
- Polak, J.; Pivalova, J.; Jokes, 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.
- Polak, J.; Pivalova, J.; Kundu, J. K.; Jokes, M.; Scorza, R.; Ravelonandro, M.; 2008: 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 Plant Path* **90**, S1 33-36.
- Ravelonandro, M.; Scorza, R.; Callahan, A.; Cordts, J. M.; Monsion, M.; Fuchs, M.; Delbos, R.; Bachelier, J.; Gonsalves, D.; Dunez, J.; 1994: Production of transgenic plants of *Nicotiana benthamiana* and *Prunus domestica* transgenic for the plum pox potyvirus coat protein and demonstration of PPV resistance in transformed *N. benthamiana*. *EPPO Bulletin*. **24**, 713-719.
- 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.; Dunez, J.; 1998a: Characterization of phenotype resistance to plum pox of transgenic plums expressing plum pox capsid gene. *Acta virologica*. **42**, 270-272.
- Ravelonandro, M.; Scorza, R.; Labonne, G.; Dunez, J.; 1998b: Challenging transgenic plums expressing potyvirus coat protein genes with viruliferous aphids. *Acta Hort.* **472**, 413-420.
- Ravelonandro, M.; Scorza, R.; Renaud, R.; Salesses, G.; 1998c: Transgenic plums resistant to plum pox virus infection and preliminary results of cross-hybridization. *Acta Hort.* **478**, 67-71.
- 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 epidemics: the plum pox (sharka) model. *Virus Research* **71**, 63-69.
- Ravelonandro, M.; Briard, P.; Scorza, R.; 2001: Significant resistance of transgenic plums against the four serotypes of plum pox potyvirus. *Acta Hort.* **550**, 431-435.
- Ravelonandro, M.; Briard, P.; Monsion, M.; Renaud, R.; Scorza, R.; 2002a: Stable transfer of the plum pox virus (PPV) capsid transgene to seedlings of two French cultivars 'Prunier d' Ente 303' and 'Quetsche 2906', and preliminary results of PPV challenge assays. *Acta Hort.* **577**, 91-96.
- Ravelonandro, M.; Minoiu, N.; and Scorza, R.; 2004: Investigation of potential environmental impacts in the release of transgenic plums. *Acta Hort.* **657**, 325-329.
- Ravelonandro, M.; Briard, P.; Scorza, R.; 2002b: Resistance to Plum pox virus resistance in transgenics is mediated by specific sequences of capsid gene. *Sănătatea* **6**, 19-21.
- Ravelonandro, M.; Kundu, J.; Scorza, R.; 2007: The effect of co-existing virus on transgenic resistant plums. *Acta Hort.* **738**, 653-656.
- Sambrook, J.; Fritsch, E. F.; Maniatis, T.; 1989: Molecular cloning: a laboratory manual, 2nd edn. Cold Springs Harbor Laboratory, Cold Springs Harbor, New York.
- Schneider, W. L.; Sherman, D. L.; Stone, A. L.; Damsteegt, V. D.; Frederick, R. D.; 2004: Specific detection and quantification of plum pox virus by real-time fluorescent reverse transcription-PCR. *J. Virol. Methods* **120**, 97-105.
- Scorza, R.; Ravelonandro, M.; Callahan, A. M.; Cordts, J.; Fuchs, M.; Dunez, J.; Gonsalves, D.; 1994: Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. *Plant Cell Repts.* **14**, 18-22.
- Scorza, R.; Callahan, A. M.; Levy, L.; Damsteegt, V.; Ravelonandro, M.; 1998: Transferring potyvirus coat protein genes through hybridization of transgenic plants to produce plum pox virus resistant plums (*Prunus domestica* L.). *Acta Hort.* **472**, 421-27.
- Scorza, R.; Fogle, H. W.; 1999: 'Bluebyrd' plum. *HortScience*. **34**, 1129-1130.
- Scorza, R.; Callahan, A.; Levy, L.; Damsteegt, V.; Webb, K.; Ravelonandro, M.; 2001a: Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Res.* **10**, 201-209.

- Scorza, R.; Callahan, A.; Levy, L.; Damsteegt, V.; Ravelonandro, M.; 2001b: Resistance to plum pox potyvirus in a transgenic woody perennial fruit tree, European plum (*Prunus domestica* L.) results from post-transcriptional gene silencing. *Acta Hort.* **550**, 425-430.
- Scorza, R.; Malinowski, T.; Minoiu, N.; Cambra, M.; Ravelonandro, M.; 2004: Potential use of transgenic plums resistant to *Plum pox virus* field infection. *Acta Hort.* **657**, 321-324.
- Scorza, R.; Webb, K.; Ravelonandro, M.; 2004: Analysis of methylation and post-transcriptional gene silencing in embryos and seedlings of C5, a transgenic *Plum pox virus* resistant plum *Prunus domestica* L. *Acta Hort.* **657**, 317-320.
- 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. M.; Malinowski, T.; Cambra, M.; Capote, N.; Zagrai, I.; Damsteegt, V.; Pascal, B.; and Ravelonandro, M.; 2007: Deregulation of plum pox resistant transgenic plum 'HoneySweet'. *Acta Hort.* **738**, 669-673.
- Smith, N. A.; Singh, S. P.; Wang, M. B.; Stoutjesdijk, P. A.; Green, A. G.; Waterhouse, P. M.; 2000: Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.
- van Engelen, F.; Molthoff, J. W.; Conner, A. J.; Nap, J. P.; Pereira, A.; Stiekema, W. J.; 1995: pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res.* **4**, 288-290.
- Vaucheret, H.; Beclin, C.; Fagard, M.; 2001: Post-transcriptional gene silencing in plants. *J. Cell Sci.* **114**, 3083-3091.
- Voinnet, O.; 2005: Induction and suppression of RNA silencing: insights from viral infections. *Nature Rev. Genet.* **6**, 206-220.
- Waterhouse, P. M.; Wang, M. B.; Lough, T.; 2001: Gene silencing as an adaptive defense against viruses. *Nature* **411**, 834-842.
- Wesley, S.V.; Helliwell, C. A.; Smith, N. A.; Wang, M. B.; Rouse, D. T.; Liu, Q.; Gooding, P. S.; Singh, S. P.; Abbott, D.; Stoutjesdijk, P. A.; Robinson, S. P.; Gleave, A. P.; Green, A. G.; Waterhouse, P. M.; 2001: Construct design for efficient, effective and high throughput gene silencing in plants. *Plant J.* **27**, 581-590.
- Zagrai, I.; Ravelonandro, M.; Scorza, R.; Gaboreanu, I.; Ferencz, B.; Popescu, O.; Pamfil, D.; Zagrai, L.; Maxim, A.; 2007: Serological and molecular variability of *Plum pox virus* in transgenic and conventional plums. *Acta Hort.* **738**, 703-710.
- Zagrai, I.; Capote, N.; Ravelonandro, M.; Cambra, M.; Zagrai, L.; Scorza, R.; 2008: Plum pox virus silencing of C5 transgenic plums is stable under challenge inoculation with heterologous viruses. *J. Plant. Path.* **S1** 63-71.