Pathogen-derived methods for improving resistance of transgenic plums (*Prunus domestica* L.) for Plum pox virus infection

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Abstract

Plum pox virus (PPV), the causal agent of Sharka disease, is considered to be one of the most serious pathogens of stone fruits including apricots, plums and peaches. This disease is of particular concern in central and southern Europe, the Mediterranean areas and North America.

The transformation of plum with viral genes, such as coat protein, can provide virus resistant varieties or gene resources for breeding new resistant varieties. In the current study we report the evaluation of two technologies for producing plants resistance to PPV, one based on co-suppression and another on RNA-silencing. Two gene constructs were evaluated; the binary vector pCamPPVcp that contained the selective *hpt* gene and *ppv-cp* gene in sense-orientation (driven by double 35S promoter) and vector pCamPPVRNAi that contained self-complementary fragments of gene *ppv-cp* (698bp) driven by double 35S promoter and the *hpt* and *gus* genes.

The fragments of the *ppv-cp* gene in pCamPPVRNAi were separated by a pdk-intron to produce a “hairpin” RNA structure in antisense-sense orientation. Seven independent transgenic lines with the sense-oriented *ppv-cp* gene and five transgenic lines with inverted repeats of the *ppv-cp* gene fragment were produced. The accumulation of coat protein in five pCamPPVcp lines was confirmed by Western blotting. Transgenic shoots were rooted and acclimatized to the greenhouse. After grafting with PPV infected buds PPV-CP was detected by Western blotting in all control and pCamPPVcp transformed plants whereas no PPV coat protein were observed in samples from plants transformed with the pCamPPVRNAi “hairpin” construct. These preliminary results confirmed the efficiency of the RNAi strategy for producing virus resistant plants in general and PPV resistant stone fruits in particular.

Keywords: RNA interference, PPV, transformation, coat protein, *Prunus domestica*.

Introduction

Stone fruit trees are valued for the quality of their fruits and as sources of hard wood worldwide. Virus and fungus diseases are main the most important pathogens of stone fruits. PPV, the causal agent of Sharka disease, is considered the most important pathogen in peach and nectarine, apricot, plum and cherry world-wide. A critical review of the literature dealing with PPV resistance, suggests that there is no source of high-level resistance or immunity to PPV in *Prunus* that protects trees against all strains of the virus. Considering the severity of the disease, the difficulty in controlling its spread, and the lack of resistant varieties, the need for novel approaches to the development of resistance is apparent.

Modern biotechnology presents new pathogen resistance strategies, especially applicable to virus resistance. The concept of pathogen-derived resistance (PDR) proposed by Sanford and Johnston (1985) has been widely used as a basis for obtaining virus-resistant plants. Since the first success with the tobacco plants transformed with the tobacco mosaic virus (TMV) coat protein (CP) gene (Powell et al., 1986), this strategy, based on transgene produced viral CP, has been the most studied application of PDR and has provided various degrees of protection (delay in the symptom development, partial resistance or immunity) against numerous plant virus groups (for review, see Beachy, 1990; Lomonossoff, 1995). This work led to the notion that the level of virus resistance was directly related to the CP expression level of transgenic lines. Further investigations by several laboratories, however, led to the surprising finding that that some transgenic lines with high virus resistance levels in fact did not express any viral CP. Moreover, the CP RNA level was very low or even not detectable in these resistant plants. Subsequent work clarified this apparent discrepancy of non-expressing transgenic plants with virus resistance. We now know that these transgenic lines were resistant to virus because the expressed CP mRNA triggered post-transcriptional gene silencing (PTGS) and provided RNA-mediated virus resistance by the siRNA pathway.

The siRNA pathway targets double-stranded (ds) RNA for degradation by DICER-like proteins (DCLs) in a sequence-specific manner through the production of siRNA. Whereas DCL2 cleaves dsRNAs from replicating viruses (Xie et al., 2004), DCL3 cleaves dsRNAs derived from endogenous transcripts through the activity of RDR2 and RDR6 (Mourrain

et al., 2000). The siRNAs produced are incorporated into RNA-induced silencing complexes (RISC), which guide cleavage of target RNAs. In RISC, siRNAs mediate sequence-specific binding and cleavage of target RNAs (Baulcombe, 2004). Once cleaved, the RNA is further degraded by exonucleases in the cytoplasm. Alternatively, siRNAs are used as primers for RDR polymerase, using target RNA as a template to generate more dsRNA and produce additional siRNAs. This RDR activity expands the pool of siRNA and amplifies PTGS resulting in more potent silencing activity and effective defense against plant viruses. Thus, in the virus-resistant lines, not only the transgene mRNAs but also the invading viral RNA (with homology to the transgene) was degraded.

In addition to CP mRNA, RNA-mediated virus resistance can be brought about by expression of satellite RNA, defective interfering (DI) RNA or even noncoding regions of viral genome RNAs that compete and interfere with virus replication (Baulcombe, 1996). This type of resistance can also be accomplished by expression of viral sequences in the sense or antisense orientation (Smith et al., 1994; Waterhouse et al., 1998) or in double-stranded forms (Helliwell and Waterhouse, 2003). In all these cases, expression triggers degradation of both the transgene RNA and the corresponding viral RNA via the siRNA pathway.

RNA interference (RNAi) is a potent method requiring only a few double stranded RNA (dsRNA) molecules per cell to silence the viral gene expression. This has made it one of the hottest topics in molecular biology in recent years. Reports from several laboratories have established that the loss of the target mRNA in steady-state accumulation is almost total if the designed transgene construct of the transgenic plant produces the nuclear transcript in the duplex conformation. Recently it was reported that the expression of self-cRNA of PPV under the control of the *rolC* promoter caused degradation of transgenic viral RNA and as a result, systemic disease resistance to challenge inoculums of PPV in transgenic *Nicotiana benthamiana* (Pandolfini et al., 2003). This evidence points out that the production of dsRNA is required to initiate PTGS in plants. Based on this, plants carrying strongly transcribing transgenes in both the sense and antisense orientations are currently being produced that show strong PTGS features (Chuang and Meyerowitz, 2000; Smith et al., 2000).

Materials and methods

Plant culture media and culture conditions: Shoot cultures of plum [*Prunus domestica* (L.)] were established *in vitro* from buds of 4-year-old trees of cultivars "Startovaya". Micro-shoots were maintained *in vitro* on JS proliferation medium (modified QL). JS proliferation medium consisted of salts supplemented with (mg/l) myo-inositol 100; thiamine HCl 0.1; nicotinic acid 0.5; pyridoxine HCl 0.5; glycine 2.0; sucrose 3 % (w/v), 0.7 % agar (w/v). The growth regulators used to plum multiplication were 2 mg/l BAP and 0.1 mg/l IBA. The medium was adjusted to pH 5.8 using 1N KOH and autoclaved for 20 min at 1.4 kg/cm^2 (121 °C). All phytohormones and antibiotics were filter-sterilized and added to autoclaved medium when required. The cultures were grown in the culture room at 24 °C ± 1 °C under a 16/8-h (light/dark) photoperiod with light provided by an equal mixture of warm-white and fluorescent lamps.

The shoot regeneration medium consisted of MS salts supplemented with (in mg/l) myo-inositol 100; thiamine HCl, 0.1; nicotinic acid 0.5; pyridoxine HCl 0.5; glycine 2.0; sucrose 3 % (w/v); agar 0.7 % (w/v). The growth regulators used to induce shoot regeneration from plum leaves were 5 mg/l BAP and 0.5 mg/l IBA. The basal rooting medium was 1/2-strength JS medium with 1 % sucrose, proliferation medium organics and 0.5 mg/l IBA.

Bacterial strains and vectors: For transformation we used the super virulent strain AGL0 (Lazo et al., 1991) with pCamPPVcp containing the *hpt* gene under the cauliflower mosaic virus (CaMV) 35S promoter and the coat protein (CP) gene of plum pox virus (PPV) driven by the double CaMV 35S promoter and with binary vector pCamPPVRNAi containing the *hpt* gene under the duplicated cauliflower mosaic virus 35S promoter (d35S), the *gus*-intron gene under the CaMV 35S promoter and self-complementary fragments of PPV-CP gene under the modified enh35S promoter (Kay et al., 1987), with a duplicated enhancer sequence (Figure 1). The fragments of the PPV-CP gene were separated by a PDK intron (from pHANNIBAL) to produce a "hairpin" RNA (hp-RNA) structure in antisense - sense orientation. The PPV-CP gene fragment contained the PPV-CP gene with the ATG codon with a length of 698 bp. The size of the PPV-CP intron-hairpin-RNA transcripts was 2220 bp.

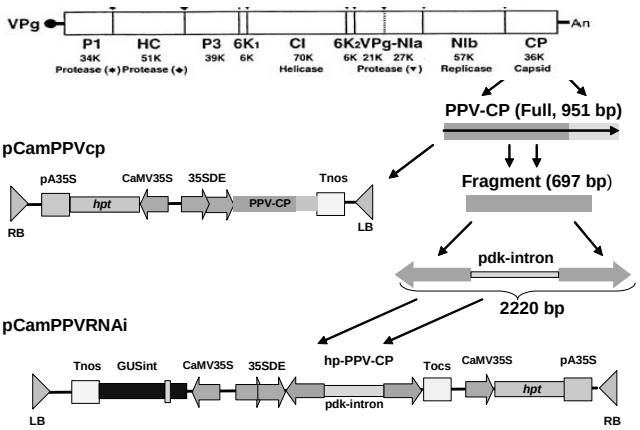

Fig. 1 Vectors construction for transfer fragments of PPV genome to plum trees. See text for details.

Plant transformation: The 1-2 youngest a fully expanded leaves of 5 to 12-week-old in vitro rooted shoots were used for experiments. The leaves were wounded by making cuts perpendicular to the midrib, not reaching the leaf edges. Leaf explants were subjected to auxin shock in liquid MS medium containing 5 mg/l indole-3-acetic acid (IAA) for a period of 5 hours. Explants were then placed in the *Agrobacterium* suspension for 30 minutes. Co-cultivation was carried out for 3 days in darkness with the adaxial side of the leaves in contact with the shoot regeneration medium. The shoot regeneration medium (SRM) consisted of MS salts supplemented with (in mg/l) myo-inositol 100; thiamine HCl 0.1; nicotinic acid 0.5; pyridoxine HCl, 0.5; glycine 2.0; calcium pantothenate 4.0; sucrose 3 % (w/v); agar 0.7 % (w/v). The growth regulators used to induce shoot regeneration from plum leaves were 5 mg/l BAP and 0.5 mg/l IBA. After co-cultivation, the leaves were transferred to the shoot regeneration medium containing 500 mg/l cefotaxime and maintained in the dark at 23 °C ± 2 °C. Immediately after co-cultivation or after 10 days, all leaf explants were transferred to the SRM supplemented selective antibiotic in various concentrations. After 2-2.5 months from the beginning of transformation, all leaf explants were transferred to the shoot elongation medium (SEM) containing 2 mg/l BAP, 0.1 mg/l IBA, 300 mg/l cefotaxime and selective antibiotic, under a 16-h photoperiod. Control explants were treated as described above, with the exception that they were not co-cultivated with *A. tumefaciens*.

Rooting and acclimatization: Three-week-old 2-3 cm long transgenic plum shoots were transferred to basal rooting medium (1/2-strength JS salts) supplemented with 10 g/l sucrose, MS vitamins and 0.5 mg/l IBA, under a 16-h photoperiod. When roots grew to at least 3 cm in length, plants were transferred to the greenhouse.

PCR analysis: The stable integration of the gene cassettes into the genome of plants was confirmed by PCR analysis. PCR was performed in Eppendorff thermocycler gradient. The primers used for amplification of a 951 bp fragment of the *hpt* gene were

5¢-CGACGTCTGTCGAGAAGTTTCTGATC-3¢ and 5¢- GTACTTCTACACAGCCATCGGTCCA-3¢.

The primers used for amplification of a 950-bp fragment of the CP-PPV gene (PPV-1/PPV-2) were

5¢-ATGGCTGACGAAAGAGAAGACGAG-3¢ and 5¢- CTACACTCCCCTCATACCGAGGAG-3¢.

The primers used for amplification of an 880 -bp fragment of the PPV gene in the "harpin" construct (PPVup - ocs-ter) were

5¢-AGACGAGGAGGAAGTTGATG-3¢ and 5¢- ACAATCAGTAAATTGAACGGAG-3¢.

The primers used for amplification of a 740 -bp fragment of the *gus* gene were

5¢-TCGTAATTATGCGGGCAACGTC-3¢ and 5¢- CGAATCCTTTGCCACGCAAG-3¢.

PCR products were separated by electrophoresis in 1.2 % (w/v) agarose-ethidium bromide gels. The absence of *Agrobacerium* contamination was shown by the PCR amplification of a fragment of the *VirB*1 gene.

Histochemical GUS assay: Histochemical GUS assay of plant tissues was performed as described in Jefferson et al. (1987).

Western blot analysis: The leaves of transgenic plum plants were ground in liquid nitrogen. Powdered material was resuspended in four volumes of extraction buffer containing 50 mM Tris-HCl, pH8.0; 10 mM EDTA, pH 8.0; 10 % glycerol (v/v); 30 mM 2-mercaptoethanol; 4 µg/ml aprotinin; 4 µg/ml leupeptin. Total proteins were extracted for 20 min at room temperature, then centrifuged for 10 min at 20 ºC and the supernatant was taken for further analysis. Thirty µl of total protein extract from each transgenic line was separated on 12.5 % SDS-PAGE and transferred onto NC membrane (BioRad, USA) by tank transfer. Western-blot analysis was performed using rabbit polyclonal antibodies to PPV coat protein; the antibody was diluted 1:1000. Anti-rabbit IgG conjugated with fluorescent label CY3 was used as secondary antibody (Amersham, USA; dilution 1:5000). The images of blots were obtained by Variable Mode Imager Typhoon 9200 (Molecular Dynamics, USA) and developed by ImageQuant program (Molecular Dynamics, USA).

Results and discussion

Seven transgenic plants with the PPV-CP gene in sense orientation were obtained after transformation of 524 leaf explants (Table 1). Transformation frequency was 1.3 %. PCR-analysis confirmed the transgenic status of plants by amplification of the predicted fragment for the *hpt* (Figure 2a) and PPV-CP genes (Figure 2b). The accumulation of coat protein was demonstrated by Western blot assay in five of six analyzed lines (Figure 3).

Tab. 1 Results of plum transformation by vector pCamPPVcp contain full length PPV-CP gene.

		Total number of lines			
Variety	**Number of explants**	**Putative**	***hpt* positive (%) transformation**	**Insertion of PPV-CP gene**	**Coat protein expression**
Startovaya	524	7	7 (1.3%)	7	5

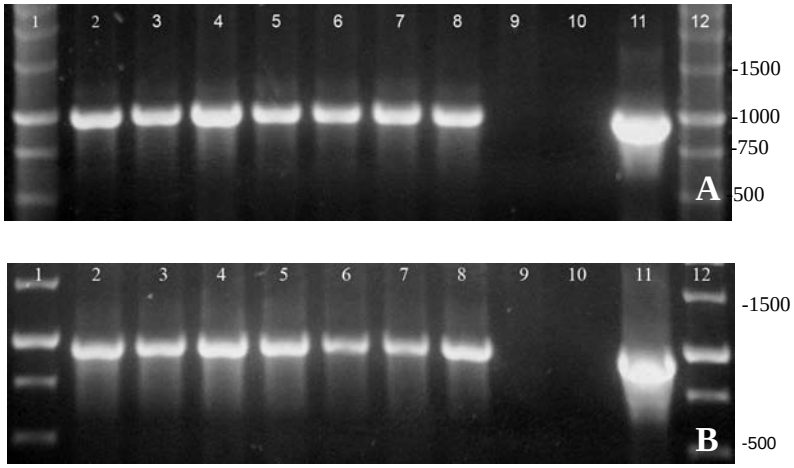

Fig. 2 PCR-analysis of genomic DNA extracted from transgenic "Startovaya" plum plants (pCamPPVcp transformed plants). **A -** agarose gel contains PCR products of the *hpt* gene. Amplification produced an 951 bp product. **B -** agarose gel contains PCR products of the fragment of the promoter and *CP-PPV* gene. Amplification produced a 950 bp product. Lines 1, 12 - molecular weight marker; 2-8- pCamPPVcp transformed plants; 9 – negative control, cv."Startovaya"; 10 – H_2O; 11 – pCamPPVcp.

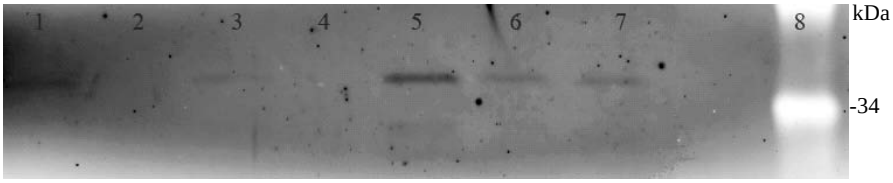

Fig. 3 Western blot assay of transgenic plants var. "Startovaya" (pCamPPVcp) with rabbit polyclonal antibodies against PPV coat protein and anti-rabbit IgG conjugated with fluorescent label CY3. Line 8 – molecular weight marker; 1-3, 5-7 – pCamPPVcp transformed plants; 4 –negative control, cv."Startovaya".

Five independent transgenic PPV-CP intron-hairpin-RNA lines (ihpRNA) were produced (Table 2). All transgenic plants were selected by on 5 mg/l hygromycin. Transformation frequency in this experiment was lower than in the previous experiments (1.1 % against 1.8-2.2 %) with the *gfp* gene (Mikhaylov et al. 2007) or *PPV-CP* gene (Mikhailov et al. 2006) probably due to the large size of the T-DNA locus (about 10,000 bp). PCR-analysis confirmed the transgenic status of plants by amplification of the predicted fragment of the *hpt* (Figure 4a) and "hairpin"-*PPV-CP* genes (Figure 4b,c).

Tab. 2 Results of plum transformation by vector pCamPPVRNAi transformed contain self-complementary fragments of PPV-CP gene.

Cultivar	Infected explants	Hyg-resistant shoots no. (%)	GUS-positive regenerants	PCR-positive		
				hpt	gus	PPV-CP
Startovaya	452	5 (1,1)	3	5	4	5

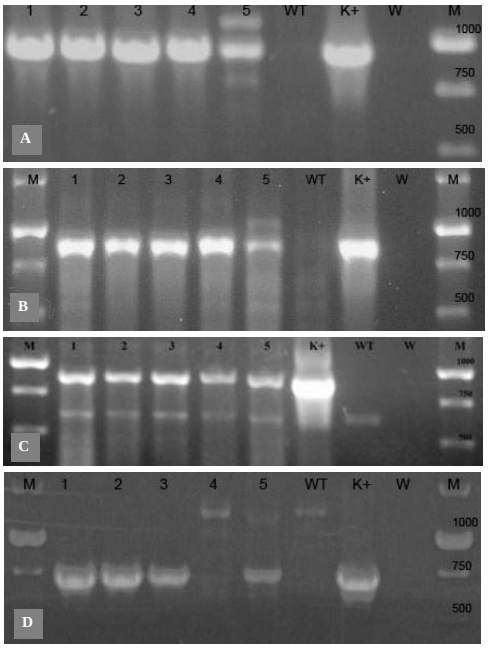

Fig. 4 PCR analysis of genomic DNA extracted from transgenic "Startovaya" plum plants (pCamPPVRNAi transformed plant). Agarose gel contains PCR products of the *hpt* (A), *PPV-CP* left (**B**), right arms (C) and *gus* (D) gene. Amplification produced a 951, 880, 880 and 740 bp product (respectively). Lines 1, 10 - molecular weight marker; 2-6 – transgenic lines; 7 – negative control, cv."Startovaya"; 8- pCamPPVRNAi; 9 – H_2O.

GUS gene expression was detected in leaves of 3 of 5 transgenic lines (fig.4d). One transgenic line (N4) had no *gus* gene; line N3 contained a transgene insertion (Figure 4d), but the protein was not expressed. Expression of GUS was detectable in all tissues, whereas no blue staining was observed in non-transformed plantlets (Figure 5).

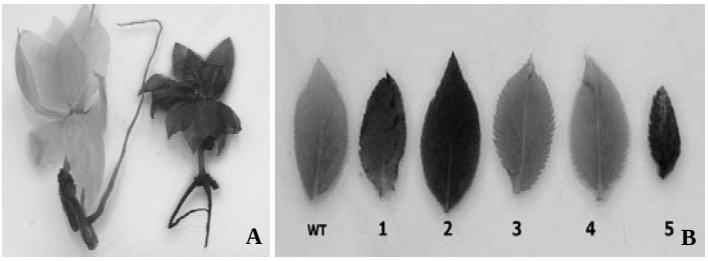

Fig. 5 Histochemical GUS analysis of plants (A) and leaves (B) of control (wt) and transgenic (1-5) plum plants cv. Startovaya. Vector pCamPPVRNAi. Scale bar on each picture represents 10 mm.

The rooted 2-month-old shoots were placed directly into a greenhouse, bypassing a stage of adaptation in a culture room. Rooted shoots had a 70 % rate of survival and successfully grew in the greenhouse. Ten plants of each transgenic line transformed by coat protein and "hairpin" construct genes and ten control plants were infected with PPV in September 2008 by grafting buds from plum shoots with Sharka symptoms. In the next spring they were cut above the grafts. Shoots that developed were tested for PPV by Western blotting (Figure 6). In all control and *ppv-cp* transformed plants PPV coat protein (39K) was detected (Figure6A,B), whereas no PPV coat protein was observed in samples from plants transformed with the "hairpin" construct (Figure 6C).

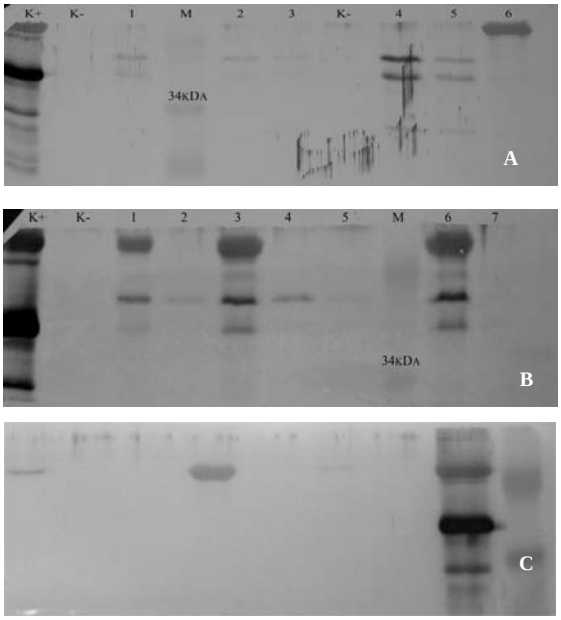

Fig. 6 Western blot assay of plums var. "Startovaya" infected by PPV with polyclonal antibodies against PPV coat protein (Loewe). **A**- nontransgenic plants lines 1-6; **B** - plants transformed by PPV coat protein gene 1-7; **C** - plants transformed by hairpin construct-lines 1-6, K- (noninfected plant) – line 7, K+ (infected plant) – line 8, size marker line - 9.

The PPV-CP gene has been transferred into the hypocotyls of the plum (*P. domestica*) (Scorza et al., 1994; Petri et al., 2008). These authors have shown that one transgenic plum line, C5, is highly resistant to PPV and remained so for over 5 years in greenhouse tests (Ravelonandro et al., 1997, 1998). Long-term field tests in Europe have confirmed that C5 is highly resistant to PPV (Malinowski et al., 1998).

Molecular analyses of the C5 transgenic clone have shown a high level of transgene transcription in the nucleus, low levels of transgenic mRNA in the cytoplasm, a complex multicopy transgene insertion with partial insert copies, transgene methylation and no detectable protein, all characteristics typical of a post-transcriptional gene silencing (PTGS) mechanism, where the expression of a transgene induces the plant to degrade RNA of the same sequence, including any RNA from an infecting virus (Scorza et al., 1994, 2001). This was initially demonstrated through nuclear run-on analysis, confirming that mRNA from the PPV coat protein is produced but does not accumulate in the plant cells (Scorza et al., 2001). The PTGS mechanism of resistance in C5 was further confirmed by the detection of short interfering RNA (siRNA) homologous to the PPV sequences (Hily et al., 2005). While the short (22nt) siRNA production has been detected in non transformed PPV-susceptible plums upon inoculation with PPV, the resistant C5 produces both the short (22nt) and the long (25-26nt) species of siRNA. This finding has lead to the suggestion that the high level virus resistance in the transgenic C5 is connected with the production of long-sized class of siRNA (Hily at al., 2005). Interestingly, the presence of the short siRNA found naturally in plum suggests that gene silencing is a natural system of reaction to PPV infection in plum. But resistant transgenic lines such as C5 developed from seedling transformation could be used only as the resistant parents in long term breeding programs. With generation cycles ranging from 3 to 6 years for *Prunus* species (Sherman and Lyrene, 1983), the time necessary to incorporate a high level of resistance to PPV plus incorporate a high levels of fruit quality, yield potential, cold-tolerance, and resistance to other diseases can be greater than the lifetime of a breeder. We also show that resistance to plum pox virus (PPV) can be induced in transgenic plants using a vector (pCamPPVRNAi) that generates PPV-CP homologous RNAi (fig.1).

Our preliminary results confirmed the efficiency of RNA strategy for protecting plants from virus attack in general, and for stone fruits from PPV particular. Our work also extends transgenic RNAi-based PPV resistance to an established plum cultivar.

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