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Research Article

The Use of Transgenic Resistant Plums in Transgrafting Fails to Confer Plum Pox Virus Resistance in Prunus

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Abstract

In order to sustain productivity, growers are implementing fruit trees to provide plum pox virus (PPV) resistant varieties. Unfortunately, classical breeding approaches have failed to develop resistant varieties. RNA interference (RNAi) silencing, as an alternate strategy, has been shown to be an efficient approach to combat PPV disease (sharka). PPV resistance based on RNAi has been demonstrated in natural conditions for over 10 years. Experiments using graft inoculation in high-containment greenhouses show that silencing protects against PPV over multiple dormancy cycles. While the virus spreads throughout the vascular tissues of susceptible hosts, PPV movement is undetectable in resistant clones. However, in this work, when PPV-susceptible cultivars were grafted onto transgenic resistant plum rootstocks, the small interfering RNA (siRNA) stopped the systemic spread of PPV into the transgenic resistant shoots but did not appear to prevent the infection of susceptible scions. Thus, we do not have convincing evidence of siRNA transfer nor the provision of resistance to the susceptible scion. Notably, *Macrolophus* insects that were experimentally fed on plums could suck RNAi from the leaves of resistant clones. While the attempt to control PPV disease through transgrafting as a new breeding technology (NBT) cannot be confirmed,

these studies provide insight into key regulatory interactions associated with RNAi accumulation and its environmentally safe use in perennial plants.

Keywords
Virus movement; resistance; RNAi; silencing; Macrolophus bug; woody plant

1. Introduction

We currently rely on the vegetative propagation of materials to ensure the availability of woody plant materials. For years, growers have produced plant materials using this grafting technology, assuming that the designated clone would have the same properties as the source [1]. Grafting techniques allow breeders to conserve and reproduce characteristics such as biotic and abiotic stress resistance or fruit production in fruit trees. Using breeding technology, researchers have improved the grafting process and identified key factors for compatibility and successful propagation. Among these were using common sources for the two components, using rootstocks and cultivars belonging to the same *genus* and, especially, using environmentally adapted rootstock.

Varying degrees of success have been achieved, highlighting the basic empirical fashion of grafting. To propagate clones, breeders have improved cultivars and developed ways to release relevant clones, ensuring the perennial availability of woody plant materials in any area of the world [2]. However, there are benefits and difficulties related to using woody plant technology. An example of how propagating can be beneficial is when French grapevines were saved from the pest *Phylloxera* by grafting the vines onto blight-resistant American rootstocks [3]. The opposite case also exists, notably, in the inadvertent transmission of the plum pox virus (PPV) through grafting onto infected rootstocks [4-7]. PPV can be efficiently preserved in woody plant tissue and can spread following ocean crossings or long-distance travel. PPV is known as a quarantine pest; hence, any transfer of *Prunus* tissues across borders should follow international rules, including a tracking permit to follow any unintended consequences of plant introduction. Not only viruses can be introduced by grafting, but proteins, viable DNA or RNA molecules, or even living organisms (e.g., through parasite eggs, *fungi*, viruses). The RNAi strategy provides a successful panel showing how several economic crops, such as rice [8], soybean [9] and tomato [10], have won the battle against plant viruses.

A new breeding concept that can be explored is the transfer of RNA silencing via grafting to confer virus resistance to non-transgenic scions (transgrafting) [11, 12]. An example is the development of a transgenic cherry rootstock that transfers *Prunus* necrotic ringspot virus (PNRSV) resistance to scions [13-15]. The siRNA from resistant rootstock has been successfully transferred to a scion through grafting [13, 15]. Using grafting technology, we attempted, in our research, to target the same objective and to better study the characteristics of silencing occurring in two PPV-resistant transgenic plums (*Prunus domestica*): 'HoneySweet' (HS) [16-21] and B14 clones [22, 23]. Here, we wanted to determine if the resistance through silencing was mobile to validate graft transmission and, if proven, to exploit the process as a new breeding technique (NBT) in crop improvement technology [24]. Breeders have argued that siRNA, via silencing, can move through

graft unions and spread efficiently to the scion [13, 15]. These papers have presented innovative results, suggesting the mobility of siRNA and its ability to genetically extend protection against virus infection. Most of these successful studies on silencing strategies have focused on annual plants [24], with few studies on perennial plants [13, 15, 24].

In order to verify an NBT concept with transgenic plum clones showing stable and durable resistance to PPV infection [16-20, 23], two resistant clones, either HS [16-20, 23, 25] or B14 plums [22, 23], were used as rootstocks but were unable to spread siRNA into the grafted scion. We saw that high accumulations of siRNA in the leaves of resistant clones triggered the viral RNA; however, the remaining virus from the scion spread through the vascular tissue. Beyond our detailed study of grafting, we challenged that RNAi can be mechanically sucked from plum leaves by insects [26]. Our results contrast reports of resistance to PNRSV infection in susceptible scions derived from transgenic RNA-expressing rootstock [13, 14]. We do not have convincing evidence of siRNA transfer, nor any alternate NBT [26, 27] to implement virus resistance in woody plants.

2. Materials and Methods

2.1 Plant Materials

Five plums and one peach clone (‘GF-305’) were utilized in these studies. Plum genotypes including an untransformed (NT) conventional (‘Brompton’) plum, an advanced selection (‘BO70146’) plum and *Prunus mariana* (‘GF8-1’), highly susceptible to PPV infection were commonly used as rootstocks. The three others are transgenics, two resistant clones HS, also known as C-5, and B14 plums and the third C-4, susceptible to PPV infection [23, 25, 28, 29]. All three transgenic clones harbored the PPV CP (capsid protein) gene. B14 plum was transformed with the intron hairpin RNA (ihRNA) construct [22, 23], and both HS and C-4 were engineered with a CP construct [23, 25, 28, 29]. Clone C-4 has high levels of CP RNA and accumulates CP however HS does not accumulate CP and very little mRNA [28].

2.2 Grafting

B14 plum was grafted onto the virus-free certified *Prunus mariana* GF8-1 rootstock. The two clones C-4 and HS were rooted plants from suckers of the respective mother trees. Using the clones above as rootstocks, we grafted scion onto each rootstock using a pair of buds collected from C-4, HS or B14 plums. Figure 1 summarizes the different combinations of plants grown in a high containment greenhouse maintained at 22-24°C with a 16h day length provided by a mix of natural and supplemental light. Reciprocal grafts were also made to further understand any transmission of resistance, HS and B14 scions respectively grafted onto the C-4 rootstocks were tip-budded either with the non-transformed (NT) peach GF-305 or the conventional Brompton plum as rootstock.

2.3 Virus Inoculation and Disease Records

The standard method of inoculating woody perennials was used [23, 25], two bark chips of GF-305 peach tree infected with PPV M were applied to young shoots. They were maintained in a high-containment greenhouse. All trees were subjected to a cold-induced dormancy cycle one to two months after the PPV inoculation. Following the routine protocol, the presence of PPV was

checked at 3 weeks following the first dormancy [16, 23, 25]. Visual symptoms of sharka can appear on leaves of susceptible scions including GF-305 peach, Brompton plum or C-4 clone.

2.4 PPV Detection

To confirm the presence of PPV, leaf samples were harvested, weighed and tested with two different methods, DAS-ELISA [23, 25, 29-31] and molecular detection of viral RNA [23, 26, 30, 32]. Positive assays were confirmed by the OneStep/RT-PCR (Qiagen kit, Gmbh, Hilden, Germany) by using the primer pairs EWD and RKSL that amplified a short fragment of 180 nucleotides (nt) spanning the COOH part of the Nuclear inclusion b (Nib) cistron (23).

2.5 DNA and Methylation Study

To verify the reliability of our study on gene silencing, genomic DNA was extracted to determine if it was methylated when silenced. Approximately 500 mg of leaf tissue was collected and genomic DNA was extracted according to Kobayashi et al.'s protocol [33]. Comparative studies of transgrafting were assayed from DNA extracted from leaf tissue (separately periderm, vascular leaf, green leaf limb, shoot apical meristem) and roots. The quality and quantity of materials were evaluated using a spectrophotometer. The DNA was digested with isoschizomeric endonucleases *MboI* and *BfuCI (*New England Biolabs, Ipswich, MA, USA) that recognize the GATC sites in the CP but are blocked when the site contains specific methylation [17, 18, 20, 23, 34]. The digested DNA was used as a template with specific primer pairs for the transgene. One pair flanks two major GATC sites and the second pair covers the large insert of 1.2 Kbp including the 3'non-coding sequence of PPV genome (not shown). The detection of an amplicon after *BfCuI* digestion points out the methylated status of the studied DNA. Primers were designed for CP target region.

2.6 RNAi Study

In addition to the above DNA study, siRNA was measured as its presence is correlated with virus resistance [19, 20]. In our attempt to study siRNA in different parts of non-inoculated perennial plants, total RNA was extracted according to Hily et al. [19]. The presence or absence of siRNA was evaluated using either RNA-blotting experiments according to [19, 20, 23, 30] or a OneStep/RT-PCR (Qiagen kit, Gmbh, Hilden, Germany) using the primers amplifying a siRNA template identified through Next Generation Sequencing (NGS) [34].

2.7 Sucking of RNAi by Macrolophus Bug

Macrolophus pygmaeus bugs, routinely used as biological agents under greenhouse conditions [35], were experimentally used for RNAi acquisition from plum leaves. They were considered harmless and purchased from Koppert Biological System (The Netherlands). Conditioned in vials, 3 pools of 20 insects were starved for a few hours prior to feeding on young plum plantlets (suckers). *Macrolophus pygmaeus* bugs were recognized as omnivorous insects fed on plum leaves during two days. Insects were collected and immediately frozen at -80°C until RNA extraction. For comparing study, 10 insects were fed on each plantlet, including one HS and one non-transformed Brompton plum as control (C). In order to reliably optimize the number of tested insects, the study was repeated three times. Two other plantlets (HS and C) used as control have not been used for

rearing bugs. To standardize our study, total RNA from either insects or the two control plums was similarly extracted using the NucleoSpin plant RNA extract kit (Macherey-Nagel GmbH, Duren, Germany) according to [22]. The results published by Calllahan et al. [34] have successfully mapped out the siRNA machinery in the HS plum, leading to the designated primers pointed out in Table 1. All these experiments, including transgenic plants and a quarantine pest, like PPV were conducted in a high containment greenhouse: Agreement 2000 of 28/10/2015, Haut Conseil des Biotechnologies, about the use of genetically modified organisms applied in Education, Research and Development.

Table 1 List of primers and conditions used

Primers	Conditions	Sequences	Target
PPV-CP 340 *Sau3AI* fwd	94°C for 2 min 30 cycles of -94°C for 20 sec -55°C for 30 sec -72°C for 1 min 72°C for 10 min	5’-CAACTCAAACGCGCTAGTCAAC-3’	CP cistron
PPV-CP 660 *Sau3AI* rev	94°C for 2 min 30 cycles of -94°C for 20 sec -55°C for 30 sec -72°C for 1 min 72°C for 10 min	5’-ATACGCTTCAGCCACGTTACTG-3’	CP cistron
EWD fwd	94°C for 2 min 30 cycles of -94°C for 20 sec -55°C for 30 sec -72°C for 1 min 72°C for 10 min	5’-GARTGGGACAGATCAAATGA-3’	Nib cistron
KSL rev	94°C for 2 min 30 cycles of -94°C for 20 sec -55°C for 30 sec -72°C for 1 min 72°C for 10 min	5’-TGAAAGAGCATTGTATGGTGC-3’	Nib cistron
Forgre fwd	95°C for 2 min 40 cycles of -95°C for 45 sec -50°C for 1 min -72°C for 1 min 72°C for 10 min	5’-TGTTCAAAATCGTTTATTT-3’	CP cistron
Revgre rev	95°C for 2 min 40 cycles of -95°C for 45 sec -50°C for 1 min -72°C for 1 min	5’-CCAAGCCAAATAAACGA-3’	CP cistron

	72°C for 10 min		
RTgre	-50°C for 30 min -95°C for 15 min	5'-CCCAGTTGGAGCCTGGGACGTAGCCAAGCCAAATAAACG-3'	CP siRNA
s5Rb fwd	94°C for 2 min 30 cycles of -94°C for 20 sec -58°C for 30 sec -72°C for 1 min 72°C for 10 min	5'-CTCTCGGCAACGGATATCTCGGCTCTC-3'	Standard gene 5S ribosome
s5Rb rev	94°C for 2 min 30 cycles of -94°C for 20 sec -58°C for 30 sec -72°C for 1 min 72°C for 10 min	5'-GGGGGCAACGGCGTGTGACGCCCAGGCAG-3'	Standard gene 5S ribosome

3. Results

3.1 Grafted Scions and Consequent Behavior of Plants

In order to evaluate and exploit the potential of transgenic resistant clones used as rootstocks, we combined through grafting the "resistant rootstock and susceptible cultivar" (Figure 1).

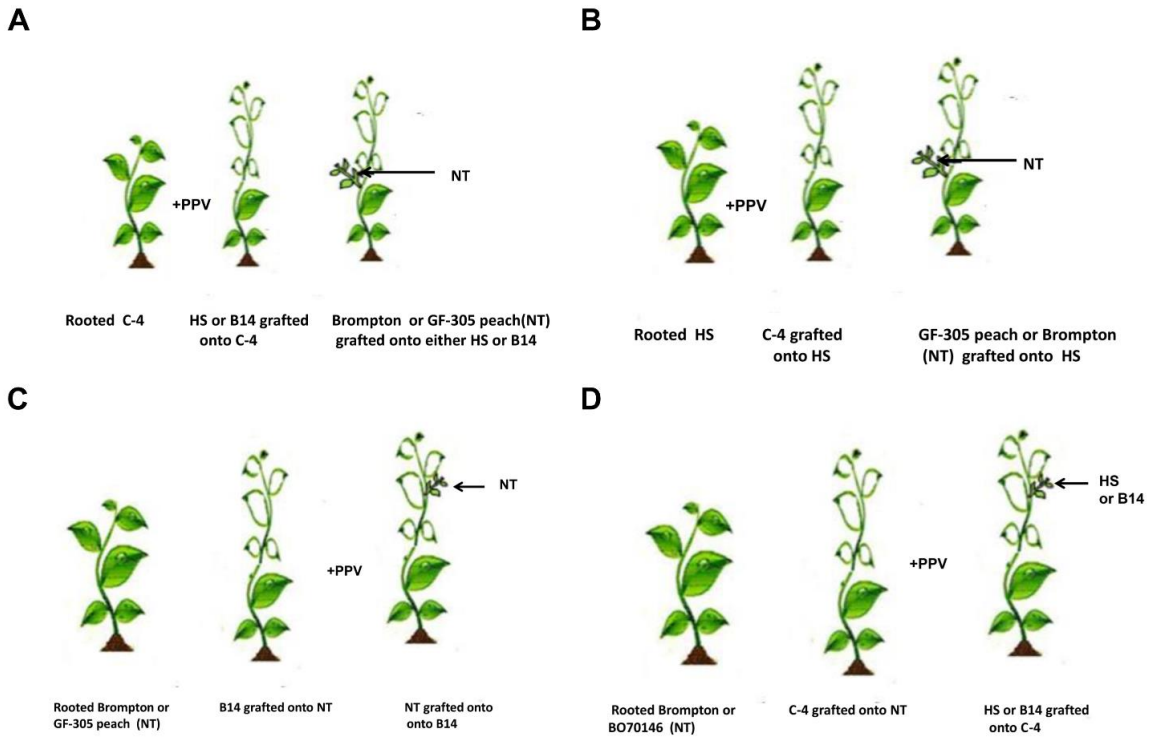

Figure 1 Schematic representation of the four scenarios using either rooted C-4 and HS clones or grafted C-4 (control) and B14 (interstocks) as rootstocks. Following to the different schemes: the untransformed bud (NT) was grafted to test for PPV movement through the transgenic plant. Either the HS clone or the B14 clone used as rootstock; either C-4 or the non-transformed GF305 peach, *Prunus mariana* GF8-1 and the conventional 'Brompton' plum were grafted. Because we did not get rooted B14 clone, it was pre-grafted onto Brompton plum prior to its use as rootstock (interstocks. Either HS or B14 were grafted onto the rooted C-4 clones that were used as rootstocks.

Six combinations were made: C4 on HS, GF305 on HS, Brompton plum on HS, C4, and B14 on C4 and C4 on B14 and *Prunus mariana* GF8-1 on plum Brompton. Table 2-6 summarizes the chronological record of PPV infection in plants detected by DAS-ELISA.

Table 2 OD value of the serological analyses for PPV detection at the different bud-breaking stages following to dormancy cycle during 2011-2016.

Dates	November 2011	October 2012	May 2013	October 2015	January 2016
Plant 1-5					
Scion C-4 tip	-[3]	0.77	0.20	1.02	0.77
Scion C-4 bot[1]	0.09	0.46	1.19	-	3.0
Rootstock HS tip	0.0	0.05	0.74	0.25	0.20
Rootstock HS bot	-	-	0.30	0.48	-
Plant II-5					
Scion C-4 tip	-	0.12	0.44	1.16	-

Scion C-4 bot[1]	-	-	0.55	1.8	3.0
Rootstock HS tip	0.0	0.0	0.18	0.0	0.0
Rootstock HS bot	0.18	0.0	0.0	0.11	-
Plant III-5					
Scion C-4 tip	0.18	0.21	0.73	1.58	-
Scion C-4 bot[1]	0.0	-	0.82	1.24	-
Rootstock HS tip	0.0	0.0	0.27	0.13	0.0
Rootstock HS bot	0.0	0.0	0.0	[4]0.10	0.0

[1] "Scion bot" means the first pair of leaves sampled above the graft union.

[2] "Rootstock tip" represents the first pair of leaves below the graft union.

[3] Not sampled.

[4] OD value measured at 405 nm that represent the backgrounds of the buffer utilized.

Table 3 Serological detection of PPV in the leaf samples collected from three scions built with HoneySweet clone as a rootstock and either Brompton plum or GF305 peach as scion during 2011-2016.

Dates	**November 2011**	**October 2012**	**May 2013**	**October 2015**	**January 2016**
Plant 1-5					
Scion GF305[1]	-[3]	0.71	1.21	-	-
Rootstock HS tip[2]	0.0	0.05	0.74	0.25	0.20
Rootstock HS bot	-	0.0	0.30	0.48	-
Plant II-5					
Scion Brompton[1]	0.34	0.68	0.57	-	-
Rootstock HS tip	0.0	0.0	0.18	0.0	0.0
Rootstock HS bot	0.18	0.0	0.0	0.11	-
Plant III-5					
Scion Brompton[1]	-	0.40	-	-	-
Rootstock HS tip	0.0	0.0	0.27	0.13	0.0
Rootstock HS bot	0.0	0.0	0.0	[4]0.10	0.0

[1] Leaves growing from the non-transformed scion.

[2] "Rootstock tip" represents the first pair of leaves below the graft union.

[3] Not sampled.

[4] OD value measured at 405 nm that represent the backgrounds of the buffer utilized.

Table 4 OD value of the serological analyses of five scions built with C-4 clone as rootstock and HoneySweet as scion for PPV detection at the different budbreaking stages following to dormancy cycle during 2011-2015.

Dates	**November 2011**	**October 2012**	**May 2013**	**October 2015**
Plant C4-1				
Scion HS tip	0.0	0.0	0.0	-
Scion HS bot[1]	0.0	-	0.0	0.0

Rootstock C-4ti p[2]	0.0	0.0	0.46	-
Rootstock C-4bot	0.0	-	0.40	3.0
Plant C4-2				
Scion HS tip	0.0	0.0	0.0	0.0
Scion HS bot[1]	-	-	0.0	0.0
Rootstock C-4tip[2]	-	0.54	0.55	0.64
Rootstock C-4bot	0.0	-	0.18	1.2
Plant C4-3				
Scion HS tip	0.08	0.41	0.0	-
Scion HS bot[1]	0.0	-	0.0	0.0
Rootstock C-4tip[2]	-[3]	0.80	0.50	-
Rootstock C-4bot	0.0	-	0.49	1.05
Plant C4-4				
Scion HS tip	0.0	0.0	0.0	-
Scion HS bot[1]	0.0	-	0.0	0.07
Rootstock C-4tip[2]	-	0.13	0.0	-
Rootstock C-4bot	0.0	-	0.043	1.48
Plant C4-5				
Scion HS tip	0.0	0.0	0.0	-
Scion HS bot	-	-	0.0	0.0
Rootstock C-4tip	0.07[4]	0.12	0.54	-
Rootstock C-4bot	-	-	0.29	0.12

[1] Scion bot" means the first pair of leaves sampled above the graft union.

[2] "Rootstock tip" represents the first pair of leaves below the graft union.

[3] Not sampled.

[4] OD value measured at 405 nm that represent the backgrounds of the buffer utilized.

Table 5 Serological detection of PPV in the leaf samples collected from three scions built with C-4 clone as a rootstock and B14 clone as scion during 2014-2016.

Dates	**January 2014**	**July 2015**	**October 2015**	**January 2016**
Plant 3B14				
Scion B14[1]	-[3]	0.01	0.0	0.0
Rootstock C-4[2]	-	0.39	2.87	0.48
Plant 4B14				
Scion B14	-	0.04	0.0	0.0
Rootstock C-4	-	0.0	3.0	1.8
Plant 5B14				
Scion B14	-	0.0	0.0	0.0
Rootstock C-4	-	0.0	3.0	2.55

[1] Leaves of the B14 scion.

[2] Leaves collected from the C-4 rootstock.

[3] Not sampled.

[4] 0.10 was the OD value measured at 405 nm that represent the background of the buffer utilized.

Table 6 Serological detection of PPV in two scions built with B14 clone as rootstock and C-4 plum as scion at the different bud-breaking stages following to dormancy cycle during 2014-2016.

Dates	January 2014	July 2015	October 2015	January 2016
Plant B14-1				
Scion C-4[1]	2.29	0.26	2.77	1.42
Rootstock B14[2]	0.31	0.0	0.0	0.1
Plant B14-2				
Scion C-4	2.55	0.10[4]	-	2.25
Rootstock B14	0.0	-[3]	0.0	0.0

[1] Leaves sampled above the graft union.

[2] Leaves collected below the graft union.

[3] Not sampled.

[4] 0.10 was the OD value measured at 405 nm that represent the backgrounds of the buffer utilized.

Out of the 16 plant combinations, three indicated that the resistant HS or B14 rootstocks exhibited a few leaves which initially showed symptoms and had a higher DAS-ELISA reading suggesting the presence of the virus (Table 2-6 and Figure 2A). These appeared to be temporal and faded with time. Interestingly all the susceptible scions as well as rootstocks were infected. Conversely plants built with a resistant scion grafted onto susceptible rootstock showed apparent differences because scions were symptomless (Figure 2B). The successful inoculation of PPV through chip-budding of the rootstock clones [25], provides evidence that PPV can move through the vascular tissue. But as exhibited here, it doesn't appear that the resistance is transmitted to the scion in the case of the resistant rootstocks, nor to the rootstock from the resistant scion.

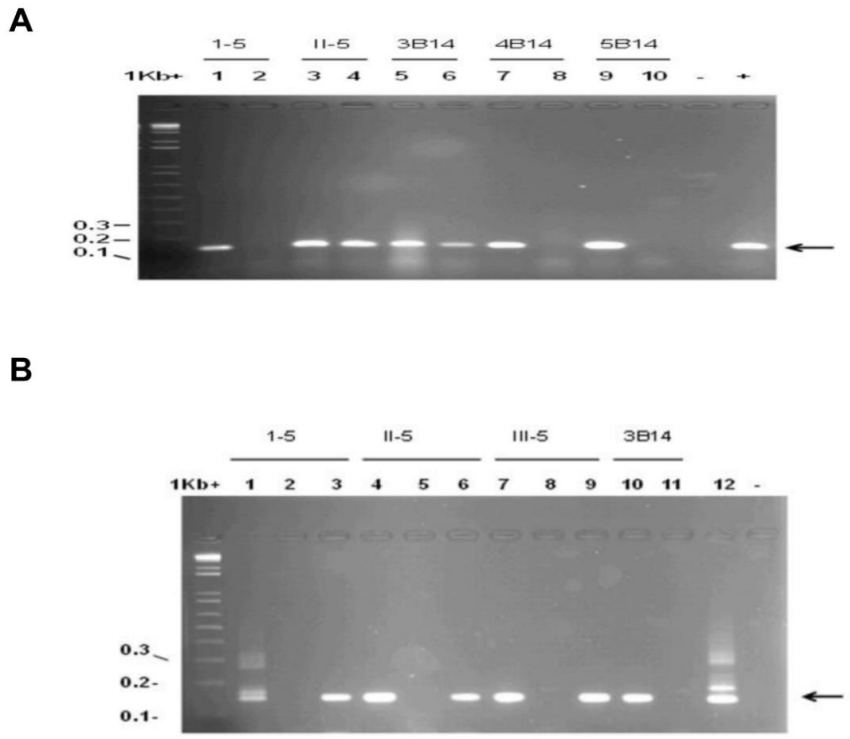

Figure 2 Detection of PPV RNA by OneStep RT/PCR by using the couple of primers EWD and KSL (Table 1) matching to the Nib cistron of PPV genome. **Panel A:** Total RNA was extracted from leaves collected from the different sections of the scions (1-5; II-5; 3B14; 4B14 and 5B14) (Tables 2-5). In panel A, odd numbers represent samples from the C-4 scion however even numbers were sampled either from the 'HoneySweet' plum utilized as rootstock (1-5 and II-5) or B14 (3B14, 4B14 and 5B14). The arrow at the right margin indicates the position of the expected amplicon. **Panel B:** After grafting of the non-transformed GF305 peach or the conventional Brompton plum, leaves were sampled from the different shoots (transgenic or not). Aliquots of total RNA collected were treated by OneStep RT/PCR by using the same couple of primers EWD and KSL. The following samples (1, 4, 7 and 10) came from the C-4 scion, samples 2, 5 and 8 from the HS rootstock, sample 11 from the B14 plum used as rootstock and the rest came from the non-transformed GF-305 peach and the Brompton plum.

3.2 Resistance Versus Susceptibility

One of the critical considerations was that PPV would be restrictively blocked in the resistant tissue. We grafted healthy GF-305 buds onto the resistant scions with infected rootstocks to see if this was the case. Following a dormancy cycle, PPV was not detected in leaves of the 'HoneySweet' resistant clone, but surprisingly GF-305 peach or Brompton plum leaves exhibited symptoms. Serological and molecular assays confirmed it (Figure 2, Figure 3). Therefore, a systemic spread of PPV through the vascular tissue of the resistant clones occurred in the grafted scion and different responses were detected between resistant and susceptible clones in the leaves (Tables 2-6, Figure 2, Figure 3), Here, the resistant clones, used as interlock, did not block

the movement of the virus in the vascular system but was able to minimize it in the leaf tissue. The grafted C-4 and the conventional plants grafted onto these resistant clones, either B14 or HS, were readily infected.

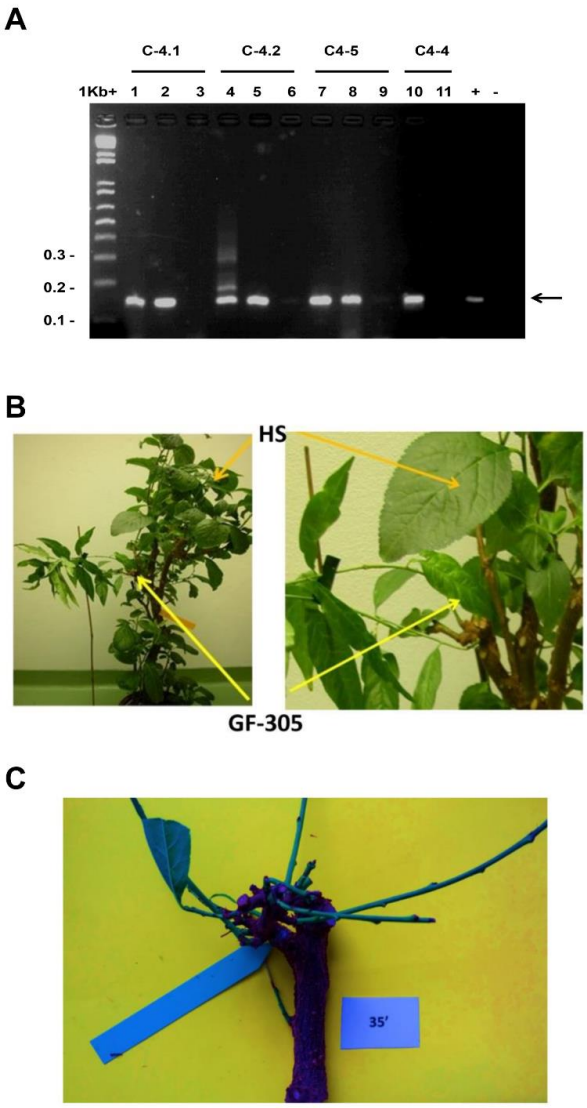

Figure 3 Panel A: PPV RNA detection by OneStep RT/PCR by using the couple of primers EWD and KSL (Table 1) matching to the Nib cistron of PPV genome. Total RNA was extracted from leaves collected from the different sections of the scions (C4-1, -2, -5 and -4) (Table 4). Lanes 1, 4, 7 and 10 came from the C-4 roostock, the following lanes 2, 5 and 8 from NT; and lanes 3, 6, 9 and 11 came from HS scions. The arrow at the right margin indicates the position of the expected amplicon. Molecular weight markers (1Kbp+) are loaded at the first lane. **Panel B:** Leaf deformation and vein clearing symptoms induced by PPV in GF-305 peach (NT) grafted onto the symptomless HoneySweet scion (HS). **Panel C:** Vigorous and multiple shoots of GF-305 peach (NT) grafted onto the symptomless HS scion.

3.3 Molecular Study

In order to understand these observations, we looked to see if the silencing process [16-22], particularly the transgene methylation and the siRNA accumulation in transgenic resistant plants was moving through the graft unions. Regardless of whether the transgenic resistant clone was rootstock or scion, no data were observed suggesting the silencing movement in the form of methylation through the graft unions. The C4 DNA was never methylated while our results suggest that the HS and B14 DNA were always methylated (Figure 4, Figure 5). The methylation indicative of silencing is consistent in resistant clones [16, 17, 19, 22, 33]. In our plum model, we did not detect any sign of silencing as has been described in herbaceous plants when non-transgenic scions are grafted onto transgenic resistant herbaceous plants [12, 36]. Our plum model does not show any movement of RNAi silencing through the grafting point. Regardless of the origin of the scion, transgenic (C-4 clone) or not (Brompton) no sign of silencing was induced nor functional resistance because PPV systemically spread in whole scion (Table 2, Figure 4). Comparing the methylation status in C-4 plum that was used as either rootstock or scion, no any sign of methylation was induced either by HS plum that was used as scion (C4-1, -2, -3 and-4 plants) or rootstock (1-5 and II-5 plants) (Figure 4B).

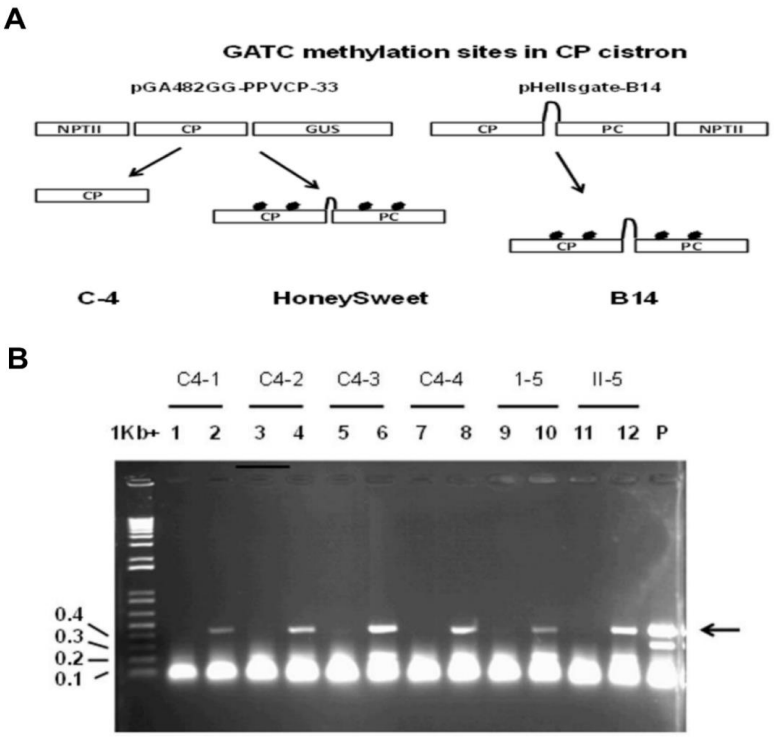

Figure 4 Study of the methylation status of the viral CP transgene, digestion of the DNA with a methylation sensitive enzyme followed by PCR of the region spanning the cut sites was done. **Panel A**: Schematic representation of the two vector cassettes utilized, pGA482GG-PPVCP33 encoding CP and pHellsgateB14 with an intron. Black excrescence indicates the potential GATC methylated sites in the CP genes of HS and B14 plums and that of the C4 clone expressing the viral CP gene. **Panel B:** Total DNA was extracted

from leaves of the different clones analyzed in Tables 2-4. After purification, aliquots were pre-digested overnight respectively with the two isoschizomers *Mbo*I and *BfuC*I. Here we represent the amplicon of 360bp resulting from the *BfuC*I digested DNA subject to PCR amplification with primers flanking the two potential sites (Table 1). The amplified fragment is indicated by an arrow at the right margin. Odd number lanes were derived from C-4 DNA and even number lanes from HS plum. P represents the positive control from the uncut DNA plasmid vector.

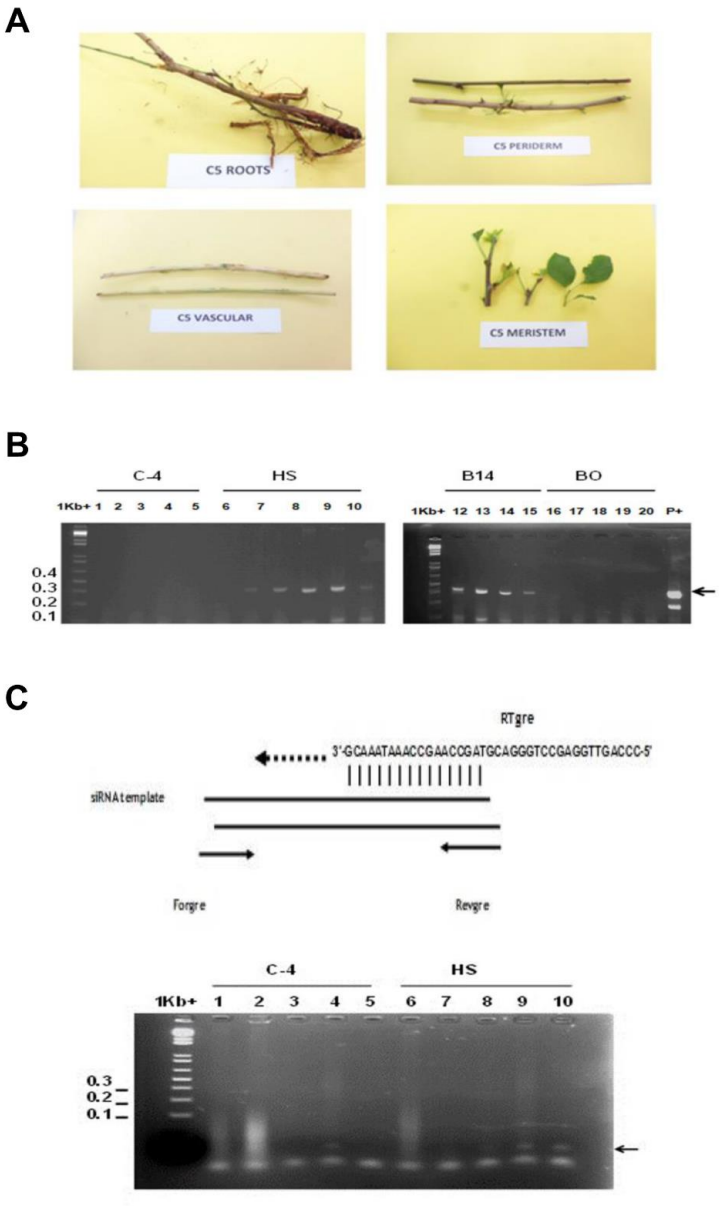

Figure 5 Comparative analysis of the methylation status and the occurrence of a siRNA in different tissues from HS and C-4 plant material either as the scion or the rootstock. **Panel A:** Different types of tissue sampled respectively from HS, C-4, B14 and BO70146 plums. They were utilized to investigate the virus transgene silencing in healthy plants grown in the greenhouse. To obtain the periderm tissue we peeled sticks as shown and to avoid any interference with the proposed analysis, we also removed the different

buds. They were lyophilized following freezing with liquid nitrogen. **Panel B:** Methylation status of the different tissue. 1, 6 and 16 represent DNA from roots, 2, 7, 12 and 17 from periderm, 3, 8, 13 and 18 from vascular tissue, 4, 9, 14 and 19 from leaves and 5, 10, 15 and 20 from shoot apical meristem. (+) represents the positive control of uncut plasmid. There is no number 11 because B14 clone is grafted onto Brompton plum. **Panel C:** A rapid siRNA detection by the OneStep RT/PCR using either the RTgre reverse transcriptase primer with the couple of primers (Forgre and Revgre) or only the aforementioned pair (Forgre and Revgre) (Table 1); Aliquotes of total RNA sampled from different tissue, roots, periderm, vascular, leaf and shoot apical meristem were used as templates. Arrow at the right margin points out an amplicon approximately fitting with the expected size (21 nt). Lanes are labeled the same as in 5B.

3.4 Differential Expression of Silencing in Perennial Plants

To investigate which tissues are involved in RNA silencing in perennial plants, we focused our study on the comparative analyses of the methylation and induced siRNAs from the transgene expression across the different tissues of the scion [33, 34]. Since little is known about the production and accumulation of siRNA in whole perennial plants [18, 19, 23, 34], we particularly wanted to examine it in tissue other than leaves. We extracted genomic DNA and total RNA from leaves. We were only able to detect transgene methylation in HS in any tissue though it may be at variable levels, notably in roots, leaf tissue and stem apical meristem (Figure 5B). Attempts to correlate methylation and siRNA accumulation in tissue were achieved through RT/PCR. QPCR targeted the 21 nt siRNA ranked sixth in the abundance of the unique siRNAs to HS CP insert [34]. There was an apparent difference in siRNA content between leaves where the highest amount of siRNA is accumulated and the other tissues (Figure 5C). The same fragment was observed in C-4 leaf extract (Figure 5C, lane 4), suggesting that this particular siRNA may not be effective in conferring resistance [34, 36]. Tested as control, a fragment of the 5S ribosome [23, 30] was similarly amplified by OneStepRT/PCR (not shown) from all RNA templates with the primer pair cited in Table 1.

3.5 Sucking of siRNA by Macrolophus Bugs

Macrolophus pygmaeus bugs, routinely used as biological agents in greenhouse conditions, were fed on plum leaves. Lane 1 of Figure 6 shows that the amplicon of RNAi detected from insect extracts matches in size with the target fragment of HS used as control (lane 3). This study supports that *Macrolophus pygmaeus* bugs enable to mechanically suck off RNAi accumulated in HS leaves.

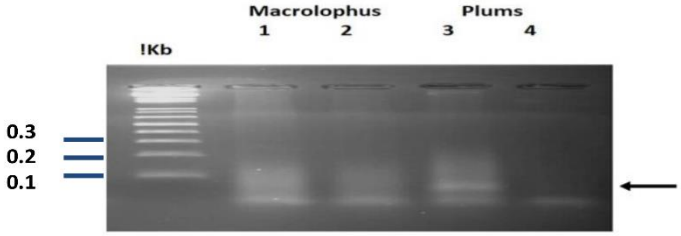

Figure 6 SiRNA detection in *Macrolophus pygmaeus* fed on plum leaves. Lane 1: RNA from insect fed on HS plum and 2: RNA from insect fed on Brompton plum. The following lanes including Lanes 3 (from leaves of HS and 4 (from leaves of plum control. Molecular weight markers (1 Kb), loaded at the first lane. Arrow at the right margin indicates the expected amplicon.

4. Discussion

Grafting techniques from centuries ago are still in use due to the benefits gained from combining two different genotypes. A few example applications (grapevines, *Prunus*, etc.) proved detrimental to the woody perennial plant industry. However, the application of transgenic genotypes as a new breeding concept [11, 12, 15] was explored by Zhao and Song [14] in their development of a transgenic cherry rootstock that was virus resistant [13, 14]. In their work, an untransformed sweet cherry cultivar grafted onto a resistant rootstock resisted PNRSV infection, apparently acquiring resistance *via* mobile siRNA from the transgenic tree. This example models one NBT [11-13, 24, 26, 27]. In our study, we grafted a susceptible clone onto rootstocks that were resistant to PPV infection (HS or B14) but saw PPV spreading and no resistance (Tables 2-6) (Figures 1-3). Silencing detected in the transgenic resistant HS and B14 plums did not spread to the grafted clones (Figures 1-5). According to a manuscript by Melnyk et al. [37], mobile molecules of siRNA are found in sink tissue, which, in the case of our plum model, would be green leaves. Unfortunately, we failed to detect siRNA mobility in our grafted clones. Among the questions raised by these studies was how and if the grafting technology affects this, and what is similar or different between the two *Prunus* genus trees (cherry (*P. avium*) and plum (*P. domestica*)), and the two plant viruses (PNRSV for cherry and PPV for plum). Recent results exploring PPV resistance sources in *Prunus dulcis* indicated that, through grafting experiments, different genes were either up- or down-regulated. Through transcriptomic and transplastomic studies, they identified small RNA involved in transcription factors and plant defenses (chitinases, acquired systemic responses…) [38, 39].

The lack of detectable transmission in plum trees is not unique; an absence of siRNA transfer *via* grafting was also noted in apple trees [40]. As noted by Sidirova et al. [41] and noted in the present results, the production of resistant plants from this NBT was ineffective. The RNAi, hypothesized to be transferred by grafting, did not move; thus, the RNAi movement that would expectedly trigger PPV RNA did not occur. Grafting did not enable RNAi transfer according to the NBT concept.

The transfer of siRNA through grafting could be an alternative way to provide a resistance trait to a conventional elite cultivar. However, the technology cannot be generalized because there is only one result of the transgrafting model (PNRSV/*Prunus avium*) from woody plants

demonstrating this movement [13]. Thus, while the concepts surrounding this NBT are exciting, its applicability remains an issue. While NBT can be considered a reliable answer [11-13, 15] to protect perennial plants against pathogens [21, 23, 25, 30, 38, 39], the suitability of NBT remains a clue for tree-growers, who will be the end users of these silencing technologies.

Two methods for transmitting PPV to woody perennials are known: natural aphid transmission and artificial inoculation through grafting. The latter is routinely used in research on the PPV inoculation of woody perennials [22, 23, 42]. Viable pathogens like PPV that can survive in bud sticks can spread through the vascular system of whole plants; likewise, any molecules in the rootstock, like virus particles or viral RNA, can reach the scion similarly (Tables 2-6, Figure 1). Related studies with transgenic cherry trees were successful had siRNAs that moved through the grafting point to confer virus resistance to the targeted host [14]. Having restricted our observations to uninfected healthy plants to look at which tissues were silenced, we did not study the post-transcriptional gene silencing machinery [17, 30, 33, 42] (Figure 4, Figure 5). The root, vascular, periderm and shoot apical meristem tissues seemed differentially methylated (Figure 5B), likely related to their developmental stages [18, 22, 23, 42]. This complicated the silencing process, as it appears to be expressed differently in woody perennial crops.

The interpretation of the results depicted here assumes that the viral transgene is transcribed as a dsRNA in any transgenic tissue; however, active silencing only occurs in the leaves [30, 42]. The correlation with the source-sink tissue of siRNA accumulation may support the correlation between siRNA and RNA-dependent DNA methylation (RdDM) in different tissues of woody perennial plants. Therefore, it is understandable that we saw in a perennial that silencing was restrictively active in the green tissues wherever the undesired virus genome was triggered by siRNA [18, 19, 22] (Figure 5). The symptomless appearances of siRNA in HS and B14 leaves demonstrate the difference between resistant and susceptible clones (Figures 2-5). When related to the scenario of the recovery reaction developed by the HS and B14 clones (Figure 2, Figure 3), silencing regulation is correlated with siRNA expression in leaf tissue. Because *Prunus* is a perennial plant, we may interpret that these molecular changes, via methylation [17, 30], support the recovery reactions [34, 42] associated with developmental stages (Figure 5B); thus, they are consequently the results of epigenetic phenomena [34, 42]. Our recent study with hybrid clones [42] obtained using cross-hybridization between a susceptible clone (clone C-2) [25] and a resistant clone (C1738) [21] harboring the hairpin RNA construct of the HoneySweet plum [23, 28] provides helpful insights. Regardless of the parental source, the virus DNA transgene of the hybrid clones was methylated [42]. These results suggest that transgene DNA methylation was involved in the RNAi machinery of the whole plant [34, 42]. In the present study, the methylation phenomenon could not be monitored in the untransformed shoot using grafting,. Similar observations have been corroborated by the unmethylated DNA status of a susceptible C-4 clone [17, 18, 22, 23] grafted onto either the HS or B14 clones (Figures 1-5).

Timing is uncertain; however, the idea of exploiting silencing in agriculture is exciting [43]. Transfer via grafting was among the targets of this NBT for improving woody plants [11, 24, 26, 27, 39]. Arguably, it is imperative that the mobilization of the 23 to 24-nucleotides (nt) siRNAs be facilitated either by vascular tissue or shoot-derived siRNA [30, 38, 39, 41]. One major difference between annual and perennial plants is the seasonal movement of the virus challenger through the vascular tissues of woody plants. Typically, in woody perennial plants, PPV can be blocked only in leaf tissues [18, 19, 41]. Therefore, the present data biased the results via grafting technology in

woody plants; hence, the new breeding plant biotechnology cannot be generalized. Trees' resistance response relies on vascular tissue's role during the developmental stage. To determine the silencing route, the apparent differential expression of silencing observed in the non-inoculated tissue of perennial plants should be further explored (Figure 5). Woody plants hypothesize that the interstock technology controlling the RNAi movement from the resistant plum clone to the untransformed scions involves several molecules, such as carbohydrates, hormones, cell wall components and transcription factors [44]. Through the comparative studies between asymptomatic and symptomatic *Prunus* leaves, Espinoza et al. [45] pointed out the involvement of different *Prunus* metabolites (sugars, phenolic compounds, organic acids…). These metabolic molecules represent some potential markers relevantly exploited for PPV detection. The leading questions surround which molecule types block RNAi movement; however, the cell wall is also known to assist in the movement of potyvirus [46].

The process is not simple, the complexity of these interactions is connected with the differences in the genetic resources of the transgenic interstock and the untransformed scions. In the present study, since the RNAi machinery was not conferred and was therefore not amplified in the untransformed tissue, the viral RNA systemically invaded the shoots *via t*he phloem tissue. Collum et al. reported that facing PPV infection, *Prunus* deployed some temporal and spatial changes around the phloem tissues [47]. Through the translatome approach, they pointed out that PPV is very active in phloem tissue. Based on our results, the effectiveness of RNAi strategies in woody plants can be improved by using a phloem-specific promoter, as recently shown with citrus trees [48].

Alternatively, gene pyramiding throughout conventional breeding combined with RNAi is the shortest way to improve crop genetic resources [49]. Unfortunately, research and development are hindered by regulations rejecting the use of genetically modified organisms [50]. It was seen in the present study that plums resistant to PPV infection could not release RNAi into the non-transformed shoot. We cannot restrict these findings to our experimental conditions because Sidirova et al. [41] obtained similar results. Because RNAi is among the key molecules involved, we did grafting techniques to envisage how the RNAi movement through transgrafting is an efficient strategy to protect a perennial plant. Unfortunately, disregarding the resistant clones utilized, the spread of PPV unexpectedly occurred in scions and broke our hope to exploit the transgrafting technology (Figure 7).

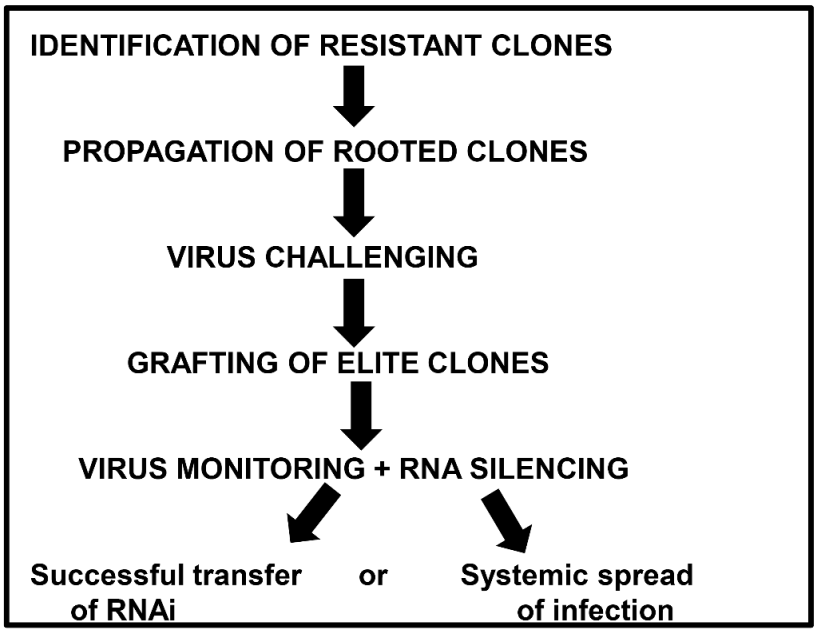

Figure 7 Guide to transgrafting technology in perennial plants

RNAi can be removed by *Macrolophus* bugs can remove RNAi *in situ*; this helps explain why small molecules like viruses, bacteria, fungi and siRNA, which are highly accumulated in the cytoplasm, can be removed. These experimental studies on the movement of siRNA through the plant sieve showed the effects of mechanical sucking by an insect not killed by the virus RNAi. This is not an issue because the accumulated RNAi in plums specifically triggers PPV RNA [19, 23, 30, 34, 41, 51]. These data indicate that RNAi may be a safe technology that will not damage natural biodiversity [51].

5. Conclusions

In addition to the different studies with transgenic resistant plums, here, we developed grafting techniques. In order to help growers in conferring virus resistance mediated by the engineered RNAi technology, the grafting strategies were used to transfer the engineered RNAi in non-transformed *Prunus*. Targeting the move of siRNA from the rootstock to the scion, we were unfortunate to observe that no engineered RNAi detected in the scion. The silencing mechanism is solely occurring in the transgenic resistant rootstock. Disregarding using the transgenic resistant clone as either rootstock (HoneySweet) or interstock (B14), the virus challenger is moving through the phloem. Based on the context analysis, challenging strategies in *Prunus* utilizing a virus transgene cassette under the control of a phloem-specific promoter should be explored.

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Author Contributions

MR conceptualized the study, actively contributed to the analytical experiments in laboratory and wrote the manuscript. PB conducted all the experiments in greenhouse including grafting, plant monitoring, tissue harvesting and performed the analytical study in laboratory (reagents, plant materials nucleic acid extraction, resistance monitoring…).

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Competing Interests

The authors declare no competing interest.

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