agronomy

MDPI

Article

Generation of Transgenic Rootstock Plum ((*Prunus pumila* L. × *P. salicina* Lindl.) × (*P. cerasifera* Ehrh.)) Using Hairpin-RNA Construct for Resistance to the *Plum pox virus*

Tatiana Sidorova [1,2,*], Alexander Pushin [1,2], Dmitry Miroshnichenko [1] and Sergey Dolgov [1,2]

[1] Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, 142290 Pushchino, Russia; aspushin@gmail.com (A.P.); miroshnichenko@bibch.ru (D.M.); dolgov@bibch.ru (S.D.)

[2] Nikitsky Botanical Gardens, Yalta, 98648 Nikita, Russia

* Correspondence: sidorovat@rambler.ru; Tel.: +7-496-773-1779

Received: 9 November 2017; Accepted: 15 December 2017; Published: 22 December 2017

Abstract: The use of *Prunus* rootstocks that are resistant to plum pox virus (PPV) is an important agronomic strategy to combat the spread of the Sharka disease in nurseries and orchards. Despite remarkable progress in developing stone fruit rootstocks to adapt to various stresses, breeding that ensures durable virus resistance has not yet been achieved. For this reason, the engineering of PPV resistant plants through genetic transformation is a very promising approach to control sharka disease. The aim of the present study is to produce transgenic plants of the clonal rootstock 'Elita', which is resistant to PPV using ribonucleic acid interference (RNAi) technology. The genetic construct containing the self-complementary fragments of the plum pox virus coat protein (PPV-CP) gene sequence were used to induce the mechanism of post-transcriptional gene silencing to ensure virus resistance. Transgenic plants have been produced after agrobacterium-mediated transformation of in vitro explanted leaves. The results of polymerase chain reaction (PCR) and Southern blotting analyses confirmed the stable genomic integration of the PPV-CP sense and antisense intron-hairpin-RNA sequence. The functionality of the introduced expression cassette was confirmed by the activity of including the *uidA* gene into the transferring T-DNA. To our knowledge, this is the first interspecific plum rootstock produced by genetic engineering to achieve PPV resistance.

Keywords: sharka disease; stone fruits; genetic transformation; leaf explant; RNA interference

1. Introduction

Plum pox virus (PPV), a member of the *Potyvirus* genus in the *Potyviridae* family, is a serious devastating disease affecting a number of the *Prunus* species. Initially discovered in Bulgaria in the early 1900s, PPV is currently found in almost all European countries, the Middle East, Caucasus, Central Asia, North Africa, China, Canada, and the United States of America [1]. Known as sharka, this disease causes chlorotic spots, irregular lines or rings on leaves, as well as the deformation and malformation of fruits [2]. When infected with PPV, many commercial cultivars of peach, plum, apricot, and nectarine produce unmarketable fruit or prematurely lose their crop [3]. In many countries traditional practices, such as periodic surveys, compulsory eradication of diseased trees and insecticide treatment to control aphids, are not often effective in constraining plum pox disease [4]. Current losses in Europe, where the virus is most prevalent, amount to $180 million each year [3]. Since the spread of the PPV virus in nurseries or orchards can occur in an unpredictable manner, it is expected that economic losses in various regions will continue to grow [1]. Taking into account the inability to cure infected plants through chemical treatment, the best approach to control sharka disease is to develop PPV resistant stone fruit trees.

Despite numerous efforts to identify the natural *Prunus* sources of PPV resistance, only a few germplasms are currently known as potential material for breeding [5,6]. In plums, however, natural sources for stable and durable resistance are not available. For this reason, only cultivars exhibiting PPV-induced hypersensitive response are considered as possible sources for the breeding of partial tolerance in plums [7]. Therefore, it is not surprising that the appearance of the first genetically engineered PPV-resistant plum 'Honeysweet' was greeted with enormous enthusiasm more than twenty years ago [8]. The produced transgenic plants were highly resistant to the plum pox virus infection due to the introduction of a sense construct of gene encoding the viral coat protein (CP) [9]. Later, it was found that the effective resistance to the virus in the 'Honeysweet' was not actually protein-mediated. In other words, the results of the unpredictable activation of RNA silencing against the PPV CP sequence that occurred was due to a duplication and rearrangement of CP gene copies during the transgene insertion [1]. By this time, it became obvious that the transgenic strategies based on the expression of sequences derived from the viral genome mainly resulted in unstable and unpredictable PPV resistance [1,4]. From the mid-2000s, the RNA silencing pathway has become a main tool for engineering virus resistant *Prunus* species. Since then the RNAi technology has been successfully used to produce transgenic plants displaying various levels of resistance to sharka disease [10–12]. In our laboratory, the PPV-derived gene constructs encoding intron-spliced hairpin RNAs (ihpRNAs) were successfully introduced and expressed in the commercial plum cultivar 'Startovaja' (*Prunus domestica* L.) [12]. The resulting transgenic plum trees showed no symptoms of sharka disease during five years of cultivation despite the long-term systemic PPV infection caused by the infected material grafted onto the transgenic shoots [13].

As long as the stone fruit trees in orchards represent the grafted commercial cultivar onto a rootstock, effective disease-control strategies should include the development of PPV resistant rootstocks. Moreover, the grafting of a non-genetically modified (non-GM) scion onto the transgenic rootstock is regarded as a very promising biotechnological tool, as it has fewer biosafety concerns [14]. Such an approach opens up the possibility of producing non-transgenic fruit while ensuring the overall resistance to viral infection using a transgenic ribonucleic acid interference (RNAi) inducing rootstock. The success of this approach, however, has largely depended on the species, the level of expression and the interactions of the small interfering ribonucleic acid (siRNA) with the target gene [15]. For example, the transfer of transgene-derived siRNAs from the transgenic cherry rootstocks to the non-transgenic scions in grafted trees was effective in inducing resistance to the *Prunus necrotic ringspot virus* [16]. In apples, however, the transmission of siRNAs from transgenic rootstock to non-transgenic scion was not observed [17]. Such contradictory data indicates that direct trials of rootstock-to-scion delivery are required to confirm the efficacy of the transgrafting technology for individual species.

It is generally admitted that plant regeneration and the genetic transformation of fruit trees is a demanding and time consuming process. Methods for stable genetic transformation of the *Prunus* genus have been developed since the beginning of the 1990s. Most plum transformation protocols involve agrobacterium-mediated gene transfer into regenerable cells of seed derived explants, such as hypocotyl [18]. It should be noted, however, that the practical use of seed derived plants is limited due to the loss of the original properties of the cultivar. The ability to regenerate transgenic plants from somatic tissues, such as leaves or stems, could significantly accelerate the application of RNAi technology for conferring PV resistance to commercial varieties and clonal rootstocks. Recently, we have described an efficient in vitro culture system initiating from leaf explants of the European plum that enables transgenic plant regeneration with retention of the original traits of the cultivar [12,19]. In this paper, we present the usefulness of this in vitro culture system for obtaining the genetically modified plum rootstock 'Elita' ((*Prunus pumila* L. × *P. salicina* Lindl.) × (*P. cerasifera* Ehrh.)). In the present study, efforts have been directed to express the genetic construct containing the self-complementary sequences of fragments of PPV-CP for the induction of plum pox virus resistance in plum rootstock.

2. Results and Discussion

In most *Prunus* species, plant regeneration and genetic transformation are not routine. Due to a high recalcitrance with respect to in vitro organogenesis, the successful generation of transgenic plum plants is limited to a few genotypes [4]. Modern interspecific rootstocks are often specially developed to withstand bacterial infection, therefore, the introduction of a transgene using an agrobacterium-mediated approach is more difficult to achieve. It is not surprising that there have not been any reports announcing the successful genetic transformation of plum rootstocks [20]. In the present study, histochemical analysis easily revealed the presence of the transient GUS activity in leaf explants starting 5–15 days after co-cultivation with AGL0:pCamPPVRNAi. However, the appearance of the antibiotic resistant organogenic calli (Figure 1a) was detected later (90–120 days of culture) than in a similar experiments with European plums 'Startovaya' [21].

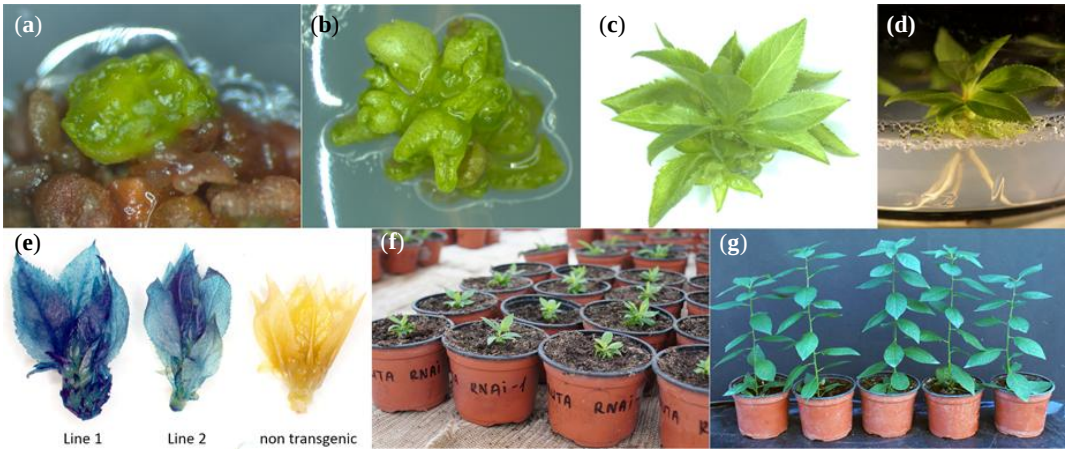

Figure 1. Generation of transgenic rootstock plants. (**a**) Formation of green organogenic callus on leaf explant; (**b**) formation of leaf-like cluster; (**c**) elongated transgenic shoot; (**d**) rooting of selected shoot on medium supplemented with hygromycin; (**e**) blue staining of transgenic shoots due to activity of *uidA* gene; (**f**,**g**) establishment of transgenic plum rootstock in greenhouse.

Although many plants survived, calluses demonstrate GUS activity and the recovery of transgenic plants of rootstock was very low. In three experiments, only two independent calli produced transgenic plants after six months of selection (Table 1). First, the shoot buds formed clusters of leaves without a main stem (Figure 1b). Then, after the detaching from callus and transferring into the proliferation medium, several clusters elongated and developed a main stem with leaves (Figure 1c). Transgenic elongated shoots could be easily maintained for several subcultures in the selective proliferation medium and readily rooted in the presence of antibiotic (Figure 1d). The resulting plants demonstrated a clear *uidA* expression in leaves and stems (Figure 1e).

Table 1. Efficiency of the genetic transformation of plum rootstock 'Elita' by CamPPVRNAi vector.

Experiment Event	Number of Explants	Number of Explants Produced Shoot Clusters	Number of PCR Positive Lines	Transformation Rate, %
1	113	1	0	0.0
2	148	2	1	0.7
3	125	2	1	0.8
total	386	5	2	0.5

PCR: polymerase chain reaction.

Both independent lines were polymerase chain reaction (PCR) positive for HPT (Figure 2a) and negative for a contamination by the *Agrobacterium* virG gene (not shown). To support the complete integration of the RNAi construct, transgenic lines were analyzed by PCR using a primer specific for the PPV-CP gene and two primers located in the CaMV35S promoter and the octopine synthase gene terminator. In both plum rootstock lines, the PPV-derived sense and antisense fragments were correctly amplified. Figure 2b shows the Southern blot of the genomic DNA of transgenic plum lines and nontransgenic plum plants treated with EcoRI, and hybridized with a probe specific to the PPV-CP sequence (Figure 3). Results confirmed that the integration of the nucleotide sequence encoded the PPVRNAi hairpin into the plum rootstock genome, while the DNA from nontransformed plant failed to hybridize the probe. Based on the hybridization profile, there is only one insertion of the expression cassette into the plant genome. Diverse hybridization patterns, however, confirmed that each line is the result of independent transformation events.

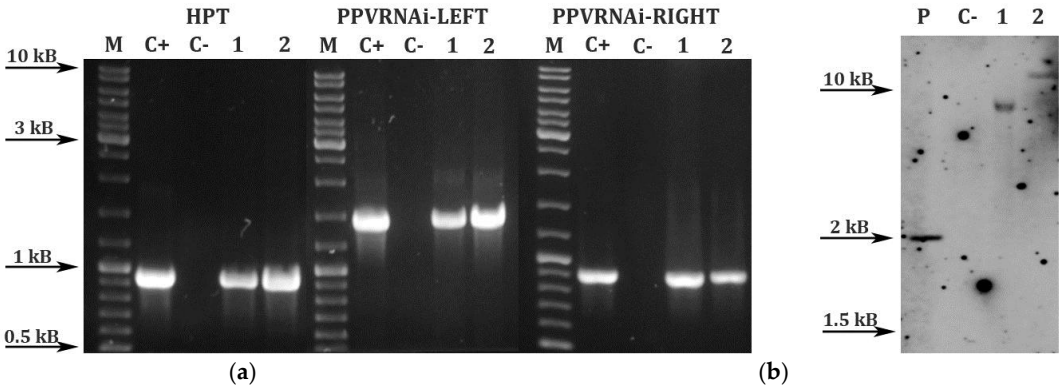

Figure 2. Analysis of transformed plum rootstock for the presence of transferred genes. (**a**) PCR analysis of AGL0:pCamPPVRNAi-transformed plum rootstock plants, where C+—pCamPPVRNAi, 1 and 2—DNA of transgenic lines; C−—genomic DNA of nontransgenic plum plant; (**b**) Southern hybridization analysis of AGL0:pCamPPVRNAi-transformed plum rootstock plants, DNA (30 µg) extracted from leaves was digested with EcoRI and hybridized with a 0.7kB PPV probe, P—DNA of pCamPPVRNAi/XbaI; 1 and 2—DNA of transgenic lines digested with EcoRI; C−—genomic DNA of non-transgenic plum plants digested with EcoRI.

In the present study, the transformation efficiency in the plum rootstock (0.4%) was lower than that of similar hygromycin selection in the European plums 'Startovaya' (on average 1.4–2.2% with different vectors) [12,21]. The resulting transformation efficiency for interspecific rootstock 'Elita' was also lower compared to *P. persica* [22], *P. dulcis* [23], *P. verginiana* [24], *P. armeniaca* [25], and *P. serotina* [26]. The similar transformation efficiencies (0.3–0.8%) were only reported for Japanese plum *P. salicina* [27], which is a species that is one of the parents of the rootstock 'Elita'. However, in the case of the Japanese plum, as well as of the other mentioned species, the transgenic plants were mainly regenerated from hypocotyl segments, which demonstrated higher regeneration abilities. In the present study, the transformation was achieved using leaf tissue, therefore providing a considerably more useful approach in the development of new traits without changing the original genetic makeup. Since the number of surviving transgenic calli was significantly higher than the number of recovered transgenic rootstock plants, better transformation efficiency may be achieved if some improvements can be made during the *Agrobacterium* infection and transformant selection. Further study concerning the modification of tissue culture technique would be required to in order to examine the regeneration abilities of developed calli. Another important factor for improvement is the timing and severity of transgenic tissue selection. Our previous research on the European plum showed that a higher transformation frequency could be obtained using a delayed selection strategy where explants were cultivated without selective pressure within three weeks after their co-culture with *Agrobacterium* [12,19]. By contrast, a shorter delay on the selection of hygromycin was more

efficient in producing transgenic plants from hypocotyl segments of the plum 'Stanley' [28]. Taking into account the significant genetic variation between interspesific rootstock and commercial plum cultivars, different selection strategies should be thoroughly investigated.

This present work is an initial report on the production of transgenic plum rootstock to control PPV resistance. Plants of two independent transgenic lines were successfully transferred to the greenhouse (Figure 1f,g) and were grafted with PPV (serotype M) (PPV-M) infected buds to examine their virus resistance. Previously, we have demonstrated that the expression of an antiviral hairpinRNA construct from a pCamPPVRNAi vector results in stable resistance to systemic PPV infection in all produced transgenic lines of the European plum 'Startovaya'. This result prompts us to believe that this strategy will be further successfully applied to prevent or reduce the PPV spread in transgenic lines of the plum rootstock 'Elita'. The presented results open the way to build mature stone fruit trees, in which both grafted scions and rootstock will show predictable resistance to PPV infection and disease.

3. Materials and Methods

3.1. Plant Material

The plant material consisted of in vitro cultured shoots of semi-dwarf plum rootstock 'Elita' ((*Prunus pumila* L. × *P. salicina* Lindl.) × (*P. cerasifera* Ehrh.)). Micropropagation and rooting of in vitro shoots were realized as described in [19].

3.2. Vector

The genetic construct pCamPPVRNAi (Figure 3), containing the self-complementary fragments (698 bp) of the PPV-CP gene sequence separated by a PDK intron from pHANNIBAL under the modified enh35S promoter, was used for the induction of PPV resistance through the mechanism of post-transcriptional gene silencing. The overall sequence size of PPV-CP sense and antisense intron-hairpin-RNA transcripts is 2220 bp. The transfer DNA (T-DNA) of binary vector pCamPPVRNAi also contains the HPT gene under the duplicated cauliflower mosaic virus 35S promoter (d35S) as a selectable marker and the GUS intron gene under the CaMV 35S promoter as a reporter marker.

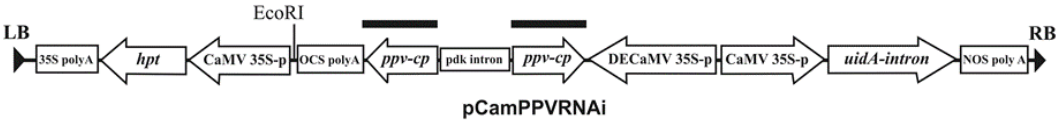

Figure 3. Schematic presentation of the T-DNA region of pCamPPVRNAi, plasmid used for plum rootstock transformation. Abbreviations: CaMV 35-p—35S RNA Cauliflower Mosaic Virus promoter; DECaMV35-p—35S RNA Cauliflower Mosaic Virus promoter (double); *uidA-intron*—intron-containing beta-glucuronidase (GUS) gene (uidA); NOS polyA—nopaline synthase terminator with polyadenylation signal; OCS polyA—octopine synthase terminator with polyadenylation signal; *hpt*—hygromycin phosphotransferase (HPT) coding sequence; 35S polyA—CaMV 35S terminator with polyadenylation signal; PPV-CP—the fragments of the PPV-CP gene of plum pox virus, pdk intron—intron from pyruvate orthophosphate dikinase of potatoes; RB—right border; LB—left border; black bar-probe binding site.

3.3. Agrobacterium-Mediated Transformation and Plant Regeneration

The transformation experiments were conducted using the super virulent Agrobacterium strain AGL0 [29]. The apical leaves excised from in vitro rooting shoots were pre-incubated for 2.5 h in culture vessels containing liquid Murashige and Skoog (MS) [30] medium supplemented with 5 mg L^{-1} indole-3-acetic acid (IAA) that was gently shaken. Incubated leaves were then cut transversely four or six times across the midrib without fully separating the segments and transferred into a agrobacterial suspension, before being gently shaken for 30 min. *Agrobacterium* was inoculated in liquid Luria–Bertani (LB) medium to OD600 of 0.8–1.0 and then mixed with a hormone-free liquid MS

medium (1:10 *v*/*v*) prior to immersing the plum explants into a agrobacterial suspension. The infected leaves were blotted dry on sterile filter paper and cultured with the adaxial side in contact with the shoot regeneration medium, which consisted of MS salts and vitamins, 3% sucrose (*w*/*v*), 0.7% agar (*w*/*v*), 500 mg/L casein hydrolysate, 100 mg/L myo-inositol, 4 mg/L calcium pantothenate, 5 mg/L benzyl aminopurine (BAP), 0.5 mg/L indole-butyric acid (IBA), pH 5.8, placed in the dark at 24 °C for three days. After the co-culture, leaves were transferred onto the same supplement with 500 mg/L cefotaxime to control *Agrobacterium* growth and remained placed in darkness. After three weeks, the explants were transferred to the light, with a 16-h photoperiod for a selection onto the regeneration medium supplemented with 6 mg L^{-1} hygromycin and 500 mg/L cefotaxime. The explants were transferred to a fresh selective medium every 10 days. When putative transgenic shoots reached 1 cm in length, they were transferred to the proliferation medium, which consisted of JS [31] mineral salts, MS [30] vitamins, 3% sucrose (*w*/*v*), 0.7% agar (*w*/*v*), 100 mg L^{-1} myo-inositol, 1.5 mg L^{-1} BAP, 0.1 mg L^{-1} IBA, and 6 mg L^{-1} hygromycin. Resulting plants were rooted in a medium containing half-strength JS mineral salt, MS vitamins, 2% sucrose (*w*/*v*), 0.5 mg L^{-1} IBA, 0.7% (*w*/*v*) agar and 5 mg L^{-1} hygromycin. Four to six weeks later, the rooted plantlets were transplanted to soil as described in [19].

3.4. Histochemical GUS Assay

The histochemical GUS assay of plant tissues was performed as described in [32].

3.5. PCR Analysis and Southern Hybridization

Genomic DNA was extracted from young leaves of three to four week old plantlets which had been growing in the selection medium using the cetyltrimethylammonium bromide (CTAB) method as described in [33]. To provide amplification, a 1476-bp fragment comprising of the sequence of the modified CaMV 35S promoter and part of the *ppv-cp* gene primers 35S712For (forward, 5′-CAGCAGGTCTCATCAAGACGATCTACC-3′) and PPVUpRNAi (reverse (for this reaction), 5′-AAGAGAAGACCTGGAGGAAGTTGATG-3′) were used. For the amplification, a 897-bp fragment comprising of the sequence of part of PPV-CP gene and the octopine synthase gene terminator primers PPVUpRNAi (forward for this reaction) and OcsTerRev (reverse, 5′-AGTAGTAGGGTACAATCAGTAAATTGAACGGAG-3′) were used. In order to provide amplification, a 951-bp fragment of the *hpt* gene primers hptIIF (forward, 5′-CGACGTCTGTCGAGAAGTTTCTGATC-3′) and hptIIR (reverse, 5′-GTACTTCTACACAGCCATCGGTCCA-3′) were used. The amplified DNA fragments were visualized under ultraviolet light after electrophoresis on 1.0% agarose gel containing a TAE running buffer and ethidium bromide.

Southern blot analysis was also performed upon digestion of the plum's genomic DNA (30 μg) with EcoRI. The enzyme was cut once with the T-DNA of plasmid and produced a diagnostic fragment as shown in Figure 3. Digested DNA was fractionated on 0.9% agarose gel and then blotted onto a positively charged Hybond N+ nylon membrane (GE Healthcare, Little Chalfont, UK) by capillary methods following the manufacturer's instructions. A 704 bp PCR amplified fragment corresponding to the coding sequence of the PPV-CP gene was used as a probe. The DNA probe was labelled with alkaline phosphatase using the Amersham Gene Image AlkPhos Direct Labelling and Detection System (GE Healthcare, Little Chalfont, UK). Prehybridization, hybridization and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling System protocol. Detection was performed with a help of CDP-Star detection reagent according to the instruction manual (Amersham CDP-Star Detection reagent, GE Healthcare, Little Chalfont, UK).

Acknowledgments: The research was funded in part by a research grant No. 14-50-00079 of the Russian Science Foundation.

Author Contributions: T.S. and S.D. conceived and designed the experiments; T.S. and A.S. performed the experiments; T.S. and D.M. analyzed the data; S.D contributed reagents/materials; T.S. and D.M. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Scorza, R.; Callahan, A.; Dardick, C.; Ravelonandro, M.; Polak, J.; Malinowski, T.; Zagrai, I.; Cambra, M.; Kamenova, I. Genetic engineering of Plum pox virus resistance: 'HoneySweet' plum—From concept to product. *Plant Cell Tissue Organ Cult.* **2013**, *115*, 1–12. [CrossRef]
2. Llacer, G.; Cambra., M. Hosts and symptoms of Plum pox virus: Fruiting Prunus species. *EPPO Bull.* **2006**, *36*, 219–221. [CrossRef]
3. Cambra, M.; Capote, N.; Myrta, A.; Llacer, G. Plum pox virus and the estimated costs associated with sharka disease. *Bull. OEPP/EPPO Bull.* **2006**, *36*, 202–204. [CrossRef]
4. Ilardi, V.; Tavazza, M. Biotechnological strategies and tools for Plum pox virusresistance: Trans-, intra-, cis-genesis, and beyond. *Front. Plant Sci.* **2015**, *6*, 379. [CrossRef]
5. Rubio, M.; Pascal, T.; Bachellez, A.; Lambert, P. Quantitative trait loci analysis of Plum pox virus resistance in *Prunus davidiana* P1908: New insights on the organization of genomic resistance regions. *Tree Genet. Genomes* **2010**, *6*, 291–304. [CrossRef]
6. Marandel, G.; Salava, J.; Abbott, A.; Candresse, T.; Decroocq, V. Quantitative trait loci meta-analysis of Plum pox virus resistance in apricot (*Prunus armeniaca* L.): New insights on the organization and the identification of genomic resistance factors. *Mol. Plant Pathol.* **2009**, *10*, 347–360. [CrossRef] [PubMed]
7. Hartmann, W.; Neumuller, M. *"Plum Breeding", in Breeding Plantation Tree Crops: Temperate Species*; Mohan, J.S., Priyadarshan, P.M., Eds.; Springer: New York, NY, USA, 2009; pp. 161–231.
8. Scorza, R.; Ravelonandro, M.; Callahan, A.M.; Cordts, J.M.; Fuchs, M.; Dunez, J.; Gonsalves, D. Transgenic plum (*Prunus domestica* L.) express the Plum pox virus coat protein gene. *Plant Cell Rep.* **1994**, *14*, 18–22. [CrossRef] [PubMed]
9. Ravelonandro, M.; Scorza, R.; Bachelier, J.C.; Labonne, G.; Levy, L.; Damsteegt, V.; Callahan, A.M.; Dunez, J. Resistance of transgenic *Prunus domestica* to plum pox virus infection. *Plant Dis.* **1997**, *81*, 1231–1235. [CrossRef]
10. Wang, A.; Tian, L.; Brown, D.C.W.; Svircev, A.M.; Stobbs, L.W.; Sanfaçon, H. Generation of efficient resistance to Plum pox virus (PPV) in Nicotiana benthamiana and *Prunus domestica* expressing triple-intron-spanned double-hairpin RNAs simultaneously targeting 5′ and 3′ conserved genomic regions of PPV. *Acta Hortic.* **2013**, *1063*, 77–84. [CrossRef]
11. Ravelonandro, M.; Scorza, R.; Hily, J.M.; Briard, P. The efficiency of RNA interference for conferring stable resistance to Plum pox virus. *Plant Cell Tissue Organ Cult.* **2014**, *118*, 347–356. [CrossRef]
12. Mikhailov, R.V.; Dolgov, S.V. Agrobacterium-mediated transformation of plum cv. Startovaya by selfcomplementary hairpin RNA of PPV-CP gene. *Acta Hortic.* **2011**, *941*, 85–90. [CrossRef]
13. Sidorova, T.N.; Vagapova, T.I.; Dolgov, S.V. Intron-hairpin-RNA construct provides stable resistance to Plum pox virus in plum cultivar 'Startovaja'. *Acta Hortic.* **2016**, *1110*, 197–202. [CrossRef]
14. Lemgo, G.N.; Sabbadini, S.; Pandolfini, T.; Mezzetti, B. Biosafety considerations of RNAi-mediated virus resistance in fruit-tree cultivars and in rootstock. *Transgenic Res.* **2013**, *22*, 1073–1088. [CrossRef] [PubMed]
15. Limera, C.; Sabbadini, S.; Sweet, J.B.; Mezzetti, B. New biotechnological tools for the genetic improvement of major woody fruit species. *Front. Plant Sci.* **2017**, *8*, 1418. [CrossRef] [PubMed]
16. Zhao, D.; Song, G.Q. Rootstock-to-scion transfer of transgene-derived small interfering RNAs and their effect on virus resistance in nontransgenic sweet cherry. *Plant Biotechnol. J.* **2014**, *12*, 1319–1328. [CrossRef] [PubMed]
17. Flachowsky, H.; Trankner, C.; Szankowski, I.; Waidmann, S.; Hanke, M.; Treutter, D.; Fischer, C.T. RNA-mediated gene silencing signals are not graft transmissible from the rootstock to the scion in greenhouse-grown apple plants *malus* spp. *Int. J. Mol. Sci.* **2012**, *13*, 9992–10009. [CrossRef] [PubMed]
18. Padilla, I.M.G.; Burgos, L. Aminoglycoside antibiotics: Structure, functions and effects on in vitro plant culture and genetic transformation protocols. *Plant Cell Rep.* **2010**, *29*, 1203–1213. [CrossRef] [PubMed]
19. Sidorova, T.; Mikhailov, R.; Pushin, A.; Miroshnichenko, D.; Dolgov, S. A non-antibiotic selection strategy uses the phosphomannose-isomerase (PMI) gene and green fluorescent protein (GFP) gene for *Agrobacterium*-mediated transformation of *Prunus domestica* L. leaf explants. *Plant Cell Tissue Organ Cult.* **2017**, *128*, 197–209. [CrossRef]
20. Gambino, G.; Gribaudo, I. Genetic transformation of fruit trees: Current status and remaining challenges. *Transgenic Res.* **2012**, *21*, 1163–1181. [CrossRef] [PubMed]

21. Dolgov, S.; Mikhaylov, R.; Serova, T.; Shulga, O.; Firsov, A. Pathogen-derived methods for improving resistance of transgenic plums (*Prunus domestica* L.) for Plum pox virus infection. *Julius Kühn Arch.* **2010**, *427*, 133–140.
22. Padilla, I.M.; Golis, A.; Gentile, A.; Damiano, C.; Scorza, R. Evaluation of transformation in peach *Prunus persica* explants using green fluorescent protein (GFP) and beta-glucuronidase (GUS) reporter genes. *Plant Cell Tissue Organ Cult.* **2006**, *84*, 309–314. [CrossRef]
23. Ramesh, S.A.; Kaiser, B.N.; Franks, T.; Collins, G.; Sedgley, M. Improved methods in Agrobacterium-mediated transformation of almond using positive (mannose/pmi) or negative (kanamycin resistance) selection-based protocols. *Plant Cell Rep.* **2006**, *25*, 821–828. [CrossRef] [PubMed]
24. Dai, W.; Magnusson, V.; Johnson, C. Agrobacterium-Mediated Transformation of Chokecherry (*Prunus virginiana* L.). *HortScience* **2007**, *42*, 140–142.
25. Petri, C.; Wang, H.; Alburquerque, N.; Faize, M.; Burgos, L. Agrobacterium-mediated transformation of apricot (*Prunus armeniaca* L.) leaf explants. *Plant Cell Rep.* **2008**, *27*, 1317–1324. [CrossRef] [PubMed]
26. Liu, X.; Pijut, P.M. Agrobacterium-mediated transformation of mature *Prunus serotina* (black cherry) and regeneration of transgenic shoots. *Plant Cell Tissue Organ Cult.* **2010**, *101*, 49–57. [CrossRef]
27. Urtubia, C.; Devia, J.; Castro, Á.; Zamora, P.; Aguirre, C.; Tapia, E.; Barba, P.; Dell'Orto, P.; Moynihan, M.R.; Petri, C.; et al. *Agrobacterium*-mediated genetic transformation of *Prunus salicina*. *Plant Cell Rep.* **2008**, *27*, 1333–1340. [CrossRef] [PubMed]
28. Tian, L.; Canli, F.A.; Wang, X.; Sibbald, S. Genetic transformation of *Prunus domestica* L. using the *hpt* gene coding for hygromycin resistance as the selectable marker. *Sci. Hortic.* **2009**, *119*, 339–343. [CrossRef]
29. Lazo, G.R.; Stein, P.A.; Ludwig, R.A. A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Nat. Biotechnol.* **1991**, *9*, 963–967. [CrossRef]
30. Murashige, T.; Skoog, F. A revised medium for rapid growth bio assays with tobacco tissue culture. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
31. Jacoboni, A.; Standardi, A. La moltiplicazione "in vitro" del melo cv. Wellspur. *Riv. Ortoflorofruttic. Ital.* **1982**, *66*, 217–229.
32. Jefferson, R.A. Assaying chimeric genes in plants: The *GUS* gene fusion system. *Plant Mol. Biol. Rep.* **1987**, *5*, 387–405. [CrossRef]
33. Rogers, S.; Bendich, A. Extraction of total cellular DNA from plants, algae and fungi. In *Plant Molecular Biology Manual*; Gelvin, S., Schiperoort, R., Eds.; Springer: Dordrecht, the Netherlands, 1994; Section 7-1; pp. 183–190.