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Short communication

A fast and efficient protocol for small RNA extraction in Japanese plum and other *Prunus* species

Evelyn Sánchez [a], David Tricon [b], Roxana Mora [a], Daniela Quiroz [a], Véronique Decroocq [b], Humberto Prieto [a,*]

[a] Laboratorio de Biotecnología, Estación La Platina, Instituto de Investigaciones Agropecuarias, Santa Rosa 11610, La Pintana, Santiago, Chile
[b] Biologie du Fruit et Pathologie UMR1332, Centre INRA Bordeaux-Aquitaine, 71 avenue Edouard Bourlaux – CS 20032 - 33882 Villenave d'Ornon Cedex, Bordeaux, France

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ABSTRACT

Background: Small ribonucleic acids represent an important repertoire of mobile molecules that exert key roles in several cell processes including antiviral defense. Small RNA based repertoire includes both small interfering RNA (siRNA) and microRNA (miRNA) molecules. In the *Prunus* genus, sharka disease, caused by the *Plum pox virus* (PPV), first occurred on European plum (*Prunus domestica*) and then spread over among all species in this genus and thus classified as quarantine pathogen. Next-generation sequencing (NGS) was used for the study of siRNA/miRNA molecules; however, NGS relies on adequate extraction protocols. Currently, knowledge of PPV-*Prunus* interactions in terms of siRNA populations and miRNA species is still scarce, and siRNA/miRNA extraction protocols are limited to species such as peach, almond, and sweet cherry.
Results: We describe a reliable procedure for siRNA/miRNA purification from *Prunus salicina* trees, in which previously used protocols did not allow adequate purification. The procedure was based on a combination of commercially available RNA purification kits and specific steps that yielded high quality purifications. The resulting molecules were adequate for library construction and NGS, leading to the development of a pipeline for analysis of both siRNAs and miRNAs in the PPV–*P. salicina* interactions. Results showed that PPV infection led to altered siRNA profiles in Japanese plum as characterized by decreased 24-nt and increased 21- and 22-nt siRNAs. Infections showed miR164 and miR160 generation and increased miR166, miR171, miR168, miR319, miR157, and miR159.
Conclusion: We propose this protocol as a reliable and reproducible small RNA isolation procedure for *P. salicina* and other *Prunus* species.

1. Introduction

Under the current scenario of climate change, several *Prunus* spp. including peach (*Prunus persica*), sweet cherry (*Prunus avium*), almond (*Prunus dulcis*) and plum (*Prunus salicina* and *Prunus domestica*), have demonstrated special sensitivity when facing environmental challenges such as chilling requirement, off-season frost, and increasing drought [1]. Also, these climate events represent improved conditions for the expansion of relevant pathogens affecting this taxonomic family [2]. Sharka disease, caused by the *Plum pox virus* (PPV), first occurred on European plum (*P. domestica*) and then spread over the last century among all Prunus species. It has therefore long been classified as quarantine pathogen. Indeed, the virus predominantly affects stone fruit crops at both agronomical and economical levels [3].

It is generally accepted that several tolerance mechanisms in plants rely on the regulation of gene expression and that they occur at both transcriptional and post-transcriptional levels. These processes involve both gene silencing and epigenetic regulations. Small interfering RNA (siRNA) and microRNA (miRNA) molecules are known to be extensively associated with these processes, and their characterization is extremely useful and informative regarding diverse tree physiology mechanisms. In this regard, siRNA/miRNA involvement in tree–environment interactions are the focus of the current study [4,5,6] and in particular, the unraveling of small RNA molecules involved in viral infection [7].

In *Prunus*, next generation sequencing (NGS) platforms have already been successfully used to detect miRNA molecules and siRNA populations. Using information from these platforms, identification and characterization of conserved and novel siRNA molecules and/or miRNAs associated with chilling events in peach [5], almond [6], and sweet cherry [4] have been proposed. Unfortunately, siRNA and miRNA characterization using NGS has relevant limitations involving time, cost, and most importantly, versatility of extraction methods. In

* Corresponding author.
E-mail address: hprieto@inia.cl (H. Prieto).
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the case of PPV-*Prunus* interactions regarding siRNAs/miRNAs, knowledge is very scarce precisely due to these technical difficulties [8].

Currently, the characterization of siRNA and miRNA profiles and populations has not been done in plum species (*P. salicina* and *P. domestica*) despite the economic relevance of this group of fruit and nut crops. Also, there are no reports about PPV–tree interactions regarding the small RNA profiles of different species generated during the interaction. In the present study, we have detailed a step-by-step small/micro RNA purification method that was used for extensive NGS experiments and *P. salicina* analyses. Experiments were performed under the PPV–plum interaction context. The protocol represents an improved method based on our previous studies done in either in woody [4,9] and/or model species [10], which did not allow for a satisfactory extraction of small RNAs in plum species.

2. Materials and methods

2.1. Plant material

In late spring, young plum leaves were collected from well-watered plants kept in pots under greenhouse conditions (*P. domestica* var. *insititia, P. salicina*, and *Prunus tomentosa*). Light regime, temperature, and humidity were according to environmental conditions. Before extraction, leaves were washed with diethyl pyrocarbonate (DEPC)-treated water (DEPC-water), collected immediately into liquid nitrogen, and stored at -80°C until used.

2.2. Reagents for RNA isolation

Reagents included several chemicals and kits: (1) PureLink® Plant RNA Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA); (2) miRNeasy Mini kit (Qiagen, Hilden, Germany); (3) RNeasy MinElute Cleanup Kit (Qiagen); (4) RWT Buffer (Qiagen); (5) RPE Buffer (Qiagen); (6) chloroform, and (7) absolute ethanol. Solutions included 5 M NaCl, 80% ethanol (*v*/*v*), 70% ethanol (*v*/*v*), and 0.1% (*v*/*v*) DEPC-water. All aqueous solutions were prepared in 0.1% DEPC-water. Water was NanoPure-distilled quality, obtained from a Synergy® Water Purification System (Merck KGaA, Darmstadt, Germany).

2.3. Laboratory utensils

Mortars and pestles were treated with RNase*Zap*® RNase Decontamination Solution (Thermo Fisher Scientific Inc.) and washed twice with 0.1% DEPC-water. Centrifuge tubes not supplied in kits were autoclaved in clean RNAse Free reservoirs. Certified filtered pipette tips were used.

2.4. Reagents for RNA analysis

Low and high molecular weight RNA, polymerase chain reaction (PCR) Microplates (Axygen Inc., CA, USA), Fragment Analyzer™ Automated CE System (AATI Advanced Analytical Technologies, IA, USA), Standard Sensitivity RNA Analysis Kit (AATI Advanced Analytical Technologies), High Sensitivity RNA Analysis Kit (AATI, Advanced Analytical Technologies), BioSpec-nano Micro-volume UV–Vis Spectrophotometer (Shimadzu, Kyoto, Japan), Ribogreen (Thermo Fisher Scientific Inc.), 10 mM Tris–HCl pH 8.0 buffer, and UltraPure DNAse/RNAse Distilled Water (Thermo Fisher Scientific Inc. MA, USA) were used.

2.5. Tissue grinding

Between 40 and 100 mg of plant tissue was ground as a fine powder in a mortar and pestle using liquid nitrogen to avoid sample thawing. The powdered sample was transferred to a 2 mL pre-cooled tube and mixed to 700 μL of PureLink® Plant RNA Reagent. The mixture was homogenized using a vortex until uniform homogeneity was achieved (usually 30 s) and incubated at room temperature for 5 min. Two hundred microliters of chloroform were added, and the capped tube was subject to vigorous vortexing for 15 s until a uniform phase was obtained. Thirty microliters of 5 M NaCl was added and mixed until a uniform phase was produced. The tube was placed horizontally and incubated for 3 min at room temperature. The mixture was centrifuged for 15 min at 12000 × g at 4°C, and the upper aqueous phase was transferred into a new 2 mL tube.

2.6. Low molecular weight (LMW) s/miRNA isolation

Using a pipette, one volume of 70% ethanol was added and thoroughly mixed with the aqueous phase from the previous step. Seven hundred microliters of this sample were recovered (including any precipitated formed) and placed into an RNeasy MiniSpin column (miRNeasy mini kit) previously assembled to a collection tube (assembly unit #1, AU1; Fig. 1). The AU1 was capped and centrifuged at 8000 × g for 15 s at room temperature. The flow-through containing the siRNA/miRNA fraction was transferred into a new 2 mL tube. The column from AU1 was kept at 4°C for high molecular weight RNA isolation (see Section 2.7). Absolute ethanol (0.6 volumes) was added to the siRNA/miRNA fraction tube and mixed thoroughly by pipetting. Seven hundred microliter aliquots from this mixture were loaded onto an RNeasy MinElute Spin Column (RNeasy MinElute Cleanup Kit) and placed in a 2 mL collection tube (AU2). The AU2 was capped and centrifuged at 8000 × g for 15 s at room temperature. This step was repeated until all the RNA was retained by the column. After whole sample processing, 700 μL of RWT Buffer (Qiagen) were loaded into the column in AU2 and centrifuged for 15 s at 8000 × g to wash the column. A new wash step was carried out by adding 500 μL RPE Buffer (Qiagen) to the column and centrifuged for 15 s at 8000 × g. A final wash was done by adding 500 μL of 80% ethanol to the column in AU2, and centrifuged for 2 min at 8000 × g. The column from AU2 was placed into a new 2 mL collection tube (AU3), and centrifuged for 5 min at 8000 × g to dry the spin column membrane. For siRNA/miRNA fraction elution from column in AU3, the column was assembled into a new 1.5 mL tube (AU4) and 14 μL of RNase-free water were loaded onto the spin column membrane. The AU4 was incubated for 1 min and centrifuged for 1 min at 8000 × g. The eluted siRNA/miRNA fraction was stored at -80°C until used.

2.7. High molecular weight RNA isolation

High molecular weight (HMW) RNA isolation was carried out using the column from AU1 (kept at 4°C from the previous step). The column of this assembly was washed with 700 μL of RWT Buffer, and centrifugation of the assembly for 15 s at 8000 × g. A second wash step was carried out by washing twice with 500 μL of RPE Buffer and centrifuged for 15 s at 8000 × g in a stepwise manner. The column was assembled in a new 2 mL collection tube and centrifuged at 8000 × g for 1 min. The column was placed into a new 1.5 mL tube and eluted by adding 30–50 μL of RNAse-free water directly onto the spin column membrane. The column was incubated for 1 min and centrifuged for 1 min at 8000 × g in order to elute the RNA. The eluted RNA was stored at -80°C until used.

2.8. Yield and quality analysis

A small volume of HMW RNA eluant was used to test the integrity and quantity with capillary electrophoresis on a Fragment Analyzer™ Automated CE System (AATI) and using the standards provided in the Standard Sensitivity RNA Analysis Kit (AATI Advanced Analytical Technologies) according manufacturer instructions. In the case of eluted siRNA/miRNA, a small volume usually between 0.5 and 1 μL was used to test the quality and quantity on a Fragment Analyzer and

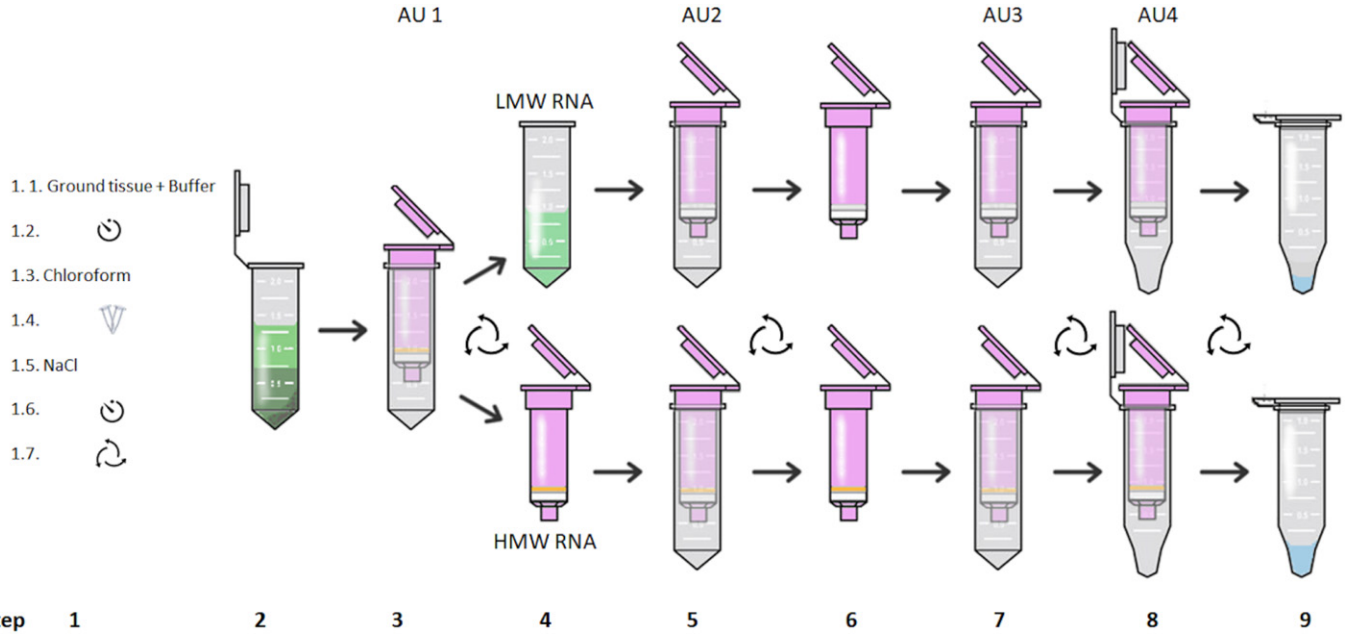

Fig. 1. Step-by-step diagram of the siRNA/miRNA extraction protocol. Plant tissue was processed (Step #1) by grinding samples using liquid nitrogen and mixing with PureLink® Plant RNA Reagent (1.1), incubating the mixture (1.2), treating it with chloroform (1.3), and vortexing it (1.4). Sodium chloride was added to the mixture (1.5), which was incubated (1.6) and then centrifuged (1.7). The upper aqueous phase (#2) was transferred into a new tube, treated with ethanol 70%, mixed, and placed into an RNeasy MiniSpin column (#3; assembly unit (AU) #1, AU1). The AU1 was centrifuged and the flow-through containing the small/micro ribonucleic acid (siRNA/miRNA) fraction (low molecular weight RNA [LMW RNA] route) was transferred into a new tube (#4), mixed with absolute ethanol, loaded onto a RNeasy MinElute Spin Column (#5), and placed in a collection tube to form AU2. The AU2 was centrifuged, washed with RWT Buffer and centrifuged. A new wash step was carried out adding RPE Buffer (#6) and centrifuging. A final wash was carried out using 80% ethanol into the column in AU2 and centrifuging. The column from AU2 was assembled, placed into a collection tube (#7; AU3), and centrifuged. For siRNA/miRNA fraction elution from column in AU3, the column was assembled into a new tube (#8; AU4). Final elution from column was achieved using RNase-free water, incubating, and centrifuging. The eluted siRNA/miRNA fraction was stored at -80°C until use (#9). In addition, the column from AU1 (#4) was used for high molecular weight RNA (HMW RNA route) isolation by assembly into a collection tube and washing with RWT Buffer (#5, lower branch) and centrifugation. A second wash step was carried out using RPE Buffer (#5) and centrifugation. The column (#6) was assembled into a new tube (#7), centrifuged, and placed into a new tube (#8). Elution for this RNA material was obtained by adding RNAse-free water directly onto the spin column (#8) and centrifuging. The eluted RNA was stored at -80°C until use (#9).

a High Sensitivity RNA Analysis Kit according manufacturer's protocol for these samples. For accurate small-RNA quantification, a fluorometric assay was assessed by using Quant-iT™ RiboGreen® RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according manufacturer's protocol.

2.9. Library construction and sequencing

Indexed libraries were built with TruSeq® Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) using 200 ng of isolated small RNA in 5 μL volume according manufacturer's instructions. Library validation, quantification, and sequencing were done according previous publications [4,9,10].

2.10. Experiment #1. Small interfering RNAs targeting the virus genome during Plum pox virus (PPV)–Prunus interaction

2.10.1. Viral infection of P. salicina

In 2012, *Plum pox virus* (strain D) infected *Prunus insititia* (Adesoto 101) trees were established in the biosafety greenhouse located at La Platina Research Station (Santiago, Chile) and used as rootstocks for *P. salicina*, *P. domestica*, and *P. tomentosa* scions. The experimental procedures for grafting, PPV challenge, and infection load determinations were as described by Wong et al. [11]. Viral infection was followed using the kit Reagent Set SRA 31505/1000 (Agdia, Elkhart, IN, USA) according to the manufacturer's instructions.

2.10.2. Bioinformatic analysis

Small RNA reads obtained from control and infected *Prunus* siRNA libraries were analyzed using the CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark) as previously reported [10]. Unique sequences between 21- and 24-nt were aligned to the PPV genome (NCBI Reference Sequence: NC_001445.1). Penalty settings for filtered read annealing to template genome sequences were previously established by Montes et al. [10]. Further analysis was performed using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA) and local scripts for plotting.

2.11. Experiment #2. Prunus miRNA species over-expressed upon PPV infection

2.11.1. Bioinformatic filtering of candidate miRNAs

Available datasets from miRbase [12] were used to define miRNAs from the small RNA reads obtained in Experiment #1. Small RNA reads from control and infected *Prunus* small RNA libraries were analyzed using the CLC Genomics Workbench software (CLC Bio) and those datasets. Specific virus-host miRNAs were selected from available setlists [13,14] and web servers [15].

2.11.2. End-point looped RT-PCR

Selected miRNA species from the previous steps were detected in the grafted materials. All procedures were as indicated in Castro et al. [9]. Stem-loop RT primers were designed according to Chen et al. [16], and the sequence data is presented in Table 1.

3. Results

3.1. RNA quality and integrity

The different steps employed for small RNA purification in plum samples are briefly explained in Fig. 1. As shown, these procedures also led to HMW RNA isolation. In both cases, RNA was eluted in

Table 1
Primers used in stem-loop PCR to validate *P. salicina* siRNA/miRNA molecules from NGS.

Name	Mature sequence	Stem-loop RT primer	Forward primer	Reverse primer
Psa-miR166-like	TCGGACCAGGCTTCATTCCCC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**GGGGAA**	GTATAC**TCGGACCAGGCTTCA**	GTGCAGGGTCCGAGGT
Psa-miR535-like	TGACAACGAGAGAGAGCACGC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**GCGTGC**	GTATAC**TGACAACGAGAGAGA**	
Psa-miR168-like	TCGCTTGGTGCAGGTCGGGAA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**TTCCCG**	GTATAC**TCGCTTGGTGCAGGT**	
Psa-miR482-like	GGAATAGGAGGATTGGGAAAA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**TTTTCC**	GTATAC**GGAATAGGAGGATTG**	

RNase free H_2O and checked for quality. The first step of the analysis included both LMW and HMW RNAs, which provided complementary information regarding small RNA status in the purification (Fig. 2). The integrity of RNA purification was evaluated with the HMW RNA fraction (Fig. 2a). Capillary electrophoresis analyses, using the Fragment Analyzer device, yielded electropherograms reporting RNA Quality Number (RQN) values. For this study, RQN values >7.5 (equivalent to an RNA Integrity Number (RIN) of approximately 7.7) were considered adequate in quality for the next steps involving LMW RNA purification steps. Accumulation of LMW RNA with particular sizes <200 nt were then determined (see peak at 126 nt; Fig. 2b) in conjunction with an rRNA occurrence <0.1% (larger signals in Fig. 2b); these requirements proved to be satisfactory for further sequencing procedures. RiboGreen-based quantifications of both LMW and HMW RNA samples showed high concentrations of starting genetic material with values >50 and 200 ng/μL for LMW and HMW RNAs, respectively. In general, concentrations exceeded the required minimum working concentrations and thus provided enough material needed to make sample dilutions to be used for extensive work.

3.2. Small RNA libraries

In Fig. 2, the necessary steps to construct small RNA libraries are briefly depicted; the most important checkpoints are included. The enriched LMW RNA fractions (<200 nt) should accumulate at least 50 ng/μL for an adequate RNA adapter ligation reaction. Immediately after this, reverse transcription and PCR amplification of the library were performed (Fig. 2c), and the fragments collected in the library

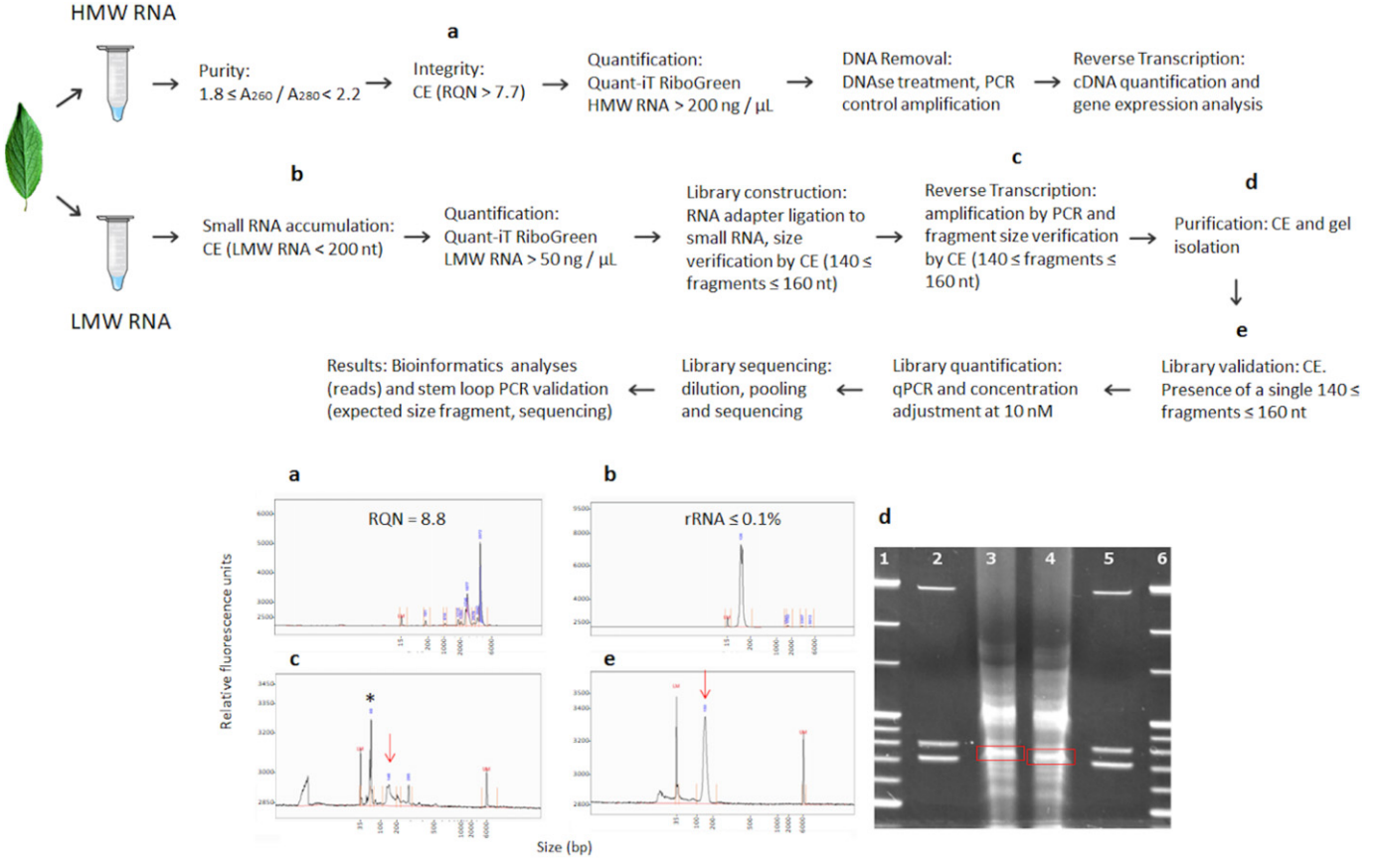

Fig. 2. Quality check pipeline of small/micro RNA purifications and next-generation sequencing analysis. The purification procedures led to both HMW and LMW isolation, which then followed different pipelines for further experimentation. Integrity of HMW (a) was used as an indicator of representative of LMW isolation to discard RNA degradation. LMW RNAs were then evaluated for their accumulation, deduced from the amount of rRNA in the samples (b; peaks over 2000 bp), and measurements of their concentrations were done. Once checked, small RNAs (siRNA and microRNA) were represented in sequencing libraries (c) which were purified from polyacrylamide gels (red boxes in d) and validated (e). Finally, successful libraries were processed for next-generation sequencing (NGS) procedures. Representative AATI Fragment Analyzer electropherograms are shown for integrity (a), accumulation (b), small RNAs reverse transcription (c), and sequencing library (e). Polyacrylamide gel electrophoresis (PAGE) of reverse transcribed cDNAs from small RNAs are shown in d (red boxes). LM, lower marker at 15 (in a and b) and 35 (in c and d) bp; UM: upper marker at 6000 bp. Red arrows indicate fragments of interest (including siRNA and miRNAs); *, RNA library adapters. Extraction procedures for fragments from PAGE are described in the library construction procedures by the manufacturer.

(which included siRNAs and microRNAs) were checked by capillary electrophoresis (arrow in Fig. 2c). If no 140–160 bp fragments resulted from these procedures, a new round of RNA adapter ligation processes was performed. Using polyacrylamide gel electrophoresis, size-specific bands were extracted (red boxes in Fig. 2d), and libraries were forwarded into a validation process for size analysis of the fragments. Again, by capillary electrophoresis, fragments between 140 and 160 nt were used as an evaluation parameter (Fig. 2e; arrow and peak at 150 nt). The library size verification by capillary electrophoresis as a repeating checking point step in library building process ensured correct library construction, which is necessary for good sequencing and data generation. Successful libraries were processed for NGS procedures.

3.3. Experiment #1. Small RNA patterns in Plum pox virus infected of 'Larry Ann' scions

Fig. 3a depicts the total reads for 21- to 24-nt siRNAs under control (left panel) and infected conditions (right panel) occurring in both scion (*P. salicina*) and rootstock (Adesoto 101) components. Specific reads raised against the virus can be deduced from Table 2 (which summarizes these results and shows the plant response caused by viral challenge) and can be judged by the read mapping on the PPV-D genome. Upon infection, these reads increased up to 64,832 molecules (control situation showed two reads). Interestingly 60,240 of these molecules fell in the range of siRNA with 21- to 24-nt in length (known as small interfering RNAs [siRNAs]). After normalization into reads per million, the distribution of siRNAs (i.e. 21- to 24-nt molecules) showed a significant trend toward the generation of 21- and 22-nt siRNAs (Fig. 3b) instead of 24-nt molecules. The distribution of these siRNAs with regard to the target virus genome is shown in Fig. 3c.

3.4. Experiment #2. Micro RNA patterns in 'Larry Ann' scions infected by Plum pox virus

Total small RNA reads were filtered, and the cleaned dataset generated a small RNA set of 21- and 22-nt molecules. These filtered molecules were subjected to miRbase dataset version 18, generating a subset of miRNAs in *P. salicina* derived from both control and PPV-infected samples. Analyses of this subset showed the occurrence of diverse miRNAs whose resulting profiles were contrasting between control and challenged situations (Fig. 4a and Fig. 4b). Infection by PPV led to the generation of miR164 and miR160 and to increased reads compared to controls for miR166, miR171, miR168, miR319, miR157, and miR159. In contrast, infection caused decreased reads for miR398 and miR408. In addition, confirmatory PCRs of some of these molecules were performed by end point stem loop reactions. Fig. 4c

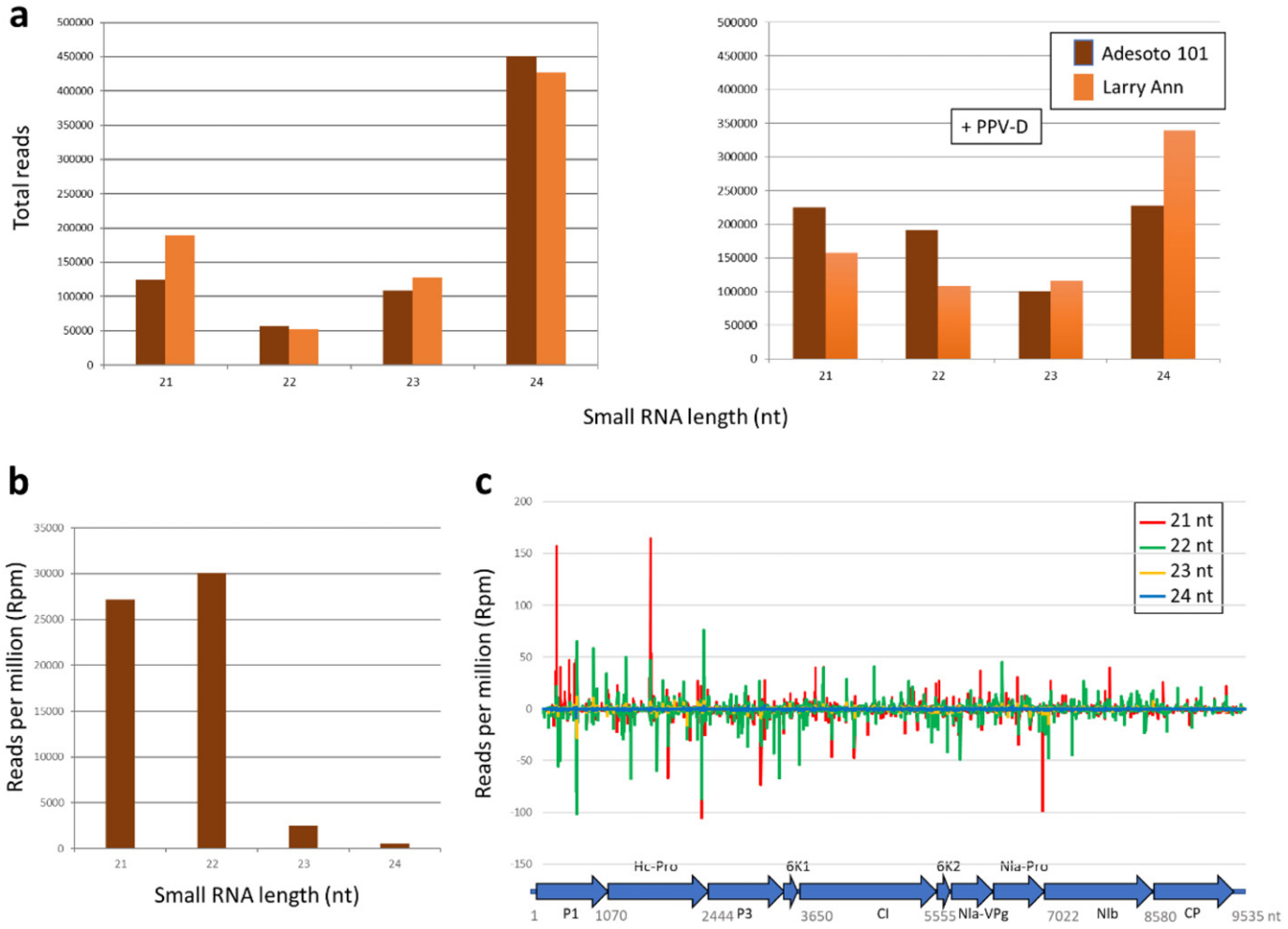

Fig. 3. Small RNA patterns during *Plum pox virus* infection of 'Larry Ann' scions (Experiment #1). Adesoto 101 trees were infected with PPV-D using chip-budding and the infected trees were then grafted with *P. salicina* scions. Control grafted units were built using non-infected rootstocks. RNA extractions were carried out and subject to NGS experiments. Total reads were filtered and 21- to 24-nt molecules deduced under control (left panel) and PPV-infection (right panel) conditions (a). Relevance of small RNA molecules was deduced by a bioinformatics pipeline which standardized filtered reads per million of filtered reads (b). Finally, 21- to 24-nt molecules were filtered for complementarity to the PPV-D genome (c) either in sense (positive *Y-axis*) or antisense (negative *Y-axis*); *X* axis in c depicts the PPV genome, open reading frames and their nucleotide position.

Table 2
Sequencing reads raised against the PPV genome in *P. salicina* scions grafted on PPV-infected *P. insititia* rootstocks.

	18–28 nt*	R-fam + TIGR**	Mapping on PPV D***	21- to 24-nt
Larry Ann + PPV	3,118,199	2,189,759	64,832	60,240
Larry Ann	1,543,343	1,237,558	2	2

* Reads filtered for length between 18- and 28-nt using CLC Genomics workbench.
** Reads filtered using R-fam and TIGR data sets available to remove contaminant small RNA reads. Computing was carried out using CLC Genomics workbench.
*** NCBI Reference Sequence: NC_001445.1.

depicts the amplified products derived from these trials, which allowed for the experimental confirmation of molecules such as miR166, miR168, and miR398 and also showed the presence other plant-pathogen involved miRNAs such as miR162, miR482, and miR535.

4. Discussion

We have described a fast, efficient, and reproducible method for isolating small RNAs from *P. salicina;* this method is also applicable to other woody *Prunus* species such as *P. tomentosa* (this study), *P. domestica*, *P. persica*, and *P. avium* (data not shown). The resulting small RNAs from this method were suitable for downstream handling such as massive sequencing and stem loop detection assays, which are technical platforms that require top quality RNA.

Whereas several other protocols have already been described for small RNA isolation in commercially relevant *Prunus* spp. such as almond [6], sweet cherry [4], and peach [17], these procedures do not allow for adequate siRNA isolation in terms of quality and quantity in Japanese plum. Extraction of high-quality small RNAs is an important procedure and can be a limiting factor for several experiments, including the current trend toward the knowledge of specific miRNAs or siRNA expression profiles using end-point RT-PCR amplification. In the present work, we have used these platforms and reviewed the potential use of the isolated RNA fractions for NGS and molecular detection experiments.

The quality control step reports critical factors such as RNA purity, yield, and integrity. Degradation of total RNA could result in an overestimation of the miRNA and siRNA molecules. RNA quality and integrity extents are broadly based on RIN [18], and values >7 are conveniently described for diverse RNA analyses including the above described platforms. The RIN index uses 18*S* and 28*S* detection curves in electropherograms; in this way, the quantity and integrity of the RNA sample are concurrently judged and RIN >7 has been declared samples adequate for NGS procedures of RNA seq and RIN > 8 indicate samples suitable for siRNA seq. In the present work, we used an RQN which is a proprietary algorithm linked to the Fragment Analyzer system; RQN also uses these factors for calculations and is automatically given by the analysis. According to our previous experience in siRNA sequencing, we used RQN values equivalent to a bottom RIN = 7.7 for siRNA NGS pipelines in woody fruit crops with no reads data indicating significant degradation of samples [4,9].

In the case of PPV–plum interaction, we evidenced a significant effect of viral infection on the generation of specific 21- and 22-nt siRNAs. These results confirmed previous observations found in herbaceous PPV-infected hosts [10]. The fact that the 24-nt siRNAs decrease upon infection supports the idea that some the outcome of the plant-virus interaction is regulated by those 24 nt molecules and that some of the 24 nt-driven biological processes are hijacked by the virus. Both 22- and 24-nt siRNAs are associated with widespread silencing [19] and mobility [19]. We confirmed the up or down regulation of previously known miRNAs. In addition, new miRNA

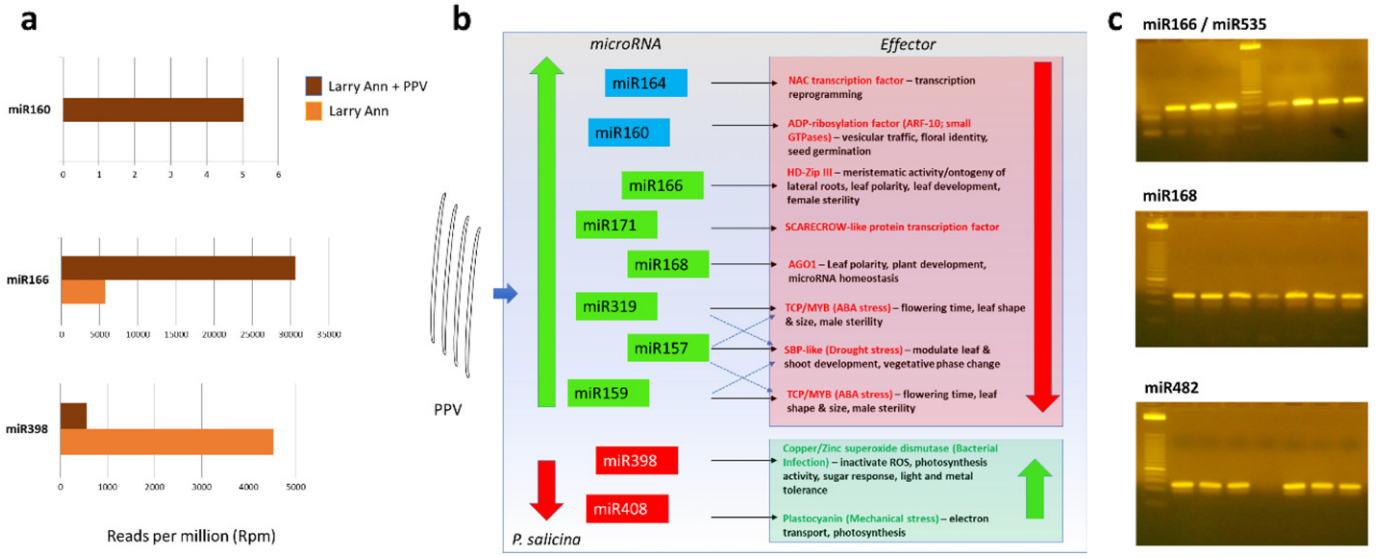

Fig. 4. Micro RNA behavior during *Plum pox virus* infection of 'Larry Ann' scions (Experiment #2). Adesoto 101 trees were infected with PPV-D using chip-budding, and the infected trees were grafted with *P. salicina* scions. Control grafted units were built using non-infected rootstocks. Small RNA extractions were carried out and subjected to next generation sequencing experiments. Total reads were filtered, and 21- and 22-nt molecules obtained under control and PPV-infection conditions were filtered and compared against the miRbase dataset version 18. A, known miRNAs associated with plant–virus interactions were identified having profiles of newly generated (miR166), induced (miR166), or repressed (miR398) upon infection (blue bars) and compared to the control (orange bars). B, Upon infection (PPV), induced (green boxes), newly synthesized (blue boxes), and repressed (red boxes) miRNAs were identified in the *P. salicina* scions. Candidate target genes were deduced (Effector red and green text boxes) based on available information [14] and also using predictive tools [15] applied on the *P. persica* and *A. thaliana* genomes; repressed (Effector red box, red fonts) and induced (Effector green box, green fonts) targets are shown including their cell function (black fonts). Green arrows indicate induction and red arrows indicate repression. C, some of the most relevant miRNA molecules were additionally checked by stem-loop polymerase chain reactions of RNA isolations from different *P. salicina* grafted scions (represented in different lanes) using polyacrylamide gel electrophoresis; 25 bp Invitrogen ladder was used in these polyacrylamide electrophoresis gels.

candidates were shown, by using psRNA Target predictive system [20], to be targeted by PPV infection. Overall, these findings encourage us to further analyze the small RNA populations under PPV infection in *Prunus* and to define the specific role of the small/micro RNA molecules in biological processes such as response to biotic and abiotic stresses.

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