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Rapid Communication

Characterization of the siRNAs associated with peach latent mosaic viroid infection

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ABSTRACT

The peach latent mosaic viroid (PLMVd) is a small, circular RNA species that has been shown to induce RNA silencing in plants. With the goal of better understanding the biological mechanism underlying this process, the siRNAs found in PLMVd infected peach leaves were isolated and sequenced. Analysis of the resulting data prompted several conclusions, including: i. PLMVd strands of both polarities are substrates for the Dicer-like enzymes found in peach leaves; ii. the more highly structured regions of PLMVd trigger the activity of the Dicer-like enzymes; and, iii. the circular PLMVd conformers appear to be favored for transport into the cytoplasm.

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Introduction

Plants have developed a highly adaptable mechanism in order to protect themselves against exogenous RNAs such as viroids. This mechanism, called post-transcriptional gene silencing (PTGS), is induced by the presence of either double-stranded RNAs (dsRNA) or structured single-stranded RNAs (Eamens et al., 2008). These RNAs are cleaved into small interfering RNAs (siRNAs) 21 to 26 nucleotides (nt) in length by an RNase III-like enzyme, more specifically a Dicer-like enzyme (DCL). siRNAs are incorporated into RNA-induced silencing complexes (RISC) and subsequently guide the sequence specific degradation of target mRNAs. In addition, siRNAs can serve as primers for an RNA-dependent RNA polymerase (RdRp), thereby causing an amplification phenomenon. Based on the detection of siRNAs of 21 to 25 nt in length, several viroids have been shown to induce PTGS (Itaya et al., 2001, Papaefthimiou et al., 2001, Martinez de Alba et al., 2002, Landry et al., 2004, Martin et al., 2007). Moreover, it has been suggested that the production of siRNAs from viroids could explain how these pathogens cause symptoms in their hosts (Wang et al., 2004).

In the case of peach latent mosaic viroid (PLMVd), characterization using wheat germ extract shed light on the silencing mechanism. First, it was shown that the long hairpin structure implicated in the replication of the viroid has the ability to preferentially trigger DCL enzyme(s) activity(ies), a much more likely situation than the highly improbable formation of a double-stranded RNA structure including PLMVd strands of both plus (+) and minus (−) polarities (Landry and Perreault, 2005). Second, these experiments showed that the RdRp could use siRNA as primers for the production of dsRNAs that should be susceptible to DCL activity, thereby leading to amplification of the RNA silencing phenomenon as well as propagation of the PLMVd derived small RNA species (Landry and Perreault, 2005). Though these experiments were instructive, they were based on *in vitro* enzymatic assays, and, consequently, needed to be confirmed *in vivo*. With the goal of better understanding this silencing mechanism, a sequencing effort of the siRNAs associated with PLMVd infection in peach leaves was performed.

Results and discussion

Leaves were collected from one healthy and one PLMVd-infected peach trees from both the west coast of Canada and the north of Tunisia (Table 1). After RNA extraction, PLMVd infection was confirmed by both RT-PCR amplification and Northern blot hybridization using a full-length PLMVd probe of either (+) or (−) polarity as described previously (data not shown) (Bussière et al., 1999, Fekih Hassen et al., 2007). It is known that PLMVd replicates in chloroplasts via a symmetric rolling circle mechanism in which there are mainly linear monomers of both polarities, almost no detectable multimeric strands and only a small amount of circular monomers present (Bussière et al., 1999). Equivalent banding patterns were obtained for the samples derived from the two infected peach trees. The content, in

Abbreviations: DCL, Dicer-like; dsRNA, double-stranded RNA; ncRNA, non-coding RNA; PLMVd, peach latent mosaic viroid; PSTVd, potato spindle tuber viroid; PTGS, post-transcriptional gene silencing; RdRp, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

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Table 1
Number of ncRNA by RNA sample

Country	State	Number of total small RNAs	Number of unique small RNAs
Tunisia	Healthy	189	165
Tunisia	Infected	156	156
Canada	Healthy	128	112
Canada	Infected	142	128

terms of PLMVd-related small ncRNAs which are the most likely to be siRNAs, was analyzed by Northern blot hybridization after fractionation of the RNA samples on denaturing 15% PAGE gels (Fig. 1A). Appropriate bands were detected only in the RNA samples isolated from infected leaves, in agreement with previous reports (Martinez de Alba et al., 2002, Landry et al., 2004).

Characterization of the small ncRNA

In order to analyze the composition in terms of the small ncRNAs, more specifically that of the PLMVd-associated siRNAs, the RNA samples were fractionated on denaturing 15% PAGE gels, the bands possessing electrophoretic mobilities that corresponded to RNAs of 18 to 26 nt in length were cut out of the gel and the RNA extracted. After the addition of linkers to the ends of the purified ncRNAs, the resulting molecules were reverse transcribed, PCR amplified, multimerized and finally cloned into pGEM-T vector according to the procedure developed for microRNA sequencing (Lau et al., 2001). A total of 615 unambiguous sequences (i.e. without any unspecified nucleotides) were obtained (see Table 1). It is not clear if the sequences found more than once represented a higher cellular abundance, or were the result of technical artifacts. In order to prevent any erroneous interpretations, all species were considered only once per sample in all subsequent analyses. This yielded a total of 561 different small RNA species, meaning that 9% of the original sequences were repeated (see Supplementary data).

Initially, ncRNAs were analyzed as a function of their size. In the samples derived from the healthy trees the 21 nt RNA species were found to be less abundant than those of 24 nt (19.7% versus 33.3%, respectively). Conversely, the 21 nt ncRNAs were slightly more abundant than the 24 nt ones in the samples derived from infected leaves (29.2% versus 26.8%) (Fig. 1B). This variation in the ncRNA levels was a good indication that the antiviral RNA silencing process, which produces 21 nt long ncRNAs, was active in PLMVd-infected leaves.

Sequence homology searches were performed for each ncRNA found using the available *Arabidopsis thaliana* plant databases since the *Prunus persica* sequence is incomplete. Additional homology searches were performed using both the available viroid subviral and miRNA databases. In all cases, the sequences were considered to be homologous when three or less mismatches existed between an ncRNA and a given reported sequence (Fig. 1C). When sequence homologies were found for more than one RNA species, the higher homology was considered for unique assignment, if possible. Briefly, most of the ncRNAs retrieved do not have any known homologous sequences reported (i.e. >70%), reflecting the fact that to date none of the peach genomes (i.e. nuclear, plastid and mitochondrial) have been sequenced. Interestingly, we noted that the proportion of the rRNA sequences was relatively low regardless of the origin of the samples. This low proportion of rRNA is a good indication of the quality of the samples. If random degradation or contamination occurred during the preparation of the ncRNA libraries, a higher ratio of rRNA would be expected due to the fact that rRNA represents >80% of the total cellular RNA. We also detected a small but significant proportion that possessed homology to the mRNA and miRNA fractions, in addition to the fact that ~20% of the ncRNA sequences possessed homology with reported DNA sequences (data not shown). A certain proportion of the small RNA sequences (~10%) exhibited homology to already reported sequences, suggesting conservation of small RNA species between organisms (Fig. 1C). Some sequences belong to two clusters, resulting in a total of 115% fractions when all are added up. For example, all miRNA were also part of the small RNA cluster. This type of result is typical of several other studies (e.g. see Ref. (Martin et al., 2007)). Importantly, no PLMVd homologous sequences were retrieved from the samples derived from the healthy leaves supporting, once again, the integrity of the samples and confirming the results from the initial Northern blot hybridizations (Fig. 1A). Conversely, 20.5% of the ncRNA sequences retrieved from the infected samples were homologous to PLMVd sequences, confirming that RNA silencing was triggered by the viroid infection (i.e. 60 out of 293 ncRNA obtained from the infected samples). This percentage is higher than those reported for both Potato spindle tuber viroid (PSTVd) and Citrus exocortis viroid (<5%), two viroids that accumulate in the nucleus (Martin et al., 2007, Itaya et al., 2007). Finally, no significant bias was

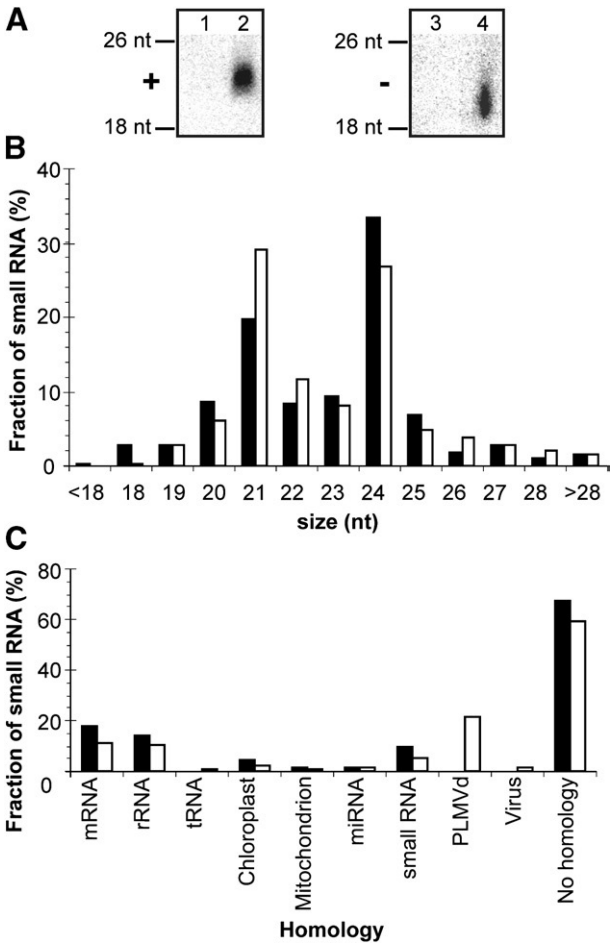

Fig. 1. Analysis of PLMVd-associated siRNA sequences and small ncRNAs retrieved in RNA samples extracted from peach leaves. (A) A representative autoradiogram of a Northern blot hybridization of RNA samples isolated from peach trees located on the West coast of Canada. RNA samples were fractionated on a denaturing 15% PAGE gel prior being transferred to a nylon filter. The hybridization was performed with ^{32}P-labeled PLMVd linear strands of either (−) or (+) polarity (i.e. left and right panels, respectively). Lanes 1 and 3 are RNA samples isolated from a healthy peach tree, while lanes 2 and 4 are RNA samples isolated from an infected peach tree. Only the portions showing the small RNA species are shown. Adjacent to the gel, the positions of the synthetic transcripts of 18 and 26 nt used as markers are indicated. (B) Histogram illustrating the size distribution of the sequenced small ncRNAs. (C) Representation of the small ncRNAs identities based on homology searches. In (B) and (C) the black and white bars represent the species retrieved from the healthy and PLMVd-infected RNA samples, respectively.

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