A Set of Novel RNAs Transcribed from the Chloroplast Genome Accumulates in Date Palm Leaflets Affected by Brittle Leaf Disease

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Accepted for publication 5 November 2007.

ABSTRACT

Marqués, J., Fadda, Z. G. N., Duran-Vila, N., Flores, R., Bové, J. M., and Daròs, J. A. 2008. A set of novel RNAs transcribed from the chloroplast genome accumulates in date palm leaflets affected by brittle leaf disease. *Phytopathology* 98:337-344.

Brittle leaf disease or *maladie des feuilles cassantes* (MFC) is a lethal disorder of date palms that has assumed epidemic proportions in the oases of southern Tunisia. After a prolonged period during which palms are declining, the disease ends with the death of the palms. Whereas no pathogen could ever be associated with the disease, leaflets of affected palms have been previously shown to be deficient in manganese. Analysis

of RNA preparations from leaflets of MFC-affected palms revealed the presence of a set of novel RNAs (MFC-RNAs) of sense and antisense polarities, which are homologous to various regions of the date palm chloroplast genome, such as the regions containing genes *rrn5S-trnR(ACG)* and *trnM(CAU)-atpE*. In the RNA preparations obtained from leaflets of affected palms, some of these RNAs are present as double-stranded species (MFC-dsRNAs), as witnessed by results from cellulose chromatography, end labeling, RNase digestion, and northern hybridization with strand specific probes. These MFC-RNAs represent a novel type of host-derived RNAs, and their presence in MFC-affected date palms is of diagnostic value.

Brittle leaf disease, known in French as *maladie des feuilles cassantes* (MFC), is a disorder of date palms (*Phoenix dactylifera* L.), which was first reported in the oases of southern Tunisia in the 1960s (29). The disease has assumed epidemic proportions and has recently been reported from eastern Algeria (21). The most characteristic symptom of the disease concerns the palm leaflets, which become brittle and break easily when flexed and squeezed. Symptoms extend to the upper fronds until the whole plant becomes affected and stunted, stops growing, and eventually dies (28). MFC epidemiology suggested a biotic etiology because affected palms appeared to cluster into foci. However, so far no pathogen could be found associated with the disease (29). In contrast, leaflets of affected palms have been previously shown to contain manganese (Mn) concentrations significantly lower than those of healthy control leaflets (18). Foliar Mn-deficiency probably results from unavailability of Mn to the palms because of high soil pH (8.0 to 8.5).

Initially, efforts to find pathogens associated with MFC included exhaustive search for nematodes, fungi, bacteria, viruses, as well as viroids within the leaf tissues of affected palms (29). Viroids are small molecules of circular RNA (246 to 401 nt), which replicate autonomously in host plants (6,2). The viroid etiology was especially attractive as two viroids are the causal agents of important diseases in the palm family (*Arecaceae*): *Coconut cadang-cadang viroid* (CCCVd) (9) and *Coconut tinangaja viroid* (CTiVd) (13). The most common strategy for determining the viroid etiology of a plant disease is based on the detection of a characteristic circular viroid RNA in affected plant material. The search for such RNA molecules in MFC-affected leaflets resulted, indeed, in the detection of RNAs (MFC-RNAs)

with the electrophoretic properties of small circular RNAs.

In the work presented here we report the isolation and molecular characterization of the MFC-RNAs, which are not viroids but RNAs of host origin. These RNAs, with sense and antisense polarities, are homologous to regions of the date palm chloroplast genome containing the genes *rrn5S-trnR(ACG)* and *trnM(CAU)-atpE*. They represent a novel type of host-derived RNAs, the presence of which in MFC-affected date palms is of diagnostic value.

MATERIALS AND METHODS

RNA extraction and purification. Total RNA was extracted from leaflets of healthy date palms (*Phoenix dactylifera*) and from leaflets of severely MFC-affected date palms showing typical MFC symptoms. Samples from MFC-affected date palms were collected in Nefta (Jouali oasis), Tunisia, and healthy controls from the Germplasm Bank of Degache, Tunisia, where diseased plants have never been observed. Typically, 10 g of tissue were homogenized using a Polytron (Kinematica) in 16.25 ml of extraction buffer (125 mM Tris-HCl [pH 9.0], 0.75% sodium dodecyl sulfate, 15 mM EDTA, 1 M 2-mercaptoethanol) and 40 ml of water-saturated and neutralized phenol. RNAs in the aqueous phase were further purified by nonionic cellulose (CF11, Whatman) chromatography (20). Briefly, the extract was brought to 35% ethanol and STE buffer (50 mM Tris-HCl [pH 7.0], 100 mM NaCl, and 1 mM EDTA) and RNA was bound in batch to 1.25 g of cellulose that was washed three times with 30 ml of 35% ethanol in STE buffer and eluted three times with 3.33 ml of STE buffer. The eluted fractions were pooled and RNA recovered by ethanol precipitation. In some experiments RNA binding to the cellulose and washing was done in buffers containing 16.5% ethanol. Carbohydrates were removed from the RNA preparations by chloroform extraction. They were brought to 1 M NaCl, 1% CTAB in a final volume of 5 ml, incubated 10 min at 65°C and

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doi:10.1094/PHYTO-98-3-0337

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extracted with 1 volume of chloroform. RNA in the aqueous phase was recovered by ethanol precipitation.

RNA analysis. RNAs were separated either by denaturing polyacrylamide gel electrophoreses (PAGE) in 5% gels in (1×) TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) containing 8 M urea or by double PAGE, the first PAGE being under nondenaturing conditions in 5% gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) and the second PAGE under denaturing conditions in 5% gels in (0.25×) TBE buffer containing 8 M urea (5). For northern blot hybridization, RNAs were electroblotted to (+)-charged nylon membranes, fixed by UV irradiation and hybridized overnight at 70°C in 50% formamide (3). Strand-specific ³²P-labeled riboprobes were obtained by in vitro transcription of conveniently linearized recombinant plasmids that contained DNA inserts corresponding to: date palm chloroplast *rrn5S* coding region and *rrn5S-trnR(ACG)* intergenic region (position 1 to 349 of EU053865), and *atpE* (position 3 to 284 of EU053866), *atpA* (position 1 to 535 of EU053867) and *rbcL* (position 3288 to 3646 of AY166799) coding regions, and date palm mitochondria *coxI* coding region (position 474 to 833 of AY166800).

For RNA end labeling, purified preparations were treated for 1 h at 37°C with 1 U of shrimp alkaline phosphatase in 10 µl of 50 mM Tris-HCl, pH 8.5, 5 mM MgCl₂ and the enzyme inactivated by heating for 15 min at 65°C. Reactions were brought to a final volume 40 µl in buffer 50 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, and containing 5 µCi of [γ -³²P]ATP (3,000 Ci/mmol),

20 U of RNase inhibitor and 10 U of T4 polynucleotide kinase and incubated for 30 min at 37°C. Reactions were stopped by heating 10 min at 70°C and nonincorporated [γ -³²P]ATP removed by Sephadex G-50 chromatography.

RNase digestions were done in 10 µl of (2×) SSC buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0) with 10 ng RNase A for 30 min at 37°C and the reactions stopped adding 1 volume of formamide loading buffer.

RNA cloning and sequencing. A preparation of the slow migrating MFC-dsRNA that was gel eluted after double PAGE separation (20 ng) was 3'-polyadenylated for 1 h at 37°C with 1 U of *Escherichia coli* polyadenylate polymerase in 20 µl of 50 mM Tris-HCl [pH 7.9], 0.25 M NaCl, 10 mM magnesium acetate, 1 mM EDTA, 2.5 mM MnCl₂, 1 mM dithiothreitol, 0.1 mM ATP and 1 U/µl RNase inhibitor. RNA was recovered by ethanol precipitation and a cDNA was synthesized for 1 h at 42°C with 20 U of avian myeloblastosis virus reverse transcriptase and 100 ng of primer PI [5'-GCTAGCAAGCTTCTCGAC(T)₁₇-3'] in 20 µl of 50 mM Tris-HCl, pH 8.5, 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, and 1 mM each dNTPs. cDNA was recovered by ethanol precipitation and 3'-polycytidylated for 1 h at 37°C with 50 U of calf thymus terminal transferase in 20 µl of 200 mM potassium cacodylate, 25 mM Tris-HCl [pH 6.6], 2.5% bovine serum albumin, and 0.75 mM CoCl₂. One microliter of this reaction product was polymerase chain reaction (PCR)-amplified with 2.5 U of *Taq* DNA polymerase and 50 pmol of each primer PII (5'-GCTAGCAAGCTTCTCGACTT-3') homologous to the 5'-half of PI and PIII [5'-(G)₂₅H-3', H is A, C or T] in 50 µl of 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, and 0.2 mM each dNTPs, by 30 cycles of 40 s at 94°C, 30 s at 60°C and 2 min at 72°C with a final extension step of 10 min at 72°C. cDNAs corresponding to the MFC-dsRNA were also reverse transcription (RT)-PCR amplified from a 3'-polyadenylated dsRNA preparation using primer PI for reverse transcription and primer PII in combination with a series of primers homologous and complementary to MFC-RNAs (described below) PIV (5'-CCAA-AATGCAAAGGTCGCTTAT-3'), PV (5'-GTTCCCGGGAGTA-CCCTCAA-3'), PVI (5'-AGGAAATGATGCGGAGATGA-3'), and PVII (5'-GCTCGTCTGAGAGCTAGATTGCG-3') for PCR amplification. Products were cloned in plasmid pGEM-T Easy (Promega). The cDNAs from the resulting recombinant plasmids were sequenced with an ABI PRISM 377 apparatus (Applied Biosystems).

RESULTS

RNAs associated with MFC. To investigate whether MFC of date palms was caused by a viroid, the presence of small circular RNA molecules in tissues of affected plants was examined. Total RNA was extracted from leaflets of healthy and affected palms, and the extracts were enriched in RNAs with the properties of viroids by nonionic cellulose (CF11) chromatography (23). Separation of the RNAs in the enriched preparations was achieved by two consecutive PAGE, first under nondenaturing conditions, and then under denaturing conditions, a technique called double PAGE (5,22) or sequential PAGE, developed essentially for viroid RNA purification and detection (4,24). After the first PAGE, the portion of the gel corresponding to the small viroid RNAs (around the 7S RNA region) is recovered and transferred to the top of the second gel. In this way, RNAs much larger and smaller than 7S RNA are removed. Also in this technique, circular RNAs display, during the denaturing or second PAGE, a slower migration than their linear counterparts of the same sizes, and they can be easily detected. Under these conditions, silver staining of the denaturing gel showed that affected palm leaflets contained two RNAs (Fig. 1, lane 3, arrows) migrating in the zone of the gel where circular RNA markers corresponding to *Citrus exocortis* viroid (CEVd, 370 nt) and *Avocado sunblotch viroid* (ASBVd, 247 nt) also mi-

Fig. 1. Double polyacrylamide gel electrophoreses (PAGE) analysis of RNA preparations from a *maladie des feuilles cassantes* (MFC)-affected date palm (second gel is shown). Lane 1, mix of RNA preparations from infected gynura and avocado that contained CEVd (370 nt) and ASBVd (247 nt) circular RNAs. Lanes 2 and 3, RNAs from healthy and diseased date palm leaflets, respectively. Lanes 4 and 5, RNAs from diseased tissues treated previously to separation with DNase or RNase, respectively. The positions of the circular forms of CEVd and ASBVd are indicated on the left of the panel. The positions of two apparently circular RNAs present in MFC-affected palms are indicated by arrows on the right of the panel.

grated (Fig. 1, lane 1). The slowest RNA moved at about the same position than CEVd and the fastest one reached a position in between the two viroid markers. These RNAs were not detected in the preparation from healthy leaflets (Fig. 1, compare lanes 2 and 3), even though total RNA concentrations in both preparations were comparable, as judged from the ethidium bromide staining of the first gel (data not shown). RNase treatment of the RNA preparation from the affected palm leaflets were carried out prior to the double PAGE separation, and resulted in the absence of both slow migrating species (Fig. 1, lane 5), while DNase treatment had no effect (Fig. 1, lane 4), confirming that they were indeed RNAs. As a whole, these results suggested that the two RNA species were apparently circular and viroid-like in nature.

Similar RNA preparations were obtained from leaf tissues from nine palms showing typical MFC symptoms and four healthy controls from a date palm germplasm bank free of the disease. The symptomatic palms corresponded to various cultivars and were collected at two different localities in Tunisia (Table 1). Double PAGE analysis of the RNA preparations showed a strict correlation between MFC and the presence of the putative circular RNAs (Table 1). These results raised the possibility that viroid-like RNAs could be associated with MFC.

Molecular characterization of the RNAs associated with MFC. The molecular characterization of the MFC-RNAs was pursued. For the purpose of cloning and sequencing, the MFC-RNAs were submitted to double PAGE separation, and eluted from the denaturing gel after ethidium bromide staining. In the case of bona fide circular RNAs, part of these molecules break during the elution step and yield linear molecules. If the eluted RNAs are subsequently analyzed by single denaturing PAGE, two partner bands are detected, one corresponding to the circular version of the molecule and the other to the linear one. Surprisingly, no such partner bands were obtained in the case of the MFC-RNA samples (data not shown). This observation suggested the possibility that the MFC-RNAs could actually correspond to dsRNAs that did not totally denature during the second electrophoretic separation. To investigate this possibility, a series of experiments were performed.

First, RNAs extracted from date palm leaflets were bound to nonionic cellulose in buffers containing decreasing ethanol concentrations and eluted with a buffer without ethanol. The MFC-RNAs bound to the cellulose at 16.5% ethanol (Fig. 2, lane 2), therefore showing the chromatographic behavior expected for dsRNAs and not for viroid RNAs that bind to the cellulose at 35% ethanol (7,23). Absence of the MFC-RNAs in healthy date palms was confirmed using these purer preparations (Fig. 2, compare lanes 2 and 3).

Second, a purified preparation of the slow migrating MFC-RNA that was obtained by gel elution after double PAGE separation

was treated with polynucleotide kinase in the presence of [γ - 32 P]ATP, with and without prior treatment with alkaline phosphatase. Double PAGE analysis of the reaction products showed radioactive bands corresponding to a species migrating slightly more slowly than the circular forms of CEVd, most probably MFC-RNA itself (Fig. 3). Similar results were obtained with the fast migrating MFC-RNA (data not shown). MFC-RNA labeling did not depend on previous treatment with alkaline phosphatase (Fig. 3, compare lanes 2 and 3). When a preparation of purified CEVd RNA was treated in the same way, no labeled bands at the position of the circular viroid RNA were seen (Fig. 3, lane 1). These results ruled out the possibility that the MFC-RNAs could be circular, and indicated that most of the 5' ends of the MFC-associated dsRNAs contain free hydroxyl groups.

Third, a purified preparation of the two MFC-RNAs obtained by gel elution after double PAGE separation was subjected to RNase A digestion at high ionic strength. Single denaturing PAGE analysis showed that these RNA species were resistant to degradation (Fig. 4, compare lane 4 and 5 with 8 and 9). Similarly, two bona fide dsRNA controls, namely those corresponding to *Citrus tristeza virus* (CTV) and *Cucumber mosaic virus* (CMV), were also RNase resistant (Fig. 4, compare lanes 3 and 7). However, a viroid RNA control corresponding to CEVd was degraded by RNase (Fig. 4, compare lanes 2 and 6). This result, again, supported the idea that the MFC-RNA species are dsRNAs.

Cloning and sequence analysis of the MFC-associated dsRNAs. To clone and sequence the MFC-associated dsRNAs (MFC-dsRNAs), the slow migrating species was eluted from the gel after double PAGE separation. It was first polyadenylated at the 3' end, and used to synthesize a cDNA by reverse transcription with primer PI consisting of a defined sequence at the 5' half and a stretch of 17 Ts at the 3' half. The cDNA product was poly-citidylated at the 3' end and used as template for PCR amplification with primers PII and PIII. PII was homologous to the 5'-

TABLE 1. Detection of RNA species with the electrophoretic mobility predicted for small circular RNAs in leaflet tissues of date palms showing *maladie des feuilles cassantes* (MFC) symptoms^a

Cultivar	Locality	Symptoms	Slow RNAs
Khouat Alig	Daklène	+	+
Deglet Nour	Daklène	+	+
Besser	Daklène	+	+
Besser	Daklène	+	+
Alig	Daklène	+	+
Tozeur Zit	Daklène	+	+
Deglet Nour	Daklène	+	+
Pollinator	Daklène	+	+
Pollinator	Hanna	+	+
Khouat Alig	Degache	—	—
Alig	Degache	—	—
Tozeur Zit	Degache	—	—
Deglet Nour	Degache	—	—

^a Samples correspond to different date palm cultivars and were collected at different localities in Tunisia.

Fig. 2. Purification of *maladie des feuilles cassantes* (MFC)-RNAs by non-ionic cellulose in a buffer containing 16.5% ethanol. RNAs were analyzed by double polyacrylamide gel electrophoreses (PAGE) (second gel is shown). Lane 1, control RNA preparation from CEVd-infected gynura obtained by nonionic cellulose chromatography with a buffer containing 35% ethanol. Lanes 2 and 3, RNA preparations from MFC-affected and healthy date palms, respectively. The positions of the monomeric circular (*mc*) and linear (*ml*) forms of CEVd are indicated on the left of the panel. The positions of MFC-RNAs are indicated by arrows on the right of the panel.

half of PI and PIII was a poly(dG) complementary to the added poly(dC) tail. When the PCR amplification was performed in the presence of [α - 32 P]dCTP, and the products were used as a probe in a northern blot hybridization, not only the slow MFC-dsRNA, but also the fast MFC-dsRNA were recognized by the probe (data not shown), confirming that PCR produced DNA homologous to the dsRNA and that both, slow and fast, species contained homologous sequences. Next, the PCR amplification products were cloned, and the resulting plasmids were subjected to a screening procedure to identify those that contained inserts homologous to the MFC-dsRNAs. Seventy plasmids in groups of 10 were used as templates for synthesizing 32 P-labeled DNA probes by asymmetric PCR using a pair of vector-specific primers that flanked the cDNA inserts. Four of the probes hybridized efficiently with both MFC-dsRNAs in the northern blot hybridization assay. The same strategy was repeated with 10 randomly chosen individual plasmids belonging to these four groups. Five of them produced probes that hybridized with the MFC-dsRNAs, and the corresponding cDNA inserts were sequenced.

Sequence analysis showed that the five cDNAs formed two groups: the first one with three cDNAs and the second one with two cDNAs. Interestingly, sequence comparisons showed the cDNAs to share similarities with the chloroplast genome. The largest cDNA in the first group was 238 bp long, and the two shorter ones corresponded to positions 7 to 226 and 1 to 212 of the largest one (Fig. 5A, black lines). The three cDNAs of the first group were homologous to an extensive region of the intergenic sequence between chloroplast genes *rrn5S*, coding for 5S rRNA, and *trnR(ACG)*, coding for Arg-tRNA(ACG) (Fig. 5A, black lines). Specifically, the 238 bp cDNA was also homologous (87% identity) to a sequence of the rice chloroplast genome (database

accession no. X15901) corresponding to position 98,642 to 98,886 (Fig. 5A). In the rice chloroplast genome, this region is repeated (11) and consequently the cDNA was also homologous to the sequence from position 116,232 to 116,476 (87% identity). The two cDNAs of the second group consisted of a large one with 162 bp and a short one with 158 bp, corresponding to positions 5 to 162 of the large cDNA (Fig. 5B, black lines). The large cDNA was homologous (86% identity) to a sequence in the rice chloroplast genome (position 51,431 to 51,592), corresponding to part of the *atpE* gene, coding for subunit CF1 ϵ of the ATP synthase (Fig. 5B). By chance, the sequence of date palm chloroplast genome is known around this region (database accession no. AY166799) and sequence comparison showed that positions 15 to 162 of the 162-bp cDNA matched exactly (100% identity) positions 2 to 149 of AY166799 (data not shown). These results indicate that the MFC-dsRNAs represent sequences from the date palm chloroplast genome, and not sequences from a hypothetical causal agent of the disease. Moreover, the slow MFC-dsRNA is not a single RNA species but a combination of at least two.

To further characterize the MFC-dsRNAs, a new preparation of the slow MFC-dsRNA, obtained by double PAGE and elution from the second gel, was 3'-polyadenylated and cDNAs were synthesized by reverse transcription using primer PI. The cDNAs were amplified by PCR using a common primer PII, homologous to the 5'-half of PI, and either one of four specific primers, PIV, PV, PVI, and PVII, homologous to different positions in both strands of the *rrn5S-trnR(ACG)* intergenic region (PIV, PV) and the *atpE* coding region (PVI, PVII) (arrows in Fig. 5). PCR products were cloned and sequenced. Three of the newly characterized cDNAs happened to be amplified with primer PIV and the longest one extended to a position homologous to 98,898 in the rice chloroplast genome, a position within the coding region of *trnR(ACG)* (Fig. 5A, PIV, gray lines). One of the cDNAs that was amplified with primer PV extended to a position homologous to

Fig. 3. Labeling the slow migrating *maladie des feuilles cassantes* (MFC)-RNA with polynucleotide kinase in the presence of [γ - 32 P]ATP. Reaction products were separated by double polyacrylamide gel electrophoreses (PAGE) and the second gel autoradiographed. Lane 1, control of purified CEVd RNA. Lanes 2 and 3, slow migrating MFC-RNA without and with a previous alkaline phosphatase treatment, respectively. The positions of the *mc* and *ml* forms of CEVd are indicated on the left of the panel. A radioactive band co-migrating with MFC-RNA is indicated by an arrow on the right of the panel.

Fig. 4. Resistance of the *maladie des feuilles cassantes* (MFC)-RNAs to RNase A digestion in a high ionic strength buffer. Lanes 1 to 5, no RNase added. Lanes 6 to 9, RNase A added. Lane 1, RNA marker with the size in nt indicated on the left. Lanes 2 and 6, control CEVd RNA. Lanes 3 and 7, control CTV (slower migrating species) and CMV (faster migrating species) dsRNAs. Lanes 4 and 8, slow migrating MFC-RNA. Lanes 5 and 9, fast migrating MFC-RNA.

98,515 which corresponded to the beginning of the *rrn5S* coding region (Fig. 5A, PV, gray line). Five of the cDNAs amplified with primer PVI extended to a position homologous to 51,593 in *atpE* coding region (Fig. 5B, PVI, gray lines). Finally, three of the cDNAs were amplified with primer PVII and the longest one extended to a position homologous to 51,287, within gene *trnM(CAU)*, coding for Met-tRNA(CAU) (Fig. 5B, PVII, gray lines).

Northern blot hybridization of RNA preparations from MFC-affected date palms. MFC-dsRNA sequence was confirmed by northern blot hybridization. RNA preparations from healthy and diseased plants were enriched by treatment with cellulose CF11 (in 35% ethanol), the RNAs were separated by double PAGE, and they were analyzed by northern blot hybridization. Strand-specific ³²P-labeled probes corresponding to the *rrn5S* coding region and *rrn5S-trnR(ACG)* intergenic region (Fig. 6A and B, *rrn5S*), and the *atpE* coding region (Fig. 6A and B, *atpE*) were obtained by in vitro transcription of conveniently linearized recombinant plasmids. A series of hybridization signals were obtained with all the probes at positions where the slow and fast MFC-dsRNAs migrate in the RNA preparation from a diseased plant (Fig. 6, lanes 2 and 4). No signal was obtained with the RNA preparation from the healthy plant (Fig. 6, lanes 1 and 3). Hybridization patterns for the detection of the (+) strands were the same than those for the (–) strands (Fig. 6A and B, lanes 2 and 4), in agreement with the double-stranded nature of the MFC-RNAs. However, the *rrn5S*-specific probe and the *atpE*-specific probe hybridized with dissimilar dsRNAs species that migrate at slightly different positions in the gel (Fig. 6A and B, compare lanes 2 and 4). These species must be co-purified when eluting the slow MFC-dsRNA for cloning purposes.

MFC-RNAs were studied so far by the double PAGE procedure, which focuses on RNAs with the properties of viroid RNAs and in which most other RNAs of larger and smaller sizes are removed. To further investigate the occurrence in MFC-affected date palms of chloroplast genome-derived RNAs in addition to those detected after double PAGE, a technique giving a wider range of RNAs was used, namely single denaturing PAGE. In the double PAGE, only a short region of a lane from the first gel is used for the second gel, while in a single PAGE the whole lane is blotted for northern analysis. In the experiment of Figure 7, RNAs from healthy and diseased plants were separated by single denaturing PAGE and analyzed by northern blot hybridization as previously, except that three different sets of strand specific probes were used: (i) two probes (Fig. 7A and B, *rrn5S* and *atpE*) specific for the two regions of the date palm chloroplast genome

from where homology with the dsRNAs was previously detected, i.e., the *rrn5S* coding region and *rrn5S-trnR(ACG)* intergenic region, and the *atpE* coding region, (ii) two probes (Fig. 7A and B, *atpA* and *rbcL*) specific for other chloroplast genes, i.e., the *atpA* gene (coding for subunit CF1 α of ATP synthase) and the *rbcL* gene (coding for the RuBisCO large subunit), and (iii) one probe (Fig. 7A and B, *coxI*) specific for a gene in the mitochondrial genome, i.e., the *coxI* gene (coding for cytochrome C oxidase), as a nonchloroplast control. For each region, two probes were obtained: one probe was specific for the sense or (+) strand RNA, and the other for the antisense or (–) strand RNA (Fig. 7A and B, (+) and (–)). With the two *rrn5S* probes, many hybridization signals corresponding to a large population of (+) RNAs (Fig. 7A, lane 2) as well as (–) RNAs (Fig. 7B, lane 2), ranging in size from less than 200 nt to more than 600 nt, were observed in the RNA preparations from MFC-affected date palm leaflets. Using the same probes, these signals were not detected in the RNA preparation from healthy date palm leaflets (Fig. 7A and B, lanes 1), even though ethidium bromide staining of the gels prior to blotting had shown that the RNA preparations from both healthy and diseased palms were comparable (Fig. 7C, compare lanes 1 and 2). Similar results were obtained with the two *atpE* probes, although in this case, apparently more (+) RNA strands than (–) were detected (Fig. 7A and B, lanes 4). Probes *atpA* and *rbcL* also revealed the presence of (+) and (–) RNAs in diseased tissues. However, these RNAs had longer sizes than those re-

Fig. 5. Schematic representation of cloned cDNAs corresponding to the *maladie des feuilles cassantes* (MFC)-associated dsRNAs. Bars represent the fragments of rice chloroplast genome A, from position 98,514 to 98,964 of sequence data base entry X15901 that includes genes *rrn5S* and *trnR(ACG)* and B, from position 51,219 to 51,817 that includes genes *trnM(CAU)* and *atpE*. Solid and open boxes represent coding and intergenic sequences, respectively. Lines represent cDNA clones corresponding to the MFC-associated dsRNAs obtained by polymerase chain reaction (PCR) amplification after tailing the 3' ends of the RNA and the cDNA (black lines) or after tailing the 3' end of the RNA and using a sequence specific primer (gray lines). Position of specific primers PIV, PV, PVI, and PVII is indicated.

Fig. 6. Northern blot hybridization analysis of RNA preparations from a *maladie des feuilles cassantes* (MFC)-affected date palm separated by double PAGE. RNAs were separated by double polyacrylamide gel electrophoreses (PAGE) and hybridized with strand-specific probes to detect the A, sense or (+) and B, antisense or (–) strands of date palm chloroplast *rrn5S* coding region plus *rrn5S-trnR(ACG)* intergenic region, and *atpE* coding regions. Lanes 1 and 3, RNA from a healthy palm. Lanes 2 and 4, RNA from an MFC-affected palm. The positions of the circular forms of CEVd and ASBVd are indicated on the left of the panels.

vealed by the other probes (Fig. 7A and B, lanes 6 and 8). No hybridization signals were detected in the RNA preparation from healthy date palm leaflets, whatever the probe used (Fig. 7A and B, lanes 1, 3, 5, and 7). Finally, no signals were detected with the probe specific for the mitochondrial *coxI* coding region (Fig. 7A and B, lanes 9 and 10).

These results indicate that MFC-affected date palm leaflets accumulate not only the (+) and (-) strands of the MFC-dsRNAs as detected in the experiments in which the double PAGE pro-

cedure was used (Figs. 1 to 3 and 6), but also additional sense and antisense RNAs strands over a wide range of sizes (≈ 200 to 600 nt), representing different chloroplast genome sequences (Fig. 7). The RNAs whose sequences correspond to the *rrn5S-trnR(ACG)* and *atpE* regions of the chloroplast genome are the most abundant.

DISCUSSION

The quest for the causal agent of the MFC of date palms led, initially, to the finding of two RNA species (MFC-RNAs) with the characteristic electrophoretic mobility of viroid-like circular RNAs. The MFC-RNAs were detected in MFC-affected date palm leaflets, and not in healthy ones, by the double PAGE technique (Fig. 1) regularly used for the diagnosis of viroid infection (5). However, MFC-RNAs were shown not to be circular viroid-like RNAs but dsRNAs (MFC-dsRNA), because (i) they bound to nonionic cellulose in the presence of 16.5% ethanol (Fig. 2), a property characteristic of dsRNAs (7,23), (ii) they could be end-labeled with ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by polynucleotide kinase (Fig. 3), a property characteristic of linear RNA molecules with free 5' ends but not shown by the circular viroid RNA molecules, (iii) they resisted degradation by RNase A at high ionic strength (Fig. 4), and finally, (iv) their (+) and (-) strands could be detected by strand specific probes (Fig. 6). Altogether, these experiments show that the MFC-RNAs, as detected in this work by the double PAGE procedure, are double stranded. However, these results do not establish whether the MFC-RNAs are also double stranded in vivo. They could eventually result from single stranded (+) RNAs annealing with complementary (-) RNA strands during the phenol homogenization procedure used for RNA preparation. Indeed, it has been established that in certain cases, the dsRNA found in extracts from plants infected with single stranded RNA viruses result from the RNA extraction procedures and did not exist in vivo (8). In addition, these dsRNAs, as detected by double PAGE, could become partially denatured as the result of the denaturing conditions prevailing in the second gel (8 M urea), and this partial denaturation would explain why they show the characteristically slow electrophoretic mobility of viroid-like circular RNAs. Consequently, dsRNAs could be a potential cause of false positive results during viroid diagnosis by double PAGE analysis.

Northern blot hybridization analysis of the MFC-dsRNAs showed that the species giving rise to the two, slow and fast, electrophoretic bands are homologous and, most probably, the small one is included in the large one. Cloning and sequencing of the MFC-RNAs giving rise to the slow electrophoretic band showed that this band corresponds in fact to a combination of two species (Fig. 5). MFC-dsRNAs derive from two different regions of date palm chloroplast genome: *rrn5S-trnR(ACG)*, coding for the 5S rRNA and the Arg-tRNA(ACG), and *trnM(CAU)-atpE*, coding for the Met-tRNA(CAU) and the subunit CF1 ϵ of ATP synthase. Under normal conditions, genes *rrn5S* and *trnR(ACG)* are co-transcribed within a large polycistronic RNA that also includes other rRNAs and tRNAs (14,25). In contrast, genes *trnM(CAU)* and *atpE* are coded from opposite strands of the chloroplast genome DNA and, therefore, they can not be co-transcribed (27).

Date palm RNAs were also studied by single denaturing PAGE, a technique yielding a wider range of RNAs than double PAGE, essentially developed for viroid RNA detection and analysis. Northern blot hybridization analysis using single strand-specific probes homologous to the *rrn5S* coding region and *rrn5S-trnR(ACG)* intergenic region, and the *atpE* coding region showed that leaflets of MFC-affected date palms accumulate a population of RNAs heterogeneous in size (from less than 200 to more than 600 nt) and comprising strands of both polarities (Fig. 7). Such RNAs do not exist in healthy date palms. A similar situation, although at a much lower extent, was also observed using probes

Fig. 7. Northern blot hybridization analysis of RNA preparations from a *maladie des feuilles cassantes* (MFC)-affected date palm with different chloroplast probes. RNAs were separated by single denaturing polyacrylamide gel electrophoresis (PAGE) and hybridized with strand-specific probes to detect the **A**, sense or (+) and **B**, antisense or (-) strands of date palm chloroplast *rrn5S* coding region and *rrn5S-trnR(ACG)* intergenic region, and *atpE*, *atpA*, *rbcL* coding regions, and mitochondria *coxI* coding regions. **C**, Ethidium bromide staining of the polyacrylamide gel previous to blotting. Lane 0, RNA marker with size in nt indicated on the left. Lanes 1, 3, 5, 7, and 9, RNA from a healthy palm. Lanes 2, 4, 6, 8, and 10, RNA from an MFC-affected palm.

specific for two other chloroplast genes, *atpA* and *rbcL*, corresponding to different operons (Fig. 7). In contrast, similar small heterogeneous RNAs, homologous to a mitochondria gene, *coxI*, were not detected in MFC-affected date palms (Fig. 7). Annealing, either in vivo or more probably during RNA preparation (8), between complementary (+) and (–) strands from the heterogeneous RNA population, is seen as resulting in the formation of MFC-dsRNAs, including those detected after double PAGE (Figs. 1 to 3 and 6).

Gene expression in the chloroplast of higher plants consists of transcription, usually of polycistronic pre-mRNAs, and posttranscriptional RNA processing of primary transcripts that includes endonucleolytic cleavage, 3'-end trimming, *cis/trans*-splicing and RNA editing (26). The accumulation in the leaflets of MFC-affected date palms of a set of chloroplast RNAs, heterogeneous in size and comprising strands of both sense and antisense polarities, suggests that the disease is associated with a severe deregulation of one or more steps in chloroplast gene expression. More specifically, the occurrence of RNAs of both coding and antisense polarities suggests defects in transcription initiation and/or termination, although an RNA-to-RNA amplification mechanism similar to that involving the cytoplasmic RNA-dependent RNA polymerase can not be discarded. Nonetheless, the MFC-RNAs do not reflect the whole chloroplast genome, but correspond to two particular regions: *rrn5S-trnR(ACG)* and *trnM(CAU)-atpE*. The possibility exists that MFC alters the level of some RNAs naturally occurring in plant chloroplasts. Recently, it has been proposed that antisense RNA could regulate RNA stability in chloroplasts (19) and that small noncoding RNAs, mostly from antisense strands and intergenic regions, could be important regulators of chloroplast gene expression (15). MFC-RNAs could be regulatory RNAs whose concentration is increased as a consequence of the disease. In this respect, it is interesting to note that two of the noncoding RNAs recently proposed for tobacco chloroplasts, Ntc-3 and Ntc-5 RNAs, are coded in *rrn4.5-rrn5S* and *trnM(CAU)-atpE* intergenic regions (15).

No pathogen could be shown to be associated with MFC-affected date palms, but the disease has been correlated with Mn-deficiency of palm leaves. Indeed, an inverse correlation between symptom severity and Mn content was observed in leaflets from healthy, apparently healthy, mild diseased, moderate diseased, and severe diseased date palms from six different oases in the southern part of Tunisia. Moreover, dot blot hybridization showed accumulation of MFC-RNAs in all these MFC-affected tissues showing different degrees of symptoms (18). Mn is an essential element, particularly in photosynthesis, because four atoms of Mn are part of the complex in photosystem II (16,30) on which water is split with the energy of sunlight to yield molecular oxygen, as well as protons and electrons needed in particular for ferredoxin and NADP reduction, and formation of ATP by coupled photophosphorylation. Thus, Mn-deficiency should seriously affect the chloroplast machinery. Indeed, leaves of MFC-affected palms are discolored and chlorotic, and the leaflets show necrotic streaks. Similarly, Mn-deficiency was implicated in needle chlorosis of silver fir trees (*Abies alba*) in Switzerland (10), and could contribute to forest decline. Mn is an essential element for living organisms also because it functions as a redox cofactor in some enzymes such as Mn superoxide dismutase (MnSOD). Interestingly, both photosystem II and the MnSOD, expected to be prime targets of Mn-deficiency in plants (31), are indeed severely impaired by Mn-deficiency, at least in *Chlamydomonas* (1). Finally, RNA elongation by RNA polymerases has also been reported to require Mn (12). How the above targets (photosystem II, MnSOD, RNA polymerases), when affected by Mn-deficiency, result in the production of the MFC-RNAs remains to be understood.

Whatever might be the biogenesis route of the MFC-RNAs, a strict correlation between their presence and MFC of date palms has been demonstrated (Figs. 1, 2, 6, and 7, and Table I), and

therefore, they can be considered as molecular markers of the disease. This conclusion is in good agreement with earlier findings in which a composite molecular probe, homologous to both *rrn5S-trnR(ACG)* intergenic region and *atpE* coding region produced hybridization signals with leaflet samples from MFC-affected date palms, regardless of cultivar, severity of symptoms, or geographical location, whereas samples from healthy date palms or date palms affected by biotic or abiotic stresses others than MFC, tested negative (17).

ACKNOWLEDGMENTS

First and second authors contributed equally to the studies presented. This work was supported by the Ministerio de Educación y Ciencia (MEC) of Spain (Programa Azahar AGL2004-06311-C02-01 and AGL2004-06311-C02-02) and Generalitat Valenciana (ACOMP06/095). J. Marqués and Z. G. N. Fadda received predoctoral fellowships from MEC and from the National Palestinian Authority and the Universidad Politécnica de Valencia, respectively.

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