Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs. I. Characterization of a new species in the genus *Chrysovirus*

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Cherry chlorotic rusty spot (CCRS) and Amasya cherry disease (ACD) display similar symptoms and are associated with a series of dsRNAs. However, a direct comparison has been lacking. Here, a side-by-side analysis confirmed that both diseases were symptomatologically very similar, as were the number (10–12) and size of their associated dsRNAs. Sequence determination of four of these dsRNAs revealed that they were essentially identical for CCRS and ACD. The largest (3399 bp), which potentially encoded a protein of 1087 aa with the eight motifs conserved in RNA-dependent RNA polymerases of dsRNA mycoviruses, had the highest similarity to those coded by dsRNA 1 of viruses belonging to the genus *Chrysovirus* and was termed CCRS or ACD chrys-dsRNA 1. The three closely migrating dsRNAs had the properties of the other components of a chrysovirus and in CCRS and ACD versions, respectively, were chrys-dsRNA 2 (3125 and 3128 bp), chrys-dsRNA 3 (2833 bp) and chrys-dsRNA 4 (2499 and 2498 bp), potentially encoding the major capsid protein (993 and 994 aa) and two proteins (884 and 677 aa, respectively) of unknown function. The four 5′- and 3′-UTRs shared internal similarities and had conserved GAAAAUUAUGG and AUAUGC termini, respectively. The 5′-UTRs contained the 'Box 1' motif followed by a stretch rich in CAA, CAAA and CAAAA repeats, characteristic of chrysovirus dsRNAs. Because species of the genus *Chrysovirus* have only been described as infecting fungi, this suggests a fungal aetiology for CCRS and ACD, a proposal supported by the properties of two other CCRS- and ACD-associated dsRNAs (see accompanying paper by Coutts *et al.*, 2004, in this issue).

INTRODUCTION

A disease termed cherry chlorotic rusty spot (CCRS) on the basis of its most conspicuous symptoms was first reported in 1996 in southern Italy. None of the known cherry viruses appeared to be consistently associated with it and initial

attempts to isolate and culture a bacterial or fungal pathogen were negative, so the disease was presumed to be caused by an undescribed virus-like agent (Di Serio *et al.*, 1996). Supporting this idea, a series of 12 double-stranded (ds)RNAs and two cherry small circular (csc)RNAs were consistently isolated from CCRS-affected tissue collected from sweet and sour cherry trees grown in the same area (Di Serio *et al.*, 1996, 1998). Cloning and sequencing of the largest cscRNA revealed that strands of both polarities could adopt hammerhead structures similar to those found previously in some viroid and viroid-like satellite RNAs and that they self-cleaved during *in vitro* transcription and after purification as predicted by these ribozymes (Di Serio *et al.*, 1997).

From a symptomatological perspective, CCRS disease resembles only the Amasya cherry disease (ACD) described previously in the region of Turkey with this name (Blodgett *et al.*, 1970; Citir, 1987). Interestingly, ACD is also associated with a complex set of presumably viral dsRNAs (Açıkgöz *et al.*, 1994). Although the symptoms as well as the number and sizes of the dsRNAs associated with both cherry disorders present clear parallels, a direct comparison of the two diseases has been lacking.

Here, we present the results of research that addressed two questions. Firstly, whether CCRS and ACD are indeed closely related diseases and, secondly, whether the molecular characterization of some of the CCRS- and ACD-associated dsRNAs could provide some insight into the nature of these RNAs. Our data showed that both diseases were very similar on the basis of symptoms and, particularly, of the sequence of six of their associated dsRNAs, which are the genomic components of two new tentative species, one in the genus *Chrysovirus* and one in the genus *Partitivirus* (see accompanying paper by Coutts *et al.*, 2004, in this issue). Since members of the genera *Chrysovirus* and *Partitivirus* typically infect fungi, these results, combined with previous observations, suggest a fungal aetiology for CCRS and ACD.

METHODS

Origin of cherry material. Leaves were collected in late summer from 15 representative sweet cherry trees (*Prunus avium* L.), cultivars 'Bigarreau-Napoleon', 'La Signora' and 'Imperiale', exhibiting typical symptoms of CCRS disease in Italy, and from 20 sweet cherry trees, cultivars 'Köroglu', 'Erkara', 'Lambert', 'Turco' and 'Tabanıyarık', exhibiting typical symptoms of ACD disease in Turkey. Single-tree and combined samples from several trees were analysed. Control samples were taken from asymptomatic trees growing in the same areas and from cherry seedlings.

Extraction and fractionation of nucleic acids. Total nucleic acid preparations were obtained by extracting leaves with buffer-saturated phenol as described previously (Pallás *et al.*, 1987). These preparations were then partitioned by chromatography on non-ionic cellulose with STE (50 mM Tris/HCl, pH 7·2, 100 mM NaCl, 1 mM EDTA) containing 16 % ethanol and the dsRNA-rich fraction was recovered by ethanol precipitation (Morris & Dodds, 1979).

PAGE and molecular hybridization analyses. The CCRS- and ACD-associated dsRNAs were separated by PAGE in non-denaturing (5 %) gels that were stained with ethidium bromide (and, when indicated, with silver). Some of the CCRS dsRNAs generating the individual bands were eluted, recovered by ethanol precipitation and used for dot-blot hybridization or for cDNA synthesis (see below). Pre-hybridization, hybridization with digoxigenin-labelled riboprobes (at 68 °C in 50 % formamide) and washing were as recommended by the supplier (Roche) and membranes were analysed by autoradiography or using a Fuji Bas 1500 apparatus.

Reverse transcription, PCR amplification, cloning and sequencing. The first cDNA clones of CCRS chrys-dsRNAs 1 and 4 were obtained by a random-primer RT-PCR protocol for cloning RNAs of unknown sequence (Navarro *et al.*, 1998), with their identity being confirmed by dot-blot hybridization with purified preparations of these dsRNAs. For the synthesis of additional cDNAs covering their complete sequence, purified CCRS chrys-dsRNAs 1 and 4 (200–300 ng) were denatured with 10 mM methyl mercuric hydroxide and reverse transcribed with Superscript II reverse transciptase as recommended by the supplier (Invitrogen) and specific primers derived from the initial clone. The resulting cDNAs were 3′-tailed with terminal transferase (Roche) and dATP or dCTP, and PCR amplified with *Taq* DNA polymerase (Roche) and the specific primers used in the reverse transcriptase reaction combined with others containing a dT_{25} or dG_{25} tail at their 3′ termini (with the last base degenerated). For obtaining cDNA clones corresponding to the 5′ and 3′ ends, denatured CCRS chrys-dsRNAs 1 and 4 were polyadenylated with yeast poly(A) polymerase (USB Corporation) and ATP, reverse transcribed with a primer containing a 3′-dT_{25} tail with the last base degenerated and PCR amplified with this primer combined with others derived from internal regions.

Complete cDNA clones of the CCRS chrys-dsRNAs 2 and 3 were obtained using, with minor modifications, the single-primer method for cloning dsRNAs of unknown sequence (Isogai *et al.*, 1998; Vreede *et al.*, 1998). In brief, the purified dsRNA was denatured, polyadenylated and reverse transcribed with a primer containing a 3′-dT_{25} tail with the last base degenerated. Following treatment with RNase H (Roche), the cDNA strands were annealed for 10 min at 80 °C, for 16 h at 65 °C and for 3 h at 30 °C and the resulting hybrid was filled in with T4 DNA polymerase (Roche) and PCR amplified using the Expand High Fidelity system (Roche) and the same primer with the 3′-dT_{25} tail and the last base degenerated. The PCR profile consisted of an initial denaturation at 94 °C for 2 min and 35 cycles of 50 s at 94 °C, 50 s at 58 °C and 3 min at 68 °C, with a final extension of 15 min at 72 °C. RT-PCR amplification products were cloned in bacterial plasmids following standard techniques (Sambrook *et al.*, 1989) and the insert sequences were determined automatically using an ABI PRISM DNA 377 apparatus (Perkin-Elmer). Proper assignation of the sequences to specific dsRNAs was established by dot-blot hybridization using purified preparations of the three dsRNAs spotted on to membranes and probes derived from internal cDNA clones.

Synthesis of cDNA clones for the ACD-associated dsRNAs was performed essentially as described for the CCRS-associated dsRNAs but used an RNA ligase-mediated (RLM)-RACE PCR (Coutts & Livieratos, 2003) procedure to determine the 5′- and 3′-terminal sequences. Sequences of the dsRNAs were completed and confirmed by overlapping of cDNA clones prepared with internal primers, and assignment to specific dsRNAs was made by comparison with the CCRS-associated dsRNAs.

Sequence analysis. The coding capacity of the nucleotide sequences was determined using the program TRANSLATE (www.expasy.ch). Comparisons with sequences deposited in databases (GenBank, EMBL, SWPROT and PIR) were executed using the programs BLASTN, BLASTP and BLASTX (www.genome.ad.jp, www.ncbi.nlm.nih.gov and

www.ebi.ac.uk). Pairwise alignments were done using the programs BESTFIT and GAP of the GCG package v. 10 and multiple alignments with CLUSTAL X v. 1.81 (Thompson *et al.*, 1997) and visualized with GENEDOC v. 2.6 (www.psc.edu). The subsequent phylogenetic analysis was obtained with the MEGA program v. 2.1 (Kumar *et al.*, 2001) and the phylogenetic relationships of the unrooted tree were based on the neighbour-joining method with a 10 000 replicate bootstrap search. The BOOTSTRAP option in the MEGA program was used for the bootstrap analysis.

RESULTS

CCRS and ACD diseases are symptomatologically similar

Early leaf symptoms of CCRS disease are translucent spots of 1–2 cm diameter that develop into chlorotic spots with small rusty areas that finally cover their complete surface. On the basis of these symptoms, which have been consistently observed in sweet and sour cherry in Italy, a name for the disease was advanced (Di Serio *et al.*, 1996, 1998). ACD leaf symptoms, reported only in some sweet cherry cultivars in Turkey, consist of brick-coloured spots following the appearance of chlorotic small lesions in the early stages of the disease (Blodgett *et al.*, 1970; Citir, 1987). In both cases, symptoms are also manifested in fruits, which do not ripen and have reduced size and elongated shapes with reddish lines on the skin, and in late stages of the disease as the decayed appearance of the trees as a result of die-back occurring in shoots.

To obtain additional evidence, CCRS- and ACD-affected leaves of Italian and Turkish origin, respectively, were examined side by side. The symptoms appeared very similar, if not identical (data not shown), and specific, suggesting that both disorders might be the same and have a common causal agent.

CCRS and ACD are associated with a common pattern of 10–12 dsRNAs

PAGE analysis of dsRNA-rich preparations from cherry trees displaying the typical ACD symptoms showed a complex profile of dsRNAs that were absent from healthy-looking trees (Açıkgöz *et al.*, 1994). Similar analysis showed ten dsRNAs, which ranged in size from approximately 1700 to 5500 bp, and two smaller dsRNAs of approximately 500 and 650 bp that were diagnostic for CCRS disease (Di Serio *et al.*, 1996) (Fig. 1a). Moreover, when CCRS-affected leaves were divided prior to extraction, with one fraction consisting of chlorotic-rusty lesions and the other the asymptomatic surrounding areas, the dsRNAs were found predominantly in the first fraction (Di Serio *et al.*, 1996). To determine whether the dsRNAs associated with CCRS and ACD had a common distribution of sizes, a direct comparison by electrophoresis on the same polyacrylamide gel was performed. This analysis revealed a remarkable similarity in the mobility of the ten individual components of higher size (Fig. 1b), thus suggesting that if they were components of the causal agent, this agent
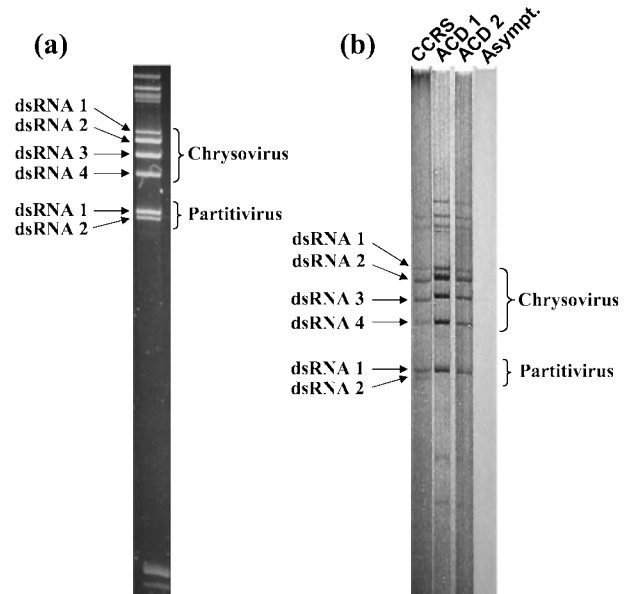

Fig. 1. PAGE analysis in non-denaturing (5 %) gels of the dsRNAs associated with CCRS and ACD. (a) Ethidium bromide staining of a dsRNA-rich preparation of CCRS-affected tissue. (b) Silver staining of dsRNA-rich preparations from CCRS- (lane 1) and ACD-affected tissues (lanes 2 and 3). A control preparation, obtained under the same conditions from an asymptomatic cherry tree, is also included (lane 4). Although the partitivirus dsRNA 2 associated with ACD disease is barely visible in lanes 2 and 3, this RNA was consistently observed in other preparations. The smallest RNAs visible in (a) ran out of the gel in (b) as a consequence of the longer electrophoresis time. In some preparations, the dsRNA with the slowest electrophoretic mobility was absent.

might be the same for both diseases. The CCRS- and ACD-associated dsRNAs could in principle represent the components of one or more viruses with a multipartite dsRNA genome(s) or the genomic and subgenomic dsRNAs resulting from infection by one or more single-stranded RNA (ssRNA) viruses.

One of the dsRNAs associated with CCRS potentially encodes a protein with the characteristics of mycoviral RNA-dependent RNA polymerases

The patterns of dsRNAs accumulating in tissues infected by typical plant ssRNA viruses are not consistent with the number and sizes of the dsRNAs associated with CCRS and ACD. On the other hand, although members of the genus *Phytoreovirus* have multipartite genomes of 12 dsRNAs, their sizes also do not correspond to those of the CCRS- and ACD-associated dsRNAs. Therefore, to get an insight into their nature, we decided to sequence some of these dsRNAs. Towards this aim, starting with a partially purified preparation of one of the CCRS-associated dsRNAs, chrys-dsRNA 4 (Fig. 2; see below for an explanation of the
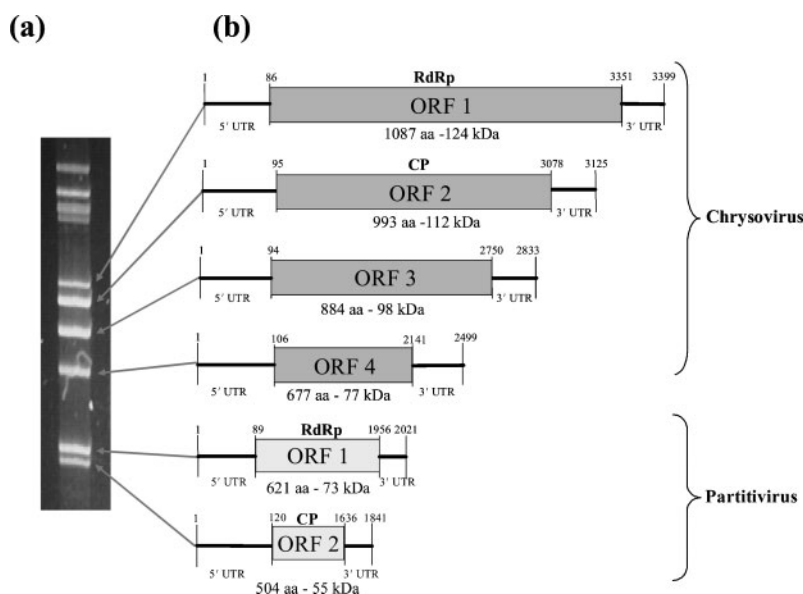
Fig. 2. Properties of the six dsRNAs associated with CCRS disease (the corresponding six dsRNAs associated with ACD disease are very similar; see Results). Identification by PAGE profile (a) and genomic organization (b) of the chrysovirus dsRNAs 1 to 4 and the partitivirus dsRNAs 1 and 2 (see accompanying paper by Coutts *et al.*, 2004, in this issue).

nomenclature), cDNAs were prepared by a PCR-based random-primer approach (Navarro *et al.*, 1998) and then cloned. Dot-blot hybridization using purified CCRS chrys-dsRNA 4 as a test identified cDNA clones corresponding to this RNA (see below).

In addition, we also identified a clone of 1572 nt that did not hybridize with CCRS chrys-dsRNA 4 but did hybridize with CCRS chrys-dsRNA 1 (Fig. 2). Analysis of the predicted amino acid sequence revealed the presence of a single ORF of 522 aa containing the eight conserved motifs typical of RNA-dependent RNA polymerases (RdRps) of fungal viruses (Fig. 3a). This prompted us to complete the sequence of this RNA by reverse transcription with primers derived from the first cDNA clone, 3′ tailing with dATP or dCTP and terminal transferase, and PCR amplification. The complete sequence of 3399 bp showed 5′- and 3′-untranslated regions (5′- and 3′-UTRs) of 86 and 49 nt, respectively, in one strand, flanking a single ORF of 1087 aa with a predicted mass of approximately 124 kDa (Fig. 2b). The complementary strand did not contain ORFs of a minimal size compatible with a functional protein (data not shown). Phylogenetic reconstruction using the fragments containing the eight conserved motifs characteristic of the RdRps of dsRNA mycoviruses, which are preceded by the conserved helicase motif GXGKS/T (Kadaré & Haenni, 1997), showed that the closest similarities were with those encoded by the dsRNA 1 of *Penicillium chrysogenum virus* (PcV), the type species of the genus *Chrysovirus* of the new established family *Chrysoviridae* (Jiang & Ghabrial, 2004; Ghabrial & Castón, 2004), and of *Helminthosporium victoriae 145S virus* (Hv145SV) (Ghabrial *et al.*, 2002), the other sequenced member of this genus (Fig. 3b). Additional data, including a 5′-UTR rich in CAA repeats (Fig. 4a) and the existence in the profile of CCRS-associated dsRNAs of bands with the sizes expected for the three other dsRNA components characteristic of chrysoviruses (Jiang &

Ghabrial, 2004; Ghabrial & Castón, 2004), strongly supported this view. Therefore, this dsRNA was named CCRS-associated chrysovirus dsRNA 1 (CCRS chrys-dsRNA 1) (Fig. 2).

Molecular characterization of the three other genomic components of a new chrysovirus species associated with CCRS disease

To confirm the presence of a chrysovirus in CCRS-affected tissue, the dsRNAs comprising the three bands immediately below that corresponding to CCRS chrys-dsRNA 1 (Fig. 2), which on the basis of their size appeared the best candidates, were eluted, polyadenylated, reverse transcribed and cloned using the single-primer approach (Isogai *et al.*, 1998; Vreede *et al.*, 1998) for CCRS chrys-dsRNA 2 and 3 and the same approach used with CCRS chrys-dsRNA 1 for CCRS chrys-dsRNA 4. Sequence analysis revealed that each dsRNA contained a single ORF in only one of the strands (Fig. 2b) and 5′- and 3′-UTRs characteristic of chrysoviruses (see below). The identity of the CCRS chrys-dsRNAs 2, 3 and 4 was established by dot-blot hybridization, using purified preparations of these three dsRNAs as targets and cDNA clones of internal regions thereof as probes (data not shown).

CCRS chrys-dsRNA 2 comprised 3125 bp and in one strand showed 5′- and 3′-UTRs of 95 and 48 nt, respectively, flanking an ORF potentially encoding a protein of 993 aa with a molecular mass of approximately 112 kDa and similarities to the major coat protein of chrysoviruses (Fig. 2b). CCRS chrys-dsRNA 3 comprised 2833 bp and in one strand showed a 5′-UTR of 94 nt, which increased to 304 nt if it was assumed that the second-most 5′-AUG codon was in a better initiation context (Kozak, 1986; Lütcke *et al.*, 1987), and a 3′-UTR of 84 nt, flanking an ORF potentially encoding a protein of 884 aa with a molecular

(a)

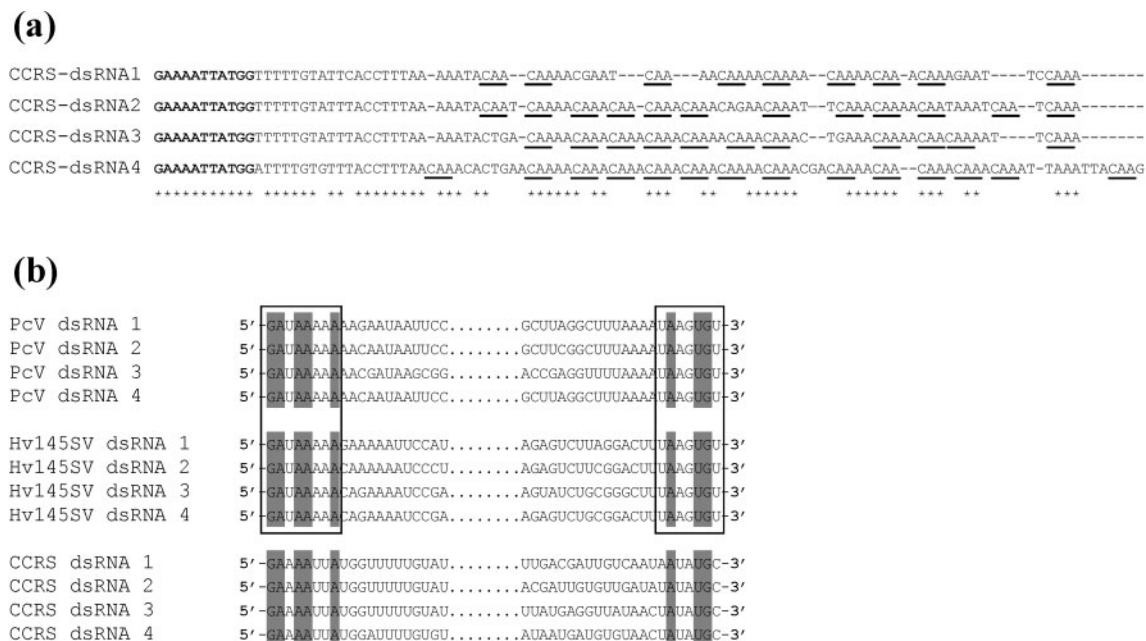| Virus	1	2	3	4	5	6	7	8
UmV-H1	LYGRG 66	WLVSGSSAG 61	GKARAIY 55	DYPDFNSMHT 63	GLYSGDRDTTLINTLLNIAYA 20	CHGDDIIT 34	EYLRI 13	RCVAT
Hv190SV	LQGRY 61	WCVNGSQNA 46	GKDRAIF 55	DYDNFNSQHS 45	TLMSGHRATTFTNSVLNAAYI 15	HAGDDVYL 34	EFLRL 12	RAIAS
ScVL-A	LMNRG 57	WVPGGSVHS 50	GKQRATY 52	DYDDFNSQHS 52	TLLSGWRLTTFMNTVLNWAYM 15	HMGDDVMI 33	EFLRV 16	RSCAT
Consensus toti	* *	* **	** ***	** *** *	* ** ** * ** **	***	* **	* *
CCRS-CV	LVGRR 73	**WLTKGSTVY** 65	YKDRTLL 52	**DWANFNAFHS** 48	GLYSGWRGTSFLNSVLNSCYT 20	HGGDDIDG 33	**EFFRI** 12	RALAR
PcV	LVGRG 73	**WLTKGGLVY** 65	KKDRTLL 52	**DWADFNEQHS** 48	GLYSGWRGTTWINTVLNFCYV 20	HGGDDIDL 33	**EFFRN** 12	RALAS
Hv145SV	LLGRR 73	**WMTKGSLVS** 65	HKDRVLL 52	**DWANFNVQHS** 48	GLYSGWRGTTWDNTVLNGCYM 20	QGGDDVDQ 33	**EFFRV** 12	RGLAT
AbV-1	LIGRR		GKNASRS 55	DYANFNEQHS 54	GLLSGWRCTAYINNLINIAQY 20	TGGDDGCA 34	**EFFRL** 12	RMLAS
Consensus chrys	* **	* *** *	*	* * ** **	** **** * * *	****	****	* **
AhV-2H			LKVRPVY 55	DWSRFDHLAP 92	GVPSGILMTQFIDSFVNLTIL 20	IMGDDNVI 42	EVLGY 8	RSISK
FsV-1			PKTRLAW 55	DFSSFDTKVP 61	GVPSGSSWTQLVDSVVNWILV 15	VLGDDSAF 36	KLLGV 8	RETEE
HmV-V70			TKIRVVF 61	DWSSFDLRSL 67	GIPSGLFTTQFLDSFYNMIMI 17	VQGDDSLI 44	NALGY 8	RDWRK
CCRS-PV			NKMRTIW 53	DWKRFDKKAY 71	GIPSGLFITQLIDSWYNYTML 17	VQGDDSII 44	EVLSY 8	RDELA
Consensus parti			* *	* *	* *** ** ** *	***	* *	*

(b)
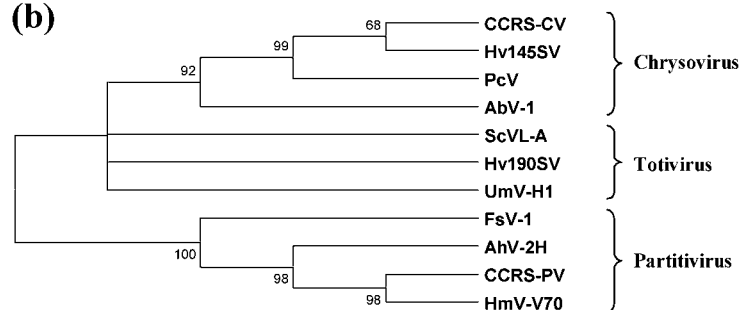
Fig. 3. Relationships between the CCRS- and ACD-associated chrysoviruses and representative dsRNA mycoviruses. (a) Multiple alignment of a fragment of the putative RdRp encoded by the CCRS- and ACD-associated chrysovirus dsRNA 1 with the RdRps of some dsRNA mycoviruses. Totiviruses: *Ustilago maydis virus H1* (UmV-H1; GenBank accession no. U01059), *Helminthosporium victoriae 190S virus* (Hv190SV; U41345) and *Saccharomyces cerevisiae virus L-A* (ScVL-A; J04692). Chrysoviruses: PcV (AF296439), Hv145SV (AF297176) and Cherry chlorotic rusty spot-associated chrysovirus (CCRS-CV; AJ781397). Partitiviruses: *Atkinsonella hypoxylon virus* isolate 2H (AhV-2H; L39125), *Fusarium solani virus 1* (FsV-1; D55668), *Helicobasidium mompa virus* strain 70 (HmV-V70; AB025903) and Cherry chlorotic rusty spot-associated partitivirus (CCRS-PV; AJ781401). Numbers 1–8 refer to the eight motifs conserved in RdRps of dsRNA viruses of lower eukaryotes (Bruenn, 1993; Ghabrial, 1998) (motifs 1 and 2 of partitiviruses have not been included because they are tentative). Numbers of nucleotide residues between the conserved motifs are indicated. Asterisks denote residue identity and bold characters denote residues conserved in chrysoviruses. (b) Unrooted phylogenetic tree based on the neighbour-joining method with a 10 000 replicate bootstrap search. Bootstrap values are indicated at the branch points.

mass of approximately 98 kDa (Fig. 2b). CCRS chrys-dsRNA 4 comprised 2499 bp and in one strand showed a 5′-UTR of 106 nt, which increased to 151 nt if it was assumed that the second-most 5′-AUG codon was in a better initiation context, and a 3′-UTR of 359 nt, flanking an ORF potentially encoding a protein of 677 aa with a molecular mass of approximately 77 kDa (Fig. 2b). The functions of the putative products of CCRS chrys-dsRNA 3 and 4, like those of the dsRNAs 3 and 4 of other chrysoviruses (Jiang & Ghabrial, 2004; Ghabrial & Castón, 2004), which are similar in size and sequence, are currently unknown, although CCRS chrys-dsRNA 3 contains a motif conserved in other ssRNA and dsRNA viruses (see below).

Assignment of numbers 1 to 4 to the dsRNAs of the CCRS-associated chrysovirus was made according to their decreasing size, following the same criterion used for PcV and Hv145SV chrysoviruses. Yet, sequence comparisons indicated that dsRNAs 3 of the CCRS-associated chrysovirus and that of Hv145SV were more closely related to PcV dsRNA 4 than to dsRNA 3. In particular, the proteins potentially encoded by these three dsRNAs contained the motif PGDGXCXXHX. This motif (I), along with motifs II (with a conserved K), III and IV (with a conserved H), form the conserved core of the ovarian tumour gene-like superfamily of predicted proteases (Makarova *et al.*, 2000). Multiple alignments showed that motifs I to IV were also present in the ORF of other viruses including *Agaricus bisporus virus 1* associated with La France disease, a tentative chrysovirus; *Potato virus M*, *Hop latent virus* and *Blueberry scorch virus*, three carlaviruses; and *Rupestris stem pitting-associated virus 1*, a foveavirus (data not shown). Whether

(a)

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CCRS-dsRNA1 GAAAATTATGGTTTTTGTATTCACCTTTAA-AAATACAA--CAAAACGAAT---CAA---AACAAAACAAAA--CAAAACAA-ACAAAGAAT----TCCAAA------
CCRS-dsRNA2 GAAAATTATGGTTTTTGTATTTACCTTTAA-AAATACAAT-CAAAACAAACAA-CAAACAAACAGAACAAAT--TCAAACAAAACAATAAATCAA--TCAAA------
CCRS-dsRNA3 GAAAATTATGGTTTTTGTATTTACCTTTAA-AAATACTGA-CAAAACAAACAAACAAACAAAACAAACAAAC--TGAAACAAAACAACAAAAT----TCAAA------
CCRS-dsRNA4 GAAAATTATGGATTTTGTGTTTACCTTTAACAAACACTGAACAAACAAACAAACAAACAAACAAACGACAAAACAA--CAAACAAACAAAT-TAAATTACAAG
            ********** ****** ** ******** *** **    ****** **    ***  **  ******    ****** *** **        ***
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(b)

Fig. 4. (a) Conserved sequence motifs in the 5′-UTRs of the coding strands of the four genomic dsRNAs of the CCRS-associated chrysovirus. The CAA repeats (and variations thereof, CAAA and CAAAA) characteristic of the 5′-UTRs of chrysovirus dsRNAs are underlined. (b) Comparison of the 5′- and 3′-terminal regions of the coding strands of the four genomic dsRNAs of the two known members of the genus *Chrysovirus*, PcV and Hv145SV, and of the CCRS-associated chrysovirus. Identical nucleotide residues between PcV and Hv145SV are boxed and those conserved in the three chrysoviruses are shaded.

the RNAs of these viruses indeed code for the predicted proteases remains to be investigated. On the other hand, a 'phytoreo S7 domain' shared with a family of several phytoreovirus S7 proteins has recently been found in PcV dsRNA 3 (Jiang & Ghabrial, 2004). This domain is also present in dsRNAs 4 of CCRS-associated chrysovirus and Hv145SV [the consensus for the three chysoviruses is X(V/I)V(M/L)P(A/M)G(C/H)GK(T/S)T(L/I)], indicating that they are the likely counterparts of PcV dsRNA 3.

The 5′- and 3′-UTRs of the four CCRS chrys-dsRNAs presented strictly conserved GAAAAUUAUGG and AUAUGC termini, respectively. Whereas the 5′ termini were similar to those of the sense strand of all four dsRNAs from PcV and Hv145SV (GAUAAAAA), the situation differed with the 3′ termini of the four dsRNAs from PcV and Hv145SV (UAAGUGU). We do not believe that these discrepancies could have resulted from the procedures used in our case not allowing proper cloning of the exact termini (see below). Additionally, the 5′- and 3′-UTRs of the four CCRS chrys-dsRNAs showed strong sequence similarities in their internal parts. This was specifically the case with the 5′-UTRs, which contained a highly conserved stretch of 36–37 nt, the so-called 'Box 1' of chrysoviruses (Ghabrial & Castón, 2004), followed by a less-conserved stretch of 63–64 nt rich in CAA, CAAA and CAAAA repeats (Fig. 4a). These repeats are also characteristic of the 5′-UTRs of

chrysovirus dsRNAs (Jiang & Ghabrial, 2004; Ghabrial & Castón, 2004) and have been previously identified in the 5′-leader sequence of *Tobacco mosaic virus* RNA as a binding site for the heat-shock protein HSP101, which is required for translational enhancement (Gallie & Walbot, 1992; Gallie, 2002). Therefore, the sequence characteristics of these four CCRS-associated dsRNAs strongly suggested that they were the genomic components of a new chrysovirus species.

Sequence analysis of the CCRS- and ACD-associated chrysovirus dsRNAs supports the close similarity of both diseases

From dsRNA-rich preparations of ACD-affected tissue, cDNA clones were synthesized using a combination of random priming, RT-PCR with internal primers and RLM-RACE PCR. Assignment of specific dsRNAs was made by comparison with the CCRS-associated dsRNAs. Comparative analysis of the four chrysovirus dsRNAs associated with CCRS and ACD showed that they had almost identical sequences (nucleotide identity greater than 93 % and predicted amino acid sequences more than 95 % identical). This was particularly the case for the dsRNAs 1 and 3, which had 3399 and 2833 bp, respectively, and contained ORFs of 1087 aa and 884 aa, respectively. The CCRS chrys-dsRNA 2 was 3 bp shorter with respect to its ACD counterpart (3125 vs 3128 bp) because of a deletion

in a region within the ORF, which was thus 1 aa shorter in the former. The CCRS chrys-dsRNA 4 was 1 bp longer with respect to its ACD counterpart (2499 vs 2498 bp) as a consequence of the insertion of 1 bp in the 5′-UTR of the former. Therefore, not only were the electrophoretic patterns of and the apparent sizes inferred for the CCRS- and ACD-associated dsRNAs very similar, but the sequences of four of these dsRNAs, which corresponded to the genomic components of a new species in the genus *Chrysovirus*, were also very similar. This similarity could be extended to other two dsRNAs (Fig. 2), which were the genomic components of a new species in the genus *Partitivirus* (see accompanying paper by Coutts *et al.*, 2004, in this issue).

DISCUSSION

The causal agent of ACD has remained an enigma since its description in Turkey more than 30 years ago (Blodgett *et al.*, 1970), as has also been the case with CCRS, a symptomatologically similar disorder reported in Italy some time later (Di Serio *et al.*, 1996). However, both diseases have been associated with a complex pattern of virus-like dsRNAs (Açıkgöz *et al.*, 1994; Di Serio *et al.*, 1996). To provide additional data supporting a close relationship between these two cherry disorders, a side-by-side examination of ACD- and CCRS-affected leaves of Turkish and Italian origin, respectively, was performed. From a symptomatic standpoint, both diseases appeared indistinguishable (and different from other known cherry disorders), a conclusion that was reinforced by similar PAGE profiles for their associated dsRNAs (Fig. 1). Furthermore, the sequences of six of these dsRNAs (this work and accompanying paper by Coutts *et al.*, 2004, in this issue) were essentially identical. Altogether, these data indicated that ACD and CCRS could be the same disease for which we suggest keeping both names, one referring to the geographical origin of the first account and the other to a symptom-based description, until definitive proof of their identity is obtained.

The molecular characteristics of four of the CCRS- and ACD-associated dsRNAs (Fig. 2) closely paralleled those of the genus *Chrysovirus* and, on the basis of their size, sequence and structural features of their 5′-UTRs, which are demarcation criteria in the genus (Ghabrial & Castón, 2004), we propose that they are the genomic components of a new species of this genus. Although the species of genus *Chrysovirus* described so far have been found infecting only fungi (Ghabrial & Castón, 2004), the possibility exists that other members could infect plants, as is the case with cryptoviruses (the members of the family *Partitiviridae* that infect plants) that are included in this family under the genera *Alphacryptovirus* and *Betacryptovirus* (Ghabrial *et al.*, 2004). However, there is circumstantial evidence suggesting a fungal origin for the CCRS- and ACD-associated chrysoviruses (and, indirectly, a fungal aetiology for the diseases). Firstly, electron microscopic examinations of thin sections have revealed a fungus-like organism in CCRS and ACD symptomatic leaves, and fluorescent microscopy observations have confirmed the presence in affected leaf areas of a 'mycelium' growing between the lower epidermis, palisade and spongy mesophyll cells that was not found in vascular bundles or in asymptomatic samples (Alioto *et al.*, 2003). Secondly, although an initial report provided some data suggesting that ACD disease was induced by a graft-transmissible agent, presumably a virus (Blodgett *et al.*, 1970), this has not been further confirmed and attempts to transmit the CCRS agent by grafting have been unsuccessful (Alioto *et al.*, 2003). Lastly, treatment with systemic fungicides diminished the adverse effects of ACD (A. Citir, unpublished data). Collectively, these results support the involvement of a fungus as the causal agent, although efforts to isolate and grow it on artificial media have failed, with the saprophytic *Alternaria alternata* being sporadically detected (Alioto *et al.*, 2003). No direct attempts to observe or isolate chrysovirus-like virions from the leaf material containing CCRS- or ACD-associated dsRNAs have been made (D. Alioto, personal communication).

Chrysovirus virions are isometric, 35–40 nm, non-enveloped particles with a capsid formed by 60 protein subunits arranged on a genuine $T=1$ icosahedral lattice that separately encapsidate the four genomic dsRNAs (Castón *et al.*, 2003; Ghabrial & Castón, 2004). From the topology of a phylogenetic tree obtained with the fragments containing the eight motifs conserved in the RdRps of a series of mycoviruses (Fig. 3b), the CCRS- and ACD-associated chrysoviruses form a separate group with PcV and Hv145SV, the two other sequenced species in the genus *Chrysovirus* (Jiang & Ghabrial, 2004; Ghabrial & Castón, 2004). Moreover, the CCRS- and ACD-associated chrysoviruses appear to be more closely related to Hv145SV than to PcV (Fig. 3b). This relationship, based on the ORFs contained in the chrysovirus dsRNAs 1, is also supported by a phylogenetic reconstruction derived from the ORFs contained in the chrysovirus dsRNAs 2 (data not shown). The 5′-UTRs of the four dsRNAs of the CCRS- and ACD-associated chrysoviruses are considerably shorter than those of their Hv145SV and PcV counterparts, whereas the 3′-UTR of the dsRNA 4 of the CCRS- and ACD-associated chrysoviruses is longer. Despite these length differences, we believe we have determined the real termini of the genomic components of the CCRS- and ACD-associated chrysoviruses because: (i) the 5′ and 3′ termini were identical for the four dsRNAs from the two diseases; (ii) diverse RT-PCR approaches were used for their amplification and cloning; (iii) the 5′ terminus of one of the dsRNAs was confirmed by primer-extension analysis (data not shown); and (iv) there are nucleotide residues strictly conserved in both terminal regions of all chrysovirus dsRNAs (Fig. 4b). In any case, the high conservation of the 5′ and 3′ termini in the four dsRNAs of the CCRS- and ACD-associated chrysoviruses (Fig. 4) suggested that they may be replicated by the same RdRp, as previously advanced for other chrysoviruses (Ghabrial *et al.*, 2002).

It must be finally noted that we do not know yet the nature of the four CCRS- and ACD-associated dsRNAs of higher size (Fig. 1). They could be the replicative intermediates of a ssRNA virus(es) or, more likely, the genomic components of additional dsRNA viruses infecting the same fungus. In support of the second view, the size of some of the largest CCRS- and ACD-associated dsRNAs is consistent with that of totivirus dsRNAs, and a mixed infection of a chryso-virus and a totivirus has been previously reported in *H. victoriae* (Sanderlin & Ghabrial, 1978; Ghabrial *et al.*, 2002). Furthermore, the possibility of more than one fungus being involved in CCRS and ACD cannot be dismissed. On the other hand, it is unlikely that the two dsRNAs of lower size found in association at least with CCRS (Fig. 1) might contain functional ORFs, because they would be at most 100 aa long. The alternative suggestion that they could represent defective or satellite RNAs appears more plausible. A proposal of this kind has been advanced for the viroid-like RNAs associated with CCRS (Di Serio *et al.*, 1997). In any case, whether the CCRS- and ACD-associated dsRNAs are avirulent for their fungal hosts, as with PcV (Ghabrial & Castón, 2004), or whether they modulate fungal pathogenicity as previously reported for other mycoviruses (Ghabrial, 1994; Nuss, 1996; McCabe *et al.*, 1999) remains an intriguing issue to be explored.

ACKNOWLEDGEMENTS

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