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Double-stranded RNA pattern and partial sequence data indicate plant virus infection associated with the ringspot disease of European mountain ash (*Sorbus aucuparia* L.)

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Summary. Double-stranded RNA (dsRNA) has been extracted from tissue of European mountain ash trees (*Sorbus aucuparia* L.) showing typical ringspot and mottling symptoms on leaves and a gradual decay in general. A characteristic dsRNA pattern was found in leaf samples of symptomatic mountain ash trees from various stands in Germany. Bands of dsRNA molecules of approximately 7 kb, 2.3 kb, 1.5 kb, and 1.3 kb, respectively, were repeatedly detected. By random primed reverse transcription cDNA was synthesised from dsRNA and amplified by degenerate oligonucleotide primed PCR. After TA cloning, the cDNA clones obtained were screened with an enhanced-chemiluminescence-labelled dsRNA probe. Positive clones were further analysed by using them as hybridisation probes in Northern blots of total plant RNA and in Southern hybridisation with genomic DNA from *Sorbus aucuparia* leaves. From cDNA clones that were found to be specific for dsRNA in Northern analysis, primers were deduced for 5′-RACE analyses and further cloning. Finally, a cDNA fragment of 3,737 bp was obtained, which showed homology to viral proteins, particularly to the RNA-dependent RNA polymerase of members of the family *Bunyaviridae*, but without high similarity to a known genus. The dsRNA pattern and the sequence information strongly indicate a virus associated with the mountain ash ringspot disease. The putative virus remains still unidentified.

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AY563040 and AY563041.

Introduction

The European mountain ash (*Sorbus aucuparia* L.), also called rowan, is an important pioneer plant, which belongs to the most unpretentious species as it grows on heavily degraded soil [1, 40]. It is found in various forest ecosystems, public and private gardens together with many other trees and shrubs, in forests particularly in the vicinity of oaks. Since 1960 it was observed that a high proportion of mountain ash trees in several parts of Europe showed severe disease symptoms [8, 18, 30, 31, 36]. The characteristic symptoms are chlorotic ringspots and chlorotic mottling on leaves (Fig. 1). In addition, affected trees often showed reduced growth and an overall gradual decay over years.

The putative agent of the mountain ash disease was shown to be graft transmissible [14], but up to now all efforts to achieve mechanical transmission were unsuccessful. The disease symptoms on leaves and the graft transmissibility are indicative of a virus infection. Virus-like particles were occasionally observed by electron microscopy [11], but so far no virus particles could be isolated from symptomatic tissues. Virus infected forest trees are discussed to be a potential source of virus diseases of cultivated crops and trees, to which the agent is transmitted by vectors, or through soil and water [3, 4, 26]. It is therefore highly important to characterise the agent of the mountain ash ringspot disease to clarify whether it could be a virus or another biotic disease agent.

Here we present the results of our investigation on the mountain ash ringspot disease. To proof the hypothesis of plant virus association with the ringspot disease we tried to isolate double-stranded RNA (dsRNA) as a typical indicator of a virus infection [9, 17, 39]. In the course of our studies we had to optimise and

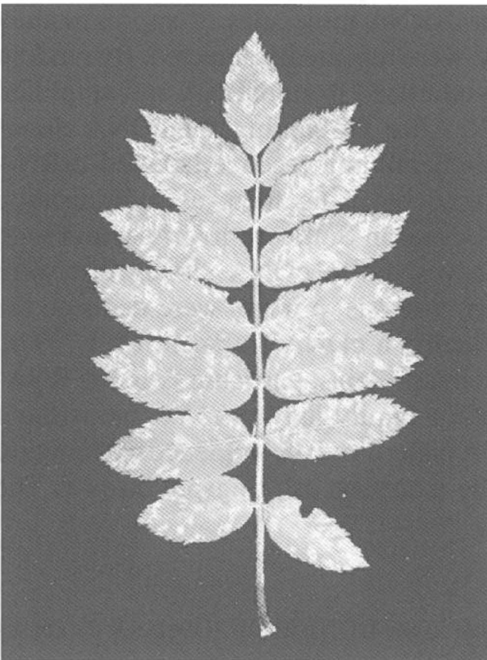


Fig. 1. Symptoms of chlorotic ringspots on a leaf of affected European mountain ash (*Sorbus aucuparia* L.)

combine existing methods for the isolation of dsRNA from mountain ash tissue and were able to obtain a reproducible dsRNA pattern in samples from symptomatic mountain ash trees, which strongly indicates a virus infection associated with the disease. First sequence analyses confirm this assumption.

Materials and methods

Plant materials

Samples of leaves and branches were collected from symptomatic and from symptomless mountain ash (*Sorbus aucuparia* L.) trees at different stands in Germany including forest areas and public gardens in Hamburg, Schleswig-Holstein, the North Sea island Amrum, the forest district "Grunewald" in Berlin as well as the Black Forest, Baden-Wuerttemberg. The investigation was carried out over two years during the period from April to June at almost all stands. In Table 1 stands and the time of taking samples are listed. Collected plant material was kept on ice until it was either immediately processed for nucleic acid isolation or stored at −70 °C. From freshly collected one and two year old branches the inner bark with phloem was carefully removed and used for RNA extraction.

Isolation of total RNA

Total RNA was isolated from leaves and the inner bark of one and two year old branches according to [5]. Samples of approximately 5 g were ground to a fine powder in liquid nitrogen and then homogenised in 50 ml GTC-buffer containing 4 M guanidiniumthiocyanate, 0.1 M β-mercaptoethanol, 1% (w/v) SDS, and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenate was treated three times for 15 s with an Ultra Turrax (Janke & Kunkel, IKA). After two centrifugation steps (10 min, 4,000 × *g*, 4 °C) the supernatant was loaded onto a 8 ml cesium chloride cushion (5.4 M $CsCl_2$, 0.1 M EDTA) and centrifuged for 18 h at 112,000 × *g*, 18 °C. The RNA pellet was resuspended in 300 μl of redistilled water. After phenol/chloroform extraction RNA was precipitated with ethanol and analysed by gel electrophoresis in a 1.5%

Table 1. Stands of affected and unaffected mountain ash trees (*Sorbus aucuparia* L.) in Germany

Number[a]	Location[b]	Sample collection	
1	Tarpenbek (Hamburg)	April 2000	April 2001
2/12	F.D. Kloevensteen (Hamburg)	May 2000	April 2001
3	F.D. Niendorfer Gehege (Hamburg)	May 2000	May 2001
4	Botanical Garden (Hamburg)	May 2000	May 2001
5/13	Island Amrum (Schleswig-Holstein)	May 2000	May 2001
6	F.D. Hahnheide (Schleswig-Holstein)	May 2000	
7	F.D. Hasloh (Schleswig-Holstein)	May 2000	June 2001
8/14	F.D. Karpak (Schleswig-Holstein)	May 2000	June 2001
9	F.D. Ravenna, Black Forest (Bad.-Wuertt.)	June 2000	
10/15	F.D. Breitnau, Black Forest (Bad.-Wuertt.)	June 2000	June 2001
11/16	F.D. Grunewald (Berlin)	June 2000	June 2001

[a]Consecutive numbers indicate the samples analysed in Fig. 3
[b]F.D., forest district

denaturing agarose gel, containing 0.3 M formaldehyde buffered with 20 mM MOPS (morpholinopropanesulfonic acid). All aqueous solutions were treated with diethylpyrocarbonate (DEPC) before use.

Isolation of double-stranded RNA

Double-stranded RNA was isolated from leaf samples and from the inner bark of young branches by a modified procedure according to [25]. All steps were carried out at room temperature. Leaf samples (10 g) were frozen in liquid nitrogen, ground to a fine powder and homogenised in extraction buffer containing 20 ml 2× STE (0.1 M Tris-HCl, pH 7.0, 0.2 M NaCl, 2 mM EDTA), 5 ml redistilled H_2O, 0.4 ml β-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone (PVP), 3% (w/v) SDS, and incubated at 37 °C for 10 min, followed by vigorously shaking at room temperature for 30 min. After centrifugation (20 min, 4,000 × *g*), 1/5 volumes of 96% ethanol and 2 g CF11 cellulose (Sigma) were added to the supernatant and the mixture was again strongly shaken for 30 min. The cellulose was sedimented by centrifugation (10 min, 4,000 × *g*), resuspended in 40 ml washing buffer (1× STE with 16% ethanol) and washed for 10 min under vigorous agitation. The centrifugation and washing steps were repeated twice. The cellulose was then filled in a 20 ml syringe and the cellulose-bound dsRNA was stepwise eluted with 13 ml elution buffer (1× STE), twice with 5 ml, and finally with 3 ml. To the combined eluates 1/5 volume of 96% ethanol and 0.5 g CF11 cellulose were added and the suspension was strongly shaken for 20 min. The suspension was filled in a new 20 ml syringe and dsRNA was eluted again with 2 ml elution buffer in two steps of 1 ml each. The eluted dsRNA was precipitated with 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes ethanol at −20 °C overnight, and resuspended in appropriate volumes of redistilled and DEPC treated water. $MgCl_2$ was added to a final concentration of 300 mM and the solution was incubated with RNase A (final concentration, 35 ng/ml) and 20 U DNase I (RNase free, Roche) at 37 °C for 40 min. The dsRNA was further purified by phenol/chloroform extraction and precipitated with ethanol as described above. The isolated dsRNA was analysed by gel electrophoresis in 1% (w/v) agarose/TBE gels and detected by ethidium bromide staining. As controls, leaves from asymptomatic mountain ash trees were treated in exactly the same way as described above.

ECL-labelling of dsRNA

To generate a dsRNA specific hybridisation probe, approximately 100 ng of dsRNA were directly labelled using the ECL horseradish peroxidase system (ECL direct nucleic acid labeling and detection systems, Pharmacia Amersham), following the instructions of the manufacturer. Specificity and labelling of the dsRNA was controlled by Northern hybridisation using isolated dsRNA of symptomatic mountain ash trees and symptomless trees as targets. For blotting 20 ng dsRNA and 15 μg total RNA were boiled for 5 min, chilled on ice and separated in a denaturing 1% agarose/formaldehyde gel followed by alkaline transfer of nucleic acids onto a nylon membrane (Hybond NX, Pharmacia Amersham) as described by [6].

Reverse transcription, DOP-PCR, and cloning of PCR products

Approximately 10 ng dsRNA were denatured by boiling for 5 min in the presence of 0.5 μg random primers in a total volume of 12 μl. The mixture was slowly cooled in the Gradient Master Cycler (Eppendorf) from 99 °C down to 25 °C with 2 s/°C and R = 0.5 °C/s and placed on ice. 1 μl dNTP-mixture (10 mM each), 5 μl 5× transcription buffer (Promega), 1 μl RNasin (40 U/μl, Promega) and 1 μl AMV reverse transcriptase (25 U/μl, Promega) were added and the mixture was incubated 10 min at 25 °C followed by 60 min at 42 °C.

Alternatively, distinct bands of dsRNA were excised from agarose gels and eluted according to [35].

The DOP-PCR (degenerate oligonucleotide primed PCR) was performed following the instructions of the Roche manual "DOP PCR master". The degenerate oligonucleotide primer 5′-CCG ACT CGA GNN NNN NAT GTG G-OH-3′ was used. A total volume of 50 μl PCR mixture contained 4 μl of the reverse transcription reaction, 1 μl dNTP mixture (10 mM each), 5 μl DOP-PCR primer (20 pmol/μl; final concentration, 2 μM), 5 μl 10× PCR buffer (Roche) and 0.5 μl *Taq* DNA polymerase (5 U/μl, Roche). The PCR program involved 5 min initial denaturation at 95 °C followed by 5 cycles of 1.5 min each at 30 °C, 3.5 min heating from 30 °C to 72 °C (R = 0.2 °C/s), 3 min 72 °C, 1 min 94 °C, followed by 35 cycles of 1 min each at 94 °C, 1 min 62 °C, 2 min 72 °C (plus additional 14 s for each cycle) and finally 7 min 72 °C. The PCR products were analysed by gel electrophoresis in 1% agarose gels stained with ethidium bromide and directly cloned into the 2.1 TOPO TA cloning vector (Invitrogen) as described by the supplier (Invitrogen TOPO™ TA Cloning®, Version E).

Characterisation of dsRNA specific cDNA clones

Modified colony screening

After initial blue white screening of bacterial colonies, white colonies were analysed by PCR with vector-specific M13 primers as described by Invitrogen (TOPO™ TA Cloning®, Version E). In addition, filter hybridisations with an ECL-labeled dsRNA probe were performed in a simplified dot blot procedure according to [35]. All white colonies containing inserts were cultured overnight at 37 °C in 2 ml LBIII medium (Luria Bertani liquid media, Sigma) with 150 μg/ml ampicillin. 100 μl of bacterial cultures and 100 μl of a 10-fold dilution in 5× SSC (750 mM NaCl, 75 mM trisodium citrate, pH 7.0) were spotted onto nylon membranes using the BRL manifold. Membranes were used for hybridisation according to the manufacturer's instructions for ECL-labelled probes.

Preparation of digoxigenin-labelled hybridisation probes

Digoxigenin-labelled RNA probes (riboprobes) were synthesised by *in vitro* transcription with T7 RNA polymerase using dsRNA-specific clones as templates. The reaction was performed exactly according to the instructions of the manufacturer (The Dig User's Guide for Filter Hybridisation, Roche). Digoxigenin-labelled DNA probes were made from dsRNA-specific TOPO TA clones by PCR with M13 primers as described by Invitrogen (TOPO™ TA Cloning®, Version E).

Northern hybridisation

For Northern hybridisation 15 μg RNA from symptomatic and from symptomless mountain ash leaves were denatured by boiling for 5 min, chilled one ice and separated in 1.5% denaturing agarose/MOPS gels containing 0.3 M formaldehyde. Blotting, hybridisation, and detection were performed as described by Roche (The Dig User's Guide for Filter Hybridisation).

Southern hybridisation

For Southern hybridisation genomic DNA was isolated from symptomatic and asymptomatic mountain ash trees with the help of the Invisorb Spin Plant Kit (Invitek) following the manufacturer's instructions. Aliquots of 10 μg genomic DNA were digested with *Hin*dIII and *Eco*RI (MBI) at 37 °C overnight and electrophoretically separated in 1% agarose/TBE

gels. Blotting, hybridisation and detection were made as described by Roche (The Dig User's Guide for Filter Hybridisation).

Sequencing and design of primers for further RT-PCR assays

Positive dsRNA specific clones were sequenced and analysed by standard molecular biological techniques.

Sequencing was carried out by using Applied Biosystems ABI Model 370A/373A with Big Dye Terminator Kit. For sequence analyses and primer search, the programs DNASIS and OLIGO 4.0 (both Pharmacia) were used. Database research was done by BLAST (NCBI).

For amplifying 3′ and 5′ ends of the putative viral RNA the SMART^{TM} RACE cDNA Amplification Kit (Clontech) was utilised. In these experiments and in further RT-PCR studies the following primers were used successfully:

2/1_120LP: 5′-CAA TGT GTG GCA ACT GTC AG-3′
2/1_4UP: 5′-GTG GGG TTA TTT AGC TGG TG-3′
195UP: 5′-CTC AAC TGT GGG GCA TAA TC-3′
519LP: 5′-CAC ACC GCT GCA GAA CAT G-3′
401LP: 5′-CCA ATG ATT CCA GAC ACG-3′

PCR was carried out by a standard protocol with 30 cycles and an extension time of 5.5 min.

Results

Isolation of dsRNA

In the course of our studies we found that standard protocols for isolation of dsRNA [7, 12, 20, 25] had to be modified in several aspects to allow the successful extraction of dsRNA from mountain ash leaves and from woody parts such as the inner bark with phloem removed from small branches. These plant tissues are rich of polysaccharides and polyphenolic compounds. The addition of 1% (w/v) PVP and of 3% (w/v) SDS to the extraction buffer from the very beginning proved to be crucial for the yield of dsRNA. Also, the amount of CF11 cellulose was raised up to 2.5 g per 20 g of plant material, as suggested by [16]. Finally, according to [12] and [7], RNase T_1 had to be replaced by RNase A and the incubation with this ribonuclease and with DNase was postponed to the very end of the extraction. All these modifications together allowed the isolation of dsRNA from affected mountain ash trees as shown in Fig. 2A. A specific dsRNA pattern with three distinct bands of approximately 2.3 kb, 1.5 kb, and 1.3 kb, as assessed from the dsDNA molecular weight marker, was seen in leaf samples of symptomatic mountain ash trees taken during early spring season in the state forest district Kloevensteen in Hamburg (Fig. 2A, lanes 1 and 2). In contrast, no dsRNA bands were detected in symptomless mountain ash trees from the same stand (Fig. 2A, lane 3).

Aliquots of the isolated dsRNA preparation were labelled with the nonisotopic ECL-labelling system and used as probes in Northern blot analyses of dsRNA preparations. As shown in Fig. 2B, hybridisation signals were only obtained with the distinct bands of dsRNA from symptomatic trees (lane 1) at approximately

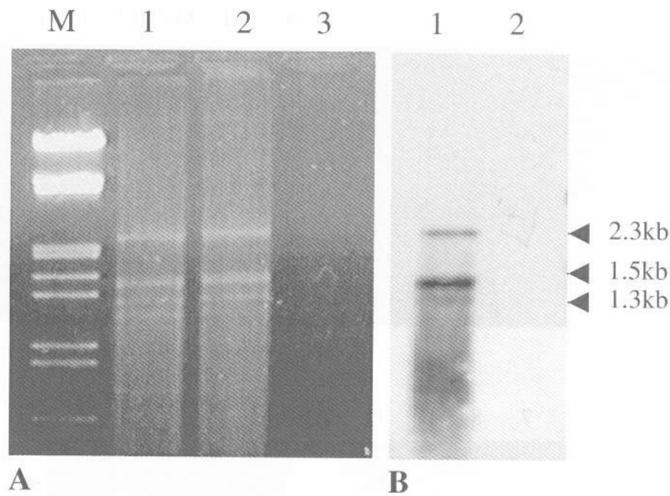


Fig. 2. Pattern of dsRNA from leaves of symptomatic and asymptomatic mountain ash trees. **A** Preparations of dsRNA were separated by electrophoresis in a 1% nondenaturing agarose gel. *1* and *2* dsRNA from symptomatic mountain ash trees; *3* fraction corresponding to dsRNA from asymptomatic trees; *M*: DNA molecular weight marker 3, λ/*Eco*RI, *Hin*dIII (MBI). **B** Northern blot analysis of dsRNA by using an ECL-labelled dsRNA probe. *1* Leaves from symptomatic trees; *2* leaves from asymptomatic trees. Arrowheads indicate the estimated size of dsRNA molecules

2.3 kb, 1.5 kb, and 1.3 kb, while no signals were visible in the RNA preparation from asymptomatic trees (lane 2). The faster migrating heterogeneous material in lane 1 may represent degradation products of the distinct dsRNA bands.

To address the question, whether a characteristic dsRNA pattern is correlated with the symptoms of chlorotic ringspots and mottling on European mountain ash leaves, we analysed the dsRNA from affected mountain ash trees at various stands in Germany. The individual stands are listed in Table 1. The dsRNA patterns from symptomatic and asymptomatic trees from the same stand revealed that only from those plants, which exhibited the typical symptoms of the mountain ash ringspot disease, dsRNA could be isolated (Fig. 3A and B, lanes 1–11). In no case dsRNA was found in samples from asymptomatic trees, collected at the same stands and at the same time (Fig. 3A and B, lanes 12–16). In this analysis, dsRNA pattern with at least three bands of approximately 2.3 kb, 1.5 kb, and 1.3 kb were present in 10 out of 11 samples of symptomatic trees. The yield of dsRNA isolated from individual leaf samples varied greatly from about 40 ng up to 400 ng per 20 g leaf material. Interestingly, an additional slowly migrating band of approximately 7 kb was found in one of the samples collected in 2000 which gave the highest yield of about 400 ng dsRNA per 20 g leaf material (Fig. 3A, lane 10). This high molecular weight dsRNA band was also found, when dsRNA was extracted one year later from symptomatic trees at the same stand (Fig. 3B, lanes 2, 5 and 10). A similar pattern could also be detected in dsRNA isolated from the inner bark of young branches of symptomatic mountain ash trees, but not from asymptomatic trees

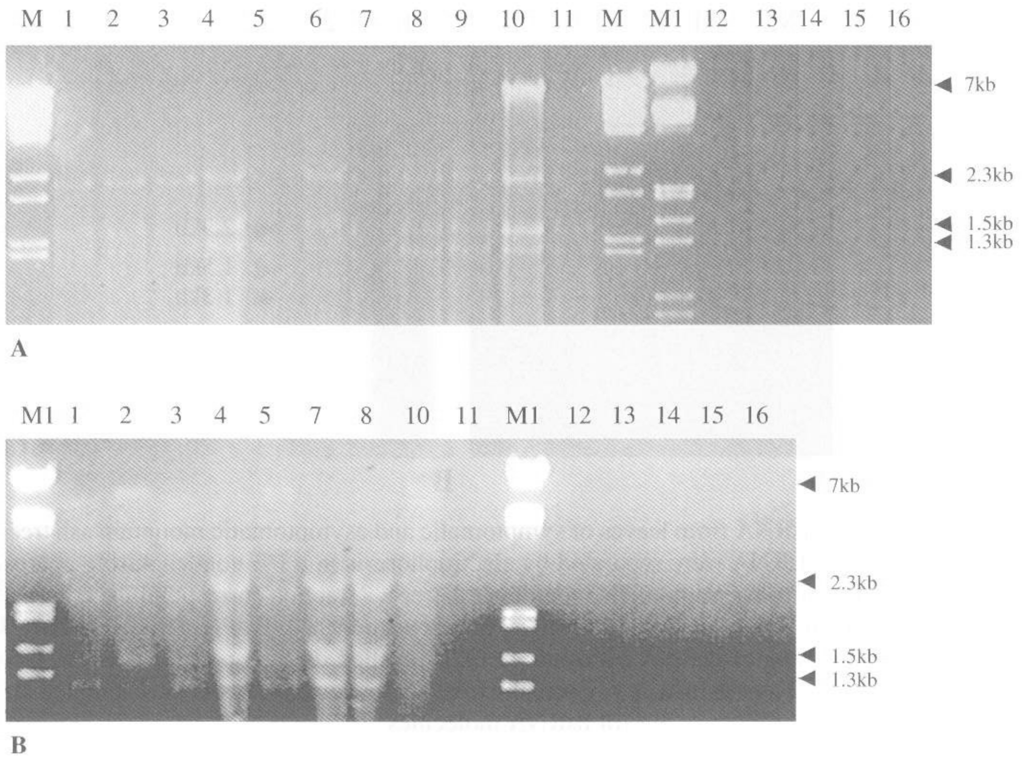


Fig. 3. Pattern of dsRNA preparations from leaf samples of symptomatic (*1–11*) and asymptomatic (*12–16*) mountain ash trees from different stands in Germany, collected during two vegetation periods in 2000 (**A**) and 2001 (**B**). Gel electrophoresis in a nondenaturing 1% agarose gel. The stands are listed in Table 1. *M* DNA molecular weight marker 15, λ/*Eco*91I (MBI); *M1* DNA molecular weight marker 3, λ/*Eco*RI, *Hin*dIII. Arrowheads indicate the estimated size of dsRNA molecules

(data not shown). Thus, a distinct pattern of three to four dsRNA bands was found to be correlated with the typical symptoms of the mountain ash ringspot disease.

cDNA synthesis and DOP-PCR

The dsRNA extracted in our routine procedure was used for reverse transcription and cDNA cloning. After some initial trials using standard cDNA cloning techniques [27, 35], which remained unsuccessful (data not shown), we decided to apply the highly efficient DOP-PCR strategy [37]. Heat denatured total dsRNA was used as template for first strand cDNA synthesis with random primers. The cDNA preparations obtained were immediately used for DOP-PCR amplification and the products were analysed electrophoretically. The size of the main fraction of DOP-PCR products usually ranged from 100 to 700 bp (Fig. 4, lane 1). In addition, also the 7 kb, 2.3 kb, and 1.5 kb dsRNA bands, excised and eluted from agarose gels, were used as templates, but no products worthy of mention could be obtained. Fractions of dsRNA preparations from asymptomatic trees were used as controls, but never yielded any PCR products (Fig. 4, lane 2). The PCR

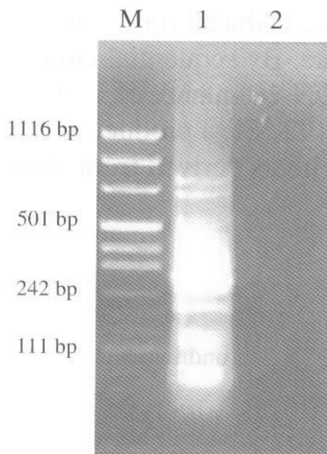
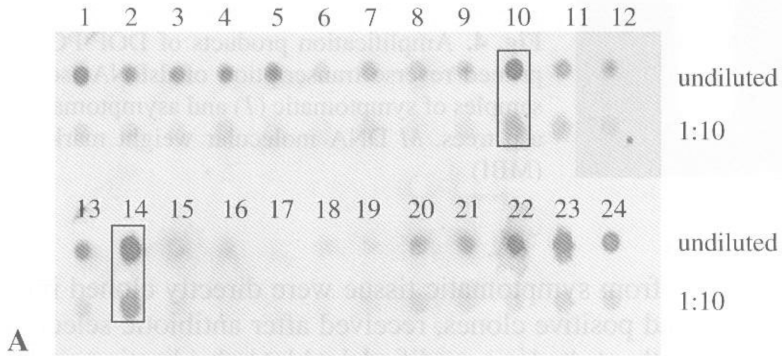


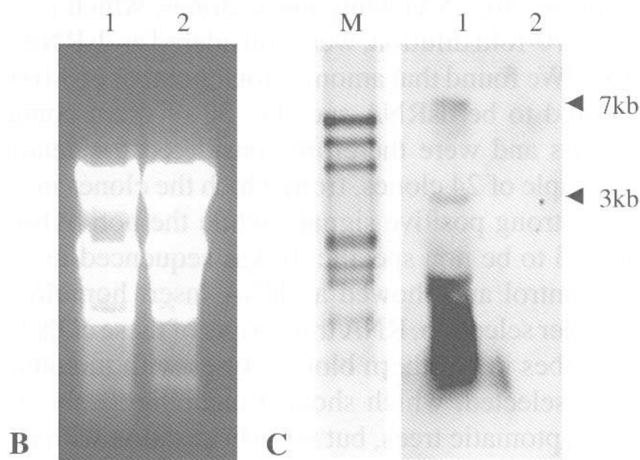
Fig. 4. Amplification products of DOP-PCR after random primed reverse transcription of dsRNA isolated from leaf samples of symptomatic (*1*) and asymptomatic (*2*) mountain ash trees. *M* DNA molecular weight marker 8, pUC Mix (MBI)

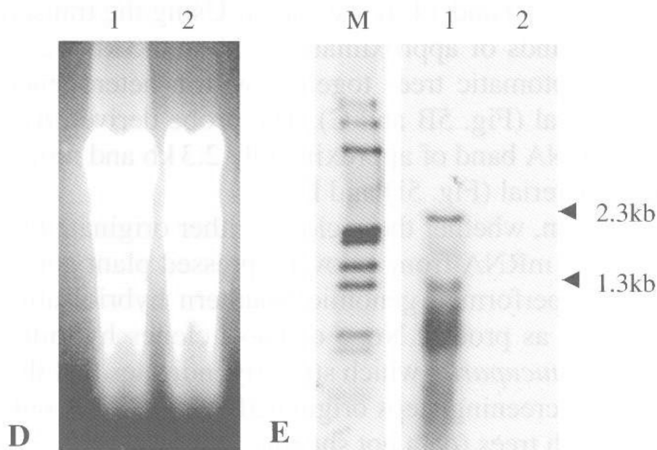
products obtained from symptomatic tissue were directly cloned into the vector PCR 2.1 TOPO and positive clones, received after antibiotic selection and PCR screening, were further tested in a modified dot blot hybridisation analysis with the ECL-labelled dsRNA probe (Fig. 5A). Only those clones, which gave significant hybridisation signals in a 10-fold dilution, were considered as dsRNA-specific and subsequently sequenced. We found that among a total number of 110 recombinant clones 41 (37%) appeared to be dsRNA-specific. 32 of them contained inserts longer than 150 basepairs and were therefore considered for detailed analysis. Figure 5A shows an example of 24 clones, from which the clones in spots number 10, 14, and 22–24 gave strong positive signals, while the non hybridising clone in spot number 17 proved to be non specific. It was sequenced in order to serve as a putative negative control and showed a cDNA insert homologous to plant ribosomal RNA. For further selection, RNA transcripts of these cDNA clones were used as hybridisation probes in Northern blot analyses with mountain ash RNA. Only those clones were selected, which showed unambiguously strong signals with total RNA from symptomatic trees, but no hybridisation with asymptomatic samples. Two clones were finally obtained, named clone 33 and clone 2/1, which are shown in spots number 10 and 14, respectively. Using the transcript of clone 33 as probe, two RNA bands of approximately 7 kb and 3 kb were detected in RNA samples from symptomatic trees together with a heterogeneous fraction of faster migrating material (Fig. 5B and C). The probe derived from clone 2/1 hybridised to a distinct RNA band of approximately 2.3 kb and more diffusely to low molecular weight material (Fig. 5D and E).

To address the question, whether these clones either originate from a disease agent or simply represent mRNA from an overexpressed plant gene in response to stress conditions, we performed genomic Southern hybridisation using the cDNA clones 33 and 2/1 as probes. None of these clones hybridised with the genomic DNA of *Sorbus aucuparia*, which strongly indicates that the two clones selected through several screening steps originated from dsRNA only present in symptomatic mountain ash trees (data not shown).

From the sequences of these two clones primers were deduced for 5′- and 3′-RACE analyses with total RNA preparations as template. By sequential cloning and PCR analyses we were able to obtain a continuous cDNA sequence of 3,737 bp (Fig. 6). 5′-RACE starting from clone 33 lead to clone 492_21, a product, which extended 962 base pairs from clone 33. RT-PCR with primers derived from clone







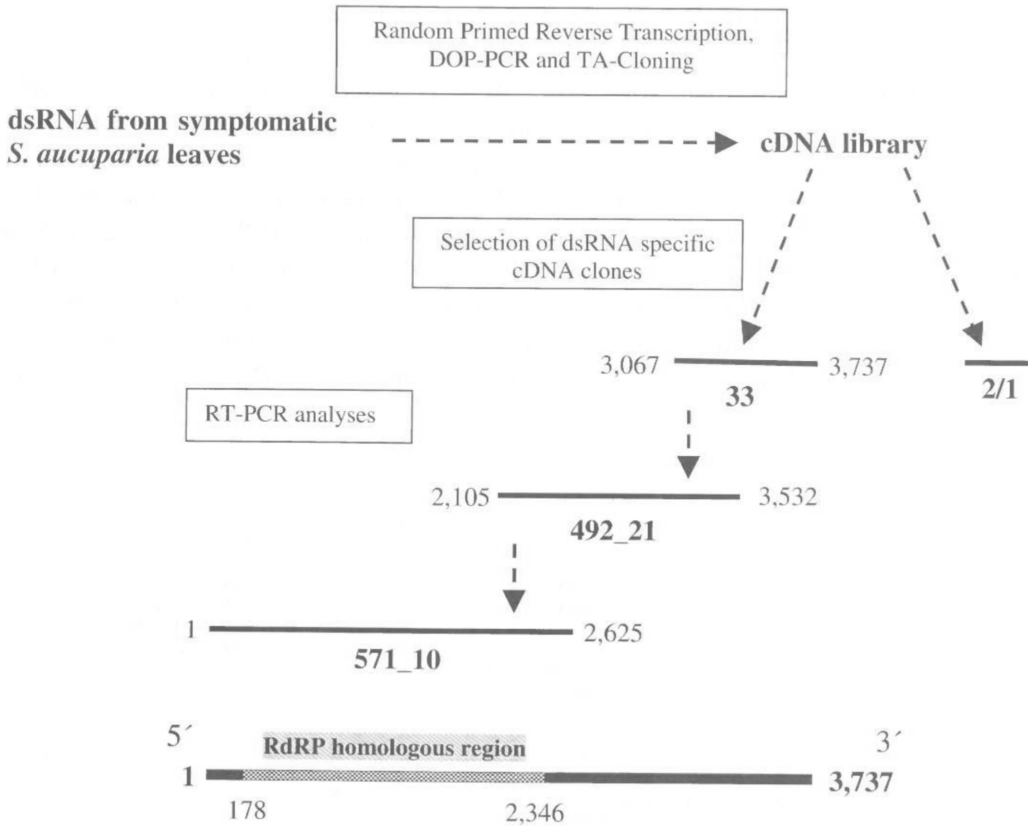


Fig. 6. Schematic representation of the generation of a 3,737-nucleotide-long partial sequence with a viral RdRp homologous segment obtained by three overlapping dsRNA-specific cDNA clones

492_21 and from clone 2/1 generated a 2,600 bp long PCR product, called clone 571_10. It overlaped perfectly with the 5′-terminal region of clone 492_21 (Fig. 6), but not with the sequence of clone 2/1. Sequence analysis revealed that the primer

Fig. 5. A–E Screening of cDNA clones derived from DOP-PCR products. **A** Filter hybridisation of 24 cloned DOP-PCR products derived from dsRNA from symptomatic mountain ash trees using an ECL-labelled dsRNA probe. Aliquots of 100 μl of bacterial cell suspensions (undiluted and in a tenfold dilution) were spotted onto a nylon membrane as indicated. Clones 33 and 2/1 that were found to be dsRNA specific in further analyses (position 10 and 14) are boxed. **B–E** Northern blot hybridisation of total RNA extracted from leaves of symptomatic (*1*) and asymptomatic (*2*) mountain ash trees using digoxigenin-labelled RNA probes derived from two different cDNA clones. Total RNA was separated under denaturing conditions in a 1.5% agarose gel and stained with ethidium bromide (**B** and **D**). After blotting onto nylon membranes, the RNA was hybridised either with a digoxigenin labelled probe of clone 33 (**C**) or of clone 2/1 (**E**). *M* DNA molecular weight marker 3, λ/*Eco*RI, *Hin*dIII, digoxigenin-labelled. Arrowheads indicate the estimated size of putative viral RNA molecules

derived from clone 2/1 had bound unspecifically to the RNA molecule, from which the cDNA clones 33 and 492_21 originated. Further attempts to extend the clones by 5′-RACE were unsatisfactory. All 3′-RACE analyses by using an oligo(dT) primer for first strand synthesis failed, in no case a PCR product was amplified.

The now obtained sequence of 3,737 bp showed over a stretch of 2,169 nucleotides (from nt 178 to nt 2,346) on the level of deduced amino acids partial but significant homology to RNA dependent RNA polymerase (RdRp) of several plant and animal viruses with a negative sense genome, especially to members of the family *Bunyaviridae*. The sequence corresponds only to a minor part of the RdRp gene of the *Bunyaviridae*, which may range up to about 11,000 nucleotides. As shown in Fig. 7, all conserved motifs A–E [28, 29] of the RdRp could be detected,

		Motif A	Motif B
put. Mountain ash V.		K T E I Y S V S S D A S K W S A R D	N W F N V R S N W L Q G N L N M T S S F V H H
	TSWV	K S R L A F L S A D Q S K W S A S G	N T Y P V S M N W L Q G N L N Y L S S V Y H S
Tospo-	GBNV	E C K M A F L S A D Q S K W S A S D	N T F P V S M N W L Q G N L N Y L S S V Y H S
	WSMV	E C K M A F L S A D Q S K W S A S D	N T F P V S M N W L Q G N L N Y L S S V Y H S
	BUNV	K A L K L E I N A D M S K W S A Q D	N Y V Q I K R N W L Q G N F N Y I S S Y V H S
Bunya-	LACV	K G L K M E I N A D M S K W S A Q D	N T V L I K R N W L Q G N F N Y T S S Y V H S
	OROV	R G L K I E I N A D M S K W S A Q D	N T V E I K R N W L Q G N L N Y T S S Y L H S
	DOBV	K R K L M Y V S A D A T K W S P G D	- - - E V R G N W L Q G N L N K C S S L F G V
Hanta-	PUUV	K R K L M Y V S A D A T K W S P G D	- - - S I K G N W L Q G N L N K C S S L F G A
	HTNV	K R K L M Y V S A D A T K W S P G D	- - - E V K G N W L Q G N L N K C S S L F G V
Nairo-	DUGV	F F K T V C I S G D N T K W G P I H	M A M N S Y N H M G Q G I H H A T S S L L T S
	UUKV	H H E T V A T S D D A A K W N Q C H	A F V Q T E T G M M Q G I L H Y T S S L L H T
Phlebo-	RVFV	P V W T C A T S D D A R K W N Q G H	T Y L E T T T G M M Q G I L H Y T S S L L H T
	TOSV	S V W T C A T S D D A R K W N Q G H	T Y L K T S T G M M Q G I L H F T S S L L H S
Tenui-	RCSV	E Y M T I S T S D D A S K W N Q G H	S Y I E T E T G M M Q G I L H Y T S S L F H A
	RGSV	S Y F T V C T S D D A S K W N Q G H	T Y I E T E S G F M Q G I L H Y I S S L F H A

		Motif C	Motif D	Motif E
put. Mountain ash V.		M V H S D D S T Y D F	I T L N E K K T Y I S	K E F L S T T I V S N
	TSWV	I V H S D D N A T S L	I T L N P K K S Y A S	V E F I S E R I S K W
Tospo-	GBNV	M V H S D D N A T S I	I T L N P K K S Y A S	V E F I S E R I I N G
	WSMV	M V H S D D N A T S I	I T L N P K K S Y A S	V E F I S E R I I N G
	BUNV	M V H S D D N Q T S L	C Q A N M K K T Y I T	K E F V S L F N L H G
Bunya-	LACV	L V H S D D N Q T S I	C Q A N M K K T Y V T	K E F V S L F N L Y G
	OROV	M V H S D D N Q T S I	N Q A N M K K T Y L T	K E F V S L F N I H G
	DOBV	A H H S D D A L F I Y	I K I S P K K T T L S	A E F L S T F F E G C
Hanta-	PUUV	A H H S D D A L F I Y	I K I S P K K T T V S	A E F L S T F F E G C
	HTNV	A H H S D D A L F I Y	I K I S P K K T T V S	A E F L S T F F E G C
Nairo-	DUGV	A G S S D D Y A K C I	Q M K D S A K T L V G	L E F Y S E F M M G N
	UUKV	L Q S S D D S G M M I	I Y S S V K S T N N T	L E F N S E F F F H I
Phlebo-	RVFV	M Q G S D D S S M L I	I Y P S E K S T A N T	M E Y N S E F Y F H T
	TOSV	M Q G S D D S S M I I	I Y P S E K S T P N T	M E Y N S E F F F H S
Tenui-	RCSV	M E S S D D S S F I I	I Y K S P K S T T Q T	M E F N S E F F F S G
	RGSV	M E S S D D S S M M I	I Y K S I K S T T G T	M E F N S E F F F A G

Fig. 7. Amino acid identity between conserved RdRp motifs **A–E** of the putative mountain ash virus and 15 members of the family *Bunyaviridae* and the genus *Tenuivirus*. Identical amino acids are marked grey. Put. mountain ash V, putative virus of European mountain ash; TSWV, *Tomato spotted wilt virus*, *Tospovirus*; GBNV, *Groundnut bud necrosis virus*, *Tospovirus*; WSMV, *Watermelon silver mottle virus*, *Tospovirus*; BUNV, *Bunyamwera virus*, *Orthobunyavirus*; LACV, *La Crosse virus*, *Orthobunyavirus*; OROV, *Oropouche virus*, *Orthobunyavirus*; DOBV, *Dobrava virus*, *Hantavirus*; PUUV, *Puumala virus*, *Hantavirus*; HTNV, *Hantaan virus*, *Hantavirus*; DUGV, *Dugby virus*, *Nairovirus*; UUKV, *Uukuniemi virus*, *Phlebovirus*; *RVFV*, *Rift Valley fever virus*, *Phlebovirus*; *TOSV*, *Toscana virus*, *Phlebovirus*; *RCSV*, *Rice stripe virus*, *Tenuivirus*; *RGSV*, *Rice grassy stunt virus*, *Tenuivirus*

but homology is too poor to allocate the putative virus unambiguously to a known genus. The clone 2/1 neither showed homology to viral RdRp sequences nor to any plant gene, but contained a short motif of conserved amino acids (GCY.C..G) similar to the glycoprotein precursor of the genus *Phlebovirus* [22].

Discussion

The presence of high molecular weight dsRNA is a valuable indication of a virus infection in plants [9, 17, 39]. Many viruses were detected and first characterised via the isolation of dsRNA rather than by purification of virus particles [15, 38]. Therefore, the detection of dsRNA bands in leaves and inner bark of symptomatic mountain ash trees strongly supports previous assumptions [11, 14] that a virus could be associated with the disease. In addition, the presence of other graft-transmissible agents such as phytoplasms could be ruled out by PCR studies using conserved primer pairs according to [21] (data unpublished).

In order to isolate dsRNA from *Sorbus aucuparia* tissue, we had to combine and modify previously published techniques. As frequently reported, polysaccharides and polyphenols as well as the formation of adducts between nucleic acids and secondary metabolites interfere seriously with the isolation of RNA from woody plant species [2, 9, 23]. Consequently, the use of additional PVP and SDS [32] as well as a higher ratio of cellulose CF-11 [16] proved to be very helpful. Yields of dsRNA in the range of 40 to 400 ng from 20 g leaf material were comparable to other reports for virus infected woody plants such as cherry leaves infected with little cherry closterovirus (LChV) [19] or grapevine infected with an isolate of *Rupestris stem pitting associated virus-1* (RSPaV-1) [24]. The observed temporal variation of the dsRNA pattern, particularly with respect to the presence of the 7 kb band, perfectly reflects the long known seasonal variation of viral dsRNAs [10] and is also consistent with the heterogeneity of virus accumulation and distribution in woody plant tissues [13, 34, 41].

The DOP-PCR strategy [37], which was reported to be a suitable and efficient method for the amplification of unknown viral sequences [33], led to only few dsRNA specific clones, most probably due to the relative low abundance of dsRNA or to insufficient denaturation of dsRNA structures. Nevertheless, two basal dsRNA-specific cDNA clones remained after selective screening, from which in subsequent RACE and RT-PCR steps a partial fragment of the putative viral sequence of about 3.7 kb could be established. Since this partial sequence, as shown by Northern blot analysis, is related to the 7 kb RNA band, it is very likely that this RNA harbours the RdRp gene. The homologies, on the level of deduced amino acids, to RdRp-sequences from the family *Bunyaviridae*, and remarkable similarities with its typical sequence motifs A, B, C, D and E [28, 29] with the genera *Tospovirus*, *Orthobunyavirus*, and *Hantavirus* strongly suggest a relationship of the putative virus to this family of negative ssRNA viruses.

The fact that clone 2/1 did not marge with the 3.7 kb fragment, further substantiates the previous conclusion, based on Northern hybridisation, that it stems from an dsRNA species which is distinguished from the 7 kb dsRNA. Although

the deduced protein sequence of clone 2/1 did not show extended homologies to data base entries, the short motif with similarity to a glycoprotein precursor of the genus *Phlebovirus* [22], another group of the family *Bunyaviridae*, suggests that clone 2/1 may originate from an additional RNA species of the same virus.

In conclusion, our study has shown that dsRNA is present in leaves and bark from symptomatic mountain ash trees showing ringspots and mottling. Four dsRNA species could be detected in a size range from 1.3 to 7 kb. Provided that the pattern of dsRNA does not reflect the presence of more than one RNA virus, the association of a virus with a multipartite genome with the mountain ash disease is most likely. Based on our partial sequence data and the homology to the RdRp gene of the family *Bunyaviridae* it is reasonable to assume that the putative virus is related to this family, but is most probably not a member of the presently known genera. Our findings are therefore in perfect agreement with previously reported electronmicroscopic studies on mountain ash leaves showing quite similar symptoms of ringspots [11]. The authors observed enveloped particles of 80–100 nm and suggested the presence of a tospovirus-like agent. Further studies are in progress in order to characterise the complete set of genomic RNAs of the putative virus associated with the ringspot disease of the European mountain ash.

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