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The occurrence of dsRNA species in apparently healthy and virus-infected *Ribes* cultivars, and evidence that one such species originates from a member of the virus family *Totiviridae*

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Abstract

Analysis was made of dsRNA in 37 cultivars and species of *Ribes*, that were healthy, naturally affected with the virus-like diseases, blackcurrant yellows, blackcurrant infectious variegation, gooseberry veinbanding or blackcurrant reversion, or graft-inoculated with scions from such diseased plants. Various dsRNA species, differing in size (from ca. 2 to 11 kbp), number and staining intensity in gels, were detected in some or all assays of all plants, including those held as virus-tested stock. In different plant tissues from individual plants, the dsRNA species were usually similar in size and number but, in some sources, the dsRNA profile from flowers and/or bark differed greatly from the profiles of dsRNA obtained from leaves. No dsRNA species were associated consistently with any of these diseases. A 499 kbp cDNA probe was obtained that in Northern blot analysis was specific to a ca. 5 kbp dsRNA species present in the blackcurrant cv. Baldwin. It also detected a similarly sized dsRNA species in plants of many other blackcurrant cultivars, but it did not react with a similarly sized dsRNA species in redcurrant and gooseberry tissues. The 156 amino acid sequence encoded by the cDNA was very similar to sequences in the RNA-directed RNA polymerases of virus species in the family *Totiviridae*, especially *Saccharomyces cerevisiae* viruses L-1 and L-A. The significance of these findings and the possible origin of these dsRNA species are discussed.

Introduction

The isolation and analysis of dsRNA has been a useful tool to detect both ssRNA and dsRNA agents in plant tissues, especially where these agents are either not mechanically transmissible or where no virus-like particles have been isolated (Dodds, 1993; Jones, 1992; 1997). This method has been especially useful for woody plant material where the high phenolic and polysaccharide content usually makes virus isolation and experimental transmission of viruses to herbaceous test plants difficult (Watkins et al., 1990; Jones et al., 1986; Jones, 1992). *Ribes* spp. are among many species of woody plants that are known to be infected with several different virus-like diseases of unknown aetiology.

The use of dsRNA analysis of *Ribes* cultivars with virus-like diseases was first reported by Jones et al. (1986). They found a dsRNA species of M_r ca. 2.9×10^6 (ca. 4.3 kbp) in plants of the blackcurrant cv. Daniel's September affected with blackcurrant infectious variegation (Adams and Thresh, 1987b) and in cv. Baldwin affected with blackcurrant yellows (Thresh, 1987). They also found this dsRNA species in symptomless plants of these two cultivars grown in the glasshouse. The role of this dsRNA species in these two diseases was therefore not clear.

In addition to viruses, plants, especially perennial plants, harbour various disease and pest organisms and studies have shown that some of these organisms may contain dsRNA viruses (Azzam and

Gonsalves, 1991; Azzam et al., 1991; Watkins et al., 1990; Zabalgogeazcoa et al., 1998; Zhang et al., 1994). The objective of the work reported here was therefore to assess in more detail the association of different dsRNA species with diseases of unknown aetiology in *Ribes*, bearing in mind the possibility that some of these species may be derived from contaminating agents. To increase the sensitivity, specificity and reliability of detecting dsRNA species, and to identify and distinguish them, attempts were made to produce dsRNA-specific labelled probes for use with *Ribes* tissues.

Materials and methods

Plant material

Where possible, all healthy *Ribes* plant material was derived from virus-tested stock plants held under insect-proof conditions at SCRI. *Ribes* material that was affected with diseases believed to be caused by virus or virus-like agents was, in most instances, either from cultures maintained at SCRI or from initially healthy plants graft-inoculated with scions from these diseased plants. All plants were held in insect-proof conditions in a gauzehouse. A few diseased plants were obtained directly from crop plants. Although fresh tissue was used where possible, due to the seasonal nature of *Ribes* plant growth, some leaf samples were collected in the spring and summer and frozen at $-20\,^{\circ}$C to await analysis during the autumn and winter. Plant tissues can usually be stored at $-20\,^{\circ}$C for months (if not years) prior to extraction without significant loss in dsRNA (Karan et al., 1991; Cox and Jones, unpublished data).

dsRNA isolation

dsRNA was isolated from *Ribes* plant tissues using a modification of a CF-11 cellulose chromatography method developed previously for strawberry tissue (Watkins et al., 1990). The modification involved a single phenol extraction of plant tissue and, after the dsRNA was obtained from the second treatment with CF-11 cellulose powder, any residual dsRNA bound to the powder was collected by repeating the elution and pooling the second eluate with the first. All other steps were the same as that described previously (Watkins et al., 1990).

Total nucleic acid isolation

To obtain clean ssRNA from *Ribes* plants, aliquots of nucleic acid from the aqueous phase following phenol extraction of dsRNA from plants was mixed with 2.5 volumes of ethanol and CF-11 powder ($0.22\,\mathrm{g\,ml^{-1}}$) for 30 min before centrifuging at 3000 g for 5 min to pellet the CF-11-bound nucleic acids. The pellets were washed three times with 1× STE/70% ethanol and centrifuged at 3000 g for 5 min each time to obtain a pellet. The final pellet, resuspended in 1× STE, was then washed in a column of CF-11 powder with 1× STE/70% ethanol and the bound nucleic acid eluted with 1× STE ($1\,\mathrm{ml\,g^{-1}}$) and precipitated with 2.5 volumes of ethanol at $-20\,^{\circ}$C overnight. Aliquots of this were then centrifuged at 14 000 rpm for 10 min and washed with 70% ethanol. The pellets were re-suspended in water or an appropriate buffer. The nucleic acids were then either blotted onto positively charged nylon membrane or, the constituents were separated by adding 1 volume of 4 M LiCl to the re-suspended pellet and keeping the solution overnight at $4\,^{\circ}$C so that ssRNA precipitated. The DNA and dsRNA remained in the supernatant fraction after centrifuging at 14 000 g for 10 min. They were precipitated from solution as for dsRNA as above and the DNA/dsRNA pellets were re-suspended in an appropriate volume of water or buffer.

Denaturing dsRNA and cDNA synthesis

dsRNA was heated to $100\,^{\circ}$C in 9 µl sterile distilled water and mixed with 2 µl random primer or 2 µl oligo (dT) primer for 8 min (all from the Boehringer-Mannheim cDNA synthesis kit). After snap-cooling on ice, reverse transcription and cDNA synthesis were done as described in the Boehringer-Mannheim cDNA synthesis kit.

Molecular cloning of cDNA

About 25% of the cDNA product was ligated with 0.05µg *Sma*I-digested, dephosphorylated pUC19 (Pharmacia), 20% of which was used to transform electrocompetent XL1-Blue cells (Bullock et al., 1987) by electroporation at 50 µF, 150 Ω and at 1500 V in 0.1 cm cuvettes. Small-scale plasmid recovery from putative recombinant colonies was done using the alkaline lysis method of Le Gouill et al. (1994).

Polyacrylamide gel electrophoresis (PAGE)

Fractionation of dsRNA and DNA components was by electrophoresis in 7% or 5% polyacrylamide gels, respectively. The sizes of dsRNAs were estimated by comparing their mobilities with those of the dsRNAs from *Vicia faba* plants infected with *Vicia cryptic virus* (VCV) (Abou-Elnasr et al., 1985) and/or *Nicotiana clevelandii* infected with *Cucumber mosaic virus* (CMV) (Jones et al., 1986; Watkins et al., 1990). In some earlier experiments, dsRNA species from particles of *Maize rough dwarf virus* were used as additional markers. dsRNA is known to migrate at different rates depending on electrophoresis conditions and our M_r estimates for some of the dsRNA species differed from those estimated previously (Jones et al., 1986). The size estimates presented here are therefore those apparent under our present experimental conditions and the definitive size of these dsRNA species must await sequence determination.

Sizes of DNA were estimated by comparing their mobility with that of a 1 kb ladder (Gibco-BRL). Gels were stained with ethidium bromide and, if they contained dsRNA, also silver nitrate (Sammons et al., 1981). DNA and dsRNA was electro-eluted from polyacrylamide gels using dialysis tubing as detailed in Sambrook et al. (1989).

Blotting of nucleic acids

Dot blotting. Nucleic acid samples (3–5 μl) were heat-denatured at 100 °C for 5 min and snap-cooled on ice for 10 min. They were then spotted onto nylon membrane and fixed using a Stratagene UV Crosslinker (245 nm for 1 min, 1.5 J $(cm^2)^{-1}$).

Sap blots. Tissue (0.25 g) was extracted in 1 ml of 0.5 M Tris–HCl (pH 8.2), 4% (w/v) PVP, 5% (v/v) Triton X-100, 0.01 M $MgSO_4$, 0.2% (v/v) 2-mercaptoethanol and 0.5% (w/v) bentonite until a slurry was obtained. This slurry was transferred to a microfuge tube and the supernatant fraction, after centrifugation for 10 min at 11 000 g, was mixed with an equal volume of 12× SSC/6% formaldehyde, heated for 1 min at 60 °C, and spotted (5 μl) onto a nylon membrane.

Making a DNA probe

The method used for radiolabelling DNA with ^{32}P was that of Feinberg and Vogelstein (1984). Membranes were pre-hybridised and hybridised at 65 °C in a Techne roller cage according to Sambrook et al. (1989).

DNA sequencing

Dideoxynucleotide chain termination sequencing was done using the Sequenase Kit, Version 2.0 (USB). Analysis of sequence data was done using the GCG package (Programme Manual for the Wisconsin Package, Version 8).

Results

dsRNA isolation

General factors. Initial analyses indicated that almost all of the *Ribes* cultivars examined contained some dsRNA species, in the size range 1.4–0.6 kbp. As these dsRNA species were of such a small size and are often present in a wide range of other plant species, including pathogen-free plants (Jones et al., 1986; Dodds, 1993), they are not discussed in these results. There were no great differences in recovery of larger sized dsRNAs among samples of fresh tissue and that kept frozen at −20 °C for varying lengths of time of up to at least 18 months, the longest period tested (data not shown). There was also little difference in dsRNA recovery among samples of 5, 7.5 and 10 g of leaves (data not shown); in most instances therefore, extracts were made from about 7 to 10 g tissue.

dsRNA from different plant tissues. dsRNA extracted from similar weights of young leaves, old leaves and flowers of individual plants of *Ribes* cultivars, generally did not differ in the major dsRNA species detected (Figure 1). However, analyses of flowers and flower buds from some blackcurrant sources, particularly cv. Ben Sarek affected with blackcurrant yellows disease (BYD) (Figure 1) and symptomless cv. Amos Black (data not shown), detected several distinct dsRNA species that were not present in their leaves. By contrast, leaves of cv. Daniel's September affected with blackcurrant infectious variegation disease (BIVD) contained a number of distinct dsRNA species, whereas no dsRNA was detected in its flowers (Figure 1). dsRNA extracts from fruit were tested only for symptomless cv. Baldwin and such extracts contained a ca. 5 kbp dsRNA species present in all other tissues (flowers, petioles, leaves,

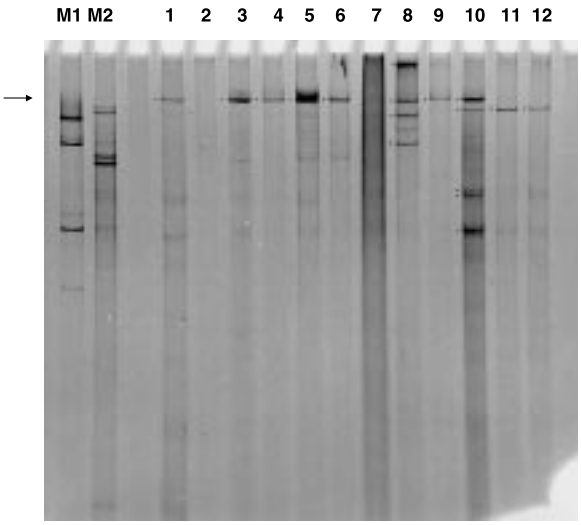

Figure 1. Polyacrylamide gel electrophoresis followed by silver staining of dsRNA from each of 4 g of old leaves (OL), young leaves (YL) or flowers (F) of different blackcurrant sources. Lanes 1 (OL), 2 (F), BIVD-affected cv. Daniel's September; lanes 3 (OL), 4 (YL), BYD-affected cv. Baldwin; lanes 5 (OL), 6 (YL), symptomless cv. Baldwin; lanes 7 (OL), 8 (F), BYD-affected cv. Ben Sarek; lanes 9 (OL), 10 (YL), cv. Amos Black; lanes 11 (OL), 12 (YL) cv. Öjebyn. The first two lanes contain dsRNA markers from CMV-infected *Nicotiana clevelandii* (M1) and VCV-infected *Vicia faba* (M2). Arrow marks the position of the ca. 5 kbp band.

bark) examined from this source (Table 1; Figures 1 and 2A).

Assays for dsRNA species in pith, cambial tissue, bark and leaf buds from dormant plants, often showed that bark and cambial tissues contained more dsRNA species than did leaves. Thus, BYD-affected cv. Baldwin contained, in addition to a major dsRNA species of ca. 5 kbp present in all tissues, dsRNA species of ca. 4, 3 and 2.6 kbp. Bark of BYD-affected cv. Ben Sarek contained 5 dsRNA species of ca. 5–8 kbp that were not detected in symptomless leaves of this cultivar (data not shown). Bark of symptomless plants of cv. Öjebyn contained, in addition to a ca. 4.4 kbp dsRNA species present in leaves, 2 major dsRNA species of ca. 4 kbp and a minor species of ca. 3 kbp (Figure 2A).

Great difficulties were encountered in obtaining significant amounts of high mol. wt dsRNA from leaves of gooseberry, and to a lesser extent, of red and white currant plants; bark proved a more suitable source of dsRNA for these plant species.

Detection of dsRNA species in apparently healthy Ribes *cultivars*

Analysis was made of the dsRNA content of leaves of a range of blackcurrant, redcurrant, white currant and gooseberry cultivars held in a screenhouse at SCRI, but acquired previously from elsewhere as apparently healthy plants. Prior to their arrival at SCRI in the late 1980s, these plants had been maintained in aphid-proof screen houses for many years. Analysis of dsRNA in these plants showed that almost all contained one or more dsRNA species (Table 1; Figure 1). By contrast, in tests on leaves of virus-tested stock plants of 7 blackcurrant cultivars maintained at SCRI, dsRNA was rarely detected in significant amounts in cvs Ben Lomond, Ben Tirran and Ben Sarek (Table 1). However, major dsRNA species of ca. 2–8 kbp were detected frequently in cvs Ben Alder, Ben More, Ben Nevis, Jet and Öjebyn (Table 1). Nevertheless, analysis of the bark of almost all cultivars detected some dsRNA (Table 2 and unpublished data). Clearly therefore, high health status plants of these cultivars that are free from all known *Ribes* viruses and virus diseases, apparently contain dsRNA species that are possibly associated with other unidentified virus-like agents.

dsRNA analysis of plants affected with virus-like diseases or inoculated with sources affected with these diseases. Plants held at SCRI, of various *Ribes* cultivars affected with BYD, BIVD, gooseberry vein banding (GVBD) and blackcurrant reversion (BRD) diseases were used as scion sources to graft-inoculate virus-tested stock plants. Only a few such plants graft-inoculated with GVBD and BRD developed obvious disease symptoms.

Blackcurrant yellows disease (BYD). Three different sources of blackcurrant naturally affected with BYD (Thresh, 1987) were assayed for dsRNA together with comparable symptomless plants. These were, cvs Baldwin and Ben Sarek from Scotland and cv. Magnus growing in New Zealand. The results showed that all the cv. Baldwin tissues tested contained a major dsRNA species of ca. 5 kbp, as well as some species of both larger and smaller sizes, regardless of whether the plant was BYD-affected or not (Tables 1 and 2; Figure 1). Furthermore, the concentration of this ca. 5 kbp dsRNA species seemed to be consistently greater in symptomless than in diseased plants (Figures 1 and 2A). By contrast, no significant amounts of dsRNA were

Table 1. dsRNA species detected in leaves of a range of symptomless *Ribes* species and cultivars by polyacrylamide gel electrophoresis followed by silver staining

Ribes cultivar	Apparent size of dsRNA species in kbp					
	> 5.6	5.0–5.5	4.0–4.9	3.0–3.9	2.0–2.9	1.5–1.9
Blackcurrant						
Amos Black	9.1, 6.3	**5.2**	4.9, 4.3	3.1	2.5	
Baldwin		**5.3**		3.8, 3.2	2.7	1.8
Ben Alder*			**4.3**		2.7, 2.4	1.9
Ben Lomond*					2.3	
Ben More*			**4.5**		**2.4**, **2.0**	
Ben Nevis*				**3.3**, **3.0**	**2.7**, **2.1**	
Ben Sarek*		5.2			2.9	
Ben Tirran						
Black Reward					2.8, 2.6, 2.1	1.6
Blacksmith					2.4, 2.1	
Cotswold Cross						
Daniel's September		**5.1**	4.0	3.4	2.6	
Greens Black		**5.3**	**4.9**,**4.2**		2.6, 2.2	1.6
Jet*	**7.9**			**3.0**		
Magnus						
Malvern				**3.0**		
Mendip			**4.1**			
Öjebyn*	6.3	5.2	**4.9**, **4.3**			
Raven		**5.0**		**3.7**		
Seabrooks Black	8.5		4.3		2.5	
Tor Cross						
Wellington	**7.4**		**4.1**			
Westwick Choice	**8.5**		**4.7**	**3.7**		
Redcurrant						
Achilles	**8.6**		**4.7**		2.8, 2.4, 2.3	
Bedford Red	**9.1**				2.9, 2.6, 2.3, 2.2	
Chenoceau	7.7, 7.3	**5.3**		3.5, **3.0**	**2.9**, 2.6, 2.1	1.8, 1.6
Fay's Prolific	**8.6**	5.2	**4.7**		2.9, 2.6, 2.1	1.9, 1.7
Jonkheer van Tets	**8.6**, 7.7	**5.5**		3.3	2.9, 2.7, 2.6, 2.1	
Laxton No. 1	**8.6**		**4.7**	3.3, 3.1	2.9, 2.6	
Red Lake				**3.0**		
Versailles				**3.4**	**2.7**	
Wilson's Long Bunch	**7.9**				**2.8**, **2.6**	
White currant						
White Smith	**7.4**		**4.4**		**2.5**	
Gooseberry						
Careless					**2.5**, **2.1**	
Green Finch			**4.5**			
Invicta			**4.1**		**2.6**, **2.2**	
Jubilee						
VBI						

Values in bold indicate those species that stained the strongest.
*Virus-tested stock plants.

isolated from leaves of either symptomless or BYD-affected plants of cvs Ben Sarek and Magnus (Tables 1 and 2). Of several cultivars of blackcurrant, redcurrant and gooseberry that were graft-inoculated with scions from the BYD-affected cvs Ben Sarek and Baldwin 5–10 years earlier, none developed BYD symptoms and most showed a dsRNA profile somewhat similar to that of ungrafted plants of these cultivars (Tables 1 and 2).

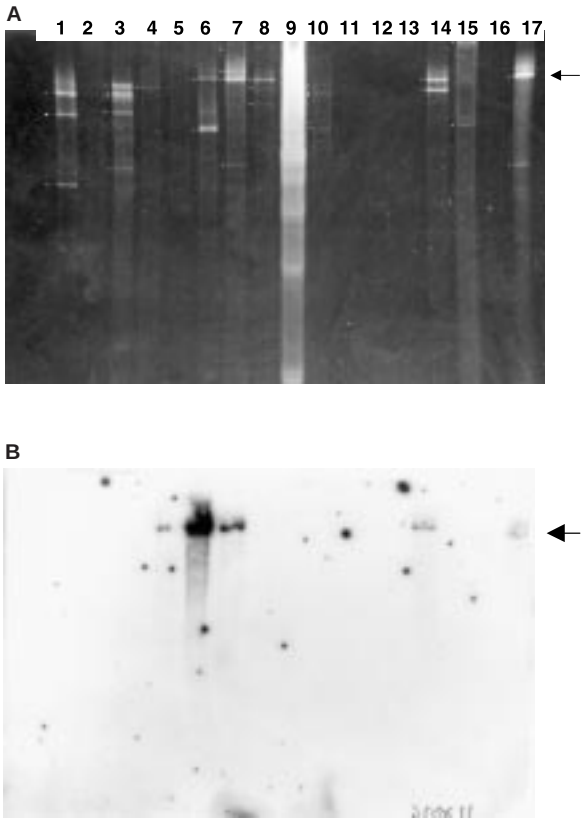

Figure 2. Polyacrylamide gel electrophoresis of dsRNA extracted from a range of *Ribes* cultivars, followed by staining with ethidium bromide (A) or by Northern blot analysis using the radio-labelled probe (BY16) (B). Lanes contain dsRNA from: 1, CMV-infected *Nicotiana clevelandii* as a control; 2–4 cv. Öjebyn – buds (2), bark (3), stem (4); 5, BIVD-affected cv. Daniel's September; 6 and 7 cv. Baldwin, BYD-affected (6) and symptomless (7); 8, cv. Amos Black; 9, ArMV-infected *Nicotiana clevelandii* as a control; 10–17, symptomless plants of cvs Black Reward (10), Ben Nevis (11), Wilson's Long Bunch (12), Versailles (13), Greens Black (14), White Smith (15), Malvern (16), Baldwin (17). Only the ca. 5 kbp species (arrow) present in lanes B, 7, 8, 14 and 17 hybridised with the probe.

Graft-inoculated cv. Ben Nevis contained a dsRNA species of ca. 6 kbp not detected in ungrafted plants of this cultivar (Tables 1 and 2).

Blackcurrant infectious variegation disease (BIVD). BIVD, is specific to blackcurrant and is believed to be caused by an agent whose mode of transmission and vector are not known (Adams and Thresh, 1987b). Initially, dsRNA analysis of leaves of plants of cv. Daniel's September (all stocks of which are reported to be infected with the BIVD agent; (Ellenberger, 1962)) detected a dsRNA species ca. 5 kbp, similar in size to that detected in both BYD-affected and symptomless cv. Baldwin (Tables 1 and 2; Figure 1). This dsRNA from BIVD-affected cv. Daniel's September was always stained less intensely than that found in cv. Baldwin sources. BIVD-affected cv. Daniel's September plants also contained a faintly stained dsRNA species of ca. 4 kbp that was detected in all tissues, except flowers (Tables 1 and 2; Figure 1). Scions from cv. Daniel's September affected with BIVD were used to graft-inoculate several blackcurrant, redcurrant and gooseberry cultivars. During 4–9 years post-grafting, no virus-like symptoms were observed in these grafted plants. dsRNA analysis of these grafted and ungrafted plants failed to detect the major ca. 5 kbp dsRNA species, present in cv. Daniel's September (Table 2; unpublished data). However, in a few blackcurrant cultivars and Fay's Prolific redcurrant, a similar sized dsRNA species was present in grafted and ungrafted plants (Tables 1 and 2) suggesting that the ca. 5 kbp species in cv. Daniel's September was not transmitted through grafts. The small amounts of some larger dsRNA species (5.5–9.6 kbp), present in the BIVD-grafted cvs Ben Alder, Ben Lomond and Ben More, were not detected in ungrafted plants of these cultivars or in cv. Daniel's September (Tables 1 and 2).

Gooseberry vein banding disease (GVBD). The uncharacterised agent of this disease infects all cultivated *Ribes* species and is transmitted by aphids (Adams and Posnette, 1987). dsRNA analysis was made of the following sources showing characteristic symptoms of the disease: a field-infected gooseberry plant, gooseberry selection VBI graft-inoculated with scions from this field-infected gooseberry plant, and a range of other *Ribes* cultivars either graft-inoculated with scions from these two gooseberry sources or naturally affected with GVBD.

dsRNA isolation from leaves of gooseberry and redcurrant was hampered by mucilaginous materials in the extracts which also affected the electrophoresis of these samples. Possibly because of this difficulty, little or no dsRNA was detected in leaves, even of plants showing very strong GVBD symptoms. However, several major dsRNA species of ca. 2–10 kbp were isolated from the bark of GVBD-affected plants, but no two isolations appeared to give exactly the same dsRNA pattern. Nevertheless, the larger (ca. 10 kbp) dsRNA species isolated consistently from the GVBD-affected gooseberry selection VBI, and the naturally GVBD-affected

Table 2. dsRNA species detected by polyacrylamide gel electrophoresis followed by silver staining in leaves (or bark*) of blackcurrant, redcurrant and gooseberry cultivars, either naturally infected or graft-inoculated with sources of virus-like diseases

Ribes cultivar	Apparent size of dsRNA species in kbp					
	> 5.6	5.0–5.5	4.0–4.9	3.0–3.9	2.0–2.9	1.5–1.9
Yellows disease						
Baldwin, infected		**5.3**				
Baldwin, grafted		**5.3**		3.8, 3.2	2.4	**1.8**
Ben Alder, grafted			**4.3**		**2.4**, 2.0	
Ben Lomond, grafted				3.2		1.9
Ben More, grafted			**4.5**		2.4, 2.0	
Ben Nevis, grafted	**5.9**			3.0	2.7, 2.2	
Ben Sarek, infected						
Magnus, infected						
Öjebyn, grafted		**5.3**				
Fay's Prolific, grafted	**8.9**, 6.6	5.7, 5.2	4.1	3.1		
Infectious variegation						
Ben Alder, grafted	**9.6**		**4.3**, **4.1**		**2.6**	
Ben Lomond, grafted	9.7,9.5	5.2	**4.0**	3.2	**2.5**	**1.9**
Ben More, grafted	9.7, 9.5		**4.5**, **4.1**	3.4	**2.6**, **2.4**, 2.0	
Ben Nevis, grafted	5.9		4.1	3.3	2.9, 2.7, **2.5**, 2.1	
Daniel's Sept., infected		**5.1**	4.4, 4.2			
Öjebyn, grafted		**5.5**			2.5	
Fay's Prolific, grafted	**8.7**, **6.3**	**5.5**	**4.9**, **4.1**	**3.3**		
Red Lake, grafted	8.9					
Reversion (E, R)						
Ben Lomond, grafted E						
Ben Lomond, grafted R						
Ben More, grafted E			**4.9**			
Ben More, grafted R						
Ben Nevis, grafted E						
Ben Nevis, grafted R	8.3		4.9			
Ben Tirran, infected E	9.1					
Ben Tirran, grafted R						
GreensBlack, infected R		**5.3**		**4.4**		
Öjebyn, grafted E	10, **7.4**					
Öjebyn, grafted R	10, **6.5**					
Fay's Prolific, grafted E	10, 9.5		**3.2**			
Fay's Prolific, grafted R			**3.2**			
Red Lake, grafted R					2.7, 2.3, 2.0	
Gooseberry veinbanding (bark)*						
Ben Lomond, healthy				3.3	2.8, 2.6	
Ben Lomond, grafted				3.3	2.8, 2.6	
Ben More, healthy			**4.2**	3.6, **3.2**	**2.7**, **2.6**, 2.0	1.9, 1.7
Ben More, grafted			**4.2**	3.6, **3.2**	**2.7**, **2.6**, 2.0	1.9, 1.7
Ben Nevis, healthy				3.8, 3.2	**2.7**, **2.6**, 2.0	1.8, 1.7
Ben Nevis, grafted				3.8, 3.2	**2.7**, **2.6**, 2.0	1.8, 1.7
Careless, healthy				**3.1**	2.6 , **2.5**, 2.3	
Careless, grafted		5.4	4.9	**3.6**, **3.4**	**2.7**, **2.6**, **2.5**, **2.4**	1.9, 1.6
Invicta, healthy		5.1	4.8, 4.4	**3.5**, **3.1**	**2.5**, **2.0**	**1.9**, **1.8**
Invicta, grafted			4.4	**3.5**, **3.1**	**2.8**, **2.5**, **2.0**	**1.8**, **1.6**
Laxton, grafted						
Red Lake, infected	**9.7**					
VBI, healthy			4.8	3.9, 3.5		
VBI, grafted	**10**			**3.9**, **3.7**	**2.8**, **2.4**	**1.8**
Versailles, infected	**10**				**2.8**, **2.5**	

Values in bold indicate those species that stained the strongest.

*Bark was used for only gooseberry veinbanding material.

redcurrant cvs Red Lake and Versailles, were very similar (Table 2). However, with the exception of VBI, none of the bark samples from grafted inoculated plants contained a dsRNA pattern or large sized dsRNA species similar to that isolated from naturally GVBD-affected gooseberry or redcurrant (Table 2). Furthermore, the dsRNA species isolated from GVBD-grafted blackcurrant and gooseberry plants were not greatly different from those isolated from the ungrafted plants of these cultivars, providing little evidence of GVBD-specific dsRNA species (Tables 1 and 2).

Blackcurrant reversion disease (BRD). There are two main forms of BRD, the common European form (BRD-E) and the more severe Russian form (BRD-R). Very recently, a virus, *Blackcurrant reversion associated virus* (BRAV), was associated with plants affected with either of these forms of reversion, and there is strong evidence that it is the causal agent of the disease (Lemmetty et al., 1997; Latvala et al., 1997). Analysis of dsRNA from three different BRD-affected sources, and from several *Ribes* cultivars graft-inoculated with scion material from plants showing symptoms of BRD-R or BRD-E, usually detected no difference in the dsRNA species isolated from these plants and from comparable ungrafted healthy ones (Tables 1 and 2). However, on a few occasions, small amounts of dsRNA species ranging from ca. 6–10 kbp were detected in some BRD-affected plants but were not detected in healthy ones (Table 2).

Cloning and characterisation of a ca. 5 kbp dsRNA from the blackcurrant cv. Baldwin

As a ca. 5 kbp dsRNA species was detected reliably in tissues of cvs Baldwin (both BYD-affected and symptomless), Daniel's September (BIVD-affected) and some other blackcurrant cultivars (Tables 1 and 2; Figures 1 and 2A). This dsRNA species from cv. Baldwin was used as a template for cDNA synthesis and the production of a labelled probe.

dsRNA Northern blots. Autoradiography of Northern blots showed that the probe hybridised specifically with the ca. 5 kbp dsRNA species present in extracts of blackcurrant cvs Amos Black, Baldwin and Greens Black but not with the other dsRNA species from these cultivars or with the dsRNA species from BIVD-affected Daniel's September, and cvs Ben Nevis, Black Reward, Malvern, Öjebyn (buds, bark and stem tissues), Versailles, Wilson's Long Bunch and White Smith or the dsRNAs from *Arabis mosaic virus* and CMV-infected *N. clevelandii* (Figures 3 and 4). However, in these tests, the ca. 5 kbp dsRNA species usually found in BIVD-affected cv. Daniel's September tissues was not apparent in the ethidium bromide-stained gel (Figure 2A).

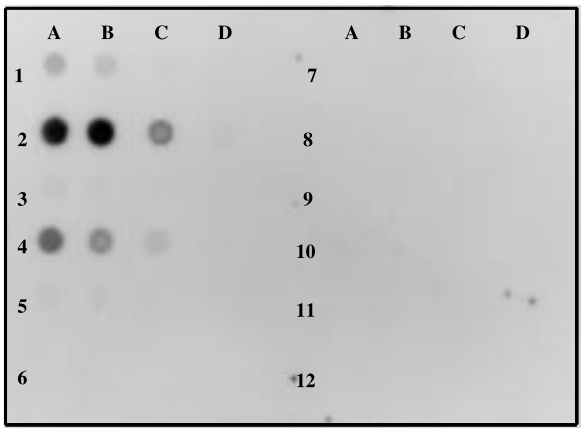

Figure 3. Dot blots of a dilution series (A–D, equivalent to 10, 5, 1 and 0.1 mg of leaf, respectively) of total nucleic acid extracts from *Ribes* or *Vicia faba* plant tissues hybridised with the radio-labelled probe (BY16). Samples were denatured before 10 μl was spotted into nylon membrane and fixed by autocrosslinking with UV. Samples were from, cv. Baldwin BYD-affected (1) and symptomless (2), BIVD-affected Daniel's September (3), cv. Amos Black (4), cv. Öjebyn (5), *V. faba* cvs The Sutton (6) and Minden (7), cv. Ben Sarek BYD-affected (8) and symptomless (9), cv. Jet (10), cv. Ben Sarek (11), water control (12). Only dots of samples 1–5 hybridised with the probe.

dsRNA dot blots. When dsRNA from plants of 19 *Ribes* cultivars was dot blotted onto positively charged nylon membrane and probed, the probe hybridised with 7 of the dsRNA extracts from the blackcurrant cvs Baldwin, Baldwin (grafted with BRD-R), Ben Tron, Daniel's September, Greens Black, Raven, and Westwick Choice showing the ability of the probe to detect the ca. 5 kbp-related dsRNA in dot blots. However, no hybridisation was detected with dsRNA from any of the 5 cultivars of redcurrant, gooseberry or white currant, even though several of these contained dsRNA species of ca. 5 kbp (Tables 1 and 2). It is noteworthy that in this test, the ca. 5 kbp dsRNA from BIVD-affected cv. Daniel's September reacted to the probe. These results extend the Northern blot data and indicate the specificity of the probe to the ca. 5 kbp dsRNA species.

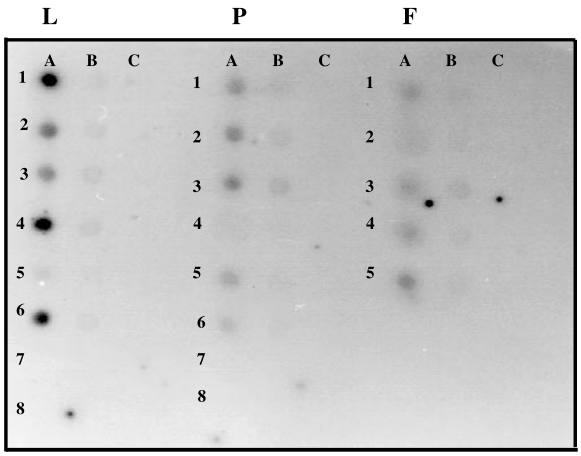

Figure 4. Autoradiography of sap blot dilutions of leaf (L), petiole (P) and flower (F) tissues hybridised with probe BY16. Sap dilutions correspond to approximately 0.6 (A), 0.06 (B) and 0.006 (C) mg of tissue. Samples (5 µl each) were spotted onto nylon membrane, fixed by microwaving and hybridised with the probe. Dot numbers refer to sap from: cv. Baldwin, BYD-affected (1) and symptomless (2); BIVD-affected cv. Daniel's September (3); cv. Ben Sarek BYD-affected (4) and symptomless (5); cv. Öjebyn (6); *V. faba* cvs The Sutton (7) and Minden (8). All except the *V. faba* material (7, 8) and flowers of cv. Öjebyn (6) hybridised with the probe.

Total nucleic acid dot blots. Total nucleic acid isolated from 9 blackcurrant plants and 2 *V. faba* cultivars was dot blotted onto nylon membrane in a dilution series equivalent to ca. 10, 5, 1 and 0.1 mg leaf tissue per dot. The probe hybridised with extracts from plants of cvs Amos Black, Baldwin (BYD-affected and symptomless), and weakly with those from cvs Daniel's September (BIVD-affected) and Öjebyn but not with those from the *V. faba* cvs The Sutton and Minden, three sources of cvs Ben Sarek and Jet, or a water control (Figure 3). Leaf tissue of symptomless cv. Baldwin contained more of the nucleic acids that reacted with the probe than any of the other cultivars tested. Indeed, the dilution series indicated that RNA from symptomless cv. Baldwin was 10–50× more reactive than that from BYD-affected cv. Baldwin and 100× more reactive than RNA from the other cultivars (Figure 3). In Northern blots, no dsRNA species from cv. Öjebyn material reacted with the probe (Figure 2) but, in this test, cv. Amos Black appeared to contain ca. 10× less reactive material than symptomless cv. Baldwin (Figure 3).

Sap blots. Serial dilutions, corresponding to ca. 0.6, 0.06 and 0.006 g of tissue per dot, of sap extracts from leaf, petiole and flower tissues of the cvs Baldwin (BYD-affected and symptomless), Ben Sarek (BYD-affected and symptomless), Daniel's September (BIVD-affected), Öjebyn and the *V. faba* cvs The Sutton and Minden were spotted onto nylon membrane. A hybridisation signal was detected with the probe in most samples except in the most dilute extracts of the blackcurrant tissues and in all of the dilutions of the tissues of the *V. faba* cultivars assayed (Figure 4).

Sequence analysis and comparisons of the insert

The probe consisted of 499 nt and its sequence was deposited as Accession No. AJ133821 in the EMBL Nucleotide Sequence Database. It showed no significant similarity with other sequences in this Database. However, an open reading frame in the probe sequence encoded a peptide sequence of 156 amino acids. In comparison with those in the OWL database, it was found that a stretch of 145 amino acids had a 42% sequence identity to the RNA-dependent RNA polymerase (RDRP) of *Saccharomyces cerevisiae* viruses L-1 and L-A (ScV-L1 and ScV-LA) that belong to the family *Totiviridae.*

The similarities between these peptides suggests that a possible source of the ca. 5 kbp dsRNA, and possibly some others, was fungi in, or on, the tissues. Attempts were therefore made to test for this possibility. Comparative analysis was made of dsRNA from mouldy and washed leaves of cvs Ben Lomond and Greens Black but no great differences among the numbers or amounts of dsRNA species were detected (data not shown). This very limited analysis suggests that the dsRNA species detected in *Ribes* may be of plant origin, the result of an endophytic organism or, an organism that was not removed in the cleaning process.

Discussion

In this study, a dsRNA species of ca. 5 kbp was isolated consistently from the blackcurrant cv. Baldwin (Tables 1 and 2; Figures 1–4). This dsRNA species is presumably the same as the 4.3 kbp species reported by Jones et al. (1986) in plants affected with BYD. As noted in the Materials and methods section, the difference in apparent size is due possibly to the different gel buffer system used in these different studies. However, this ca. 5 kbp species, and none of the many other dsRNA species detected in the different

Ribes sources analysed, were associated consistently with BYD or with any of the other virus-like diseases studied (Tables 1 and 2).

Nevertheless, the dsRNA size and profile from the bark of some plants naturally affected with GVBD may be characteristic of a filamentous virus (Table 2), and this finding would be in keeping with the fact that closterovirus-like particles were seen in ultra-thin sections of leaves of GVBD-affected plants (Roberts and Jones, 1997). Closteroviruses are usually phloem-restricted, which might possibly explain our more efficient recovery of dsRNA from bark and cambial tissue than from leaves of plants. However, none of the bark samples from cultivars graft-inoculated with GVBD, or from cultivars that later showed transient GVBD symptoms, contained such large sized dsRNA species (Table 2). This may suggest that the dsRNA species detected in plants naturally affected with GVBD are not derived from the causal agent of the disease or, that this disease is caused by more than one agent.

The causal agent of BRD is probably BRAV, which has a genome of two linear polyadenylated ssRNA species, ca. 7.7 and 6.7 kb in size (Lemmetty et al., 1997; Latvala et al., 1997). The dsRNA analysis of material affected with BRD, either naturally or by grafting, provided no consistent evidence of disease-associated dsRNA species (Tables 1 and 2). Nevertheless, a field-infected cv. Ben Tirran plant, and some plants infected by grafting, contained small amounts of dsRNA species that ranged in size from ca. 6–10 kbp and that were not present in healthy plants (Table 2). It is not known if any of these species are derived from the genome of BRAV, but the known erratic distribution of BRD symptoms and of BRAV in affected plants (Adams and Thresh, 1987a; Lemmetty et al., 1997; Latvala et al., 1997) may explain the difficulty and inconsistency of obtaining BRAV-specific dsRNA species.

In Northern blot assays, the radio-labelled probe to the ca. 5 kbp dsRNA species present in cv. Baldwin, detected less than 100 ng of the ca. 5 kbp dsRNA. When used in hybridisation analysis with dsRNA from a range of *Ribes* cultivars, the probe reacted with only those blackcurrant cultivars known to contain a ca. 5 kbp dsRNA species (Figure 3). However, it did not react with a ca. 5 kbp dsRNA species present in redcurrant and gooseberry cultivars despite the fact that in these tests, this dsRNA was readily detectable in gels by silver staining. Consequently, although difficulties were often encountered in isolating detectable quantities of dsRNA from the leaves of gooseberry and redcurrant, the detection of these dsRNAs in gels in these tests, indicates that a low concentration of dsRNA is unlikely to be the reason for the lack of reaction to the probe. This suggests that the ca. 5 kbp dsRNA species in blackcurrant is distinct from similarly sized dsRNA species in these other *Ribes* species.

Hybridisation analysis of sap extracts improved detection of the ca. 5 kbp species presumably because of an increased concentration of the target (Figures 3 and 4). The maximum amount of tissue assayed in the total nucleic acid blot experiment (Figure 3) was 10 mg compared to 600 mg (Figure 4) in the sap assays. In assays of different tissues, the strongest signals were given by isolations from leaves, the weakest from flowers, with petioles being intermediate in strength. However, these differences may reflect the ease with which the ca. 5 kbp dsRNA can be extracted from certain tissues and not differences in the amount of this dsRNA in these tissues. A minimum of 0.6 g of tissue was necessary to give a consistent hybridisation signal in sap extracts.

Comparisons of the amino acid sequence of the insert used for the probe showed similarities to the RNA-directed RNA polymerase (RDRP) of ScV-LA, the type member of the genus *Totivirus* of the family *Totiviridae* of dsRNA viruses. Comparisons of the putative peptide sequence of the BY16 insert, the probable RDRPs of species in the genera *Giardiavirus* and *Leishmaniavirus* of the family *Totiviridae* and of a 5.2 kbp dsRNA of the flax rust (*M. lini*, strain I) (kindly donated by Dr Matt Dickinson, University of Nottingham), showed that they all have the RDRP conserved motifs 7 and 8. The strain I of *M. lini* has isometric particles of 40 nm diameter and one large (5.2 kbp), 6 medium (2.0–2.7 kbp) and 4 small (1.1–1.5 kbp) sized dsRNA species. The 5.2 kbp dsRNA shows significant homology to the RDRPs of yeast and Leishmania viruses (Zhang et al., 1994).

In New Zealand, dsRNAs and virus-like particles in *Botrytis cinerea*, an important *Ribes* pathogen, were catalogued (Howitt et al., 1995), and of 200 isolates taken from a diverse range of crops, 72% contained dsRNA species. However, the number (1–8 species per isolate) and size (0.8–1.5 kbp) of these dsRNAs suggest that they are not responsible for the ca. 5 kbp dsRNA species found in *Ribes*. In a study in Moldova, *Ribes* species were found to be hosts for 126 fungal species, 110 of which were found on blackcurrant (Popusoi and Stratu, 1993). In theory therefore, one or more of

a large number of fungi could be responsible for the ca. 5 kbp dsRNA we have detected in blackcurrant. Very preliminary analyses of dsRNA species associated with exophytic fungal infection on blackcurrant leaves did not indicate that either the ca. 5 kbp or any of the other major dsRNA species commonly isolated from blackcurrant, were from this origin. However, endophytic fungi are also known to contain dsRNA viruses (Zabalgogeazcoa et al., 1998) and these could be a possible source of at least the ca. 5 kbp dsRNA in *Ribes*.

The range of dsRNA species present in the many cultivars and species tested shows that very few *Ribes* plants appear to be dsRNA-free. The vegetative propagation of commercial *Ribes* cultivars and species, and their perennial nature, provides many opportunities for infection by many different pathogenic and non-pathogenic agents. In this study, none of the dsRNA species could be associated consistently with any of the virus-like diseases studied. However, the affinity of at least one of these dsRNA species with viruses in the family *Totiviridae*, suggests that it may be derived from fungi infecting *Ribes*, including apparently healthy virus-tested stock. This finding identifies a factor to be considered, additional to those already noted (Abou-Elnsar et al., 1985; Watkins et al., 1990), in the interpretation of dsRNA analysis of plants, and especially of woody perennials.

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