Diagnosis of Flame Chlorosis, a Viruslike Disease of Cereals, by Detection of Disease-Specific RNA with Digoxigenin-Labeled RNA Probes

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ABSTRACT

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Flame chlorosis (FC) is a novel, viruslike disease of cereals for which FC-specific double-stranded RNAs (FCdsRNAs) have been identified. Visual symptoms of the disease are distinctive, but definitive diagnosis requires examination of cytopathology and/or the detection of FCdsRNA. FC-specific RNA (FC-RNA) was detected in 1.5-mm leaf disks using labeled RNA probes made from cDNA clones of the FCdsRNAs. Crude leaf extract was prepared in a citrate/formaldehyde buffer, heated at 95 C for 10 min, cooled on ice, and briefly centrifuged, and the supernatant was applied to a nylon or polyvinylidene fluoride membrane with a dot or slot blotting manifold. Blotted samples were fixed by being heated for 3 min in a microwave oven and hybridized with digoxigenin-labeled RNA probes made from cDNA clones. After incubation with antidigoxigenin-phosphatase and reaction with chromogenic substrate, the intensity of colored spot or band formation was relative to the level of FC-RNA in the original sample. The assay is useful as a research tool as well as for clinical diagnosis because the small sample size allows repeated multiple sampling from plants or comparison of adjacent chlorotic and green areas on the same leaf.

Flame chlorosis (FC) is a soil-transmitted, viruslike disease of cereals that was first observed in western Manitoba, Canada, in 1985 (6). Until now, its routine diagnosis has been based on visual symptoms (3), but these may be confused with certain forms of herbicide injury by inexperienced observers (5). Moreover, a definitive diagnosis based on physical evidence rather than a subjective interpretation of visual symptoms may be required, for example, in an insurance claim to show that crop losses were attributable to disease (such as FC) rather than to improper herbicide application.

Serology, an approach widely used to diagnose plant virus diseases, requires the preparation of suitable disease-specific immunogen, usually purified virus particles. No virus particles have yet been associated with FC (6) (J. Chong and S. Haber, *unpublished*), so we have developed a technique for diagnosis of FC that is based on the detection of disease-specific RNA.

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The patterns of double-stranded RNA (dsRNA) bands visualized by ethidium bromide staining in agarose gel electrophoresis characteristic for FC (4,6) are used to confirm visual diagnoses (5). There are two problems with applying this approach in routine clinical diagnosis. First, there are variations in the dsRNA patterns among isolates from different hosts and geographic locations (4). Second, extracting sufficient dsRNA of adequate purity for clear visualization on gels is too laborious for routine clinical use.

Northern analysis has shown that there is a high degree of homology among the numerous flame chlorosis-specific dsRNA (FCdsRNA) fragments of an individual isolate, as well as among fragments of similar but distinct patterns that were purified from different hosts and geographic isolates (4) (S. Haber, *unpublished*). Therefore, dot blot hybridization with labeled probes made from cDNA clones of FCdsRNAs could provide the basis for reliable and rapid diagnosis. To facilitate routine use of the assay by disease clinics as well as research laboratories, a label other than radioisotope is desirable. We report the development of a dot blot hybridization assay for FC-specific RNA (FC-RNA) that employs specific RNA probes labeled with alkaline phosphatase in a two-step procedure using a commercially available kit.

MATERIALS AND METHODS

Preparation of purified FCdsRNA. FCdsRNA of the barley "type" isolate (4) was purified using methods described previously (6). Twenty grams of FC-infected leaf tissue was pulverized in liquid nitrogen, and the frozen tissue was covered with 20 ml of boiling extraction buffer (2% cetyltrimethylammonium bromide [CTAB], 2% mercaptoethanol, and 1.4 M NaCl) and then incubated with gentle stirring at 60 C for 5 min in a fume hood. After the buffer was cooled to room temperature, 20 ml of chloroform/isoamyl alcohol (24:1) was added and stirring was continued for 5 min. The aqueous component was removed by centrifugation at 10,000 *g* for 15 min at 20 C. A one-tenth volume of 10% CTAB, 1.4 M NaCl, was added to the measured volume of the aqueous phase, followed by 15 ml of chloroform/isoamyl alcohol (24:1), and the mixture was emulsified by vigorous vortexing. Once more, the aqueous component was removed by centrifugation at 10,000 *g* for 15 min at 20 C, and the volume was measured. An equal volume of TE buffer (50 mM Tris-HCl, pH 8.0, and 10 mM disodium ethylenediaminetetraacetic acid [EDTA]) was added slowly with stirring and the mixture was incubated at 20–25 C for 1 hr. Nucleic acids then were precipitated with ethanol and pelleted by centrifugation at 15,000 *g* for 20 min at 4 C. The vacuum-dried pellet was resuspended in 40 ml of STE (50 mM Tris-HCl, pH 7.2, 1 mM disodium EDTA, 100 mM NaCl, and 16.5% ethanol), and the dsRNA component was isolated by cellulose column chromatography as described by Morris and Dodds (8).

The concentration of purified FCdsRNA was determined spectrophotometrically (using E_{1cm} at 260 nm = 25), divided into aliquots of 100 μl at 1 μg/μl, and stored at −70 C.

Preparation of FC-specific cDNA clones. Complementary DNA species were prepared from gel-purified FCdsRNA of the "type" western Manitoba barley isolate following the method of Skipper (10). Resulting dsDNA molecules were cloned in plasmid pTZ18U (United States Biochemical, Cleveland, OH) following the method of Teeri and co-workers (11). The presence of FC-specific inserts was determined by colony hybridization with FCdsRNA that had been labeled at the 5′ end with ^{32}P by partial hydrolysis (9), followed by kinase reaction (7). The presence of FC-

specific DNA in the clones identified by colony hybridization was confirmed by hybridization to end-labeled FCdsRNA in Southern blots of minipreps made from these clones.

Preparation of FC-RNA probes. Two FC-specific cDNA clones with the longest inserts, FCcDNA-21 and -23 (631 and 623 base pair, respectively) were used in the protocols supplied with the Genius SP6/T7 RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) for preparing digoxigenin-labeled RNA (digRNA) runoff transcripts. After determining that both sets of digRNA probes were effective, all assays were performed using digRNA probes made from FCcDNA-21.

Preparation of plant extract for dsRNA dot blotting. Small (1.5 mm) disks were punched from either fresh, frozen, or dried leaf tissue into 1.5-ml microvials with a mini-borer made from a 16-gauge disposable syringe needle. After each disk was punched, the needle was dipped in alcohol and flamed. Disks were punched from healthy control leaves, from chlorotic and green areas of FC-infected leaves, or from leaves infected with barley yellow dwarf virus (BYDV), brome mosaic virus (BMV), barley stripe mosaic virus (BSMV), or wheat streak mosaic virus (WSMV). Leaf disks were either stored frozen in microvials for later use or immediately macerated against the microvial wall with a Teflon plunger that had been dipped in alcohol and flamed. Ten microliters of autoclaved, distilled water was added to the small volume of macerate, followed immediately by 9 μl of 20× SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0). The mixture then was vortexed, 8 μl of 37% formaldehyde was added, and the mixture was briefly vortexed again. At this point, sample microvials were either frozen at −20 C until further processing the next day or immediately heated to 95 C in a water bath for 10 min, chilled on ice, and briefly centrifuged; 20 μl of supernatant was withdrawn from each microvial for application to membrane.

Preparation of plant extract for dsRNA slot-blotting. The procedure was identical to that for dot blotting, except the sample disk was processed in 100 μl of autoclaved, distilled water, 90 μl of 20× SSC, and 80 μl of 37% formaldehyde, and a supernatant of 200 μl was withdrawn for application to membrane.

Application of sample to membrane. A nylon (NyTran, Schleicher & Schuell, Keene, NH) or polyvinylidene fluoride (Immobilon, Millipore, Bedford, MA) membrane was wetted and placed dull side up in a dot blot or slot blot manifold (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Samples were applied to the membrane (20 μl for dot blot and 200 μl for slot blot), taking care in the case of the dot blot not to let the sample touch the plastic wall of the manifold. After air-drying the membrane, nucleic acid was fixed by irradiation in a 450W microwave oven for 3 min (2). Membranes then were either processed or stored desiccated at room temperature until prehybridization and hybridization with a labeled probe.

Prehybridization of membrane. Membranes were wetted with distilled water, the excess water was blotted, and then membranes were transferred to a preheated shaking water bath for incubation at 68 C for 2 hr in a prehybridization solution (50% formamide, 6× SSC, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS], and 5% blocking agent) prepared according to manufacturer's instructions (Boehringer Mannheim).

Hybridization of membrane with digoxigenin-labeled probe. Hybridization solution was prepared by adding 0.5 μl of a digRNA probe to 5 ml of prehybridization solution and heating to 95 C for 10 min. The prehybridization solution was decanted from the membranes immediately before adding the hybridization solution. Membranes were incubated at 68 C with gentle shaking overnight (16–20 hr). Hybridization was terminated by decanting the hybridization solution and thoroughly washing the membranes. The membranes were washed twice for 5 min at room temperature in 2× SSC and 0.1% SDS, and then twice for 15 min at 68 C in 0.1× SSC and 0.1% SDS. Membranes were then either processed or stored desiccated at room temperature until incubation with labeled antibody.

Reaction with antidigoxigenin-phosphatase. Dried membranes were wetted with distilled water and then membranes were equilibrated in 50 ml of buffer A (0.1 M Tris-HCl, pH 7.0, and 0.15 M NaCl) for 1 min before blocking by incubation for 3 hr at room temperature (with gentle agitation) with 50–100 ml of buffer B (buffer A containing 2% [w/v] Boehringer Mannheim blocking agent). Near the end of the 3-hr incubation period, 20–30 ml of a 1:5,000 dilution of antidigoxigenin-phosphatase (Boehringer Mannheim) in buffer B was freshly prepared. After the blocking solution was decanted, the antibody solution was added, and the dish with membranes was gently agitated for 30 min at room temperature. The antibody solution was decanted to stop the incubation with antibody and the solution was washed twice for 15 min by gentle agitation with 200–250 ml of buffer A.

Reaction with chromogenic substrate. Membranes were first equilibrated in 50 ml of buffer C (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, and 0.05 M MgCl) for 2 min. One membrane per dish then was incubated with chromogenic substrate. Typically, one 4 × 8 cm strip of membrane (32 dots) was incubated in a 100 × 15 mm petri dish. The chromogenic NBT/X-phosphate substrate solution was prepared freshly according to manufacturer's instructions (Boehringer Mannheim), and 5 ml of substrate was placed in each dish with membrane. After overnight development in the dark at 20–25 C, the membrane was transferred to a dish with 50 ml of buffer D (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and incubated for 5 min at room temperature to stop the reaction. Membranes were air-dried and stored wrapped in cellophane away from direct light. If the blue or purple spots of positive reactions faded during storage, color was restored by rewetting membranes in buffer C.

End-point determination and quantitation of assay results. Aliquots (200 μl) of purified FCdsRNA were serially diluted from 2 μg to 500 pg (Table 1) with 200 μl of 6× SSC/2.2 M formaldehyde, heated and chilled as described above, and slot blotted onto nylon or polyvinylidene fluoride membranes using the Bio-Rad slot blot apparatus. After the assay, the developed membrane was photographed through an orange filter using Polaroid Type 55 positive/negative film, and the negative was scanned with an ISCO Model 1312 densitometer.

RESULTS

Dose-response relationship. The difference between slot and background

Table 1. Dose-response relationship between amount of flame chlorosis-specific dsRNA (FCdsRNA) and signal strength[a]

Dilution (four-fold)	FCdsRNA amount	Signal strength[b]		$\log_{10}$ (signal)	
		1	2	1	2
	0 (blank)	0	0	...	...
0	2,000 ng	68	60	1.83	1.78
1	500 ng	67	64	1.82	1.81
2	125 ng	54	56	1.73	1.75
3	31 ng	30	32	1.48	1.51
4	7,800 pg	8.0	8.0	0.90	0.90
5	1,950 pg	2.0	2.0	0.30	0.30
6	490 pg	0.5	0.45	−0.30	−0.35

[a] Signal strength is the maximum difference in optical absorbance at 580 nm, multiplied by 100, between band and background in a densitometer scan of a photographic negative of the developed slot blot.
[b] Duplicate data set.

absorbance in the densitometer scan of the developed slot blot was tabulated as a function of amount of FCdsRNA (Table 1) and the logarithm of this value plotted vs. logarithm of FCdsRNA amount (Fig. 1). As little as 500 pg of purified FCdsRNA diluted in the extract made from healthy plants could be detected (Table 1). The relationship between logarithm of FCdsRNA amount and logarithm of densitometer reading was linear from the lowest detectable level of 500 pg to 30 ng. At higher levels of FCdsRNA, the densitometer reading did not increase proportionally with increased FCdsRNA and the response was saturated from approximately 500 ng to 2 μg, the highest level tested (Table 1 and Fig. 1). With this calibration curve, relative levels of FCdsRNA could be determined within a 1,000-fold range from the limit of detection to saturation. Most disks sampled from FC-infected tissue gave results that fell within the linear portion of the dose-response curve, making it possible to estimate levels of FC-RNA in different samples.

Detection of FC-RNA in plant samples. The assay detected FC-RNA in disk samples from the three FC hosts—barley, wheat, and oat (Fig. 2). Positive results were obtained only when FC-infected tissue was used; there was no detectable reaction when tissue from healthy plants or from cereals infected with other viruses was used (Fig. 2). Positive results were always obtained when disk samples were taken from chlorotic and early necrotic areas of FC-infected leaf tissues. However, samples taken from nonsymptomatic leaves of FC-infected plants or from the green areas immediately adjacent to chlorotic areas on the same leaf usually gave much less intense reactions or were indistinguishable from healthy controls (Fig. 3).

DISCUSSION

Analysis of dsRNA provides evidence for infection with an RNA virus (8). When studying RNA plant virus infections for which causal virus particles have been identified, dsRNA analysis is an additional tool to serology, transmission studies, and microscopic examination of infection-induced inclusions. FC (6) and whitefly-mediated silvering in squash (1) are examples of plant diseases with indications of viral etiology for which no disease-specific, viruslike particles have been identified, but which are associated consistently with specific dsRNAs. For such diseases, detection of this disease-specific dsRNA may offer the only feasible approach to routine, definitive diagnosis.

Our assay detected FC-RNA in crude extracts from small leaf specimens of FC isolates from barley, wheat, and oat (Fig. 2). In "blind" tests, coded specimens in which FC had been positively identified earlier on the basis of visual symptoms and/or cytopathology always gave a positive result with this assay. The manner of tissue preservation did not affect the assay's ability to detect FC-RNA, as similar results were obtained from fresh, frozen, or air-dried tissue (*data not shown*).

For the assay to work properly, the labeled FC-specific ssRNA probe must encounter its single-stranded complement bound on the membrane. However, we cannot be certain whether ssRNA is derived solely from melting of FCdsRNA present in the extract or whether it also reflects the presence of FCssRNA in the tissue itself. Comparisons of bulk

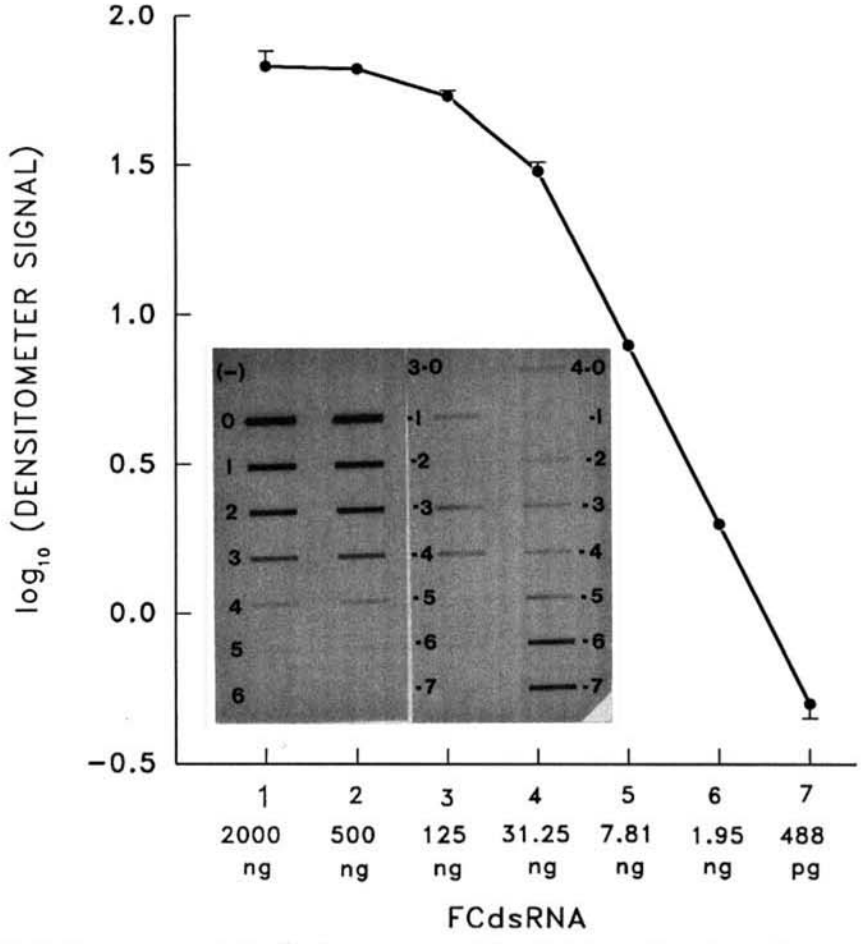

Fig. 1. Dose-response relationship between amount of purified flame chlorosis-specific double-stranded RNA (FCdsRNA) and densitometer reading of slot blot. Slot blot of FCdsRNA titration (see Table 1) and FCdsRNA in disk samples (see Table 2) is shown in inset.

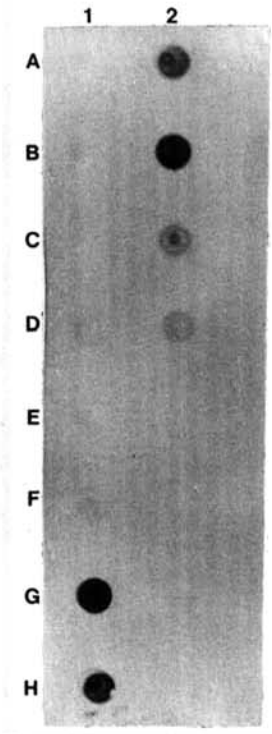

Fig. 2. Detection assay specifically detecting flame chlorosis (FC)-specific RNA in disks from FC-infected barley, oat, and wheat. 1A = healthy oat; 1B = healthy barley; 1C = healthy wheat; 1D = FC-infected oat, yellow area; 1E = FC-infected oat, green area; 1F = FC-infected oat, yellow area (same plant but different leaf from 1D); 1G = FC-infected barley, yellow area; 1H = FC-infected barley, yellow area (near 1G on same leaf); 2A = barley, yellow area (same plant but different leaf from 1G and 1H); 2B = FC-infected wheat, yellow area; 2C = FC-infected wheat, yellow area (near 2B on same leaf); 2D = FC-infected wheat, yellow area (same plant but different leaf from 2B and 2C); 2E = oat infected with barley yellow dwarf virus; 2F = barley infected with brome mosaic virus; 2G = barley infected with barley stripe mosaic virus; and 2H = wheat infected with wheat streak mosaic virus.

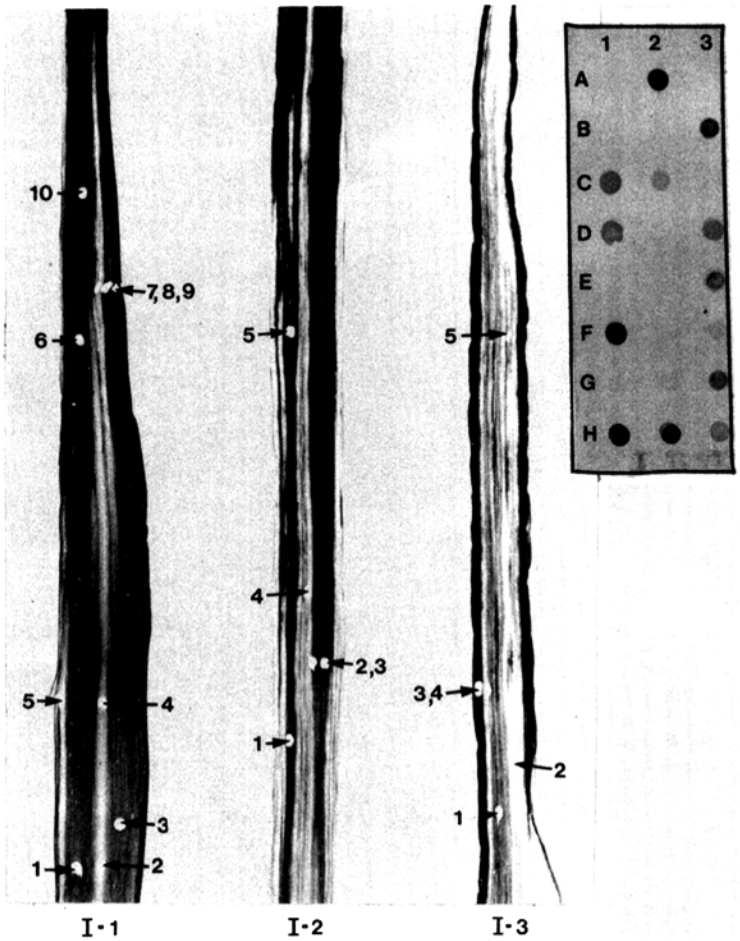

Fig. 3. Close association between macroscopic symptoms (chlorosis and necrosis) and level of flame chlorosis (FC)-specific RNA. Leaf disk samples were taken from a four-leaf stage FC-infected barley plant. I-1 is the youngest leaf and has early FC symptoms. I-2 is the next youngest leaf; chlorosis is more extensive. I-3 is the oldest leaf with FC symptoms; areas of earlier chlorosis have started to become necrotic. Inset shows assay results from disk samples. 1A and 1B = healthy controls; 1C and 1D = purified FC-specific double-stranded RNA (positive controls); 1E = disk 1, leaf I-1; 1F = disk 2, leaf I-1; 1G = disk 3, leaf I-1; 1H = disk 4, leaf I-1; 2A = disk 5, leaf I-1; 2B = disk 6, leaf I-2; 2C = disk 7, leaf I-1; 2D = disk 8, leaf I-1; 2E = disk 9, leaf I-1; 2F = disk 10, leaf I-1; 2G = disk 1, leaf I-2; 2H = disk 2, leaf I-2; 3A = disk 3, leaf I-2; 3B = disk 4, leaf I-2; 3C = disk 5, leaf I-2; 3D = disk 1, leaf I-3; 3E = disk 2, leaf I-3; 3F = disk 3, leaf I-3; 3G = disk 4, leaf I-3; and 3H = disk 5, leaf I-3.

Table 2. Estimates of amounts of flame chlorosis-specific double-stranded RNA (FCdsRNA) in disk samples

Disk[a]	Signal	Estimated amount[b] (ng)
3-0	0.0	0.0
1	9.5	10
2	3.2	3.2
3	17.5	17
4	15.5	15
5	2.0	2.0
6	3.5	3.4
7	3.0	3.0
4-0	6.0	6.0
1	2.0	2.0
2	6.5	6.2
3	11.5	11
4	10.0	10
5	19.0	18
6	53.0	110
7	45.0	72

[a] Sample disks (1.5 mm diameter) were taken from third and fourth leaf of a flame chlorosis (FC)-infected barley seedling. Disks 3-0, -2, -5, -6, and -7 and 4-0, -1, and -2 were from green areas adjacent to chlorotic areas; 3-1, -3 and -4 and 4-3, -4, -5, -6, and -7 were from chlorotic areas. Developed slot blot of the assay of these samples is shown in the inset in Figure 1.

[b] Amount of FCdsRNA estimated by interpolation from dose-response relationship (see Fig. 1) between FCdsRNA and signal strength.

extracts of healthy and FC-infected tissues by analysis of dsRNA and northern analysis of ssRNA and dsRNA using radiolabeled FCdsRNA as a probe have shown that the predominant FC-specific nucleic acid is dsRNA (4,6) (S. Haber, *unpublished*), but the proportion of these two topological forms of FC-specific RNA might be different in certain small areas of tissue. For this reason, we can only assume that our probe detected FC-RNA rather than just FCdsRNA.

An unexpected result was the finding that the distribution of a probe-specific target (presumably FCdsRNA) coincided so precisely with the macroscopic symptoms of chlorosis and necrosis. If one assumes that most of the FC-RNA is dsRNA (and the FCdsRNA dose-response relationship may therefore be applied), the levels of FC-RNA in a chlorotic area of a leaf can be estimated to be up to 1,000 times higher than in the immediately adjacent green tissue (Table 2 and Fig. 1). Under this assumption, a 1.5-mm disk (containing 10–15 μg dry mass of tissue) from the most intense FC-infected leaf areas contains 1%, and possibly as much as 10%, FCdsRNA by mass. This evidence is also consistent with the observation that the chlorotic areas of FC-infected leaf tissue have much higher levels of FC-specific ultrastructural alterations than the adjacent green areas (6) (J. Chong and S. Haber, *unpublished*). Such a close association between FC-RNA, macroscopic disease symptoms, and the extent of disease-specific cytopathology is consistent with this RNA being the disease agent. We are now using the FC-RNA probe to determine directly in ultrathin sections the subcellular locations of FC-RNA.

The variegated distribution of FC-RNA in FC-infected leaf tissues requires that special care be taken to sample the tissue correctly. We recommend assaying disk samples from at least one chlorotic and one green leaf area in each specimen. A positive signal from the chlorotic area combined with a weaker or undetectable signal from the green area provides a further indication of flame chlorosis and constitutes an additional internal control in the assay.

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