Diagnosis of Flame Chlorosis by Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

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ABSTRACT

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Flame chlorosis (FC), a viruslike disease of cereals, is associated specifically with double-stranded RNAs (FCdsRNAs). The FCdsRNAs can be detected by dot blot hybridization assay that is adequate for detecting FC-RNA in symptomatic areas of infected leaf tissue, but has proved insufficiently sensitive to detect FC-RNA in small (≤10 mg) quantities of suspect root tissue or mycelium of candidate fungal vectors. A reverse transcription-polymerase chain reaction assay to detect FC-RNA (FC-RT-PCR) was developed to improve sensitivity. Total RNA was extracted from milligram quantities of test tissue, reverse transcribed, and amplified by the PCR. The primer pairs #86 [F:5′-CTATTCGCTTGGCTCAGATCG-3′ and R:5′-CCAGAGTAGTGACTAGAACAGC-3′] or #307 [F:5′-GTGAAAGTCTTGAGGATGC-3′ and R:5′-TTCATCTCTATTGGCACCACG-3′] were used. These primer pairs had been determined from a consensus 821-bp FC sequence covering an open reading frame (GenBank No. X59248), and were predicted to yield 358- and 347-bp DNA fragments, respectively. Sensitivity of specific FC-RNA detection was further enhanced by hybridization of the RT-PCR product to digoxigenin-labeled riboprobe (digFC-RNA) used in the earlier FC dot blot hybridization assay; the digoxigenin label was subsequently reported with enzyme-linked antidigoxigenin antibody and chromogenic substrate.

Flame chlorosis (FC) is a soilborne, viruslike disease of cereals (5) that is associated with specific double-stranded RNAs (FCdsRNAs) (6,7). We previously demonstrated a diagnostic assay that exploited stringent hybridization of labeled probes to disease-specific RNA (FC-RNA) (7). Diagnosis based on serology was not feasible because we had not been able to find virus particles in diseased tissues (1). The dot blot hybridization assay (FC-DBA) showed clearly that FC-RNA (predominantly in the form of FCdsRNA) was present at much higher levels in the areas of yellow variegation on symptomatic leaves than in adjacent green areas (7). Indeed, FCdsRNA made up as much as 1% of the dry cell mass in small, localized areas of FC leaves that were intensely chlorotic, and was readily detected in crude extracts made from 1.5-mm disks (10 to 15 µg dry tissue mass) (7). By contrast, similar quantities of root tissue from FC plants did not usually give positive results in FC-DBA even though electron microscopy showed that some root cells had FC-specific cytological alterations as extensive as those found in mesophyll cells of symptomatic leaf tissue (1). In root, unlike leaf, tissue there are no visible symptoms to guide the selection of samples.

In addition to addressing the need for more sensitive and definitive diagnosis, we used the approach of detecting FC-RNA to search for a fungal vector of the soilborne FC agent (4). Based on the observation that FC symptoms and FC-RNA appeared as early as the one-leaf stage, we hypothesized that a candidate FC vector should readily infect leaf and root initials in the earliest stages of seed germination (4). *Pythium* spp. meet this requirement in that they infect cereal embryos within 24 to 48 h after planting (3), and FC-DBA showed that certain *Pythium* isolates possessed FCdsRNA (4; S. Haber, *unpublished*). The amount of FC-RNA in the *Pythium* isolates, however, was much lower than in chlorotic areas of FC leaf tissue, so it was necessary to culture fungal mycelium in liters of medium in order to obtain sufficient nucleic acid extract for analysis. To analyze diverse fungal isolates from FC and non-FC soils more rapidly, it was clearly desirable to be able to detect FC-RNA in extracts prepared at the scale of

When part or all of the sequence of the diagnostic RNA is known, it is straightforward to exploit the coupled reactions of reverse transcription–polymerase chain reaction (RT-PCR) for amplification (15). Making a reverse transcript from dsRNA, however, may be problematic given its high melting temperature (14). This difficulty can be overcome if disease-specific single-stranded RNA (ssRNA) also is present, even if it constitutes only a small proportion of the total ssRNA pool. We demonstrate here that tissues with FCdsRNA (6,7,12) also contain FC-specific ssRNAs (FCssRNAs), which hybridize to riboprobes made from cDNA clones of FCdsRNAs.

The cDNA clones of FCdsRNA that were developed for the dot blot hybridization assay, FCcDNA-21 and -23 (7), establish together with other FCcDNA clones a consensus sequence of 821 bp (GenBank No. X59248) that contains an open reading frame. This sequence has no significant homology with any sequence deposited to date in the GenBank and EMBL nucleic acid sequence databases. Therefore, strong evidence for the presence of FCssRNA in the original RNA extract is shown when specific primers from X59248 amplify reverse transcripts under stringent annealing conditions and yield fragments of predicted sizes. Subsequent stringent hybridization of the DNA product with FC-specific digoxigenin-labeled riboprobe (digFC-RNA) confirms the presence of FCssRNA in the original RNA extract and further enhances the sensitivity of detection.

MATERIALS AND METHODS

Preparation of FC-specific nucleic acids. For developing the RT-PCR protocols, FC-RNA, quantified by spectrophotometry, was prepared by run-off transcription of cDNA clones using T7 DNA-directed RNA polymerase and the protocols supplied with the Boehringer Mannheim DIG-labeling system (Boehringer Mannheim Canada, Laval QC). FCssRNA was also prepared from infected leaf tissue and from cultured *Pythium* mycelium using a method adapted from a protocol for the purification of wheat RNA (10). Tissue was lyophilized, then ground to a fine powder with dry ice in a small mortar. About 10 mg was transferred to a 1.5-ml microvial containing 400 µl of a freshly

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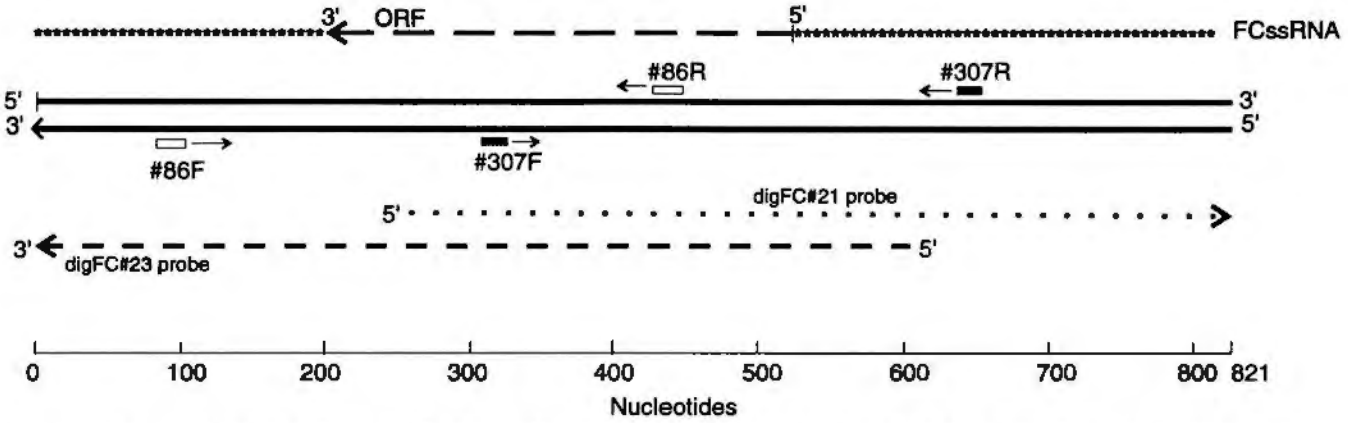

Fig. 1. Schematic diagram showing relationship of flame chlorosis–specific primer pairs and digoxigenin-labeled RNA probes to the consensus 821-nucleotide flame chlorosis sequence (GenBank No. X59248).

1 part 8-hydroxyquinoline (wt/vol/wt), saturated with Solution B (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% [wt/vol] napthalene-1,5-disulphonic acid sodium salt). This emulsion/tissue powder mixture was then continuously inverted at 4°C for 10 min before separating the phases by microcentrifugation (12,000 rpm) for 5 min. The upper aqueous phase was removed, the volume estimated, and 4-aminosalicylate sodium salt (Cat. No. A3505, Sigma Chemical Co., St. Louis, Mo.) added to a final concentration of 6% (wt/vol). After two more extractions with Solution A, the RNA in the aqueous phase was precipitated by adding 2.5 volumes of ice-cold ethanol, incubating at –20°C for 10 min, and centrifuging at 12,000 rpm for 20 min at 4°C. The precipitate was resuspended in 100 µl of sterile, RNAse-free water, extracted with 20 µl of water-saturated phenol, and the aqueous phase collected for subsequent RT-PCR (9).

RT-PCR. Conditions for RT-PCR were adapted from the protocol of Zimmermann (15), which describes the use of a single heat-stable enzyme from *Thermus thermophilus* (*Tth*) for both RT and PCR. The following components were added in sequence to reaction vials in a PCR thermocycler (Model TC1, Perkin-Elmer Cetus, Norwalk, Conn.) at 70°C: 1) 11.4 µl RNA/water mixture from the above RNA microextraction; 2) 2.0 µl reverse transcription buffer (100 mM Tris-HCl, pH 8.3, 900 mM KCl); 3) 1.0 µl (adjusted to 100 ng/µl) each of the forward and reverse primer of pair #86 [F:5′-CTATTCGCTTGGCTCAGATC-G-3′ and R:5′-CCAGAGTAGTGACTAGA-ACAGC-3′] or pair #307 [F:5′-GTGAAAG-TCTTGAGGATGC-3′ and R:5′-TTCATCT-CTATTGGCACCACG-3′] (cf. Fig. 1); 4) 2.0 µl of 9 mM $MnCl_2$; 5) 1.6 µl of a mixture of 10 mM dGTP, 10 mM dATP, 10 mM dTTP, and 10 mM dCTP. After overlaying this RNA/primer mix with 50 µl of mineral oil, 4 units (= 1 µl) of *Tth* enzyme (Amersham "Tet-z," Boehringer Mannheim "Tth" or Perkin-Elmer "rTth" may be used) 70°C for 10 min. To change the enzyme's activity from reverse transcriptase to DNA polymerase, 80 µl of DNA polymerase buffer (10 mM Tris-HCl, pH 8.3, 100 mM KCl, 1.88 mM $MgCl_2$, 0.75 mM EGTA and 5% glycerol) was added to each 20-µl RT reaction, the combined 100-µl volume briefly centrifuged to ensure mixing, and an additional 50 µl of mineral oil added. This mixture was then subjected to the following regime in the PCR thermocycler: 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 75°C (extended 1 s for each cycle). After the final cycle, there was an additional 5 min at 75°C, before cooling to 4°C.

Agarose gel electrophoresis of PCR products. Products of FC-RT-PCR reactions were electrophoresed in 1% agarose gels (Mini Sub-Cell, or Wide Mini Sub-Cell, Bio-Rad Laboratories, Richmond, Calif.) in Tris-acetate EDTA (TAE; 13) buffer at 40 V for 2 h, and stained with ethidium bromide (13). Sizes of FC-RT-PCR products were estimated by comparison with the migration of the 511-, 396-, 344-, 298-, and 220-bp bands of a 1-kb DNA ladder (Life Technologies, Gaithersburg Md.).

Combined FC-RT-PCR and dot blot assay (FC-RT-PCR-DBA). Products of FC-RT-PCR reactions were denatured in 0.03 N NaOH, neutralized with an equal volume of 1 M Tris-HCl, pH 7.5, and dot blotted to nylon membranes using a manifold (Pierce Easy-Titer, Model 77000, Pierce, Rockford, Ill.). The membrane was probed with digFC-RNA, and developed with anti-digoxigenin alkaline phosphatase (antiDIG-AP) and chromogenic substrate (7).

Southern blotting of FC-RT-PCR DNA and detection with DIG-labeled FC-riboprobe (digFC-RNA). After agarose gel electrophoresis, FC-RT-PCR DNA fragments were blotted to nylon membrane (Nytran, Schleicher & Schuell, Keene, N.H.) with a vacuum-blotting apparatus (Model 785, Bio-Rad) following the manufacturer's instructions. The blotted

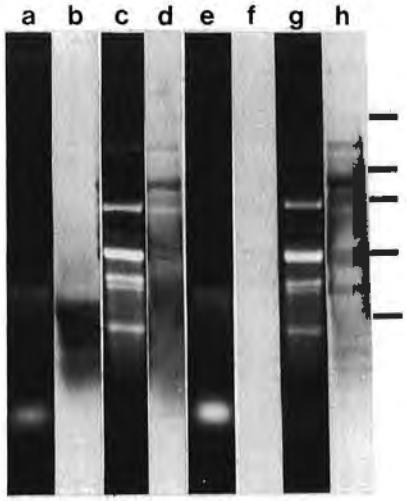

Fig. 2. Presence of flame chlorosis–specific double-stranded and single-stranded RNA (FC-dsRNA, FCssRNA) in diseased leaf tissue. Topologies of RNA had been confirmed as described (6,12). a) ssRNA stained with ethidium bromide; b) Northern blot of (a) probed with anti-message (cf. Fig. 1) digoxigenin-labeled flame chlorosis–specific riboprobe, digFC#21-RNA; c) dsRNA stained with ethidium bromide; d) Northern blot of (c) probed with digFC#21-RNA; e) ssRNA stained with ethidium bromide; f) Northern blot of (e) probed with message-sense digoxigenin-labeled flame chlorosis–specific riboprobe, digFC#23-RNA; g) dsRNA stained with ethidium bromide; h) Northern blot of (h) probed with digFC#23-RNA. Bars on right indicate positions of marker bands from 1-kb ladder: 3,054, 2,036, 1,636, 1,018 and 517/506 bp (top to bottom).

oven (Litton-Moffat 450 W). The blot was then hybridized with digFC-RNA riboprobe (cf. Fig. 1), and developed with antiDIG-AP and chromogenic substrate (7).

RESULTS AND DISCUSSION

FCssRNA as effective template in RT-PCR. The two primer sets, #86 and #307 (cf. Fig. 1), were effective in the RT-PCR

cDNA clones FC#23 and #21 (7), respectively, (data not shown). In symptomatic plant tissue, FCssRNA as well as FCdsRNA is present, as demonstrated by a comparison of Northern blots of ds- and ssRNA extracted from FC leaves (Fig. 2). The message-sense digFC-RNA riboprobe made from clone FC#23 (cf. Fig. 1) hybridizes only with FCdsRNA, while the anti-message sense riboprobe made from clone FC#21 hybridizes with both FCdsRNA and FCssRNA (Fig. 2).

The FCssRNA present constitutes only a small proportion of the ssRNA pool in diseased leaf tissue (Fig. 2A,B), whereas FCdsRNA accounts for all dsRNA detected in FC leaf tissue (6). Moreover, FCdsRNA bands may be discerned directly in gel electrophoresis of total nucleic acid extracted from FC leaf tissue (6). Only FCssRNA present in the pool of total extracted RNA can be amplified by FC-RT-PCR, as FCdsRNA cannot be melted and reverse-transcribed with our protocol (data not shown). When RNA extracted from FC leaves was used as substrate in RT-PCR with primer pairs #86 and #307, unique fragments were produced of the predicted sizes, 358 and 347 bp, respectively (Fig. 3A). The specificity of the amplification was further confirmed by Southern blot hybridization at high stringency (7) with FC#21 (digFC-RNA) riboprobe (Fig. 3B).

Sensitivity of detection, and potential for diagnosis from tissue containing low amounts of FC-RNA. In young, healthy cereal plant leaves, total RNA is (to the limit of detection) all in ssRNA form, and constitutes about 3×10^{-4} of the dry tissue mass (J. D. Procunier, *unpublished*). Although FCdsRNA may constitute as much as 1% of the dry cell mass in small localized areas of infected leaves (7), it does not greatly increase the total amount of nucleic acid that can be isolated from bulk leaf tissue (cf. Fig. 4A in reference 6). When primer pair #307 was used, FC-RT-PCR followed by dot or Southern blotting and hybridization to FC#21 riboprobe detected FCssRNA in as little as 100 fg RNA purified from FC leaf tissue (Fig. 4A,B). FCssRNA could therefore be expected to be detected from as little as 1 mg of test tissue if FCssRNA made up as much as 10^{-6} of total RNA. Indeed, FC-RT-PCR followed by hybridization with FC#21 riboprobe detected FC-RNA in 10 µl of a 100-µl extract made from 10 mg of lyophilized tissue of *Pythium arrhenomanes* Drechs. (Fig. 5; cf. reference 4).

Primer pair #86 was less effective than #307 (Figs. 3A,B). Computer analysis of X59248, the sequence on which the selection for FC-RT-PCR primers is based, indicates that reverse transcription must proceed through more RNA stem structures (not shown), and that the stem structures predicted to exist in the sequence covered by the #86 primers are more stable (11) than is the case with primer pair #307. To the extent that reverse transcription is impeded in the first step of RT-PCR, the differences in RNA secondary structure may contribute to the clear differences seen in overall efficiency of amplification. Even if the initial differences in the relative efficiencies of the RT step are small, the exponential effect of subsequent PCR amplification can result in substantial differences in yield.

An alternative explanation may be simply that the primers themselves account for the differences in sensitivity. This has been demonstrated recently in attempts to optimize amplification of gene elements of

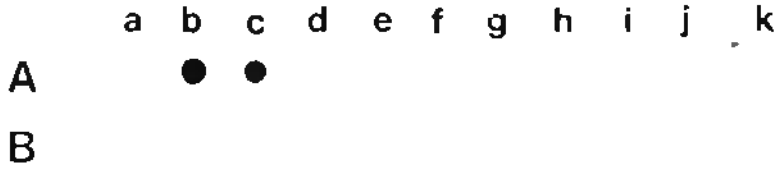

Fig. 3. Flame chlorosis–specific primer pairs #86 and #307 effective in amplifying flame chlorosis single-stranded RNA (FCssRNA) by reverse transcription-polymerase chain reaction (RT-PCR). (**A**) DNA product of RT-PCR amplification of FCssRNA extracted from diseased leaf tissue: a) RT-PCR with #86 primer; b) 1-kb DNA ladder; c) RT-PCR with #307 primer. (**B**) Southern blot of gel shown in (A) probed with digoxigenin-labeled flame chlorosis–specific riboprobe, digFC#21-RNA. The 1,636-bp and 396-bp bands of the 1-kb DNA ladder hybridize

Fig. 4. Titration of flame chlorosis–specific reverse transcription–polymerase chain reaction (FC-RT-PCR). Aliquots (10 µl) of PCR reactions employing primer pair #307 (cf. Methods) containing the equivalent of a) 10 ng RNA; b) 1 ng; c) 100 pg; d) 10 pg; e) 1 pg; f) 100 fg, g) 10 fg; h) 1 fg; i) 100 ag, j) 10 ag; k) no RNA were (**A**) spotted onto a nylon membrane, or (**B**) separated on an agarose gel

mycobacteria (8). In amplifying the same sequence region, new combinations of primers derived from slightly different positions of the original target sequence achieved 100- to 1,000-fold higher sensitivity (8).

Variants of FC-RT-PCR for different diagnostic formats. One of the advantages of the approach outlined here is that it combines sensitivity of detection with specificity of diagnosis, and allows choice among several variants offering different combinations of speed, convenience, and sensitivity. The simplest, fastest but also least sensitive variant is the direct visualization by gel electrophoresis and ethidium bromide staining of a DNA band of the predicted size. In the course of developing the RT-PCR protocols we established that this variant of FC-RT-PCR could detect 10 pg and 1 pg of run-off transcript FC-RNA using primer pairs #86 and #307, respectively (data not shown). When these FC-RT-PCR products are dot blotted and probed with digFC-RNA riboprobe (7) it is the stringent hybridization to a known probe, rather than the predicted size of an amplified product, that ensures that specific FC-RNA is being detected (Fig. 4A). Southern blotting of FC-RT-PCR products followed by hybridization with digFC-RNA riboprobe combines the sensitivity of dot blotting and two independent criteria, predicted size and specific hybridization, in ensuring specificity (Fig. 4B). The additional sensitivity afforded by dot blotting and Southern blotting of FC-RT-PCR products in practice allows FC-RNA to be diagnosed from 1 mg of lyophilized mycelium, compared with the 200 mg required for the conventional hybridization assay (4,7).

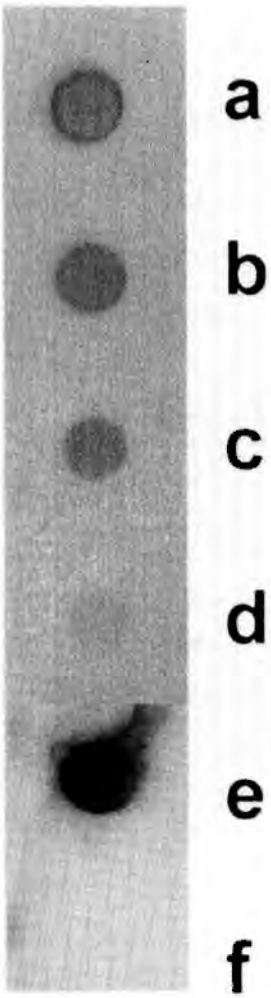

Fig. 5. Detection of single-stranded flame chlorosis RNA in *Pythium* mycelium. RNA extracted from 10 mg of lyophilized mycelium powder of *Pythium arrhenomanes* Drechs. (a through d) (cf. reference 4), or negative control *Pyrenophora teres* Drechs. (f) was subjected to flame chlorosis–specific reverse transcription–polymerase chain reaction (FC-RT-PCR) as described in Methods. From the 100-µl RT-PCR reaction volume: a) 50 µl; b) 5 µl; c) 1 liter; d) 0.1 µl and f) 50 µl were assayed by dot blot hybridization (6); e) 1 µl plasmid DNA contain-

In some applications, one may be willing to sacrifice some sensitivity to obtain the information of Southern analysis more quickly and easily. Instead of identifying the FC-specific DNA product by blotting and hybridizing to DIG-labeled probe, the DNA product can be labeled in the PCR reaction itself using DIG-labeled deoxyribonucleotides (Boehringer Mannheim). After agarose gel electrophoresis, DIG-labeled FC-specific DNA fragments can be blotted to nylon membrane and, after blocking, reported directly with enzyme-labeled antiDIG antibody. Alternatively, DIG-labeled FC-specific DNA fragments can be detected in situ, eliminating both the blotting and hybridization steps (S. Haber, *unpublished*). In preliminary trials we have found these variants of FC-RT-PCR to be intermediate in sensitivity between simple ethidium bromide staining and dot or Southern blot hybridization to DIG-labeled riboprobe (S. Haber, *unpublished*). The results of the in situ variant are available within 4 h of completing agarose gel electrophoresis, and the purple bands against a clear background are easily quantified with inexpensive gel-scanning apparatus (e.g., ISCO Model 1520, ISCO Instruments, Lincoln, Nebr.).

If, on the other hand, much greater detection sensitivity is desired, the FC-RT-PCR product could be further amplified in a second round of PCR using nested primers and *Taq* polymerase (15), and the products of the nested PCR hybridized to labeled probe in dot or Southern blots.

Use of RT-PCR to provide evidence that dsRNA is a product of viral infection. The use of RT-PCR extends the principle of diagnosing disease by detecting specific RNA. Detection and characterization of dsRNA has been advanced as a general approach for diagnosing infection viruses or viruslike agents not associated with the production of protein-coated virus particles (6,7). If the disease-specific dsRNA is a replicative intermediate of an infectious RNA rather than, for example, the product of simultaneous transcription off both strands of circular dsDNA (14), an ssRNA counterpart must also occur. Thus, when the nucleotide sequence of cDNA of the disease-specific dsRNA is at least partially known, RT-PCR can be used to amplify portions of the ssRNA counterpart to the disease-specific dsRNA. Detection of the amplified dsDNA product, on the basis of its predicted size and homology to specific probes, confirms the presence of disease-specific ssRNA when it might not have been detectable otherwise. An example of a specific question that this approach might shed light on is whether the dsRNA composing the putative avocado virus 1 is linked to viral infection, or perhaps the product of unusual transcription from the avocado genome (2).

We are now exploiting FC-RT-PCR to screen root tissue, and isolates of *Pythium* spp. and other fungi for RNA sequences specific to the FC viruslike agent.

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