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THE POLYMERASE CHAIN REACTION AND PLANT DISEASE DIAGNOSIS

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INTRODUCTION

The polymerase chain reaction (PCR) provides a simple, ingenious method to exponentially amplify specific DNA sequences by in vitro DNA synthesis. Three essential steps to PCR (Figure 1) include (a) melting of the target DNA, (b) annealing of two oligonucleotide primers to the denatured DNA strands, and (c) primer extension by a thermostable DNA polymerase (123). Newly synthesized DNA strands serve as targets for subsequent DNA synthesis as the three steps are repeated up to 50 times. The specificity of the method derives from the synthetic oligonucleotide primers, which base-pair to and define each end of the target sequence to be amplified. The use of PCR grew rapidly in plant pathology, as in other disciplines, with the introduction in 1988 of *Thermus aquaticus* (*Taq*) DNA polymerase. This enzyme exhibits relative stability at DNA-melting temperatures, which eliminates the need for enzyme replenishment after each cycle of synthesis, reduces PCR costs and allows automated thermal cycling.

PCR offers several advantages compared to more traditional methods of diagnosis: organisms need not be cultured prior to their detection by PCR;

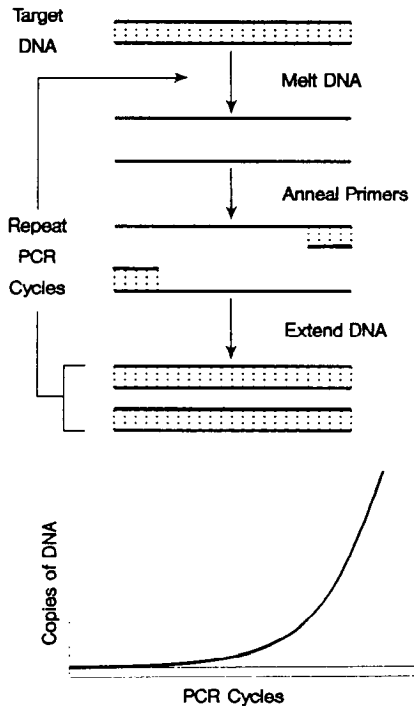


Figure 1 The stages of PCR and the resultant amplification of DNA copies of the target region. (Reprinted from Ref. 137 with permission.)

the technique possesses exquisite sensitivity, with the theoretical potential to detect a single target molecule in a complex mixture without using radioactive probes; and it is rapid and versatile. Similar to serology, both narrow and broad selectivities are possible and, depending on the choice of primers, the method facilitates the detection of a single pathogen or many members of a group of related pathogens. Unlike serology, the development of reagents with narrow or broad specificities is accomplished almost at will with lower cost. Synthesis of hundreds of different PCR primers generates costs comparable to those of developing only a few monoclonal antibodies.

Molecular genetic protocols in a variety of other disciplines employ PCR. Several reviews on its use and methodology in fields other than plant pathology have been published recently (4, 10, 11, 46, 46a, 91, 137). This review therefore focuses on the use of PCR for diagnosis of plant diseases and other applications in plant pathology.

DETECTION OF PHYTOPATHOGENS OR PLANT-COLONIZING MICROORGANISMS

The composition of a PCR mixture is quite simple—it consists of sample, water, buffer, salts, deoxyribonucleotide triphosphates (dNTPs), primers, and DNA polymerase. Despite this simplicity, the dynamics and physical chemistry of the PCR reaction itself are complex and not well understood. The major research emphasis to date has been development of new PCR applications rather than examination of PCR as a process. Many factors affect the specificity and efficiency of DNA amplification by PCR. As a result, optimization of reaction mixture composition and temperature cycling regime necessitates an empirical process. One often begins by designing a set of primers and then adjusting primer and buffer salt concentrations, as well as thermal cycle times and temperatures, until reaching the desired sensitivity and selectivity. Nevertheless, a wide range of plant pathogens in various hosts or environmental samples are detected using PCR (Table 1). In this section we review approaches to developing new diagnostic PCR procedures. It should be emphasized, however, that while there are many rules of thumb in the PCR literature, they should only be taken as initial guidelines.

Primer Selection

Although DNA or RNA fingerprinting is accomplished by several methods, random amplified polymorphic DNA (RAPD) analysis is appealing because it does not require genome sequence information or radiolabeling, and it distinguishes between organisms or even between different strains or isolates of the same organism (22, 22a, 31, 57, 127a, 131, 135a, 149, 159). RAPD fingerprinting uses PCR and a set of short, random-sequence oligonucleotide primers that produce characteristic profiles of amplified products for each organism. However, because DNA sequences of plants and other organisms are also amplified with random primers (22, 39), plant pathogens must first be purified or cultured from their hosts or reservoirs to obtain fingerprints. Hence, RAPD analysis is not useful for direct detection of plant pathogens in complex environmental samples, or for organisms that are difficult or impossible to culture. In contrast, with longer, more pathogen-specific primers, PCR allows detection of target sequences in crude specimens, without culturing.

Specific primers are derived from sequences of either amplified or cloned DNA (cDNA) or RNA from the microorganism to be detected. Several factors affect primer specificity for the target sequences, including primer length, annealing temperature, magnesium concentration, and secondary structure of target and primer sequences (114, 132; reviewed in 4, 10, 11, 46, 137).

Table 1 Amplification of plant pathogen/colonizer target sequences by PCR

Organism/Agent	Disease(s)/Host(s)	Target sequence/Sample treatment	Comments/References
<i>Viroids</i>			
Hop stunt	Hops	Viroid sequence; Total nucleic acid extracted from infected host tissue	Amplification and sequencing of viroid cDNA (112)
Apple scar skin Dapple apple Pear rusty skin	Pome fruit	Viroid sequence; Total nucleic acid extracted from infected host tissue	Amplification of viroid cDNA (58)
Citrus exocortis Cachexia Citrus viroid IIa	Citrus	Viroid sequence; Total nucleic acid extracted from infected host tissue	Amplification of viroid cDNA (165)
Grape viroids	Grapes	Viroid sequence; Total nucleic acid extracted from infected host tissue	Amplification of viroid cDNA (113)
<i>Viruses</i>			
Cauliflower mosaic virus (CaMV)	Cabbage	CaMV sequence; DNA extracted from single aphids fed on infected plant tissue	Detection of viral DNA in a single aphid; caulimovirus (92)
Bean golden mosaic virus (BGMV)	Bean	BGMV sequence; DNA extracted from infected bean	Detection and sequencing of viral DNAs; geminivirus (54)
Maize streak virus (MSV)	Maize and other grasses	Conserved geminivirus sequence; DNA extracted from infected maize	Detection and sequencing of viral DNAs; geminivirus (120)
Tomato yellow leaf curl virus (TYLCV)	Tomato	TYLCV; DNA extracted from whiteflies or infected tomato	Detection of viral DNA in a single whitefly; geminivirus (101)

Tobacco mosaic virus (TMV)	Tobacco	Transport protein gene sequence; RNA extracted from leaf tissue	Sequences amplified with <i>Tth</i> polymerase; tobamovirus (41)
Beet pseudo-yellows virus (BPYV)	Beet	BPMV sequences; Total nucleic acid extracts from infected host tissue	Detection of viral cDNA; closterovirus (29)
Beet western yellows virus (BWYV) and beet mild yellowing virus (BYMV)	Sugar beet oilseed rape	BMV- and BWYV-specific sequences; Total plant nucleic acid extracts	Detection of BWYV and viral cDNA; differentiation of BWYV and BMV luteoviruses (72)
Luteoviruses	Cereals, sugar beet, and potato	Conserved luteovirus sequences; Total plant nucleic acid extracts	Detection of viral cDNA of barley yellow dwarf, beet western yellows and potato leafroll luteoviruses Differentiation by RFLP analysis (115)
Bean yellow mosaic virus (BYMV)	Gladiolus	BYMV-specific sequences; Total plant nucleic acid extracts	Detection of viral cDNA in leaves and corms; potyvirus (154, 155)
Pea seedborne mosaic virus (PSBMV)	Pea	PSBMV-specific sequences; Total plant or seed nucleic acid extracts	Detection of viral cDNA; potyvirus (78)
Plum pox virus (PPV)	Pome fruit	PPV-specific sequences; Total plant nucleic acid extracts	Detection of viral cDNA using biotin-label in PCR reaction; potyvirus (79)
PPV	Pome fruit	PPV-specific sequences; Nucleic acid extracts of antibody-bound virus	Combines selectivity of both serology and PCR (161)
Potyvirus	Various	Conserved potyvirus sequences Extracts from purified virus or total plant nucleic acid extracts	Detection of viral cDNA, used to generate primers (80)

Table 1 (continued)

Organism/Agent	Disease(s)/Host(s)	Target sequence/Sample treatment	Comments/References
Cherry leafroll virus (CLRV)	Walnut	CLRV-specific sequences; Crude tissue extract	Detection of viral cDNA; sample not treated with phenol or protease; nepovirus (16)
Fiji disease virus (FDV)	Sugarcane	FDV-specific sequences; Total plant nucleic acid extracts	Detection of viral cDNA; plant reovirus (135)
Fungal viruslike particle (VLP)	<i>Cryphonectria parasitica</i>	VLP-specific sequences; Total fungal nucleic acid extracts	Detection of viral cDNA; (26)
<i>Bacteria</i> <i>Agrobacterium tumefaciens</i>	Tumors (galls) <i>Vitis</i> spp. (grape)	<i>iaaH</i> and <i>iaaM</i> pathogenicity genes of Ti plasmid; DNA extracted from purified isolates	With most isolates the presence of Ti pathogenicity genes (positive PCR results) correlated with pathogenicity tests (40)
<i>Erwinia amylovora</i>	Pome/ fire blight	Plasmid sequences; boiled bacteria and infected plant tissue; inclusion of Tween 20 in reaction buffer increases sensitivity	<i>Tth</i> polymerase used (12)
Mycoplasma-like organisms (MLOs)	Various	Conserved rDNA sequences; DNA extracted from infected plants	Detects many MLOs; use RFLP to distinguish among isolates (1, 34, 82-84)
Mycoplasma-like organisms (MLOs)	Periwinkle	Cloned MLO sequences; DNA extracted from infected plants	Detection of MLO DNA (37)
<i>Pseudomonas solanacearum</i>	Bacterial wilt/wide host range	Specific sequences cloned by subtractive hybridization; boiled bacteria and infected plants	Detection of 5-116 bacterial cells, depending on the strain; also sensitive detection in potato field trials (128)

<i>Rhizobium leguminosarum</i>	Nitrogen fixation/beans	<i>npt</i> gene of Tn5; boiled nodule tissue; DNA extraction from soil	Detection in nodules and soil (110); other bacteria harboring Tn5 would also be detected
<i>Xanthomonas campestris</i> pv. <i>citri</i>	Citrus/bacterial canker	Plasmid sequences with high G+C content; either purified bacterial DNA or boiled, crude water extracts in buffer containing Triton-100	Target sequences detected in lesions, from which viable bacteria could not be cultured (61)
Fungi			
<i>Colletotrichum gloeosporioides</i>	Various/wide host range	ITS1 and conserved rDNA; DNA extracted from infected tomato plants	(98a)
<i>Gaeumannomyces graminis</i>	Root and crown diseases/cereals and grasses	Mitochondrial (mt) DNA; boiled, infected plant tissue	Use of nested primers to increase specificity, but assay does not distinguish between three fungal varieties (45, 63)
<i>Glomus intradices</i> and <i>Gigaspora margarita</i>	Vesicular-arbuscular endomycorrhizae (VAM) cultured on carrot roots	rDNA of mt DNA; DNA extracted from purified isolates	Amplified DNA used as a probe to distinguish RFLPs among the three fungal varieties (158)
<i>Glomus vesiculiferum</i>	VAM colonizing leeks	18S rDNA; spores crushed with chelex resin followed by 4 freeze-thaw cycles	Primers specific for VAM fungi (133, 134)
<i>Laccaria</i> spp.	Ectomycorrhizae colonizing conifers	18S rDNA; DNA extracted from colonized roots	(133)
		ITS of nuclear rDNA repeat; mitochondrial large subunit ribosomal RNA gene; DNA extracted from pure cultures or from colonized roots	(158)

Table 1 (Continued)

Organism/Agent	Disease(s)/Host(s)	Target sequence/Sample treatment	Comments/References
<i>Leptosphaeria maculans</i>	Black leg/crucifers	ITS 1 and conserved region of nuclear rDNA repeat; DNA extracted from infected canola leaves	Pathotype-specific detection (164)
<i>Phoma tracheiphila</i>	Wilt/lemon	Unknown, but <i>P. tracheiphila</i> -specific; DNA extracted from infected tree branches, or boiled mycelium from tree branches	Fungus detected in lignified plant tissue (118)
<i>Phytophthora</i> spp.	Various/wide host range	ITS of rDNA; extracted fungal DNA	Amplified DNA probed with labeled, species-specific oligonucleotides (85a)
<i>Pyrenophora teres</i>	Net, spot blotch/barley	Unknown, but low-copy, <i>P. teres</i> -specific; boiled, infected barley tissue	B. M. Baltazar, A. L. Scharen, V. Raboy (unpublished data)
<i>Verticillium albo-atrum</i> and <i>V. dahliae</i>	Wilt diseases/wide host range	ITS 1 and 2 of nuclear rDNA; DNA extracted from fungal spores or infected hosts	Fungal biomass quantitated (66, 102)
<i>Mycosphaerella musicola</i> and <i>M. fijiensis</i>	Leaf spots/banana, plantain	ITS 1 and conserved region of rDNA; DNA extracted from purified isolates or from infected leaves	(70)
<i>Nematodes</i>	Root-knot/wide host range	Mitochondrial DNA from individual eggs and juveniles	Restriction polymorphisms in amplified DNA used to distinguish species and populations (60)
<i>Meloidogyne incognita</i>			
<i>M. hapla</i>			
<i>M. javanica</i>			
<i>M. arenaria</i>			

Specific oligonucleotide primers are typically 18–30 nucleotides in length, about 50% in G+C content, and without complementary 3' ends or inverted repeats. However, PCR can be effectively executed with specific primers as short as 14 and 15 nucleotides in length (115) or of only 25% G+C content (R. French & N. L. Robertson, unpublished observations). Primer “melting” or “annealing” represents a bimolecular association/disassociation of oligonucleotide and target DNA so that T_m (melting temperature) depends on both primer and target concentration, which change during amplification. Rychlik et al (122) considered the thermodynamic nature of primer melting to develop a formula to predict optimum annealing temperatures that agree with experimental results over a variety of primer/template combinations. Other formulae or computer programs that help in selecting appropriate primer sequences are also available (64, 97, 121).

There are several useful controls to use when first developing a PCR detection method. Besides known positive and negative controls, it is helpful to set up parallel reactions containing each primer individually to determine if PCR amplification is truly dependent on the presence of both primers. This is particularly important if the size of the PCR product is larger or smaller than expected. If PCR yields little or no product, one can test the reaction and thermal cycle conditions with a known set of reagents, primers, and template that are available commercially in kits. Adding aliquots of test samples to such reactions is also an easy method to detect inhibitors in samples. Lack of specificity can often be corrected by raising annealing temperature or reducing the initial concentration of template or primers.

PCR amplifies DNA sequences up to 10 kilobase pairs in length (73), but shorter sequences on the order of 100 to 1000 base pairs (bp) are most efficiently amplified and easily resolved by agarose electrophoresis. Hence, DNA sequences within a few hundred base pairs are usually chosen as primer annealing sites. Single-stranded oligonucleotides are routinely used to prime amplification reactions. However, Sarkar & Sommer (127) recently used “megaprimers,” i.e. double-stranded DNA sequences up to several hundred bp in length, to amplify human gene sequences. Megaprimers, which are PCR products themselves, could also add specificity to PCR tests because of their additional length.

While almost any nucleic acid may be amplified by PCR, amplification of mRNA and single- or double-stranded viral RNA requires modification of the procedure shown in Figure 1 (135, 141). RNA is typically reverse-transcribed into cDNA before amplification by *Taq* polymerase, but more direct RNA amplification is also possible using the manganese-dependent reverse transcriptase activity of *Thermus thermophilus* (Tth) polymerase (41, 46). Because of extensive genetic heterogeneity with many RNA viruses, the use of highly conserved sequences for primer design is recommended for detecting all

individuals of a particular virus (20, 117). Alternatively, all members of a group of viruses may be detected using degenerate primers derived from conserved, reverse-translated viral amino acid sequences (80, 103, 115).

Any DNA or RNA sequence that is specific for a particular organism can be used for PCR detection of that organism. For example, sequence data from a cloned mitochondrial (mt) DNA fragment specific to *Gaeumannomyces graminis* facilitated primer design for fungal DNA amplification in infected wheat or bermudagrass (63). Primers have also been based on pathogen-specific plasmid sequences, such as those used to develop PCR diagnostic assays for *Agrobacterium tumefaciens* (40), *Erwinia amylovora* (12), and *Xanthomonas campestris* pv. *citri* (61). Other potential targets for specific amplification include the short, interspersed repetitive elements present in bacteria (35, 94), and the species-specific tandem DNA repeats in many eukaryotes, including potato cyst nematodes (139). A strain or isolate can also be marked, or transformed, with a known gene and primers specific for the reporter gene used to detect the isolate. For instance, primers specific for the *npt* gene of bacterial transposon Tn5 detect *npt* sequences in soil and root nodules inoculated with a Tn5-marked strain of *Rhizobium leguminosarum* (110).

DNA fragments specific for *Pseudomonas solanacearum* have recently been cloned by subtractive hybridization (128). This technique enriches for nucleic acid sequences specific for a particular organism or strain by hybridization and subsequent removal of sequences that are in common with other organisms. In this study for example, DNA sequences from *P. solanacearum* in common with sequences from *Xanthomonas campestris* pv. *vesicatoria* were removed by hybridization with excess, sheared DNA of *X. campestris*, and the remaining fragments, enriched for sequences specific to *P. solanacearum*, were cloned. Primers derived from these cloned fragments specifically amplify DNA from all except three of the 85 strains of *P. solanacearum* tested, and from field potatoes infected with *P. solanacearum*. Subtractive hybridization has also expedited isolation of strain-specific DNA from *Rhizobium leguminosarum* bv. *trifolii*. DNA fragments recovered from a strain of *R. leguminosarum* following subtractive hybridization failed to hybridize with genomic DNA from seven closely related strains of *R. leguminosarum* bv. *trifolii* (15). Sequences derived from these strain-specific fragments could be used to design primers for detection of this particular strain.

Ribosomal genes and the spacers between them provide attractive targets for molecular detection and phylogenetic studies because they occur in high copy numbers, possess conserved as well as variable sequences, and can be amplified and sequenced with universal primers based on their conserved sequences (7, 19, 83, 136, 157). Genes for 5.8S, 17S (18S), and 26S (28S) fungal nuclear ribosomal RNA genes (rDNA) are usually organized in

head-to-tail tandem, identical repeats with 60–200 copies per haploid genome (discussed in 19, 166). Despite conservation of fungal rRNA genes, sufficient sequence variation can exist to allow their use as targets for differential amplification. Simon et al (133) describe the use of primers based on 18S rRNA gene sequences to specifically amplify vesicular-arbuscular endomycorrhizal (VAM) fungal DNA from complex samples that included other fungal and plant DNAs.

Considerably greater sequence variation is found in the internal transcribed spacer (ITS) regions between the rRNA genes within a rRNA repeat unit (rDNA; 70, 102, 106, 164, 166). Even more sequence differences are in the nontranscribed spacer (NTS) regions between the rDNA repeat units and still more in the intergenic spacer (IGS or IGR) regions, or noncoding sequences that occur within the rDNA repeat unit of some fungi (75). In principle, any organism or even different strains or isolates of one organism that has rDNA repeats may be specifically detected by selecting primer sequences based on variable spacer regions. Nazar et al (102), for example, found adequate sequence differences in the ITS regions of the wilt fungi, *Verticillium dahliae* and *V. albo-atrum*, to design primers that specifically amplify the DNA of each species (Figure 2). Primers based on differences in ITS 1 sequences of *Leptosphaeria maculans* allow specific amplification of either weakly or highly virulent isolates of this fungal pathogen, the latter being detectable in infected canola leaves even before symptom development (164).

Less specific, or even degenerate primers often amplify similar-sized DNA

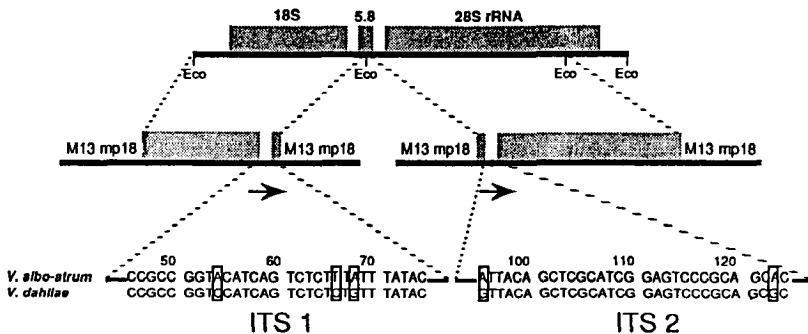


Figure 2 Sequence differences in the intervening transcribed sequences of rDNA from *Verticillium albo-atrum* and *V. dahliae*. Genomic libraries of *EcoRI*-digested fungal DNA were prepared and inserts complementary to the 5.8S rRNA were subcloned in M13 mp18 for DNA sequencing. Shaded regions indicate DNA corresponding to the mature rRNA sequences and arrows indicate the direction and extent of DNA sequencing. Regions of sequence difference that were subsequently used for hybridization probes and PCR primers are indicated below each subclone; the nucleotide differences are enclosed in boxes. (Reprinted from Ref. 102, with permission.)

fragments of related organisms that are distinguished by restriction enzyme analysis or fragment length polymorphisms (RFLPs; 9, 32, 35, 75, 90, 100, 158, 160). For example, related plant viroids or viruses have sequences in common that are exploited to amplify group-specific sequences subsequently differentiated by RFLP analyses (58, 80, 103, 115, 120,165). For PCR products greater than 500 bp, restriction endonucleases with four-base recognition sites are usually adequate, since any particular four-base sequence occurs an average of once per 256 bases. However, amplified DNAs of less than 500 bp in length are unlikely to contain sufficient recognition sites to allow differentiation between particular isolates. Recently, a number of restriction endonucleases have been isolated from virus-infected eukaryotic green algae (151). One of these, *CviJI*, has the recognition sequence purine-A-G-pyrimidine, statistically equivalent to a three-base recognition site, which should occur an average of once per 64 nucleotides. *CviJI* allowed distinction between the PCR products of barley yellow dwarf virus (BYDV) field isolates (Figure 3; R. French & N. L. Robertson, unpublished data). By

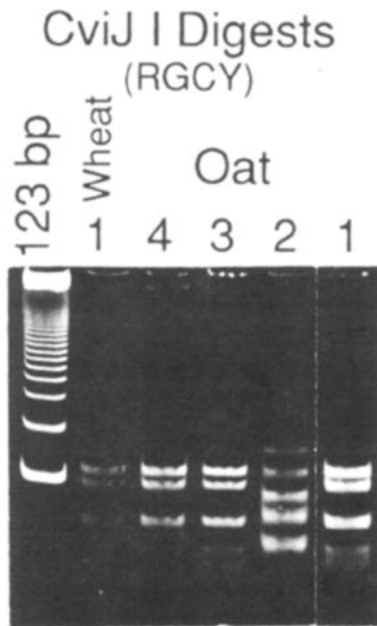


Figure 3 RFLP mapping of PCR products of barley yellow dwarf virus (BYDV) with *CviJI*. Luteovirus-specific primers (115) were used to amplify BYDV cDNAs from one sample from wheat and three from oats. The rightmost lane contains *CviJI*-digested PCR products of the type isolate of BYDV-PAV and the leftmost lane contains the 123-base pair ladder (BRL) DNA size markers.

hybridization analysis, these isolates are indistinguishable from the PAV strain of BYDV (115), but *Cvi*JI digestion readily differentiated an oat isolate from the others (Figure 3).

Sensitivity and Quantitation

The sensitivity of detection by PCR varies, and for diagnostic tests of prevalent phytopathogens, reduced sensitivity may be desirable. Target sequences and sample treatments should be chosen accordingly. That is, multi-copy target sequences allow greater sensitivity than single- or low-copy target sequences. PCR reaction conditions also affect sensitivity. For example, target sequences of *X. campestris* pv. *citri*, which have a high G+C (61%) content, are amplified in a buffer with pH 9.0 and 1% Triton X-100 (61) but not in standard buffer (98).

PCR sensitivity can be increased with the use of labeled probes or primers (48, 61, 72, 153), but single-copy detection is sometimes achieved without their use (28). Additional rounds of amplification add sensitivity (10, 11, 110, 137; R. Warren & J. M. Henson, unpublished observations); however, this is costly and increases the risk of contaminating reactions. It is also possible to achieve greater sensitivity with more thermostable polymerases, such as the Stoffel fragment of *Taq* or the DNA polymerase of *Sulfolobus acidocaldarius*. These enzymes have longer half-lives at denaturation temperatures and therefore withstand more DNA amplification cycles than *Taq* polymerase (reviewed in 4, 10, 11).

Sample complexity also influences detection sensitivity. For instance, as few as five cells (from 1 μ l of broth culture) of *P. solanacearum* produced positive PCR reactions, even with a 20- or 200-fold excess of cells of *Erwinia herbicola* or *Xanthomonas campestris* pv. *campestris*; however, 2,000 and 20,000-fold excesses of other bacteria decreased detection by tenfold (128).

PCR is capable of quantifying relative differences as well as absolute amounts of scarce target RNA or DNA sequences, and is often more sensitive than traditional methods for nucleic acid quantitation (reviewed in 17, 50). One of the most facile quantitative methods is the inclusion of known amounts of reporter or internal control sequences in PCR reactions (17, 39a, 66, 67, 76, 134, 142), and there are simple procedures for generating these competitor DNA fragments (39a, 53, 148). The internal control sequence is usually amplified with the same primers and is identical to the target sequence except for its slightly different size that distinguishes it from target DNA amplification product on gels (39a, 53, 148). However, use of a heterologous internal control sequence that is amplified with the same primer pair as the target sequence can avoid the generation of artifactual PCR products caused by heteroduplex formation between the target sequences and the internal control sequences during amplification (66). It is assumed that the amplification

efficiency of control sequence is the same as that of the target sequence; the amount of target sequence in the initial sample is calculated from the amount of initial concentration of control sequence and comparison of the ratio of the quantities of amplified target and control amplified products.

The number of target sequences in samples are also quantitated with limiting dilutions and Poisson statistics (140). That is, at the limit of dilution of a specific sequence, some PCR reactions will be negative and some positive. The number of targets present in the undiluted sample is calculated by the Poisson distribution. Although it requires several replicate reactions for each sample, this method is simple and quantitates the number of sequences initially present in samples instead of calculating initial amounts based on amounts of amplified product.

Quantitation of phytopathogens in diseased plants is desirable, especially with ubiquitous phytopathogens that are present on healthy plants or in healthy soils, and disease is a matter of degree of infection or infestation. Changes in pathogen inoculum levels (e.g. in soil) could also be monitored by quantitative PCR. For example, it would be helpful to predict the probability and severity of take-all disease of wheat caused by the soilborne fungus *G. graminis*, based on PCR-determined estimates of the amount of *G. graminis* in field soil.

Robb and colleagues (66) have recently monitored the spread of *Verticillium dahliae* and *V. albo-atrum* in individual, infected host plants by PCR quantitation of fungal biomass. Although the traditional plating method for detecting this fungus generally correlates with PCR detection, PCR is more sensitive. Target sequences were detected by PCR immediately after host inoculation (spore infusion) in top stem segments of host plants, but the pathogen was undetected by the traditional assay until 24 hr after inoculation. In addition, fungal quantitation by plating methods requires 3–4 days, with up to six weeks for positive fungal identification, whereas PCR detection requires only one day. Quantitative PCR analyses of resistant and susceptible host cultivars also suggests that *V. albo-atrum*-resistant alfalfa limits initial fungal colonization, whereas *V. dahliae*-resistant sunflower rapidly eliminates the fungus after initial colonization (66). Quantitative PCR will likely be helpful for determining what stage of pathogenesis is inhibited in other resistant host cultivars. Recently, quantitative PCR has also been used to measure the resistance of human immunodeficiency virus to antiviral drugs (47), and similar drug susceptibility testing of phytopathogens is feasible.

Quantitating target sequences is especially useful for estimating the biomass or number of cells of unculturable microorganisms or obligate biotrophs, such as the endomycorrhizal fungi, which are difficult to enumerate in colonized

plants. For example, in competitive PCR reactions with internal rDNA sequences, Simon et al (134) determined the number of vesicular-arbuscular mycorrhizal 18S rRNA genes (an indicator of VAM biomass) in leek roots colonized by *Glomus vesiculiferum*.

It is difficult to confirm the presence of viable pathogens in diseased plants that produce positive PCR reactions but from which the pathogen cannot be cultured. For example, plasmid DNA sequences from *X. campestris* pv. *citri* were detected in 7-month old dried lesions on inoculated grapefruit leaves, but viable organisms were not recovered (61). Even free-living bacteria that are typically culturable can exist in viable but unculturable states (30, 163a). *G. graminis* is also difficult to culture from soil known to be infested with the fungus based on wheat infection assays (33). Atlas and colleagues (95) used quantitative PCR to observe an increase in giardin mRNA after excystation induction in viable, but not killed, *Giardia* cysts. Similarly, viability of plant pathogens detected by PCR could be confirmed by measuring induction of pathogen-specific mRNA.

Microbial detection methods that combine antibody binding and PCR are especially sensitive, and they detect microbial antigens in addition to, or instead of, their nucleic acids. Hence, these methods are possibly better indicators of microbial viability. For instance, Wetzel et al (161) used specific antibody to concentrate plum pox potyvirus from diseased trees before amplifying a specific RNA sequence of the "captured" viral particles. Antibody-coated magnetic particles were recently used to extract *Salmonella* bacteria from samples prior to PCR amplification (162). Immuno-PCR is another extremely sensitive antigen detection technique (125). In this method a DNA fragment is molecularly linked to antigen-antibody complexes. Protein A and streptavidin portions of the linker molecule bind antibody and DNA, respectively. Antigen present in a sample binds specific antibody, which, in turn, binds the linker molecule. The latter is bound to a nonspecific, biotin-labeled DNA sequence that is subsequently amplified by PCR.

In each of these studies the sensitivity of detection was improved dramatically. For example, immunocapture PCR was 250 times more sensitive than direct PCR for detection of plum pox potyvirus (161). Immuno-PCR detected 580 antigen molecules, or was 10^5 times as sensitive as the enzyme-linked immunosorbent assay for detection of antigen. In principle, it would allow detection of a single antigen molecule (125). Immuno-PCR requires only antigen-specific antibody, whereas immunocapture requires antigen-specific antibody and nucleic acid sequence information from the microbe being detected. Soon it may be feasible to combine immunocapture and RAPD fingerprinting, which would also require specific antibody but not specific primers.

Other Considerations

Viability of an organism is not required for detection or amplification of target nucleic acid sequences, making PCR an extremely powerful tool for the analysis of ancient DNA (77), or for the identification or fingerprinting of hazardous, infectious microorganisms. Even autoclaved material containing target sequences can produce specific amplified PCR products (8, 43)! However, because it is possible for a single copy of contaminating target sequence to produce a positive PCR result, contamination problems occur easily. Thus, diagnostic PCR assays require numerous negative controls. False-positive reactions result from contamination introduced by aerosols, hair, skin, gloves that have touched a surface with target sequences, or contaminated reagents, including commercial preparations of *Taq* DNA polymerase (28, 116, 124). Some PCR operators apparently become target DNA “carriers” or “shedders” and frequently contaminate reactions (28). Methods to reduce or eliminate contamination include ultraviolet irradiation of reagents (43, 49) and treatment of reagents or PCR reactions with exonucleases (99, 116). Also, dUTP may be substituted for dTTP in PCR reactions. When amplification is followed by uracil DNA glycosylase treatment, reaction products cannot serve as templates for further amplification, thereby reducing contamination due to carryover of PCR products (143). However, each of these treatments has associated problems or expense, and the best defense against false-positive reactions is meticulous laboratory technique and clean-room standards (28, 111).

Another potential problem with PCR reactions is the amplification of products other than those predicted. Background amplification not only confuses test results, it interferes with amplification of predicted products by consuming reaction reagents. Additional products include single-stranded DNA (150), or result from mis-priming or amplification of primer artifacts (“primer dimerization”). Background is reduced or eliminated by using “hot start” (28) or “heat-soaked” (119) PCR—procedures that ensure initiation of reactions at denaturation temperature. Mis-priming, or annealing of primers to alternative sequences is also influenced by reaction conditions such as magnesium concentration or unknown factors in samples containing target sequences. For example, expected products were amplified in *G. graminis* PCR reactions if hyphae were picked from Luria or potato dextrose agar medias, but not if they were picked from SM7 agar, a selective medium for *G. graminis* that contains L-DOPA and several antibiotics (45).

Increasing primer annealing specificity reduces background amplification (114, 126, 132; reviewed in 4, 11, 46, 137). The use of nested primers in PCR diagnostic tests also improves specificity (2, 63, 168). For example, PCR detection of *G. graminis* DNA using one set of outside primers amplified

a mitochondrial fragment of approximately 450 bp. Reaction products were subsequently diluted into a second reaction with nested primers that specifically amplified sequences within the 450-bp sequence. Reactions with only the outside or inside primers were less specific, and they amplified products from other fungi as well as *G. graminis* (63, and references therein). Unfortunately, diagnostic tests that use nested primers and two rounds of amplification increase expense as well as specificity. Twice as much DNA polymerase, primers, and thermal cycling time are required for nested primer tests. In addition, reactions are more easily contaminated because of additional reaction tube handling, although recently a method has been described in which nested primers are used in a single reaction tube (168). Finally, first-round primers can interfere with second-round amplification, necessitating their removal. Exonuclease VII efficiently eliminates oligonucleotide primers without interfering with subsequent amplification (86), but this treatment increases costs.

A related method, hybrid-selected template amplification, has been used to increase specificity and allow detection and cloning of an influenza viral DNA sequence that was previously difficult to detect after PCR amplification due to high background (93). First-round amplification products are diluted into a second reaction with a set of nested primers to amplify a smaller product. Second-round product is then used to select by hybridization the larger, desired product of the first round of amplification. Hybrid-selected first-round products are finally amplified in a third reaction to produce the initial amplification product of larger size without background amplification.

The expense of *Taq* polymerase, primers, high quality agarose, and thermal cyclers is prohibitive for routine diagnostic testing of some phytopathogens, especially those easily cultured and identified. However, because of the speed and relative simplicity of PCR assays, a comparative reduction in labor costs offsets some of these expenses. As additional diagnostic procedures are developed, it should be possible to assay for several pathogens simultaneously using multiple sets of pathogen-specific primer pairs, or "multiplexing" (reviewed in 10, 11), which would further reduce costs per assay. Also, other polymerases are now commercially available and offer additional advantages; for example, *Thermococcus litoralis* polymerase has a lower error rate during DNA synthesis than *Taq* polymerase, *Thermus thermophilus* (*Tth*) polymerase has both DNA- and RNA-dependent DNA synthetic activities, and *Sulfolobus acidocaldarius* DNA polymerase is more thermostable than *Taq* (reviewed in 4, 10, 11).

Sample Preparation

Target DNA or RNA has been amplified from seemingly intractable material, including autoclaved bacterial culture slants (8), patient stools (2), mummies and ancient sediments (reviewed in 77), insects or their saliva or hemolymph

(52, 92, 101, 107), insects fossilized in amber (23, 38), paraffin-embedded tissue (138), fixed cells on microscopic glass slides (167), sorted chromosomes (25), agarose gels (14), soil (18, 68, 109–110, 130, 147), sewage/sludge (147a), large volumes of water (10, 144, 157), fungal hyphae or spores (24, 63, 133), and either healthy or diseased plant tissue (36, 81; Table 1). Target sequences are usually purified or treated to remove DNA polymerase inhibitors, such as polysaccharides (36) or phenolic compounds or humic substances (24, 68). Some purification or treatment is probably required for maximum sensitivity of target sequence detection in most environmental samples. However, if the sample contains many copies of the target sequence, simply boiling the sample for a few minutes is often adequate preamplification treatment for qualitative detection of the target sequence. Target sequences from as few as five boiled, bacterial cells of *P. solanacearum* from pure culture are amplified sufficiently to generate products visible on agarose gels (128). Rollo et al (118) used boiled mycelium collected from infected lemon trees to detect the fungal pathogen *Phoma tracheiphila*, and boiling infected barley leaf tissue was sufficient to detect specific DNA sequences of *Pyrenophora teres* (B. Baltazar, A. Scharen, V. Raboy, unpublished data). Boiled wheat roots or crowns infected with *G. graminis*, infested oat seeds used as field inoculum, or even a single, boiled ascospore (containing 100–1200 copies of mitochondrial DNA target molecules) apparently released enough target mtDNA to produce visible amplified products on agarose gels (63).

This PCR test for *G. graminis* uses two rounds of amplification and nested primers, and it is possible that any DNA polymerase inhibitors present in the first round of amplification are removed or reduced by dilution during the subsequent second round of amplification. However, inhibition of DNA polymerization by soil is not completely overcome by such dilution. Amplified products are not produced from samples containing up to 40 ascospores when 1 gm of soil is added. Yet samples with entire perithecia containing up to 100 ascospores are positive, indicating that even soil inhibition is overcome if large numbers of target molecules are present in the sample (63).

Removal of PCR inhibitors from samples is frequently accomplished by simple procedures, including treatment with cation-exchange resins (68, 133, 138) or polyvinyl pyrrolidone (PVP), which binds polyphenolic compounds (24, 71). PVP treatment also removes inhibitors from yeast, insect, and nematode samples (M. E. John, personal communication). Detection of *G. graminis* in soil was improved by grinding samples with insoluble PVP before boiling, but detection was most sensitive with samples that were boiled in buffer and centrifuged through Sephadex G200 columns (147; T. Goins & J.

M. Henson, unpublished observations); five boiled ascospores in 1 gm soil are detectable using the latter method. Borja & Ponz (16) find that simply diluting crude walnut bark extracts with reverse transcriptase buffer containing dilute ionic and nonionic detergents allows efficient cDNA synthesis and subsequent PCR detection of cherry leafroll virus. Sample dilution alone permits amplification of previously inhibited reactions from host plants infected by but resistant to *V. dahliae* (66).

Additional Amplification Procedures

Besides their expense, thermal cyclers present other problems in the development of PCR diagnostic tests. Their designs vary and can affect product yields and sizes, background, and well-to-well reproducibility (65, 74). Recently developed alternative amplification techniques are isothermal, and these techniques could reduce overall costs as well as variable amplification results (11, 13, 56, 91, 156). One of these, called self-sustained sequence replication, or 3SR, mimics the replication of retroviruses (13, 56). It is similar to PCR in that two primers are used, but in addition to gene-specific sequences, primers also incorporate promoter sequences for a phage RNA polymerase. Using a combination of reverse transcriptase, RNA polymerase, and RNase H, target-specific cDNAs and RNAs are continually produced. Another approach uses *cis*-acting RNA phage sequences coupled to a target-specific RNA probe (13,91). After hybridization, unbound probe is washed off and any bound probe is amplified to detectable concentrations by adding phage replicase. Although most of the isothermal amplification techniques detect RNA sequences, one called strand displacement amplification targets specific DNA sequences (156). The ligase chain reaction (LCR; reviewed in 11, 13, 163) still employs the alternating melting and annealing cycles of PCR, but may be less sensitive to variation in heating and cooling times. LCR detects single base-pair differences in target DNA sequences; for example, an LCR assay based on single base-pair differences in their 16S rRNA genes distinguishes closely related *Listeria* species (163). However, LCR requires four synthetic oligonucleotides per reaction.

OTHER APPLICATIONS AND CONCLUSIONS

PCR is often employed in phylogenetic studies to amplify genetic material, usually ribosomal or transfer RNAs or the spacers between them. Amplified sequences are compared and used to discern evolutionary relationships of organisms, including plant pathogens or soil microorganisms (7, 77, 96, 107, 129, 136, 157). For example, Lee & Taylor (85) use the internal transcribed spacers of the rDNA region to infer a phylogenetic tree of several

Phytophthora species. Recently, analysis of sequences of ribosomal-protein genes demonstrated that aster yellows-type mycoplasma-like organisms (MLOs), which are pathogenic for members of the genus *Oenothera*, are more closely related to *Acholeplasma laidlawii* (a nonsterol-requiring acholeplasma) than they are to sterol-requiring animal mycoplasmas (88). Phylogenetic studies facilitated by PCR are also used to measure biodiversity in particular ecosystems; reviewed in 21, 157). In addition, denaturing gradient gel electrophoresis of amplified 16S ribosomal RNAs from complex ecosystems has recently been employed to profile microbial communities (99a).

Classification of pathogen isolates may also be simplified using a combination of morphological and molecular characteristics. For instance, obtaining the sexual stage of *G. graminis* var. *graminis* takes weeks in the laboratory, and it would be faster to identify this fungus based on its morphologically characteristic adhesive cells (hyphopodia) and a positive PCR test (45).

PCR is already being used to advance studies of host-pathogen interactions. Cloning or gene-synthesis strategies for pathogen or pathogen-induced host genes or cDNAs often include PCR procedures (5, 27, 62, 69). PCR could also be used to construct pathogen genomic or cDNA libraries (169), or it could be used to construct libraries of host or pathogen genes that are differentially expressed during the infection process (42, 59). In addition, genetic mapping is facilitated by PCR-generated markers used in linkage studies (96, 145, 152) that will likely assist in mapping and cloning disease resistance loci of host plants.

Studies of gene expression during plant infection will be advanced with techniques that use PCR. Expression of a gene is detectable by PCR even when only a few cells among thousands are actively transcribing it (44, 146), and the onset of expression of a particular gene can be determined more precisely (55). Differentially expressed mRNAs, such as those synthesized during infection, can now be specifically amplified by PCR and displayed on gels (87). In addition, chromatin structure, DNA-protein interactions, or DNA methylation patterns, all of which affect gene expression, can be analyzed by a recently developed PCR method called ligation-mediated PCR (LMPCR; 51, 108).

More sensitive monitoring of microbial infection or colonization of individual plants can be accomplished with PCR, especially since it is now possible to amplify target sequences *in situ* in tissue or individual cells (26, 105). Natural microbial populations or genetically engineered organisms or their nucleic acids can be sensitively monitored in soil, insect vectors, water, or air by PCR. Observing the movement of genetic elements through plants or microbial populations will also be facilitated by PCR. For example, PCR

could be used to follow fungal dsRNAs or linear dsDNA plasmid migration during hyphal anastomosis. Some fungal dsRNA elements are possible determinants of virulence (6), while others confer hypovirulence to their host fungi. For instance, one viral dsRNA confers hypovirulence to its host, the chestnut blight fungus, *Cryphonectria parasitica*, and rapid and sensitive monitoring of this genetic element will facilitate its use as a biological control agent of chestnut blight (27).

Because of its sensitivity, PCR will continue to be used diagnostically to detect genomes or antigens of microorganisms that are scarce, difficult to culture, or difficult to identify once cultured. Pathogen screening of seeds, stored grain, micropropagated tissue culture, or vegetatively propagated plants will be assisted by PCR or related techniques. PCR or immuno-PCR may prove to be more sensitive and reliable methods for detection of toxins, pesticides, or other undesirable chemicals, microorganisms, or ingredients in our food. For example, PCR was used to detect wheat contamination (wheat-specific DNA) in dietary nonwheat products (3), and it was used to detect the human pathogen, *Listeria monocytogenes*, in naturally contaminated food samples (104).

The sensitivity, speed, and versatility of PCR are primary factors in its wide acceptance in plant pathology as well as many other fields of biology. In its impact on both basic and applied research, PCR is unsurpassed. It is adaptable to many experimental objectives, and it is used with a wide range of starting material, including purified nucleic acids, intact cells or tissues, or complex environmental samples. Research to date has just begun to develop specific applications, and it is likely that the implementation of PCR-based diagnostic tests will grow rapidly in the near future. Central questions in plant pathology can be addressed at a level of precision that was impossible just a few years ago. As PCR methods for detection of pathogens become available, more research will focus on using these as tools to study pathogen populations, biology, ecology, variability, and host-pathogen interactions.

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