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Case Study

ROLE OF POLYMERASE CHAIN REACTION IN PLANT PATHOLOGY

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ABSTRACT
In plant pathology, cultural and biochemical characteristics have been used widely for the identification of the plant pathogens. For effective management of a plant disease proper identification and characterization of plant pathogens is very important. With the advent of polymerase chain reaction and molecular methods, diagnosis of plant diseases has become more feasible. The present review focuses on the basics of Polymerase chain reaction and its application in plant pathology. In this review we discuss the various developments made in plant disease identification using PCR.

KEY WORDS- PCR, plant pathogens, diagnosis, plant disease.

INTRODUCTION

Morphological and biochemical characters have been widely used in the past to investigate relatedness, phylogeny, and inheritance of genetic material in plant pathogens. Identification of plant pathogens based on morphology is a tedious, time consuming, difficult task, needs mycological expertise and may lead to wrong identification sometime. Thus, conventional phytopathological methods have limitations in developing quarantine methods, disease resistance breeding and disease management purposes. The various tests based on nucleic acid offer greater sensitivity, specificity, reliability and may be quicker than many conventional methods used to detect plant-pathogens in different plant hosts and environments. With the development of polymerase chain reaction (PCR) such high sensitivity is achieved, improving the accuracy of pathogen detection and identification (Mullis, 1987, Holland *et al.*, 1991, Vincelli and Tisserat, 2008). The advent of genome analysis has made it possible to accomplish the same task directly at the molecular level. Different biochemical and molecular approaches like isozyme analysis (Anderson *et al*., 1995), RAPD (Zimand *et al.*, 1994), ITS marker (Braun and Takamatsu, 2000) and Restriction Fragment Length Polymorphism (RFLP) (Mondal *et al*., 2004) are being applied in plant pathogens identification. The evidences available clearly indicate that these molecular markers are useful in separating morphologically similar species. Molecular markers are nucleic acid segments that behave as landmarks for genome analysis and are based on naturally occurring genetic variability (usually termed polymorphisms). Molecular markers can be categorized in two groups according to the way they are generated *i.e.* Hybridization-based techniques and Amplification-based techniques. Hybridization-based techniques are based on the Watson-Crick complementary rules of base pairing and require the use of labeled nucleic acid molecules as hybridization probes. Probes can be short single-stranded nucleic acid segments (oligonucleotides) of synthetic origin or cloned DNA segments that bind to complementary nucleic acids, forming hybrid molecules. Eg.- Restriction fragment length polymorphic markers (RFLP). Amplification based techniques use oligonucleotides (DNA or RNA) to get the exponential accumulation of specific sequences from defined regions in a genome or transcriptome (Saiki *et al*., 1988). PCR-based techniques have been used to amplify single targets such as ribosomal RNA (rRNA) genes, mitochondrial and chloroplast DNA sequences, and repetitive DNA.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a powerful, extremely sensitive technique employed in the field of Molecular biology, agriculture diagnostics, forensic analysis, medical and biological research lab and population genetics (Saiki *et al*., 1985). It was developed by Kary Mullis in 1983 (Mullis, 1987). It is a technology which is used to amplify a single copy or a few copies of a piece of DNA, generating thousands to millions of copies of a particular DNA sequence (Bartlett and Stirling, 2003). PCR is based on the enzymatic amplification of DNA fragments that is flanked by oligonucleotide primer hybridizing to opposite strands of the target sequence. Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR. This invention brought him a number of scientific awards; among them most important were the Japan Prize and the Nobel, both awarded to him in 1993.

Constituents of PCR reaction

Several components and reagents are required for a basic PCR set up (Cheng *et al*., 1994). These components include: **Primer**- The most essential requirement of PCR is the availability of short oligonucleotides called primers having sequence complementary to either ends of the target DNA segment. Primers are short strand of nucleic acid serves as starting point of DNA synthesis.

Template DNA

DNA segment to be synthesized in large amount. Genomic DNA or RNA is used as a template.

Taq DNA Polymerase

Thermus aquaticus DNA polymerase (Taq DNA polymerase) is a thermostable enzyme that replicated DNA at 72-74°C and remains functional even after incubation at 95^0C. The enzyme has 5'-3' polymerase activity and 3'-5' exonuclease activity. Availability of thermostable DNA polymerase-Taq (from the bacteria *Thermus aquaticus*) (Chien *et al*., 1976) has facilitated automation of the PCR.

DNTP's

The deoxynucleotidetriphosphates are dATP, dGTP, dCTP, dTTP (used as 10 mM each). These are the building-blocks from which the DNA polymerase synthesizes a new DNA strand.

Assay Buffer (10X)

10X assay buffer for Taq polymerase enzyme. Assay buffer contains 10 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin. It provides a suitable chemical environment for optimum activity and stability of the DNA polymerase. (Pavlov et al, 2004).

Procedure

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. It relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for melting of DNA and then enzymatic replication of the DNA. PCR consists of a series of 20-40 repeated temperature changes known as cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. Three basis steps which constitute a single cycle of a PCR are as follows:

(i) Denaturation of the target DNA at 92-94°C, is the first step of PCR cycle. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules (Sharkley *et al*., 1994).

(ii) Annealing of the primers to the template DNA, this step happens at a lower temperature *i.e.* 50 to 65°C for 20-40 seconds. This temperature needs to be low enough to allow hybridization of the primer pair but high enough in order to specifically hybridise the primer on the complementary site. For the formation of stable DNA–DNA hydrogen bonds the primer sequence should very closely match the template sequence. The polymerase enzyme binds to the primer-template hybrid and begins DNA formation (Rychlik *et al*., 1990).

(iii) Primer extension by addition of nucleotides to the 3' end of the primers by the enzyme DNA polymerase. The temperature of this step should be based on the polymerase enzyme used. Most commonly extension is carried out at 72°C. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand (Lawyer *et al*., 1993).

Final elongation is a step occasionally performs at 70-74°C for 5-15 minutes to ensure elongation of the entire remaining single stranded DNA. After that an indefinite time may be employed for final hold of the product for storage at 4°C. As the number of PCR cycle increases, the amount of target DNA synthesized increases exponentially.

Preparation of PCR mixture

A master mix containing all the above mentioned components and no template DNA should be prepared in laminar airflow under sterile conditions for total number of PCR tubes to be used. This will reduce the pipetting errors. Then distribute the master mix in each tube (24 µl each) and finally add 1 µl of different DNA template in each tube. Gently mix and centrifuge the mixture for 10 sec. Annealing temperature should be standardized using T-gradient programme (Carr *et al*., 2012).

Agarose Gel Electrophoresis

To check whether the PCR generated the anticipated DNA fragment or amplicon, agarose gel electrophoresis is employed. The size (s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. Agarose Gel Electrophoresis is a submerged gel electrophoresis unit on which DNA and RNA can be separated on the basis of size by running the DNA through an agarose gel.

Significance of PCR

The genetic material of each living organism, plant or animal, bacterium or virus possesses sequences of its nucleotide building blocks (usually DNA, sometimes RNA) that are unique and specifically present only in its own species. These unique variations make it possible to trace genetic material back to its origin, identifying with precision at least what species of organism it came from, and often from which particular member of that species. However, such an investigation requires that enough amount of the DNA under study should be available for analysis, which is where PCR comes in. PCR exploits the remarkable natural function of the enzymes known as polymerases. These enzymes are present in all living things, and their job is to copy genetic material (and also proofread and correct the copies). Sometimes referred to as "molecular photocopying," PCR can characterize, analyze, and synthesize any specific piece of DNA or RNA. It works even on extremely complicated mixtures, seeking out, identifying, and duplicating a particular bit of genetic material from blood, hair, or tissue specimens, from microbes, animals, or plants, some of them many thousands-or possibly even millions of years old (Dickinson, 2005).

Application of PCR in Plant Pathology

PCR technology has become an essential research and diagnostic tool for improving knowledge regarding identification, characterization, detection and diagnosis of plant pathogens. PCR technology allows scientists to take a specimen of genetic material, even from just one cell, copy its genetic sequence over and over, and generate a test sample sufficient to detect the presence or absence of a specific virus, bacterium or any particular sequence of genetic material. Therefore, it is hard to exaggerate the impact of the polymerase chain reaction. PCR, the quick, easy method for generating unlimited copies of any fragment of DNA, is one of those scientific developments that actually deserve timeworn superlatives like "revolutionary" and "breakthrough" (Gelsomino *et al*., 2011). Molecular approaches developed over the last ten years to detect many bacteria, *Spiroplasma*, phytoplasmas, viruses, and viroids in plant or environmental samples

(Louws *et al*., 1999, Alvarez, 2004) can be grouped as follows, according to Bonants *et al*. (2005), a) RNA level: RT-PCR, NASBA or AmpliDet RNA; and b) DNA level: hybridization, FISH, and PCR variants (conventional PCR, nested PCR, cooperative PCR, multiplex PCR, real-time PCR).

Diagnosis and characterization of the pathogen

PCR utilises specific oligonucleotide primers, which are designed based on nucleic acid sequences that are diagnostic for the pathogen. These may be sequences identified by genomic sequencing or techniques such as DNA fingerprinting of pathogens. To improve the sensitivity of these techniques, it is sometimes an advantage to use nested PCR methods, in which the products from the initial PCR amplification are diluted and re-amplified with a second set of primers internal to the original primer set in the pathogen sequence (Cai *et al*., 2014). These conventional PCR-based techniques have proved to be useful for diagnosis of fungal, bacterial and phytoplasma-associated diseases with a number of good taxon-specific primers developed for example from the rRNA subunit genes. Because of the sequence variation between these regions in different isolates, it has sometimes been possible to identify restriction endonucleases that give different restriction patterns upon digestion of the PCR products depending upon the isolate (Schaad and Frederick, 2002).

Primers have also been developed based on more specific sequences such as the *argk-tox* gene of *P. syringae* pv. *phaseolicola* which encodes a gene involved in phaseolotoxin biosynthesis and can be used to identify bacteria that possess this trait, or the *aflR* gene of *Aspergillus flavus* which regulates aflatoxin production in these fungi. In addition, PCR-based techniques have proved useful for identifying the vectors for insect-transmitted diseases. For example, DNA extracted from leafhoppers that are potential vectors for phytoplasma diseases can be PCR amplified using phytoplasma-specific primers to identify which species are the true vectors.

Single-strand RNA viruses can be detected by modifying the PCR to include a reverse transcriptase step (RT-PCR) (Salis, 2009). In this, the reverse transcriptase uses a viral specific primer to make a cDNA copy of the viral RNA which is then amplified using Taq polymerase through conventional PCR. RT-PCR has the potential for use in diagnostics of bacterial and fungal pathogens. Through identification of particular genes that are expressed during pathogen growth, or at specific stages of development, it may be possible to use RT-PCR to identify the developmental stage of the pathogen in infected material. It has also been possible to produce 'multiplex' PCR kits capable of detecting more than one pathogen present in a particular plant or soil sample. Kits are commercially available to unravel the cereal stem-based complex of fungi comprising *Tapesia yallundae* and *T. acuformis* (eyespot fungi), *Fusarium culmorum, F. avenaceum, F. graminearum* and *F. poae* (ear blights), and *Microdochium nivale* (snowmould of cereals).

CONCLUSION

Conventional PCR, its variants and more recently, realtime PCR provides the advantages of nucleic-acid based technology in the sensitive, specific and rapid diagnostic of plant pathogens. The optimised protocols should be simple and robust enough so that reliable and reproducible results can be obtained. Furthermore, appropriate sampling protocols and systems as well as sample preparation need to be developed and carefully studied and evaluated for each combination of pathogen, plant material, molecular technique and protocol (Lopez *et al*., 2011). The future will bring more novel tools to detect plant pathogens, probably based on the new sequences available and because of them novel and improved reagents, linkers and molecular technologies are expected. However, there acceptability by plant pathologists will be based on not only the quality of test results, but also on their sensitivity and specificity as well as their cost-effectiveness (López *et al*., 2006).

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