General Principles of Real-Time PCR: A Technology for Quantitative Detection of Phytopathogens

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Abstract-Real-time PCR provides a rapid and specific method for detection of plant pathogens in various environmental samples. The technique incorporates traditional PCR efficiency with the production of a specific fluorescent signal, providing quantification of specific targets. There are four main chemistries, which can be clustered into the non-specific DNA-binding fluorophores and the specific fluorophore-labelled oligonucleotide probes. In the present review, we describe the general background of major real-time chemistries as well as some considerations for assay development. Basically, Scorpion and SYBR green have the most and least sensitivities between available chemistries, respectively. However, with accurate optimization, real-time PCR can provide reliable and high throughput detection of target DNA in various environments.

Index Terms—alternative chemistries, plant pathology, realtime PCR

I. INTRODUCTION

One of the most important strategies for controlling plant diseases is accurate identification and detection of plant pathogens [1]. The traditional detection methods based on morphological characteristics often require extensive knowledge of classical taxonomy and are frequently laborious and time-consuming [2]. Polymerase chain reaction (PCR)-based technology is a rapid and sensitive method that offers advantages over the traditional diagnosis methods. The development of realtime PCR technology allows detection and quantification of pathogens that cannot be extracted or cultured easily from host tissue and also provides conclusive results as it can discriminate between closely related organisms [3], [4]. In recent years, real-time PCR has been used in most agricultural fields such as plant protection as a valuable tool for phytopathogens diagnosis with accuracy and high throughput quantification of specific target DNA. The present review focuses on four basic chemistries used in

real-time PCR and some considerations for routine detection and quantification of plant pathogens.

II. REAL-TIME PCR TECHNOLOGY

The most influential characteristic of real-time PCR is its suitability for quantitative analyses. Generally, realtime PCR is based on the detection of the fluorescence produced by a reporter molecule that binds to the doublestranded DNA which increases as the reaction proceeds [1]. The benefit of real-time PCR compared with cPCR is determined by three main features; firstly, data are available in real time, on screen, do not require time consuming post-PCR processing (e.g., electrophoresis, colorimetric reaction or hybridization). Secondly, realtime PCR commonly amplify the short DNA fragments (70-100 bp), which favors a higher level of efficiency and sensitivity [5]. Lastly, several target DNAs can be simultaneously detected from different microorganisms in real-time PCR assays. In fact, simultaneous detection of more than one organism provides significant benefits particularly for diagnostic programs dealing with a lot of samples [3]. There are two common methods for the detection of products in real-time PCR: first, nonspecific fluorescent dyes that intercalate with any doublestranded DNA; and second, sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary Generally, sequence specific methods sequence. guarantee higher specificity levels which are particularly important when they are used for detection and quantification of pathogens in natural samples or within symptomless tissues [6].

A. Non-Specific Detection Using DNA Binding Dyes

SYBR Green Dye: SYBR Green is an intercalating dye, which binds to a minor groove of the double-stranded DNA and is the dye most widely used for real-time PCR assays. SYBR Green does not emit fluorescence in its free form, emitting the fluorescence signal only when binding to the dsDNA (Fig. 1).

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Figure 1. When SYBR Green binds unspecifically to double-stranded DNA (A), it is able to emit green light as fluorescence (B). The amount of fluorescence is directly proportional to the amount of DNA amplified. Taq: Taq polymerase; SG: SYBR Green Dye.

Because of simplicity, flexibility, reliability and the low-cost of SYBR Green, this method is widely utilized for the quantification of plant pathogens [4]. Since unmodified oligonucleotide primers can be used with SYBR Green, its application is cheaper than other detection forms. However, the principal drawback to intercalation-based detection of product accumulation is that specific and nonspecific products produce signal. So, the formation of non-specific amplicons can lead to false positive results in the quantification [7].

B. Specific Detection using Target Specific Probes

1) TaqMan probe

TaqMan probes consist of a sequence 25–30 nucleotides in length which is labeled with a donor fluorophore (as reporter) at the 5' end, and an acceptor dye (as quencher) at the 3' end. In the extension step, the probe cleaves by the 5'-nuclease activity of Taq DNA polymerase when the enzyme reaches the probe, resulting in separation of the fluorescent reporter dye from the quencher, thus generating a fluorescent signal (Fig. 2).



Figure 2. Because the primer is bound, Taq polymerase can create a complementary strand (A). The reporter dye is released from the extending double-stranded DNA created by the Taq polymerase (B). Taq: Taq polymerase; Q, Quencher Dye; R, Fluorescent Reporter Dye.

One advantage of the TaqMan probe over SYBR Green dye is that specific hybridization between probe and target DNA sequence is required to produce fluorescent signal. Also, a TaqMan real-time PCR assay can be multiplexed, because it can amplify several distinct sequences in a single PCR reaction tube due to possibility of labeling of the fluorogenic probes with different detectable reporter dyes [8].

2) Molecular beacon

Molecular beacons are single-stranded oligonucleotide hybridization probes that form a hairpin-like. When the probe sequence in the loop anneals to a complementary nucleic acid target sequence, the stem portion of the beacon separates out and hybridizes to the target, resulting the fluorescence emission. In the absence of a complimentary target sequence, the beacon remains closed and there is no appreciable fluorescence (Fig. 3). So, the amount of fluorescence at each cycle depends on the amount of specific product.



Figure 3. When the beacon binds to the PCR product (A), it is able to fluoresce when excited by the appropriate wavelength of light (B). Q: Quencher Dye; R: Fluorescent Reporter Dye.

In comparison with linear probes, molecular beacons are especially suitable for identifying point mutations, because the hairpin-like structure makes mismatched hybrids thermally less stable than hybrids between the corresponding linear probes and their mismatched target [7].

3) Scorpion probe

Scorpion primers are bi-functional molecules in which a primer is covalently linked to a specific probe sequence that is held in a hairpin–like form with a fluorophore at one end and a quencher at the other. At the 5' end, the Scorpion primer sequence contains a non-target sequence as PCR blocker that prevents polymerase read-through. In the annealing step, scorpion primer combines to the PCR product and then the probe sequence in the tail curls back to hybridize with the sequence of target, separating the fluorophore and the quencher, which leads to an increase in the fluorescence emitted (Fig. 4).



Figure 4. During annealing, the hairpin primer binds to the template, and is then extended (A). The reporter separates from the quencher, and the loop sequence binds to the internal target sequence (B). Q, Quencher Dye; R, Fluorescent Reporter Dye; B, Blocker.

In Scorpion primers, the probe is physically coupled to the primer which means that the reaction leading to signal generation is a unimolecular event which is efficiently instantaneous [8]. So, this leads to stronger signals, enhanced discrimination and shorter reaction times compared with molecular beacons and TaqMan probes. Relative sensitivities of the real-time PCR chemistries in increasing order are: SYBR green I < TaqMan < molecular beacons < Scorpion [9].

C. Real-Time PCR Considerations

1) DNA extraction

A critical pre-analysis step for real-time PCR assays is DNA extraction. The purpose of a DNA extraction procedure is to provide a sufficient quality of DNA with a low concentration of substances inhibiting PCR reactions for subsequent analyses. Typically, the inhibitory substances can be removed using different columns and resins such as gel filtration resins, agarose gel electrophoresis, and template dilution [10]. Unlike dilution plating methods, a number of commercial kits are available to extract RNA or DNA from plant tissues and soil samples. Also, several methods such as isopropanol, silica-columns, magnetic beads, lyophilization, Freeze– grind and heat treatment have been used for extraction of high-quality DNA from samples.

2) Target genes selection

Sequences of the target primer must be unique in order to recognize virulence genes or a particular organism. Sequencing of conserved genes has been used to develop PCR-based detection with varying levels of specificity for plant pathogens [11]. Among the variable regions, ITS region within prokaryotic and eukaryotic rDNA operons is the most widely sequenced in phytopathogens. Also, intergenic spacer (IGS) sequences are more difficult for amplification and sequencing, but they can be more variable than the ITS sequences. So, IGS regions of bacterial 16S ribosomal RNA genes and ITS regions of the fungal ribosomal RNA genes have been used most commonly for PCR-based identification of plant pathogens. Moreover, the b-tubulin gene has been used for diagnosis purposes of plant pathogens when variation of ITS sequence is not appropriate for production of a taxon-specific diagnostic [8].

3) Primer and probe design

Primer design is aimed to obtain a balance between efficiency and specificity of amplification. So, the most important issue for designing efficient PCR primers is that they must bind to the target site efficiently. Specificity can generally be defined as the tendency for a primer to hybridize to its intended target and so primers that only amplify one product will provide the best assay sensitivity [12]. Specific primers and probes can be properly designed for SYBR Green, TaqMan probes, Molecular beacons and Scorpion PCR assays using different primer design softwares. After primers and probes designing, their specificity should be checked by in silico analyses using the Basic Local Alignment Search Tool (BLAST) in GenBank to confirm the existence of similar sequences.

4) Other considerations

As mentioned above, the problem with PCR inhibitors is frequently occurred when environmental samples are assessed. For detecting inhibitor effects, an internal positive control such as the amplification of a housekeeping gene can be included in the assays to distinguish false negatives caused by inhibitors [4]. Another problem is the risk of nucleic acids contamination by external sources [9]. The risk of contamination can be reduced by precise activities such as using negative control, positive control and reagent control in each PCR run. Totally, in order to obtain the best results, primer and probe design, amplification conditions and amplicon length should all be optimized.

III. CONCLUSION

Real-time PCR is extremely promising in order to diagnose and quantify pathogen populations, whereas other PCR-based techniques qualify only for the identification/detection of the microbial communities. With accurate optimization, real-time PCR can provide accurate, reliable and high throughput detection and quantification of target DNA in various environments. In fact, real-time PCR is an ideal technique to measure levels of inoculum threshold, which has a positive impact on epidemiological studies, and for evaluating the efficacy of methodologies used to prevent distribution of the pathogens into non-infected agricultural fields.

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