Advancements in Real-Time and Quantitative LAMP for Detection of Plant Pathogens

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Loop-mediated isothermal amplification (LAMP) stands out as one of the most advanced and widely used techniques for detecting plant pathogens. Numerous LAMP-based methods have been developed to identify plant pathogens, incorporating various improvements such as portable devices, enhanced visualization methods, and standardized approaches for multiplex detection to optimize field diagnostics. Since Notomi et al.'s seminal publication in 2000, over 250 research articles on LAMP assays for plant pathogen detection have been published, with a steady annual rise in such studies observed since 2010 (Le and Vu, 2017). This article will focus on the application of LAMP technology for identifying plant pathogens.

Loop-mediated isothermal amplification (LAMP) offers an efficient alternative to PCR due to its faster processing time, reduced labor demands, and comparable or even superior sensitivity and specificity compared to other RNA/DNA amplification techniques (Panno et al., 2020). The LAMP method utilizes six primers designed to recognize eight distinct regions of the target DNA, along with the Bst DNA polymerase enzyme, which exhibits strand displacement activity (Notomi et al., 2000; Nagamine et al., 2002). One of LAMP's significant advantages is its ability to function at a constant temperature between 60°C and 65°C, requiring only a simple water bath. Additionally, it is compatible with crude DNA extracts and direct tissue samples (Nagamine et al., 2002). The forward inner primer (FIP) combines F1c and F2, while the backward inner primer (BIP) combines B2 and B1c (Notomi et al., 2000).The outer primers, forward (F3) and backward (B3), act only in the non-cyclic phase (Parida et al., 2008). Loop primers, forward (LF) and backward (LB), bind to additional DNA regions, enhancing amplification and speeding up the reaction (Nagamine et al., 2002; Parida et al., 2008). The reaction begins with the forward inner primer (FIP). The target DNA is first denatured by heating to 95°C and then rapidly cooled on ice, allowing the FIP to anneal to its target sequence (Notomi et al., 2000). The F2 region of the FIP binds to the complementary F2c segment on the target DNA, initiating the synthesis of a complementary strand. This process is carried out by a strand displacement polymerase at a constant temperature of 65°C. The outer primer F3 then anneals to its complementary F3c sequence, triggering strand displacement synthesis and producing a FIP-linked complementary strand. This forms a loop structure at one end when the F1c region of the primer anneals to its F1 complement. The resulting single-stranded DNA serves as a template for further DNA synthesis. This starts with the B2 region at the 3′ end of the backward inner primer (BIP), followed by strand displacement synthesis initiated by the B3 primer. This process releases a BIP-linked complementary strand, which forms a dumbbell-shaped structure. The dumbbell shape is then converted into stem-loop DNA through self-primed DNA synthesis. The stem-loop DNA formed during the non-cyclic phase acts as the substrate for LAMP cycling. The process begins when the forward inner primer (FIP) anneals to the F2c sequence within the loop of the stem-loop DNA, initiating strand displacement DNA synthesis. The addition of the LB primer enables its binding to the loop connecting the B1 and B2 regions of the stem-loop DNA, generating additional stem-loop structures. These structures allow binding by the LB and LF primers, driving exponential amplification. If present, the LF primer binds to the loop between the F1 and F2 regions of the stem-loop structure, functioning similarly to the LB primer to enhance LAMP efficiency. From the structure in step 12, two intermediate products are generated: a double stem-loop DNA, which acts as the primary substrate for LAMP cycling, and a novel stem-loop DNA with a stem twice the original length. These intermediates serve as templates for FIP-primed strand displacement reactions during subsequent elongation and recycling cycles, ultimately producing more elongated DNA structures. To mitigate this, various closed-tube visualization techniques are available. Strand displacement from the 3′ end of the F1 region

opens the 5′ end loop, while a second strand displacement at the 3′ end loop of the B1 region produces two intermediate structures. One is a complementary double stem-loop DNA to the original, and the other is a new stem-loop DNA with a stem twice the length. These products serve as templates for subsequent strand displacement reactions, initiated by the backward inner primer (BIP) in the elongation and recycling phases. During stages 11a and 11, BIP anneals to the loop of the stem-loop DNA, initiating synthesis of complementary double stem-loop DNA. Among these, HNB performs particularly well for visualizing LAMP products, offering detection sensitivity comparable to SYBR Green while being compatible with in-reaction use, thereby minimizing cross-contamination risks (Goto et al., 2009).

The primer strand, following the same mechanism as with the original stem-loop DNA. Calcein and HNB detect amplified products by monitoring changes in metal ion concentration during LAMP. The reaction generates large amounts of pyrophosphate ions, which form insoluble salts by binding with metal ions such as manganese. This reduces the manganese ion concentration, and the calcein-manganese complex responsible for quenching turns the solution orange. When LAMP is initiated in the presence of DNA, the pyrophosphate ions strip manganese from the calcein complex. Calcein then binds to the remaining magnesium, resulting in fluorescence (Tomita et al., 2008). Detection of LAMP products can be achieved visually by observing turbidity in the solution or quantitatively using a photometer. Real-time LAMP tracks DNA amplification by quantifying Mg^{2+} ion concentrations in the solution (Parida et al., 2005; Goto et al., 2009; Zhang et al., 2013). Recent advancements include the use of fluorescent assimilating probes, which enhance real-time LAMP accuracy and mitigate non-specific detection issues inherent to dye-based methods (Gadkar et al., 2018). This device maintains the ideal temperature for the LAMP reaction (60°C–65°C) while simultaneously monitoring the turbidity of multiple samples (Mori et al., 2004; Panno et al., 2020). However, the sensitivity of turbidity-based detection is ten times lower than that of real-time LAMP using fluorescent probes (Quyen et al., 2019). The Fluorescence Resonance Energy Transfer (FRET) method enhances the LAMP technique for quantitative detection by using two labeled oligonucleotide probes. One probe is a fluorescent strand, and the other is a quenching strand. During DNA synthesis, the quenching strand is displaced from the fluorescent strand by the amplification process, resulting in the emission of fluorescence. This displacement and fluorescence emission are directly related to the amount of target DNA, allowing for the quantitative measurement of the amplification process in real-time. Several portable devices support the FRET-based LAMP technique, making it adaptable for field and on-site applications. Notable devices include the "Bioranger" by Diagenetix, Inc., and the "Genie II and III" models by Optigene Ltd. These devices are equipped with built-in systems to monitor fluorescence emissions, enabling real-time analysis of LAMP reactions. The portability of these devices allows for the convenient detection of pathogens in various environments, especially in situations where laboratory facilities are not accessible. In addition to FRET-based real-time detection, LAMP products can also be quantified using traditional fluorescent dyes with a real-time PCR thermal cycler. Real-time PCR instruments, which are typically used for quantitative PCR applications, can be adapted for LAMP by incorporating fluorescent dyes such as SYBR Green or EvaGreen. These dyes intercalate with amplified DNA, emitting fluorescence that can be measured to assess the amplification process.For field applications, the ESE-Quant Tube Scanner, a portable fluorescence scanner, provides a simple and effective option for rapid on-site detection of LAMP products. This device is designed for use in various settings, offering easy handling and quick results, making it an excellent tool for detecting plant pathogens or other targets in the field. Its compact size and ability to scan multiple samples in a short period make it ideal for high-throughput screening in resource-limited environments. Furthermore, the ESE-Quant Tube Scanner is user-friendly, with real-time monitoring and minimal need for technical expertise, offering significant advantages for on-site diagnostics, such as detecting pathogens in agricultural settings. Thus, these innovations in portable devices and real-time detection methods significantly enhance the efficiency and practicality of LAMP for pathogen detection, allowing for quicker, on-site analysis with high sensitivity and specificity. Real-time LAMP has also been used for quantifying and identifying pathogens

in wheat, including Wheat Dwarf Virus (Trzmiel and Hasiów-Jaroszewska, 2020; Hao et al., 2021), *Pyricularia oryzae*, *Triticum* lineage (Yasuhara-Bell et al., 2018), *Tilletia* species (Pieczul et al., 2018), *F. graminearum* (Gupta et al., 2020), and *Fusarium* mycotoxins (Denschlag et al., 2014). Moreover, a quantitative assimilating probe-based LAMP was used to detect airborne inoculum of *Magnaporthe oryzae*, the pathogen causing grey leaf spot in turfgrass fields. This method was integrated with a spore trap device for field application (Villari et al., 2017).

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