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Short communication

Development of multiplex reverse transcription-ligase detection reaction-polymerase chain reaction (MRLP) mediated universal DNA microarray for diagnostic platform

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ABSTRACT

A multiplex reversal transcription-ligase detection reaction-polymerase chain reaction (MRLP) based universal microarray for the simultaneous pathogens detection was established by using potato viruses as a model. The proposed procedure integrated LDR for multiplicity and specificity, PCR amplification by universal primers for sensitivity, which required design of upstream and downstream LDR probes specific for each virus, and subsequent Zip-code microarray for multiplex and specific identification. Each MRLP fragments carried a unique sequence (complementary Zip-code sequence, *c*Zip-code) which identified a virus by addressed to the location on the microarray where the Zip-code sequence has been spotted. Such Zip-code microarray and universal primers are therefore "universal" being unrelated to a specific molecular analyte. With this technique, target RNAs of ten potato viruses were reversal transcribed by random primer in a single reaction, then subjected to LDR and asymmetric labeling PCR as template, finally, the MRLP amplicons were analyzed by microarray hybridization and subsequent scanning. The technique platform was optimized and evaluated by using reference samples and artificial samples, which can specifically detect down to 3 copies of single or mixed plasmid templates. Due to its universality, multiplexing, specificity and sensitivity, this method can be recommended for simultaneously detecting a large number of different target types in related fields.

1. Introduction

The outbreaks of some pathogens are related to huge economic and social impact in many countries (Gall et al., 2009). Furthermore, some pathogens infections are virtually impossible to eradicate, and once established, the only effective means of control is the use of resistant varieties (Li et al., 2008). Traditionally classical techniques are known for their limitations, such as laborious procedures, time-consuming, and inability to differentiate pathogenic organisms from closely related non-pathogenic ones (Lee et al., 2009; Wang et al., 2004). The recent development of PCR-based detection technique has greatly improved the sensitivity, specificity, and speed of detecting pathogens. However, one of the major drawbacks of the PCR method is that the number of species that can be analyzed in each reaction is limited (Suo et al., 2010). Multiplex PCR accommodates several pairs of primers in one reaction, resulting in reduced material costs and time (Nie and Singh, 2000; Sachse et al., 2009; Wang et al., 2007). Multiplex PCR yet have several disadvantages such as complex system with many primers, low amplified efficiency and no identical efficiency on different templates, which restricted the commercial application in detection (Xiao et al., 2009).

DNA microarrays technology has revolutionized molecular diagnostic techniques for pathogen detection not only enhancing assay capability of DNA microarray but also allowing for high-throughput detection. However, one important problem of DNA microarrays technology is the limitation of the specificity and sensitivity for the hybridization of DNA samples to the respective capture probes (Gall et al., 2009; Hultman et al., 2008; Song et al., 2005).

The LDR allows accurate target identification due to the feature of the Taq DNA ligase which allows only ligated LDR products to be captured, thus avoiding background signals and enhancing specificity (Cheng et al., 2006). It can also be configured to do highly multiple assays and uses a thermally stable ligase to linearly amplify the LDR product (Hashimoto et al., 2005). But the amplification yield of LDR is not enough for practical applications, thus LDR-PCR was developed (Wiedmann et al., 1994; Xiao et al., 2006). However, the gel electrophoresis limits the number of targets and several pairs of primers used to amplify different ligation products followed LDR may interfere each other. Universal micorarrays have been introduced in some studies (Busti et al., 2002; Girigoswami et al., 2008). Significant advantages of universal

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microarray lie in uniform hybridization conditions of all Zip-code sequences which can be used with multiple ligation probe sets for the same microarray platform (Hultman et al., 2008). Nevertheless, all discriminating probes were labeled with fluorescence in these papers, which increased the cost of experiments. Szemes et al. (2005) have designed padlock probes for simultaneous detecting several pathogens by universal amplification. Although this method increases sensitivity and multiplexing capabilities without limiting the range of potential target organisms, to achieve combination is not an easy thing (Lohmann et al., 2007). Padlock probes about 100-nucleotide long are difficult to synthesize and much expensive. Furthermore, nuclease treatments make it somewhat more time consuming (Eason et al., 2004).

In this study, we report the development and evaluation of an MRLP based universal microarray platform for simultaneous identification of multiple target templates (Fig. 1). Ten potato viruses, which can infect potato and cause substantial yield losses in different regions of the world, are selected as a model assay for multiplex pathogen diagnostics.

2. Materials and methods

2.1. Materials

Ten plant viruses used in this study, alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), potato leafroll virus (PLRV), potato virus A (PVA), potato virus M (PVM), potato virus S (PVS), potato virus X (PVX), potato virus Y (PVY), tobacco mosaic virus (TMV), tobacco ring spot virus (TRSV), each contained in positive potato leaves tissue samples known to be infected with one of ten viruses respectively, were purchased from Bioreba AG (Switzerland) and stored at −80°C. Four non-target viruses, tomato aspermy virus (TAV), watermelon mosaic virus (WMV), Dasheen mosaic virus (DsMV), soybean mosaic virus (SMV), used to determine the specificity of the assay, were kept in our laboratory. Health potato leaves identified to be negative for above ten viruses by PCR, were collected from Xiasha, Hangzhou and used as negative control.

2.2. Methods

2.2.1. Zip-code microarrays, primers, and LDR probes design

Thirteen Zip-code sequences (Supplementary Table 1) were selected in a way to minimize secondary structures from a set of tetramer sequence units as described (Szemes et al., 2005). One of which chosen for potato housekeeping gene (18S rRNA) was used as a positive control and served as a direct indication of whether or not the hybridization conditions were adequate and also as a spatial marker for ease of viewing.

Ten kinds of potato viruses' genome sequences were downloaded from NCBI database and aligned by using the Clustal W (DNAStar Inc., Madison, USA) algorithm to select optimal primer and probe binding regions characterized by a high degree of conservation among different virus strains of each virus. Additionally, the potato housekeeping gene (18S rRNA), as internal amplification control (IAC) to check whether the potato viral RNAs isolation and the RT-PCR are successful, was also included in the designs (Supplementary Tables 2 and 3). The Zip-code sequences, primers, and LDR probes were designed and optimized by using Primer Premier 5.0 (Primier Biosoft International, CA, USA) and DNAStar software (Supplementary Tables 1–3). All Zip-codes, primers, and LDR probes sequences were subsequently blasted against the complete GenBank database to ensure that they represented unique sequences avoiding non-specific PCR products and unwanted cross-hybridizations. Primers, probes and Zip-codes were synthesized from Sangon (Shanghai, China).

2.2.2. Zip-code universal microarray preparation and layout

Universal microarray was fabricated according to Jiang et al. (2010). The microarray layout was showed in Supplementary Table 1 and Fig. 2.

2.2.3. Viral RNAs isolation and quantification

Total RNAs of the positive tissue samples (50–100 mg) were extracted using Trizol reagent (Takara, Dalian, China), following the procedure recommended by the manufacturer. The quantity and quality of the purified RNAs were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE) by measuring A260 and the ratio of A260/A280, respectively.

2.2.4. The single potato virus cDNA synthesis and standard plasmid templates construction

Ten extracted RNAs were reversal transcribed following the procedure recommended by manufacturer (Takara). Non-template negative controls (NTC) were included in each round. After agarose gel electrophoresis proved the exactitude of the RT products, 11 LDR plasmid templates were constructed by amplification, purification, and cloning procedures recommended by the manufacturer (Promega, Madison, USA). The resulting plasmids were verified by sequencing and quantitated by using the NanoDrop ND-100 spectrophotometer.

2.2.5. Simplex LDR coupled with asymmetric labeling PCR

LDR was performed in a 20 μl reaction containing 1 μl of each upstream probe (0.1 μM), 1 μl of each downstream probe (0.1 μM), 3 μl simplex cDNA products or plasmids, 2 μl of 10× Taq DNA ligase buffer (New England Biolabs, MA, USA), 0.5 μl Taq DNA ligase (1 U/μl, New England Biolabs) and 12.5 μl distilled water. NTC reactions contained dH_2O instead of cDNA or plasmids. The ligation reaction was cycled for 35 rounds at 94 °C for 30 s and at 58 °C for 4 min on a PCR system (Bioer, Shanghai, China). The first cycle was preceded by 3 min denaturation step at 94 °C.

The resulting ligation product was used as asymmetric labeling PCR amplification template. The reaction mixture contained 2 μl of the LDR product, 5 μl 10× PCR buffer, 4 μl $MgCl_2$ (25 mM), 0.5 μl Taq polymerase (5 U/μl, Sangon), 4 μl dNTP (2.5 mM, except dCTP 0.125 mM, Takara), 8 μl Cy5-dCTP (0.01 mM, Amersham Biosciences UK Ltd., Little Chalfont, UK), 1 μl universal upstream primer (25 μM), 1 μl universal downstream primer (2.5 μM) and 24.5 μl distilled water. The cycling condition for asymmetric labeling PCR was denaturating at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s for a total of 35 amplification cycles. The first cycle was preceded by 3 min denaturation step at 94 °C. The last cycle was followed by an additional 10 min extension step at 72 °C and then cooling at 4 °C for preservation.

2.2.6. MRLP

The MRLP was performed using the same method as that in Section 2.2.5, except that individual plasmid or cDNA template was replaced by pooled plasmid or cDNA templates for LDR. The MRLP was optimized by varying the concentrations of LDR probes (10–0.01 μM), the LDR annealing temperature (65–45 °C), the quantity of Taq DNA ligase (5–0.1 U), the total of LDR cycle (60–5), the concentrations of universal upstream primer (0.5–0.05 μM) and downstream primer (5–0.5 μM), and the quantity of Taq polymerase (5–0.5 U). Ultimately, the optimum reaction conditions were found to be similar to those described in Section 2.2.5, except that the final concentration of universal upstream primer was 5 μM, universal downstream primer concentration was 0.5 μM, the quantity of Taq DNA ligase was 2 U, and Taq polymerase quantity was 4 U.