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Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan® real-time RT-PCR

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Abstract

The requirements of sprouting dormant potato tubers for biological or serological assays or RNA extraction for nucleic acid and PCR assays add to the cost of virus screening. Recently, cheaper, reliable and more rapid methods for the screening of potato tuber-seed pieces for viruses have been developed that do not require sprouted tubers for indexing, including TaqMan® real-time RT-PCR. Although the assays are often designed for minimal time and reagent use, they still require a time-consuming and laborious RNA extraction step. This paper describes an assay where four common potato-infecting viruses, *Potato leafroll virus*, *Potato virus A*, *Potato virus X* and *Potato virus Y*, were detected simultaneously from total RNA and saps of dormant potato tubers in a quadruplex real-time RT-PCR. Factors critical for the detection of these viruses in saps of dormant potato tubers included: optimum dilution and inhibition of RNAses, and the optimization of the reverse transcription and PCR steps. Potato virus detection directly from tuber saps was comparable to that from purified total plant RNA, and this represents significant savings of time and expense. The TaqMan® system developed in this study detected between 200 and 400 copies of potato virus RNA.

Keywords: Multiple virus detection; Potato tubers; TaqMan®; Real-time RT-PCR; Multiplex detection

1. Introduction

In the field, potato (*Solanum tuberosum* L.) is infected frequently with several viruses during a growing season (McDonald, 1984), which leads to reduced yield and quality tubers. Among the most common viruses affecting potato crops are: *Potato leafroll virus* (PLRV, genus *Polerovirus*), *Potato virus X* (PVX, genus *Potexvirus*), *Potato virus A* and *Potato virus Y* (PVA, PVY, both genus *Potyviruses*), and *Potato virus S* (PVS, genus *Carlavirus*) (Singh, 1999). These viruses can occur in single or as mixed infections within the potato crop.

Planting seeds that are free or/and resistant to viruses is a way of controlling viral diseases. Reliable and sensitive indexing techniques are indispensable tools for determining the virus status of potato seeds. There are several systems extant for seed indexing and certification, but most utilize a combination of field-based inspections for visual symptoms and serological testing using enzyme-linked immunosorbent assay (ELISA) (Singh and Singh, 1996). However, these methods are time consuming, expensive and generally cannot be carried out on dormant tubers (Huttinga, 1996). Consequently, several reverse transcription polymerase chain reaction (RT-PCR) protocols have been developed for detection of individual viruses from dormant tuber-derived RNA (Singh and Singh, 1997, 1998).

Detection of several individual viruses separately by RT-PCR reactions is also expensive and time-consuming. Multiplex reverse transcriptase polymerase chain reaction, which accommodates several pairs of primers in one reaction, has been investigated as a means to reduce cost and increase efficiency (Singh and Nie, 2003). For example, duplex RT-PCR detection of PLRV and PVY has been reported (Singh et al., 2000), as has multiplex detection of PVY strains (Nie and Singh, 2002; Singh and Nie, 2003). Multiplex RT-PCR detection of five potato viruses (PVA, PVS, PVX, PVY and PLRV) has also been reported (Nie and Singh, 2000). Most recently, multiplex RT-PCR was used to differentiate strains of PVY (Lorenzen et

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al., 2006). Increased efficiency can also be gained from these methods because the results of RT-PCR tests of dormant potato tubers compared well with the results of winter grow-out test (Russo et al., 1999). However, these methods rely on agarose gel electrophoresis of amplicons, which is not well suited for high-throughput applications.

PCR, utilizing real-time, closed-tube, fluorescent detection chemistry, have streamlined significantly the way PCR is carried out in many situations (Heid et al., 1996; Lie and Petropoulus, 1998; Holland et al., 1991). First, closed-tube amplicon detection reduces the risk of pre-PCR contamination from post-PCR products, a problem associated with traditional methods. Second, fluorescent detection of amplicons obviates the resources needed to analyze the sample after the reaction, including the need to undertake agarose gel electrophoresis, which is a time consuming and potentially hazardous procedure. Real-time PCR reduces the cost of virus detection and facilitates, especially high-throughput virus detection.

Multiplex real-time PCR is being developed because it is possible to label probes with different compatible fluorescent dyes. For potato viruses, TaqMan® duplex RT-PCR have been used for the detection of *Tobacco rattle virus* (TRV) and *Potato mottle top virus* (Boonham et al., 2000), and for PLRV and PVY (Klerk et al., 2001). The simultaneous detection of two targets of interest by real-time PCR is relatively common, however reliable detection of three or more targets is not common partly because optimization of these reactions is challenging and fluorescent reporter dyes may have overlapping excitation/emission spectra. However, some methods have begun to explore expanding multiplex assays to three or four targets with the availability of new dyes with resolvable excitation/emission spectra. For example, four pathogenic retroviruses were detected using molecular beacons (Vet et al., 1999). In addition, a four-color multiplex PCR assay for the simultaneous detection of four allelic variants based on TaqMan® chemistry has also been reported in Biorad bulletin no. 2955.

One potential disadvantage in developing multiplex detection systems for plant viruses in field situations is that of optimizing primer concentrations in relationship to constituent template concentrations (Singh et al., 2000; Mumford et al., 2000). The titre levels of each of the viruses co-infecting a plant can be very variable in the field, and a robust multiplex detection system must have a fixed optimum concentration of the constituent primers regardless of the titres of co-infecting viruses.

Reverse transcription PCR for potato viruses typically requires purification of total RNA from infected tissues. This step is expensive and time consuming. Automated systems for high-throughput RNA extraction are available, but few have been adapted for the special conditions needed to process plant-based samples. Any RT-PCR system that can bypass this extraction step will reduce greatly the cost of virus detection.

In this report, the development of a real-time multiplex RT-PCR method using TaqMan® chemistry is described for simultaneous detection of four potato viruses. The effects of various reaction components on the sensitivity, specificity, and robustness of virus detection are detailed. Furthermore, methods are also described for potato virus assay that gives suitable results directly from tuber sap. These new methods were compared to conventional methods using total RNAs and sap from infected dormant potato tubers.

2. Materials and methods

2.1. Uninfected, virus-infected tubers and RNA purification

Total RNAs and sap were obtained from dormant potato tubers (cv. Russet Burbank) derived from either certified virus-free tissue culture plants or certified plants infected singly with PLRV, PVA, PVX or PVY.

Total RNA was extracted from 200 mg of dormant potato tuber tissue using RNeasy Plant Mini Kit (Qiagen Inc., USA). The RLC buffer provided with the kit was used and the extraction was done following the manufacturer's protocol. Using the same kit, viral RNAs were also extracted from virions of PLRV, PVA, PVX and PVY, purified previously by sucrose density gradient centrifugation (Berger and Shiel, 1998; Huisman et al., 1988; Thomas et al., 1997). In addition, total RNAs were extracted from leaf tissues of potato plants (cv. Russet Burbank) singly infected with these viruses.

2.2. Primer and probe design

Full genome sequences of these viruses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) to obtain primers and TaqMan® probes that could detect the widest possible range of strains of PLRV, PVA, PVX and PVY. Multiple alignments were done with the sequences of each of the viruses with Clustal-X, and conserved regions identified. Primers and probes were designed to target the conserved regions of the viruses, using Primer Express (Software, version 1.5, Applied Biosystems). Parameters were set so that the T_m of primer pairs of was about 60 °C, probe T_m about 70 °C, and predicted amplicon sizes between 70 and 200 bp. Primer and probe sequences for each virus were tested *in silico* using BLAST on GenBank to minimize the likelihood of non-specific amplification. Primer 3′-end sequences and probes of each virus that showed no sequence homology with other viruses, or other sequences in GenBank were selected. Primers and probes were synthesized at Integrated DNA Technologies, USA (Table 1).

2.3. Treatment of sap from dormant potato tubers

One gram of tissue taken from different parts of a washed dormant potato tuber infected with either PVX, PVY, PVA, PLRV or virus-free, was ground into powder in liquid nitrogen, and then extracted with 5 ml extraction buffer [DEPC-treated PBS buffer (pH 7.4) containing 2% (w/v) PVP-40, 0.2% (w/v) egg albumin and 1% (w/v) Na_2SO_3]. Four microlitres RNA-secure reagent (20×) (Ambion) was added to 36 μl tuber sap, mixed and incubated at 60 °C for 10 min. Throughout this experiment, tuber sap was diluted in the extraction buffer.