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A multiplex RT-PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B

Claudio Ratti[a,*], Gerard R.G. Clover[b], Concepcion Rubies Autonell[a], Valerie A. Harju[b], Christine M. Henry[b]

[a] *DiSTA, Via Fanin, 44, 40127 Bologna, Italy*
[b] *CSL, Sand Hutton, York YO41 1LZ, UK*

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Abstract

A multiplex reverse-transcription polymerase chain assay (mRT-PCR) was developed, based on primers designed to distinguish the A and B types of beet necrotic yellow vein virus (BNYVV). RNA was extracted from 72 BNYVV isolates from Asia, Europe and North America, and the type of each isolate determined using an established detection method based on single strand conformation polymorphisms (SSCPs). An area of the 'triple gene block' region on RNA 2 was amplified and sequenced from 16 isolates of the A and B types. These sequences were aligned and two sets of PCR primers were designed to amplify unique areas common to each type. The A type assay produced a single 324 base-pair RT-PCR fragment when positive samples were amplified. The B type assay produced a 178 base-pair product from positive samples. No amplification was observed from healthy *Chenopodium quinoa* or sugar beet plants and from plants infected by others sugar beet soil-borne viruses.

Fragment length differed sufficiently to allow both assays to be run in a single PCR tube. The results obtained using the new multiplex RT-PCR assay were consistent with those from the established SSCP method for all 72 reference samples.

Keywords: *Benyvirus*; BNYVV; Multiplex-RT-PCR; Diagnosis; Characterisation.

1. Introduction

Beet necrotic yellow vein virus (BNYVV) is the type member of the genus *Benyvirus* and causes the disease known as rhizomania. From an economic point of view, it is the most important sugar beet virus transmitted by *Polymyxa betae* Keskin. BNYVV was first described in Italy (Canova, 1959) and has since been reported from many sugar beet growing countries in Europe (Austria, Belgium, Bulgaria, Croatia, Czech Republic, France, Germany, Greece, Hungary, Iran, Kazakhstan, Kyrgyzstan, The Netherlands, Poland, Romania, Russian Federation, Slovakia, Spain, Sweden, Switzerland, Turkey, Ukraine, UK and Yugoslavia) (Asher, 1993; Tamada, 1999; Lennefors et al., 2000), Asia (China, Japan and Syria) (Gao et al., 1983; Miyanishi et al., 1999; Mouhanna et al., 2002) and in North America (California, Colorado, Idaho, Minnesota, Nebraska, Texas and Wyoming and in the United States of America) (Duffus et al., 1984; Duffus and Liu, 1987; Rush and Heidel, 1995).

The genome of BNYVV consists of five (4 + 1) plus-sense strands of RNA and on the basis of restriction fragment length polymorphism (RFLP), SSCP or sequence analysis of RT-PCR products from RNA 1–5, three major types of the virus have been identified. Types A and B are distributed worldwide and contain only four RNA species and type P, identified in Japan, France (near Pithiviers), Kazakhstan and UK, which

* Corresponding author. Present address: Alma Mater Studiorum, Università di Bologna, Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA), Area di Patologia Vegetale, Via Fanin, 44 40127 Bologna, Italy. Tel.: +39 051 2096733; fax: +39 051 2096722.
E-mail address: crubies@agrsci.unibo.it (C. Ratti).

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contains RNA 5 (Tamada et al., 1989; Kruse et al., 1994; Koenig et al., 1995; Koenig and Lennefors, 2000; Harju et al., 2002). Variations from 3% to 6% have been observed between nucleotide sequences of A and B types but a sequence identity of more than 99% suggests a highly conserved genome among isolates within A and B types (Koenig and Lennefors, 2000). To date, the molecular discrimination between A and B types does not seem to be related to different pathogenicity (Heijbroek et al., 1999). In contrast the P type is known to be responsible for severe rhizomania symptoms even in resistant varieties (Heijbroek et al., 1999).

Recent studies on the molecular properties of RNA 3 type A isolates identified a hypothetical P type in Belgium and the Pithiviers area which underlined the importance of being able to rapidly discriminate between A and B types for epidemiological and/or evolutionary studies, especially in areas where severe rhizomania symptoms occur (Lemaire et al., 2003).

Various different molecular protocols for BNYVV detection have been developed and many isolates have been characterized using RFLP, SSCP or sequence analysis methods (Kruse et al., 1994; Henry et al., 1995; Koenig et al., 1995; Lennefors et al., 2000; Morris et al., 2001; Lemaire et al., 2003; Meunier et al., 2003). Our goal in this work was to develop a multiplex (m)RT-PCR protocol for the simultaneous detection and direct characterisation of both BNYVV A and B types.

2. Material and methods

2.1. Plant material and RNA extraction

Single stranded RNAs were extracted using the CTAB method as described by Boonham et al. (2001) from leaf material (*Chenopodium quinoa*) or sugar beets roots grown on infected soil from 72 Asian, European and North American sources (Table 1). RNA 2 and 3 were analysed by RT-PCR and SSCP as described by Koenig et al. (1995).

2.2. Primer design

PCR products from an area of the 'triple gene block' (TGB) region on RNA 2 from 16 isolates were amplified using rhizoTGBR1/rhizoTGBF1 primers and then cloned into the pGEM-T vector (Promega). Sequencing was done by Sequiserve (Vaterstetten, Germany) and sequences were analysed using the Clustal V method (Megalign, DNAStar, Madison, WI, USA). The gene bank accession numbers for the 16 RNA 2 sequences produced in this study are given in Table 1. Two sets of primers were designed and used in RT-PCR and mRT-PCR: rhizoAR/rhizoAF and rhizoBR/rhizoBF (Table 2).

2.3. RT-PCR and multiplex RT-PCR assays

Deoxynucleoside triphosphates (dNTPs) were used at final concentration of 1 mM in RT and PCR reactions. RT reaction was performed at 37 °C for 1h in a total volume of 5 μl using 0.5 μl of RNA extraction, 50 units of reverse transcriptase (M-MLV Promega, Madison, WI) and 0.5 μg of random primers. PCR and mPCR reactions were carried out in a 25 μl volume using 1.25 units of *Taq* polymerase (Promega) and 3 mM $MgCl_2$. All primers were used at final concentration of 5 pmol per 25 μl in PCR reactions and 1.25 pmol (rhizoAR–rhizoBF) and 1.75 pmol per 25 μl (rhizoAR–rhizoBR) in mPCR reaction.

Cycling conditions consisted of an initial denaturation step at 94 °C for 10 min, followed by 40 cycles at 94 °C for 40 s, 58 °C for 35 s and 72 °C for 40 s followed by a final elongation step at 72 °C for 10 min in an T3 Thermocycler Biometra. PCR products were separated by electrophoresis in 2.0% agarose gels and visualized under an ultraviolet light after staining with ethidium bromide. Nucleotide sequence analysis of RT-PCR products from two well-characterised isolates of A and B types was under taken.

2.4. Nested multiplex RT-PCR assay (nmRT-PCR)

Single-tube RT-PCR reactions were carried out using primers pairs rhizoTGBF1/rhizoTGBR1 in 25 μl of total volume. The reaction mixture contained 0.5 μl of RNA extraction, 1 × Taq reaction buffer (Promega; 10 mM Tris–HCl, 50 mM KCl, pH 9.0), 1.5 mM $MgCl_2$, 1 mM dNTPs, 5 pmol of each primer, 50 units of M-MLV reverse trascriptase (Promega) and 1.25 units of *Taq* polymerase (Promega). Thermo-cycling was performed as follows: 37 °C for 30 min, 95 °C for 5 min then 30 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 45 s, followed by 72 °C for 10 min. RT-PCR products 1/25 diluted (2 μl) were added to the nmPCR mixture: 1 × Taq reaction buffer (Promega), 3 mM $MgCl_2$, 1.5 μg bovine serum albumen, 1 mM dNTPs, 1.25 pmol of rhizoAR and rhizoBF primers, 1.75 pmol of rhizoAR and rhizoBR primers, and 1.25 units of *Taq* polymerase (Promega). Following this, amplification was carried out under the follow cycling conditions: initial denaturation step at 94 °C for 1 min, followed by 45 cycles at 94 °C for 20 s, 66 °C for 20 s and 72 °C for 20 s followed by a final elongation step at 72 °C for 10 min in an T3 Thermocycler Biometra. PCR products were separated as described above.

3. Results

3.1. BNYVV diagnosis

BNYVV was detected in all 72 sources amplifying viral genome regions between nucleotides 2811–3241 on RNA 2 and between nucleotides 409–1268 on RNA 3 (Koenig et al., 1995) by standard RT-PCR reaction. SSCP analysis on RNA 2 and 3 distinguished A and B type isolates (Table 1) observing, for each type, similar patterns to those obtained by Koenig et al. (1995).