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The use of real-time RT-PCR (TaqMan®) and post-ELISA virus release for the detection of Beet necrotic yellow vein virus types containing RNA 5 and its comparison with conventional RT-PCR

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Abstract

Real-time RT-PCR (TaqMan®) assays were developed for the specific detection of Beet necrotic yellow vein virus (BNYVV). The two assays designed were a broad-spectrum one that detected RNA 2 from all types and a second designed to detect types containing RNA 5. The assays were validated against a range of different isolates from Europe and the Far East. These real-time assays were compared to a conventional RT-PCR assay for the detection of RNA 5. Sensitivity comparisons showed that for the detection of RNA 5, TaqMan® was 10,000 times more sensitive than the conventional RT-PCR assay. Further improvements were made to the test procedure by using post-ELISA virus release (VR), as an alternative to RNA extraction. This significantly increased the speed of processing samples and reduced the staff input required, allowing the TaqMan® assay to be used routinely as part of an annual survey of UK field samples.

Keywords: Sugar beet; Rhizomania; BNYVV; P-type

1. Introduction

The disease rhizomania of sugar beet (*Beta vulgaris*) is caused by Beet necrotic yellow vein virus (BNYVV) (Tamada and Baba, 1973)—the type member of the genus *Benyvirus* (Koenig and Lesemann, 2000). BNYVV is a soil-borne pathogen, transmitted by the soil protist *Polymyxa betae* (Keskin, 1964; Fujisawa and Sugimoto, 1977; Richards and Tamada, 1992). The disease was first reported in Italy (Canova, 1959) and has since been detected in many other countries (Tamada, 2002). Severe economic losses can be caused by the disease, through reductions in both crop yield and beet sugar content (Henry, 1996).

BNYVV is characterised by rod-shaped particles, 20 nm in diameter and of four different modal lengths: 85, 100, 265 and 390 nm (Putz, 1977). These particles have been shown to contain four separate single-stranded genomic RNAs of 1467, 1774, 4612 and 6746 base pairs (bp), respectively. A single protein of molecular weight 21,000 Da is found associated with virus particles, which is the virus coat protein (Steven et al., 1981). Two major strain groups of BNYVV, A and B, were revealed by restriction fragment length polymorphisms (RFLP) analysis (Kruse et al., 1994). A third type was detected using single-strand conformation polymorphism (SSCP) analysis (Koenig et al., 1995) and was later defined by nucleotide sequence analysis and the presence of a fifth RNA (Koenig and Lennefors, 2000). Other isolates containing a fifth RNA have been found in Japan (Tamada et al., 1989; Kiguchi et al., 1996), China (Li et al., 1999) and the UK (Harju et al., 2002); BNYVV containing RNA 5 from the Pithiviers area of France (Koenig et al., 1997) and

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from Kazakhstan (Koenig and Lennefors, 2000) have been termed P-types, (after Pithiviers, France where it was found originally).

In Japan (Tamada et al., 1996) and China (Liu et al., 2003), it has been reported that BNYVV isolates containing RNA 5 induced more severe symptoms and yield reduction in sugar beet, than BNYVV isolates not containing RNA 5. It was also shown that RNA 5-containing isolates caused increased damage in partially resistant cultivars, such as Rizor (Tamada et al., 1996). Evidence was found that sugar beet cultivars with varying degrees of resistance, varied in their responses to different types of BNYVV, with those infected with P-types having higher virus content than those infected with the common types without RNA 5 (A- or B-types) (Heijbroek et al., 1999). The possibility that isolates containing RNA 5 can overcome conventional resistance is of considerable concern, as BNYVV-resistant varieties are now widely grown in rhizomania-affected regions and increasingly provide the main means of disease control.

Given the potential additional risk posed by RNA 5 and the P-type having already been found in France, the decision was made to carry out surveys in the UK for field samples containing RNA 5. In order to achieve this it was necessary to develop a reliable diagnostic method that could be used as part of routine surveys, to detect RNA 5 in sugar beet infected with BNYVV. This paper describes the development of such a method, based upon real-time (TaqMan®) RT-PCR and its comparison with a conventional RT-PCR method.

2. Materials and methods

2.1. Plant material and virus isolates

Infected material containing known isolates of BNYVV (Table 1) was obtained by bait testing from infected soils (Henry et al., 1992). Sugar beet seedlings (cv. Roberta) were grown in infested soils for 6 weeks at 20 °C. After this period, the plants were harvested and their roots washed, prior to being tested. All of these known isolates were typed using the SSCP method of Koenig et al. (1995).

In addition, 157 UK field samples of BNYVV (75, 42 and 40 in years 2001, 2002 and 2003, respectively) were obtained from mature sugar beet samples sent to the Central Science Laboratory (CSL), as part of the annual rhizomania survey.

All type isolates and field samples used were confirmed as BNYVV-positive using enzyme-linked immunosorbent assay (ELISA) (see below).

2.2. ELISA

Triple antibody sandwich (TAS) ELISA was performed using BNYVV-specific polyclonal (CSL H3) and monoclonal (CSL MAFF 9) antibodies (Torrance et al., 1988). These antibodies are available from Adgen Ltd., Auchincruive, Scotland. Assays were performed essentially as described by Clark and Adams (1977), with modifications as described in Henry et al. (1992).

2.3. Post-ELISA virus release (VR) for TaqMan® RT-PCR

Following ELISA, trapped virus was released using the method of Harness et al. (2003). Positive or potentially positive ELISA plate wells were washed with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 and tapped dry. These wells were then filled with 50 μl of virus release buffer (VRB); (10 mM Tris–HCl, pH 8.0 with 1.0% (v/v) Triton X-100), covered to prevent evaporation and then shaken for 5 min at 65 °C. After incubation, the extracts were decanted and stored at 5 °C prior to testing the same day or frozen at −80 °C for testing. For testing by TaqMan®, 5 μl of extract was used.

2.4. RNA extraction

Total RNA was extracted from samples using a CTAB method, modified from that described by Chang et al. (1993). Leaf or root tissue (100 mg) was ground in 1 ml of grinding

Table 1
Isolates of BNYVV used during assay development

Derivation of isolates	Research contact	Type	RNA 5 (RT-PCR)	RNA 5 (TaqMan®)	RNA 2 (RT-PCR)	RNA 2 (TaqMan®)
Baotou, China (C)	H. Chenggui	P	+	+	+	+
Pithiviers, France (P)	P. Houdmon	P	+	+	+	+
(T101), Japan (J)	T. Tamada	P	+	+	+	+
Siena, Italy	C. Ratti	A	–	–	+	+
Ravenna, Italy	C. Ratti	A	–	–	+	+
Tompladony, Hungary	L. Potyondi	A	–	–	+	+
Celldomak, Hungary	L. Potyondi	A	–	–	+	+
Blaxhall, UK	C. Henry	B	–	–	+	+
Boyton, UK	C. Henry	A	–	–	+	+
Barton Mills, UK	C. Henry	A	–	–	+	+
Noord Beveland, NL	W. Heijbrooek	A	–	–	+	+
Poland	M. Jezewska	A	–	–	+	+

Type was determined by SSCP for A- and B-types (Koenig et al., 1995), and RNA2 and RNA5 testing was performed using TaqMan® and RT-PCR for P-types.