Detection of Sugar Beet–Infecting Beet Mild Yellowing Luteovirus Isolates with a Specific RNA Probe

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ABSTRACT

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A complementary RNA (cRNA) probe, BM1, was prepared by transcription of a 1,061 nucleotide cDNA fragment complementary to nucleotides 1 to 1,061 (open reading frame [ORF] 1) within the sequence of a French sugar beet–infecting beet mild yellowing luteovirus (BMYV) -2ITB isolate. This probe detected specifically the homologous isolate as well as 14 other BMYV isolates collected from sugar beet grown in various areas, mainly in Europe. It did not hybridize with non-beet-infecting isolates of the closely related beet western yellows luteovirus (BWYV) or cucurbit aphid-borne yellows luteovirus (CABYV) -N isolate, but reacted weakly with two English BMYV isolates that do not infect *Capsella bursa-pastoris* or *Montia perfoliata*. The probe BM1 detected BMYV in single *Myzus persicae*, giving no reaction with nonviruliferous individuals. As a comparison, a second BMYV probe (BM2) was produced to the coat protein gene (ORF 4) of the French BMYV-2ITB isolate. This probe detected all BMYV, BWYV and CABYV isolates, highlighting the closer sequence homology within this region among Subgroup 2 luteoviruses. The dilution end-point for the detection of virus from infected material by radioactively labeled probes was 1:500, and about 250 fg of viral RNA could be detected from purified virions preparations. Non-radioactively labeled (digoxigenin [DIG]) probes were found to be 30-fold less sensitive than radioactive cRNA probes. Probe BM1 has potential for large-scale screening with applications in epidemiology and sugar beet-breeding programs. This report shows that heterogeneity at the 5′ proximal regions of the genomes of BMYV and BWYV offers the potential for discriminating between the two viruses and identifying the sugar beet–infecting BMYV isolates.

Additional keywords: aphid, chemiluminescence, dot blot hybridization.

Beet mild yellowing luteovirus (BMYV), first reported by Russell (32,33), causes a yellowing disease of sugar beet (*Beta vulgaris* L.) (11,16) that is common within European sugar beet growing areas. Major outbreaks occur sporadically, but can cause serious economic damage: epidemics have been observed, e.g., in the mid-1970s, when over 50 % of the sugar beet in England was affected by virus yellows (mainly incited by BMYV, and less frequently by beet yellows closterovirus) by late summer. A similar situation arose across the rest of north-west Europe (38).

The disease is mainly controlled by preventive spraying with aphicides directed against the aphid vectors. For optimum efficiency, this approach requires a reliable and sensitive method to forecast the potential threat by measuring BMYV levels in overwintering host plants, which are believed to play an important part in the survival and spread of this virus (15,20,34,41), and in aphid migrants, which contaminate sugar beet in spring.

Detection of BMYV has been routinely based on enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies (2,3,37). Unfortunately, it is very difficult to discriminate by ELISA between BMYV and beet western yellows luteovirus (BWYV). BWYV was first described by Duffus (6,7) and is the most widespread member of the luteovirus group (49), infecting more than 146 plant species (6,8). Indeed, BMYV is often considered to be a narrow-host-range strain of BWYV (12); BMYV has a more limited host range than have the U.S. sugar beet–infecting BWYV isolates (9). Most European BWYV isolates either do not infect sugar beet or do not induce yellowing symptoms and yield losses (15), but infect lettuce (*Lactuca sativa*) (22, 25,47) and crucifers such as oilseed rape (*Brassica napus* subsp. *oleifera*) (39,48). Both viruses, however, are transmitted in a persistent, circulative manner by several aphid species, mainly *Myzus persicae*, and share many source plants, rendering difficult their specific detection without time-consuming biological tests. Thus, both epidemiological studies and forecasting would benefit from the development of simple and reliable methods to discriminate between BMYV and BWYV.

Monoclonal antibodies (MAbs) raised against BMYV could not discriminate between BWYV and BMYV (18,29). Recently, it has been shown that BMYV and the non-sugar-beet-infecting BWYV can be distinguished using a specific MAb raised against barley yellow dwarf luteovirus (BYDV-PAV-IL1) (4,41). However, this MAb does not detect all strains of BMYV (42) and therefore other methods are required.

Another approach to luteovirus detection is based on RNA-sequence-specific procedures such as hybridization with specific nucleic acid probes (18,21,31) or polymerase chain reaction (PCR) (30,35). Sequence data are now available for many luteoviruses, including a French isolate of BWYV (46). Based on genome organization, the luteoviruses have been divided into two major subgroups: Subgroup 1, including BYDV PAV and MAV isolates, and Subgroup 2, which includes most of the other characterized luteoviruses, including BYDV RPV, potato leafroll virus (PLRV), cucurbit aphid-borne yellows virus (CABYV) (13), BMYV, and BWYV (28). The differences between the two subgroups are situated principally within the 5′-half of the genome, where the polymerase genes in both subgroups have different evolutionary affinities (27,28,46). The Subgroup 2 viruses pos-

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sess an "extra" open reading frame (ORF) (ORF 1, also referred to as ORF 0 by some authors) encoding a 29-kDa protein, which is lacking in the Subgroup 1 viruses (28). The 3′-terminal half of the genome of both subgroups, on the other hand, has a cluster of three ORFs encoding the 22-kDa coat protein, a 17 to 19 kDa protein (whose ORF is embedded within the coat protein gene in another reading frame) and the 54-kDa "readthrough domain" (Fig. 1). This set of genes displays considerable intrasubgroup sequence similarity and less but still highly significant similarity between subgroups, particularly within the coat protein gene (22 kDa). This sequence conservation has permitted design of both "universal" and subgroup- or virus-specific primers for detection of luteoviruses by reverse transcription (RT)–PCR (30). RT-PCR has been employed to detect BWYV and BMYV in crop plants (21) but, so far, has not been used to discriminate between the two viruses. Another approach to discriminate between luteoviruses is to employ nucleic acid probes from heterologous parts of their genome. Sequence comparison among the Subgroup 2 viruses has revealed that the greatest sequence variability occurs within the 5′-proximal ORF 1 (H. Guilley, *unpublished*). We have previously shown that a riboprobe encompassing this region of BWYV does not hybridize detectably with BMYV (18).

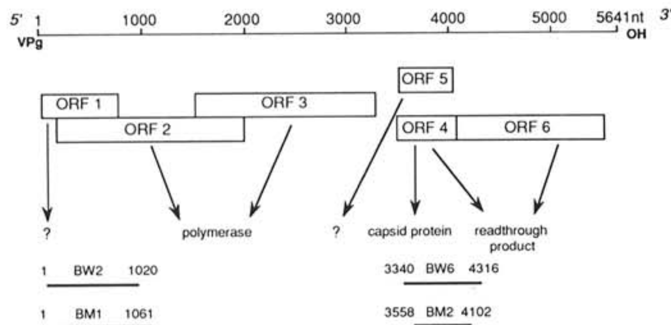

Fig. 1. Schematic representation of the BWYV-FL1 and the BMYV-2ITB genomes (H. Guilley, *personal communication*) and localization of the riboprobes used for dot blot hybridization. BW 2, 6 and BM 1, 2 are probes transcribed from BWYV-FL1 and from BMYV-2ITB cDNAs, respectively. For each probe, the corresponding position in nucleotides has been reported.

In this paper, we describe use of a riboprobe derived from the 5′-terminal region of a French isolate of BMYV for the specific detection of sugar beet–infecting BMYV isolates in both plant sap and viruliferous aphids. We have also investigated the possibility of applying this technique for routine tests using riboprobes labeled with digoxigenin (DIG), a hapten that is detected by a highly specific antibody.

MATERIALS AND METHODS

Virus isolates. The virus isolates used in this study are listed in Table 1. All the isolates, except those from England and the CABYV-N isolate (provided by H. Lecoq, INRA, Mont Favet, France), were maintained in a climatic cabinet at 20 to 22°C in Colmar by serial transmission using *M. persicae* as the vector.

The French isolates were maintained on fodder beet (cv. Trestel). BMYV-2ITB, the isolate used for sequence analysis and riboprobe synthesis, was an isolate collected in September 1980 from diseased sugar beet near Baudreville (Eure et Loir). Isolates J and L were collected in Alsace in 1981 and 1990, respectively. All these isolates induced characteristic symptoms and loss of yield in sugar beets. Stof, Roufl, and RoufR were collected from symptomatic sugar beets in the surrounding area of Colmar in October 1993.

The English isolates were maintained at Broom's Barn and include a standard BMYV isolate (BMYV-Stand), which has been maintained in glasshouse-grown sugar beet and consists of isolates collected from different sugar beet growing regions and mixed to produce a stock "culture"; the Ipswich and York isolates (BMYV-Ips, BMYV-York) are field isolates; the isolate BMYV-Camb was collected from an infected field in Cambridgeshire; BMYV 2, 8, 20, and 56 are field isolates from sugar beet col-

TABLE 1. Reaction of luteovirus isolates that infect or do not infect sugar beets, to four ^{32}P-labeled riboprobes

				BWYV-FL1 probes		BMYV-2ITB probes	
Virus isolates	Host plant	Symptoms on sugar beet	Geographic origin	BW2	BW6	BM1	BM2
BMYV-2ITB	Sugar beet	+[a]	Eure et Loir, France	–[b]	+[c]	+	+
BMYV-J	Sugar beet	+	Colmar, France	–	+	+	+
BMYV-L	Sugar beet	+	Colmar, France	–	+	+	+
BMYV-Stof	Sugar beet	+	Colmar, France	–	+	+	+
BMYV-Roufl	Sugar beet	+	Rouffach, France	–	+	+	+
BMYV-RoufR	Sugar beet	+	Rouffach, France	–	+	+	+
BMYV-Iran	Sugar beet	+	Iran	–	+	+	+
BMYV-Stand	Sugar beet	+	Broom's Barn, U.K.	–	+	+	+
BMYV-Ips	Sugar beet	+	Ipswich, U.K.	–	+	+	+
BMYV-York	Sugar beet	+	York, U.K.	–	+	+	+
BMYV-Camb	Sugar beet	+	Cambridgeshire, U.K.	–	+	+	+
BMYV-2	Sugar beet	+	Suffolk, U.K.	–	+	+	+
BMYV-20	Sugar beet	+	Suffolk, U.K.	–	+	+	+
BMYV-8	Sugar beet	+	Suffolk, U.K.	–	+	+/–[d]	+
BMYV-56	Sugar beet	+	Suffolk, U.K.	–	+	+/–	+
BWYV-FL1	Lettuce	ND[e]	Avignon, France	+	+	–	+
BWYV-Col	Oilseed rape	–[f]	Colmar, France	+	+	–	+
BWYV-Fev	Field bean	–	Colmar, France	+	+	–	+
BWYV-Stand	Oilseed rape	–	Broom's Barn, U.K.	+	+	–	+
BWYV-OSR	Oilseed rape	–	Broom's Barn, U.K.	+	+	–	+
BWYV-Som	Oilseed rape	–	Somerset, U.K.	+	+	–	+
BWYV-USA	Sugar beet	ND	California, U.S.	–	+	–	+
CABYV-N	Melon	–	Nérac, France	–	+	–	+

[a] Yellowing symptoms on cultivated sugar beet.
[b] Negative results by dot blot hybridization.
[c] Strong positive results by dot blot hybridization (1/50 to 1/500 end-point dilution).
[d] Faint positive results by dot blot hybridization (1/5 end-point dilution).
[e] Not determined.
[f] Absence of symptoms on sugar beet.

lected in Suffolk; the BWYV-OSR isolate is a mixture of isolates collected from infected oilseed rape and maintained on oilseed rape and *Montia perfoliata*; and the BWYV-Som isolate is a field isolate collected from oilseed rape in Somerset.

The other virus isolates were provided by G. Wisler (BWYV-USA, Salinas, CA) and by M. R. Hajimorad (BMYV-Iran, Tehran, Iran) (14).

Virion purification. For virion purification, BMYV-2ITB was multiplied on *M. perfoliata*. Two months after inoculation, randomly selected plants were tested by ELISA using an anti-BMYV antiserum and dot blot hybridization using the BWYV-specific riboprobe 2 (18) to ensure that no cross-contamination had occurred with BWYV. Thereafter, the plants were harvested and frozen at –20°C.

Virion purification followed the method described by Lecoq et al. (23) for purification of CABYV. Two batches of 300 g of frozen leaves were used for each purification. The concentration of virus was estimated using an extinction coefficient of 8.6, as determined for PLRV (43). About 20 µg of purified BMYV was generally obtained per 100 g of leaf tissue. Another procedure employed the method of Van den Heuvel et al. (44) for purification of PLRV. Using 0.5% Cellulase and 0.5% Macerozyme (Yakult Honsha Co., Tokyo, Japan) instead of 1.5% Extractase P20X (Genencor, Schaumburg, IL) the yield was increased 4 times (80 µg of purified BMYV per 100 g of leaf tissue). The purified virus preparations were again tested by dot blot hybridization with the BWYV-specific riboprobe 2 (BW2) to rule out contamination with BWYV.

Preparation and spotting of leaf and aphid samples. We compared 3 extraction buffers and 2 membranes to determine the best conditions for cRNA dot blot hybridization. The procedure described here gave us the best signal-to-noise ratio and the highest sensitivity.

About 200 mg of infected leaf material was ground on ice in 1 ml of cold MOPS buffer (0.2M 3-(N-morpholino)-propane sulfonic acid (Pharmacia, Uppsala, Sweden), 0.05 M sodium acetate, 0.01 M EDTA, pH 8.0). Cellular debris were eliminated by centrifugation for 5 min at 6,000 × *g*. The supernatant was kept on ice and 20 µl was spotted immediately onto a positively-charged nylon membrane (Hybond N+, Amersham, Little Chalfont, U.K.) presoaked with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), using a Hybridot apparatus (BRL, Gaithersburg, MD) essentially as described previously (18). Aphid samples consisted of 1 or 5 wingless adults of *M. persicae* taken from a culture reared on healthy peppers. The aphids were allowed to acquire BWYV-Fev for an indeterminate period from infected field bean (*Vicia faba*), and BMYV-2ITB for 24 h from *M. perfoliata*. They were then placed in ELISA microtiter plates and crushed in 50 µl of MOPS buffer on ice, using a multiple insect homogenizer as described previously (17). Twenty microliters of the homogenate was pipetted onto the filter.

Riboprobe synthesis. The BWYV-cDNA clones, obtained from the BWYV-FL1 lettuce isolate (46), were used to transcribe riboprobes BW2 and BW6 (previously referred to as riboprobes 2 and 6), as described previously (18).

The clone pBM1, used to prepare the BMYV-specific riboprobe BM1, contained cDNA corresponding to residues 1-1080 of BMYV-2ITB RNA (H. Guilley, *unpublished*) inserted between the *Sal*I and *Bam*HI sites of PBS(-) (Stratagene, La Jolla, CA) with antisense RNA synthesis under control of the bacteriophage T7 RNA polymerase promoter. For ^{32}P-labeled riboprobe synthesis (18), pBM1 DNA was linearized by digestion with *Sal*I.

A second cDNA clone (pBM2) corresponded to residues 3,080 to 4,102 of BMYV-2ITB RNA inserted into the *Eco*RI site of PBS(-) with antisense RNA synthesis under control of the bacteriophage T3 RNA polymerase promoter. pBM2 DNA was digested with *Pvu*II before riboprobe synthesis. The resulting riboprobe contains, in addition to ^{32}P-labeled antisense viral RNA, a ~250 residue "tail" of RNA derived from the vector sequence between the end of the cDNA insert and the downstream *Pvu*II site.

Procedures for synthesis of ^{32}P-labeled riboprobes were as described previously (18). The probe coordinates for BM1 were nucleotides 1 to 1,061 and for BM2, nucleotides 3,558 to 4,102, based on the BMYV-2ITB sequence (Fig. 1) (H. Guilley, *personal communication*).

Nonradioactive riboprobes were synthesized from *Sal*I-digested pBM1 and *Pvu*II-digested pBM2 using digoxigenin-11-uridine-5'-triphosphate (DIG-11-UTP, Boehringer, Mannheim, Germany) instead of ^{32}P-UTP and following the supplier's instructions. About 4 µg of DIG-labeled riboprobe was obtained from 0.4 µg of plasmid. The DIG-labeled riboprobes were stored at –20°C in 50 µl of sterile water with the addition of 20 units of RNasin for long-term storage.

Hybridization and detection. For virus detection with ^{32}P-labeled riboprobes, after spotting, RNA was cross-linked to the membrane by treatment with UV light (254 nm) for 3 min. Prehybridization was for 3 h at 60°C in prehybridization buffer: 50% formamide, 5× SSC, 8× Denhardts, 0.1 M phosphate buffer pH 6.5, 0.1% sodium dodecyl sulfate (SDS), and 250 µg of denaturated salmon sperm DNA per ml. Hybridization was overnight at 55°C in 10 ml of the same buffer supplemented with 2.5 ml of 50% dextran sulfate and about 0.8 µCi of riboprobe per ml. The membrane was washed twice for 15 min with 2× SSC, 0.1% SDS and once for 30 min with 0.2× SSC, 0.1% SDS at 65°C, dried and exposed to film for 12 to 48 h at –80°C. In cases of faint autoradiographic signals, the exposure was prolonged to 7 days.

For detection with DIG-labeled probes, the samples were blotted onto the same kind of membrane and immobilized by baking for 2 h at 80°C. Prehybridization, hybridization (with about 40 ng of riboprobe per ml) and membrane washing conditions were as described above, except that prehybridization was carried out at 65°C and hybridization at 58°C.

Chemiluminescent detection of DIG-labeled probes was performed as recommended by the supplier (Boehringer) using 4-methoxy-4- (3-phosphatephenyl) spiro-(1,2-dioxetane-3,2′-adamantane) (Lumigen PPD). The anti-DIG alkaline phosphatase conjugate was diluted to 1:10,000 in the supplier's buffer 2. The membrane was incubated in 5 ml of this solution for 30 min at room temperature with agitation. The membrane was then exposed to a Hyperfilm ECL film (Amersham, Little Chalfont, U.K.) at room temperature for 5 to 60 min, depending on the strength of signal and background.

RESULTS

Probe synthesis and sample preparation. The four ^{32}P-labeled riboprobes, BM1, BM2, BW2, and BW6, and the two DIG-labeled riboprobes, DIG-BM1 and DIG-BM2, were synthesized by in vitro transcription. The yield of ^{32}P-UTP incorporation was similar to previous results (18). The labeling efficiency of the DIG-labeled riboprobe was as high as the control-labeled probe provided by the supplier (about 10 µg of DIG-BM1 or BM2 was obtained from 1 µg of plasmid DNA). Of the various RNA extraction procedures tested, the method described in Materials and Methods for virus detection in plants or aphids gave us very faint background signals after dot blot hybridization with both radioactively labeled and DIG-labeled probes.

Detection of different BMYV isolates and discrimination between BMYV and other luteoviruses. Dot blot hybridization analysis with probes BW2 and BM1 demonstrated that probe BM1 detected all BMYV isolates, without any cross-hybridization with the three BWYV isolates maintained at Colmar, whereas the results obtained with the BW2 probe were consistent with those previously described (18). As previously shown, BW2 hybridized specifically to the BWYV isolates from

lettuce, oilseed rape, and field bean, but not to the BWYV-USA isolate. BW6 and BM2 hybridized to all the luteoviruses tested, including CABYV-N, providing a useful tool to identify closely related luteoviruses belonging to Subgroup 2.

These results demonstrate that probe BM1 can specifically detect BMYV (Fig. 2A), and complement our previous results showing that 5′ end cRNA probes (i.e., probe BW2) specifically detected BWYV (Fig. 2B). A faint cross-hybridization between BWYV and probe BM1, as well as between BMYV and probe BW2, was detectable with 1 µg/ml or more of purified virions (Fig. 3), suggesting some sequence homologies within ORF 1 between BMYV and BWYV. However, none of the BMYV- and BWYV-infected plant or aphid samples gave any cross-hybridization with specific probes BW2 and BM1, even after a 4-day autoradiographic period (Figs. 2 and 3). None of the healthy samples tested gave any background, regardless of the probe.

To examine the ability of probe BM1 to detect other sugar beet–infecting BMYV isolates, a wide range of BMYV and BWYV isolates, collected mainly in France and England, were tested. The autoradiogram profiles of these isolates using the four riboprobes BM1, BM2, BW2, and BW6 are summarized in Table 1. The results show that all BMYV isolates assayed, which induced yellowing symptoms on sugar beet, were readily and specifically detected using probe BM1, except isolates BMYV 8 and 56 from the U.K., for which only faint autoradiographic signals were observed.

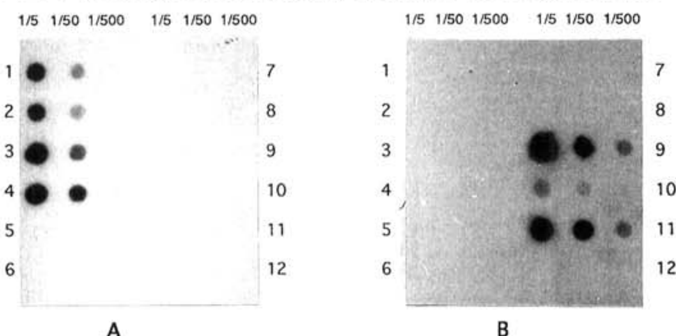

Fig. 2. Detection of BMYV and BWYV isolates by dot blot hybridization using ^{32}P-labeled riboprobes, after 4 day autoradiography. Ten-fold dilution series (1/5, 1/50, 1/500) of crude extracts were hybridized with specific cRNA probes; BM1 (**A**) or BW2 (**B**). Samples were: (1) BMYV-J infected sugar beet, (2) BMYV-L infected sugar beet, (3) BMYV-2ITB infected sugar beet, (4) BMYV-Iran infected sugar beet, (5) uninfected sugar beet, (6) uninfected *Montia perfoliata*, (7) uninfected oilseed rape, (8) uninfected field bean, (9) BWYV-FL1 infected *M. perfoliata*, (10) BWYV-Col infected oilseed rape, (11) BWYV-Fev infected field bean, (12) BWYV-USA infected sugar beet.

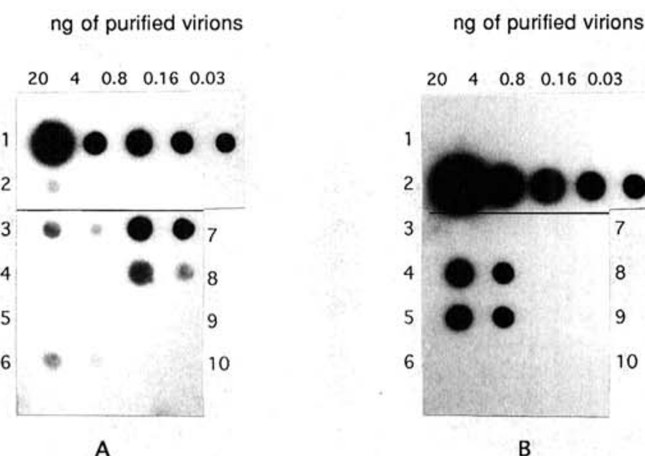

Fig. 3. Specific detection of BMYV and BWYV isolates in adult apterous aphid samples (*Myzus persicae*) by dot blot hybridization using BMYV and BWYV ^{32}P-labeled riboprobes. Rows 3 to 10 represent 2 successive spots of aphid samples: five (first dot) or one crushed aphids (second dot), hybridized with probes BM1 (**A**) or BW2 (**B**). Samples were: (1) and (2), respectively, serial dilutions of purified BMYV-2ITB and BWYV-FL1; rows (3), (6), (7) and (8) were *M. persicae* kept for 24 h on a BMYV-2ITB infected leaf of sugar beet; (4) and (5) were *M. persicae* reared on BWYV-Fev infected field bean; (9) and (10) were nonviruliferous aphids reared on healthy sugar beet and field bean, respectively.

These results confirm the viral specificity of the assay and demonstrate that most of the sugar beet–infecting BMYV isolates are detectable by dot blot hybridization using the probe BM1.

Detection in aphids and sensitivity of dot blot hybridization. BMYV and BWYV could be detected specifically in single wingless *M. persicae* adults using probes BM1 and BW2, respectively (Fig. 3). The faint signals obtained with two BMYV-carrying aphids showed that the quantity of virus within one aphid may be less than 5 pg after 24 h acquisition access period, near the detection threshold (Fig. 4).

To estimate the sensitivity of the dot blot hybridization assay, serial dilutions of purified BMYV-2ITB and BWYV-FL1 were used. All the probes tested, BW2, BW6, BM1, and BM2, showed that the dilution end-point was about 50 pg of purified virions per ml, equivalent to 1 pg of virus spotted (Fig. 4). In most cases, we could readily detect the virus in leaf sap at a 1:500 dilution, depending on the virus isolate and host (Fig. 2).

Comparison between nonradioactively and radioactively labeled probes. The utility for detection of the DIG-labeling system linked to chemiluminescence was examined. The DIG-BM1 probe hybridized to purified homologous virions and virus-infected leaf or aphid extracts, respectively, to an end-point dilution of 30 pg (1.5 ng/ml), 1:50, and 5 aphids (data not shown). We obtained the same amount of 30 pg of purified virions detected, when using a BMYV preparation that had been stored for 3 months in 50% glycerol at –20°C; we did not notice a detectable decrease of the RNA content (data not shown). The sensitivity decreased about 30-fold in comparison with radioactive labeling. The specificity of the DIG-BM1 probe was comparable to that obtained with the ^{32}P-labeled BM1 probe.

DISCUSSION

The radioactively labeled probe BM1 detected specifically the BMYV isolates with a detection limit for dot blot hybridization of about 1 pg of virions, the equivalent to 250 fg of viral RNA. Under optimal conditions, ELISA can detect about 2 ng of BMYV or BWYV virions per ml (19). Previously, we could not detect less than 120 pg and 2.4 ng of purified virions per ml by hybridization and ELISA, respectively (18); the new extraction procedure using MOPS buffer thus increased the sensitivity of detection by greater than twofold (50 pg/ml). The use of formamide or formaldehyde denatured plant or aphid samples might further improve the sensitivity of detection of viral RNA by dot blot hybridization as shown by Smith et al. (40). However, preliminary experiments using formamide-denatured plant extracts with radioactively labeled probe BM1 did not increase the assay sensitivity. Nevertheless hybridization is a highly sensitive detection procedure and many samples can be easily probed in a short period of time, which is ideal for large scale survey. Transcribed probes were chosen for this study because of their higher sensitivity and specificity than oligo-labeled or nick-translated cDNA probes to detect RNA viruses (45); it has been demonstrated that, at least for luteoviruses, cRNA probes were more sensitive than cDNA probes for homologous virus detection (31).

To overcome the disadvantages of radioactive probes (short half life and health hazards), non-radioactive labeling systems have been tested. In previous studies, the biotin-streptavidin system has been tested, but nonspecific reactions with endogenous biotin in host plants were experienced along with a marked adherence of streptavidin even on blocked filters, causing unac-

ceptably high background levels (10,36). In a previous paper (36), we have successfully used 2-acetylaminofluorene (AAF)-labeled probes to detect beet necrotic yellow vein furovirus in sugar beet. The health hazard linked to the possible presence of carcinogenic N-acetoxy-2-acetylaminofluorene (AAAF) present in association with the AAF-labeled probe led us to choose the DIG-system. Our results show that DIG-labeled BM1 probe linked to chemiluminescence could be used to replace radioactive labeling. However, the sensitivity decreased and probe DIG-BM1 detected only 30 pg of BMYV (1.5 ng/ml). Similar results were reported by Dietzgen et al. (5), detecting 10 pg of peanut mottle potyvirus with DIG-labeled riboprobes. However, it would be useful to further test the possible increase of sensitivity with formaldehyde denaturation as described by Smith et al. (40), attaining the sensitivity of ^{32}P-labeled riboprobes. Improved methods for crude sap preparation and extraction of viral RNA are currently under investigation. To increase the detection threshold, RT-PCR may be used. It was shown that RT-PCR could detect BWYV and BMYV with a detection limit of about 3 fg viral RNA without background (21). This has the advantage of amplifying a target sequence in a variable region (i.e., ORF 1 or ORF 2), which can be analyzed further for more subtle changes.

The riboprobe BW2, produced in an earlier study, was shown to be specific to all of the BWYV isolates tested, whereas the riboprobe BW6 cross-reacted with a wide range of luteoviruses, including BMYV, PLRV, BYDV-RPV and -PAV, and CABYV (18,23). A specific, sensitive, and reliable method for the detection of sugar beet–infecting BMYV isolates in plants or aphid vectors has been developed using riboprobe BM1 in vitro transcribed from such an isolate. This probe also detected isolates collected from a wide range of geographic regions and there was an absence of nonspecific background, with healthy plants or aphids, or cross-hybridization with BWYV isolates or CABYV. However, there was a weak cross-hybridization between BM1 and purified BWYV at high concentration of BWYV (over 1 µg/ml). This is due to a domain of weak homology between the nucleotide sequences of ORF 1 of BMYV and BWYV presenting 46% identity (H. Guilley, *personal communication*). This, however, should not be a problem with probe BM1 and BWYV or closely related luteoviruses in plant sap under our stringency conditions, because luteoviruses are limited to the vascular system and to the immediately surrounding tissues, and thus are present at low concentration within plants.

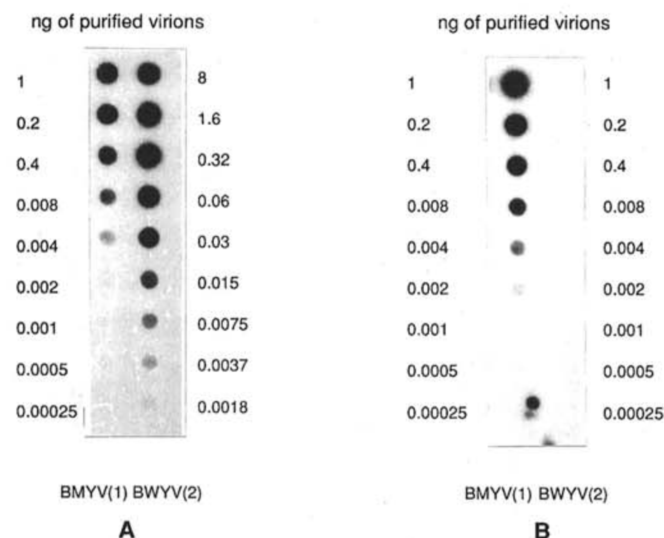

Fig. 4. Detection of BMYV-2ITB and BWYV-FL1 virions in purified preparations by dot blot hybridization using BMYV ^{32}P-labeled riboprobes. Dilution series of BMYV-2ITB (1) and BWYV-FL1 (2) virions were hybridized with BMYV-cRNA probes corresponding respectively to riboprobes BM2 (**A**) and BM1 (**B**). Virions concentrations (ng) were estimated spectrophotometrically.

The choice of the genome region covered by the probe had a strong influence on the range of heterologous viruses to be detected. The use of a shorter probe corresponding to a more variable region within the sequence of ORF 1 could provide an even more specific tool to estimate the variability among BMYV isolates. It is now possible to assay any portion of the BMYV genome by choosing the right probe in sequence and length from a collection of cloned and mapped cDNA.

The availability of a BMYV-specific probe has great potential in epidemiological studies that require virus assessment in winter hosts and migrant aphid vectors. Current polyclonal antisera react with BMYV and BWYV as well, and hence tend to overestimate the risk for sugar beet crops. MAbs have the same disadvantage, with the exception of BYDV-PAV-IL1 (4,41), which is specific to most, though not all (42), BMYV isolates. With our BMYV-specific probe, it is now possible to clearly identify BMYV or BWYV, as well as mixed infections or vectors carrying both viruses. This will therefore improve the forecasting of BMYV within Europe and the advice given to growers regarding the need for or timing of treatments.

We showed previously (18) that BYDV-RPV, PLRV, BWYV, and BMYV form a cluster of more related viruses within the luteovirus group, all of them belonging to Subgroup 2. This study is in agreement with that conclusion, and suggests that BMYV-2ITB is closely related to CABYV-N, as well as to BWYV-FL1. Furthermore, the combination of ELISA using specific monoclonal antibodies and hybridization with BM1 or shorter probes within the 5′ region of BMYV RNA, would be useful for analyzing both similarities and differences among various sugar beet–infecting BMYV isolates collected worldwide and for comparing BMYV with other related luteoviruses such as BWYV or CABYV.

Dot blot hybridization using probe BM1, covering the entire ORF 1 of BMYV, is effective for detecting geographically diverse sugar beet–infecting BMYV isolates in plants or aphids. Therefore, ORF 1 seems to be linked to a common feature for almost all sugar beet–infecting BMYV isolates tested that induce yellowing symptoms. However, we did not obtain the same results with two English isolates, BMYV 8 and 56, that induced only faint signals on autoradiograms with probe BM1; this is not due to a lower concentration of these isolates, as these reacted with probes BM2 and BW6 with the same intensity as other BMYV-isolates. Their host range differs from that of other BMYV isolates as they do not infect *Capsella bursa-pastoris* and *M. perfoliata* (42). Furthermore, we did not observe any signals using probes BM1 and BW2 under our hybridization stringency conditions for the BWYV-USA isolate, which replicates in sugar beet and presents a wider host range than does BMYV. This suggests that this U.S. isolate belongs to Subgroup 2, but differs from the others in ORF 1 nucleotide sequence. ELISA and hybridization data using the BM2 probe have shown that the virus titer was about the same for these isolates, confirming the hypothesis of little or no homology within sequence of ORF 1. It has been previously suggested, for PLRV, that ORF 1 may be involved in host specificity (26). This study provides results in agreement with that hypothesis. Further studies involving directed mutagenesis, agroinoculation (1,24) and sequencing of ORF 1 from BMYV-BWYV isolates differing in their host range will help to elucidate the function of ORF 1.

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