Detection of Potato Leafroll Virus in Dormant Potato Tubers by Immunocapture and a Fluorogenic 5′ Nuclease RT-PCR Assay

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ABSTRACT

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A gelfree, reverse-transcription polymerase chain reaction-based fluorogenic detection method for potato leafroll virus (PLRV) in dormant potato tubers has been designed. A PLRV sequence-specific oligonucleotide (TaqMan probe, Roche Molecular Systems, Inc., Roche Molecular Systems, Inc., Alameda, CA), containing a 5′-terminal 'reporter' fluorescein and a 3′-terminal rhodamine 'quencher,' was specifically degraded during amplification, resulting in a relative increase in reporter-associated fluorescence. The system was coupled to immunocapture of PLRV from tuber sap by paramagnetic beads coated with an antiserum against the virus. Addition of cell wall-degrading enzymes, cellulase, and macerozyme to tuber sap was essential to make detection of PLRV possible on tubers after a storage period. The potential for application in routine detection of PLRV in seed potatoes was investigated by testing the method on primarily infected, dormant tubers of four potato cultivars. Results were validated both by gel electrophoresis and enzyme-linked immunosorbent assay of shoots from sprouted tubers, which is the current assay for postharvest control of PLRV. All methods showed complete agreement in discriminating between PLRV-infected and noninfected samples. Detection took place in microtiter plates and was rapid, reproducible, semi-quantitative, and amenable to automation. The system offers the possibility of reducing the inspection time of seed potatoes for PLRV infection from 5 weeks to 1 day.

Although polymerase chain reaction (PCR) technology has a widespread application for virus detection and diagnosis in specialized laboratories (4), it still has a low impact in routine applications for certification and indexing programs. Some of the drawbacks of PCR technology compared to enzyme-linked immunosorbent assay (ELISA)-based detection methods lie in the difficulties encountered when dealing with high-throughput sample analyses and automation of pre- and post-PCR processing steps (2). Current methods of detecting PCR products include ethidium bromide (EtBr)-stained agarose gel electrophoresis and various assays involving hybridization and capture techniques (17). Because these assays are heterogeneous, the product detection process is still labor-intensive, and its automation is difficult or impractical.

A homogeneous assay for detecting the accumulation of specific PCR products in which no post-PCR separation is needed recently has been described (9). The assay takes advantage of the 5′ nuclease activity of *Taq* DNA polymerase (6,10) in combination with fluorogenic probes (8). The fluorogenic 5′ nuclease PCR assay is based on digestion by *Taq* DNA polymerase of a probe that is labeled with two fluorescent dyes and that hybridizes specifically to the target PCR product during amplification. The probe (TaqMan, Roche Molecular Systems, Inc., Roche Molecular Systems, Inc., Alameda, CA) carries both a reporter and a quencher fluorescent dye. During PCR amplification, the hybridized probe is cleaved by *Taq* DNA polymerase during strand elongation (10), resulting in a semiquantitative increase in reporter fluorescence (9). Repeated cycles of the PCR process result in exponential synthesis of the amplicon, with a concomitant increase in fluorescence intensity, generating a quantifiable signal.

Here we report the development and application of such a homogeneous, fluorogenic PCR-based method to detect potato leafroll virus (PLRV) in dormant potato tubers. The direct detection of PLRV and other viruses in dormant potato tubers by ELISA is not reliable (5,16). For this reason, the current seed-potato certification program is carried out by performing ELISA tests on leaf extracts of sprouted tubers. This procedure is labor-intensive and requires at least 5 weeks for completion. An assay that could be performed directly on dormant potato tubers and that is suitable for high-throughput analyses would be beneficial to simplify the inspection of seed potatoes. PCR technology is a good candidate because the nucleotide sequence and genomic organization of PLRV isolates from Europe, North America, and Australia have been determined (7,11,18) and primers as well as reverse-transcription PCR (RT-PCR) procedures specific for PLRV have been designed (3,15,16).

This paper describes how an RT-PCR assay specific for PLRV was designed for 5′ nuclease PCR assay detection and analysis. We have coupled this system to an optimized pre-PCR, immunocapture (IC) method that captures PLRV particles with paramagnetic beads carrying an antiserum against surface epitopes of the virus. Finally, we have assessed and verified the potential of the method for routine detection of PLRV in seed potatoes by testing the procedure on primarily infected, dormant tubers of different potato cultivars and comparing it with the current postharvest inspection method.

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MATERIALS AND METHODS

Plant material and virus. Healthy tubers of potato cultivars Bintje, Désirée, Kennebec, and Spunta were obtained from the Dutch General Inspection Service (NAK), Ede, the Netherlands. Pieces of tuber, each bearing one eye, were planted in pots in a greenhouse with an ambient temperature range of 20 to 24°C. At 10 to 11 days after planting, the young leaves of the developed sprouts were inoculated with PLRV by 20 viruliferous *Myzus persicae* Sulzer aphids per plant. The aphids were subjected to an acquisition-access period of 24 h on PLRV (isolate Wageningen)-infected *Physalis pubescens* plants before inoculation. The inoculation-access period was 96 h. Some plants were not inoculated to produce PLRV-free tubers. About 2 months after inoculation, the new tubers were harvested and tested by RT-PCR as described below. The first completely developed leaves at the top of each plant were tested by ELISA as described below. Tested tubers were treated with 3 nM gibberellin (4 µl/liter of water), planted, and sprouted. After 5 weeks, the top leaves of the shoots were tested by ELISA to verify whether they were infected by PLRV (shoot test). Tubers not used for tests were stored at 4°C. Purified PLRV virions were a gift from D. Maat (IPO-DLO, Wageningen, the Netherlands). RNA of PLRV was isolated from purified virions by repeated phenol/chloroform extractions in the presence of 0.5% sodium dodecyl sulfate.

Capture of PLRV virions from infected potato material and RT. Two procedures for the capture of PLRV virions from potato tuber material were compared: (i) IC in microfuge tubes, a slight modification of the method reported by Nolasco et al. (13) and (ii) immunocapture using paramagnetic beads (IMC). For the latter method, monodisperse, silanized iron-oxide superparamagnetic beads, 0.5 to 1.5 µm in diameter, with covalently linked goat anti-rabbit immunoglobulin G (IgG; Advanced Magnetics, Cambridge, MA) were employed.

In the optimized IMC procedure, potato material (0.8 g) was sampled at the heel-end of tubers with a razor blade. Each tuber piece was ground with a roller press, adding 1 ml of sample extraction buffer containing phosphate-buffered saline (PBS; pH 7.4), 0.05% Tween 20, 2% polyvinylpyrrolidone 25,000, 0.2% ovalbumin, and 0.5% bovine serum albumin (BSA). After every extraction step, the press was rinsed with tap water, 0.2 M NaOH, and a second rinse with tap water.

Tuber extracts (50 µl) were transferred to microtubes containing paramagnetic beads at a final concentration of 50 µg/ml. The extracts were brought to 1% cellulase Onozuka R-10 and macerozyme R-10 (Yacult Pharmaceutical Ind. Co., Ltd., Tokyo) and to 1 µg of PLRV antiserum (PLRV-Bi 9266 IgG, 1 mg/ml in PBS) per ml. The tubes were placed on a roller for 2 h at room temperature to maintain the beads in suspension. The beads were trapped along the side of the tubes with a magnet, and supernatants were removed. The beads were washed once in 100 µl of PBS, 0.1% Tween 20, and 0.1% BSA and once in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ (annealing reaction mixture). Between the washing steps, beads were trapped, and supernatants were discarded. Finally, the beads were resuspended in 30 µl of annealing reaction mixture containing 4 units of RNase inhibitor (Perkin-Elmer Corp., Norwalk, CT) and 0.1 µM downstream primer ([3]; Table 1). The samples were incubated for 5 min at 65°C and for 30 min at 23°C, and 20 µl of RT reaction mixture was added, consisting of annealing reaction mixture containing 0.5 mM each dNTP and 200 units of M-MLV reverse transcriptase (Gibco BRL Life Technologies, Gaithersburg, MD). Samples were incubated at 37°C for 2 h.

5′ nuclease PCR assay. The PCR conditions were a modification of the assay described by Hadidi et al. (3). The location and sequence of primers and the TaqMan probe are given in Table 1. The probe was developed as reported by Livak et al. (9). A 5-µl aliquot of the RT reaction was mixed with 45 µl of PCR mixture containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 to 5.0 mM $MgCl_2$, 0.05% W-1, 0.2 mM each dNTP, 0.3 µM downstream primer, 0.3 µM upstream primer (Table 1), 1.25 units of *Taq* DNA polymerase (Perkin-Elmer), and 40 nM TaqMan probe. A thermocycler (GeneAmp PCR system 9600, Perkin-Elmer) was used for amplification. All PCR assays began with 5 min at 95°C, followed by 35 to 45 cycles at 94°C for 20 s, 60°C for 45 s, and 72°C for 10, 30, or 60 s. All assays were done in triplicate.

Post-PCR analysis. The fluorescence spectra of each RT-PCR reaction were measured in a Perkin-Elmer LS-50B luminescence spectrometer equipped with a microtiter plate reader. Each reaction was transferred to an individual well of a white, 96-well microtiter plate (Perkin-Elmer). Each plate was scanned at 518 nm for the reporter (R) value (FAM, 6-carboxyfluorescein) and at 582 nm for the quencher (Q) value (TAMRA, 6-carboxytetramethylrhodamine), with a 10-nm slit width and at an excitation wavelength of 488 nm with a 5-nm slit width.

Normalization of data, to estimate the degree of probe cleavage, was as applied by Livak et al. (9) for calculation of the ΔRQ: $\Delta RQ = RQ^+ - RQ^-$, where

RQ^+ = emission intensity of reporter (FAM)/emission intensity of quencher (TAMRA)

for PCR with target and

RQ^- = emission intensity of reporter (FAM)/emission intensity of quencher (TAMRA)

for PCR without target.

To determine whether amplification had occurred in each sample, the threshold ΔRQ level was calculated by the standard deviation obtained from at least three no-template controls multiplied by the t distribution value for a 99% confidence level. Samples with a ΔRQ value greater than the threshold are considered positive.

TABLE 1. Characteristics of primers and TaqMan probe used for amplification and detection of potato leafroll virus (PLRV) RNA

Primer/probe	Sequence	Length (nt)	Position[a] (nt)	Type[b]
DS1	5′-gcactgatcctcagaagaatcg-3′	22	4103–4124	Down[c]
DS2	5′-ggcactgatcctcagaagaatc-3′	22	4104–4123	Down
US1	5′-aagaaggcgaagaaggcaatcc-3′	22	3638–3659	Up[c]
US2	5′-acaaccaagaaggcgaagaa-3′	20	3632–3651	Up
US2m	5′-acaaccaagaatgcgaagaa-3′	20	3737–3756[d]	Up
P1	5′-cagaagaggaggcaatcgccgc-3′	22	3734–3755	Probe[e]

[a] PLRV coat protein open reading frame (18).
[b] Down = downstream; Up = upstream.
[c] From Hadidi et al. (3).
[d] PLRV coat protein open reading frame (11).
[e] The probe carries a FAM dye (reporter fluorescein) covalently attached at the 5′-terminal nucleotide, a TAMRA dye (terminal rhodamine quencher) attached at the 3′-terminal nucleotide, and is 3′-terminal phosphate blocked.

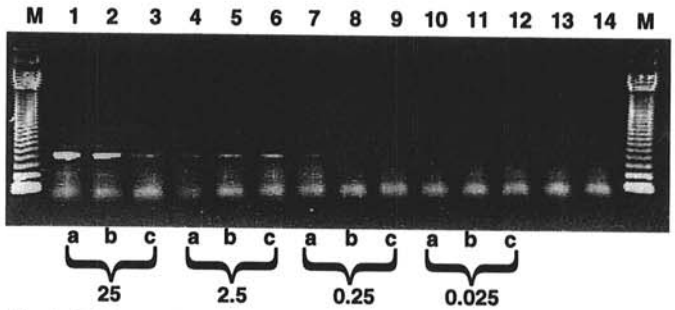

Fig. 1. Gel electrophoretic analysis of amplification product (486 bp) after immunocapture (IC) reverse-transcription polymerase chain reaction of potato leafroll virus (PLRV) from uninfected potato cv. Bintje tubers spiked with different amounts of purified PLRV virions (25, 2.5, 0.25, and 0.025 ng). The thermal disruption of the virions after IC was performed at **a**, 65°C; **b**, 65°C in the presence of 1% Triton X-100; and **c**, 90°C. Lanes M, 123-bp DNA ladder; lane 13, water control; and lane 14, water control plus 1% Triton X-100.

The presence of a specific PCR product was verified by electrophoresis of 10 µl of each PCR reaction on a 1% agarose gel containing 0.5 µg of EtBr per ml. Bands were visualized by UV excitation and photographed with a DocuGel-V digital photodocumentation system (Scanalytics, Billerica, MA).

ELISA. Indirect double-antibody sandwich-ELISA (IDAS-ELISA) with a PLRV-specific rabbit antiserum produced at IPO-DLO and conjugated with alkaline phosphatase was used for detection of PLRV in potato material. Absorbance of samples at 405 nm (A_{405}) was read in an ELISA microplate reader (EL 312e, Bio-Tek Instruments, Winooski, VT).

RESULTS

Optimization of PLRV capture from potato-tuber extracts. To permit a direct RT-PCR assay of PLRV in tuber sap without the need for nucleic acid extraction involving chemical treatments, capture of PLRV particles was performed by IC or IMC. With IC, the virions are captured in wells coated with a specific antiserum raised against PLRV, whereas with IMC the virions are captured from the extracts by paramagnetic beads carrying the antiserum on their surface. The advantages of using magnetic beads over coated wells are that they have a relatively large surface area, which allows more efficient capture, and bound material can be manipulated by its magnetic properties to allow efficient concentration and purification of virions from dilute mixtures.

Extracts from uninfected Bintje tubers were brought to different concentrations of PLRV virions (in the range of 25 ng to 25 pg), and these were used to optimize IC or IMC, followed by an RT-PCR assay. To optimize the IC/RT-PCR procedure, disruption of virions at different temperatures and the presence of detergent were tested. Figure 1 shows that the presence of 1% Triton X-100 during the 65°C treatment of the samples or a 90°C heat treatment did not improve sensitivity. With this procedure, the detection limit was 0.25 ng of PLRV virions in tuber extracts.

To optimize IMC/RT-PCR, three concentrations of magnetic beads were used (200, 50, and 5 µg of tuber extract per ml). Thermal disruption of the virus was performed at 65°C. Figure 2 shows that after IMC/RT-PCR, the overall detection of PLRV was about 10 times more sensitive compared to IC/RT-PCR. Using 50 µg of magnetic beads per ml in the potato extracts, the equivalent of 25 pg of PLRV virions in tuber extracts could still be detected in agarose gels. Because the RT-PCR procedure was the same as that used after IC, the improvement obtained with IMC can be attributed to more efficient PLRV capture by the magnetic beads.

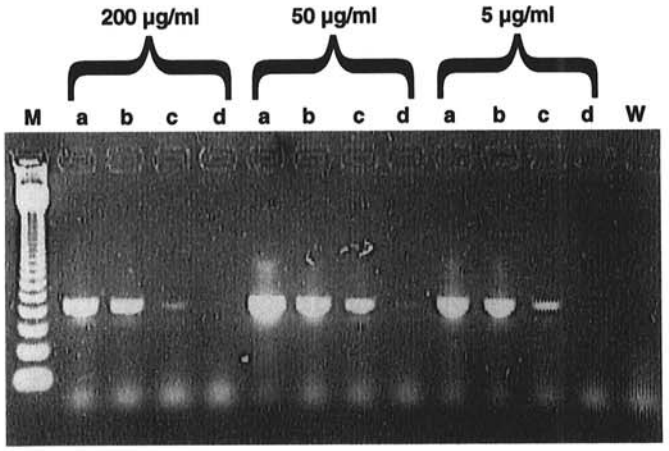

Fig. 2. Gel electrophoretic analysis of amplification product (486 bp) after immunomagnetic capture, reverse-transcription polymerase chain reaction of potato leafroll virus (PLRV) from uninfected potato cv. Bintje tubers spiked with different amounts of purified PLRV virions (25, 2.5, 0.25, and 0.025 ng, **a, b, c, and d,** respectively). PLRV virions were captured with different amounts of paramagnetic beads in the tuber extracts (200, 50, and 5 µg/ml). Lane M, 123-bp DNA ladder; and lane W, water control.

To assess the robustness of IMC/RT-PCR as a tool for routine screening of PLRV, we produced a set of primarily PLRV-infected and uninfected tubers of three potato cultivars. As soon as tubers were obtained, they were tested by IMC/RT-PCR. The results were verified in a grow-out test that assessed PLRV infections by ELISA tests on leaves of primarily infected plants and on shoots grown from tested tubers (i.e., secondarily infected material: shoot test). The latter test corresponds to the current postharvest inspection method for certification programs. The results and performances of the different methods are given in Table 2. In each case, ELISA tests on leaves of primarily infected plants underestimated the incidence of PLRV in tubers compared to IMC/RT-PCR and ELISA after shoot emergence. Except for one discrepancy with cultivar Désirée, the results indicated that IMC/RT-PCR was as efficacious in detecting PLRV as ELISA after the shoot test. Uninfected material always scored negative for PLRV with all the assays employed (data not shown).

The results of the previous experiment showed that, in general, the IMC/RT-PCR procedure was able to reliably detect PLRV in potato tubers within a few days after the tubers were harvested. In practice, however, seed-potato lots to be inspected are stored for unknown periods of time after harvest. Normally, this storage period covers about 4 months throughout the postharvest inspection season. Therefore, it is important that any new detection method be able to reliably assess the presence of virus in dormant tubers that have been stored for different periods of time. To test this, primarily infected tubers, produced as described above, were stored at 4°C, analyzed by IMC/RT-PCR, and replanted for verification by ELISA after shoot emergence. Table 3 shows the results obtained by testing dormant tubers stored for 8 weeks. Compared to the current inspection method, IMC/RT-PCR did not detect all in-

TABLE 2. Detection of potato leafroll virus (PLRV) by immunomagnetic capture, reverse-transcription polymerase chain reaction (IMC/RT-PCR) in primarily infected tubers of different potato cultivars and verification of infection by enzyme-linked immunosorbent assay (ELISA) tests on leaves of corresponding plants and after the shoot test[a]

Cultivar	No. of tubers tested	Positive by ELISA[a] on leaves[b]	Positive by IMC-RT-PCR[c]	Positive by ELISA[a] (shoot test)[d]
Bintje	12	7	11	11
Désirée	11	1	8	9
Kennebec	9	5	9	9
Total	32	13	28	29

[a] A_{405} for uninfected controls was 0.20 ± 0.03 SD (n = 6). Samples were positive when A_{405} was above 0.25.
[b] Leaves from the PLRV-inoculated plants that produced the tubers tested.
[c] Samples in which a PLRV-specific 486-bp band (3) was detected by ethidium bromide after gel electrophoresis.
[d] Shoots grown from the tested tubers (i.e., secondarily infected material).

TABLE 3. Detection of potato leafroll virus (PLRV) in primarily infected, dormant potato tubers after 8 weeks of storage at 4°C by immunomagnetic capture, reverse-transcription polymerase chain reaction (IMC/RT-PCR) and verification by enzyme-linked immunosorbent assay (ELISA) after the shoot test

Cultivar	No. of tubers tested	Positive by IMC/RT-PCR[a]	Positive by ELISA[b] (shoot test)[c]
Bintje	4	2	4
Désirée	4	0	4
Kennebec	4	4	4
Total	12	6	12

[a] Samples in which a PLRV-specific 486-bp band (3) was detected by ethidium bromide after gel electrophoresis.
[b] A_{405} for uninfected controls was 0.21 ± 0.01 SD (n = 6). Samples were positive when A_{405} was above 0.25.
[c] Shoots grown from the tested tubers (i.e., secondarily infected material).

fected tubers. This discrepancy was most evident with cultivar Désirée. The results indicate that the capability of IMC/RT-PCR to detect PLRV in dormant tubers decreases during the storage period of the tubers, possibly in a cultivar-dependent way. Uninfected tubers always were scored as negative by all detection methods (data not shown).

Conditions to improve both the efficacy of the virion capture step from tuber extracts and the course of the RT step were investigated. The tubers used, stored for 10 weeks at 4°C, were chosen from the same group in which detection by IMC/RT-PCR was problematic in the previous experiment. Factors and variables tested included (i) the addition of Triton X-100 (0.5%, vol/vol) and the cell wall-degrading enzymes, cellulase and macerozyme (1%, vol/vol), to the tuber extracts; (ii) the addition of a nonfat milk cocktail (Blotto, 0.2% vol/vol) to the RT mixture; and (iii) the incubation time of the RT reaction (1 or 2 h). The results are shown in Figure 3.

Figure 3B shows all controls performed. As expected, in uninfected material no PLRV-specific amplified fragment was found after electrophoresis. The IMC/RT-PCR assay was dramatically improved by the addition of cellulase and macerozyme in the tuber extracts (Fig. 3A, C, and D). When these enzymes were added to the extracts, a clear PLRV-specific amplification product could be detected in the tubers of those cultivars in which PLRV could not be detected previously after 8 weeks of storage (Table 3). No other factors or variable tested had such a prominent effect on the outcome of the assay. The macerating and clarifying action of the enzymes positively influenced PLRV capture from the tuber extracts. Overnight incubation of tuber extracts with magnetic beads without cell wall-degrading enzymes did not improve the efficacy of the virus capture (data not shown).

Optimization of detection of PLRV by a fluorogenic 5′ (TaqMan) nuclease RT-PCR assay. In the 5′ nuclease RT-PCR assay with a fluorogenic probe, production of a detectable signal occurs concurrently with target amplification. The formatting of such an assay requires the optimization of probe annealing and target amplification conditions to obtain the most satisfactory performance. In TaqMan PCR, fluorescence of the reporter group at the 5′ terminus is quenched by the 3′-terminal TAMRA dye. During PCR amplification of probe-specific template, the release of FAM dye during probe cleavage causes an increase in emission intensity at 518 nm, whereas the emission intensity of the TAMRA dye at 582 nm remains relatively unchanged. To optimize the TaqMan reactions, we evaluated the effect on fluorescence yield (ΔRQ) of different concentrations of $MgCl_2$ and primers as well as different extension times and numbers of cycles.

Figure 4 shows the effects of the different conditions investigated. Different downstream and upstream primers were tested in combination with the fluorogenic probe. Figure 4A shows that the combination US1-DS1 (Table 1) gave the highest ΔRQ, whereas US2m-DS1 gave the lowest. Although combination US2-DS1 also performed satisfactorily, in all further experiments only the primer

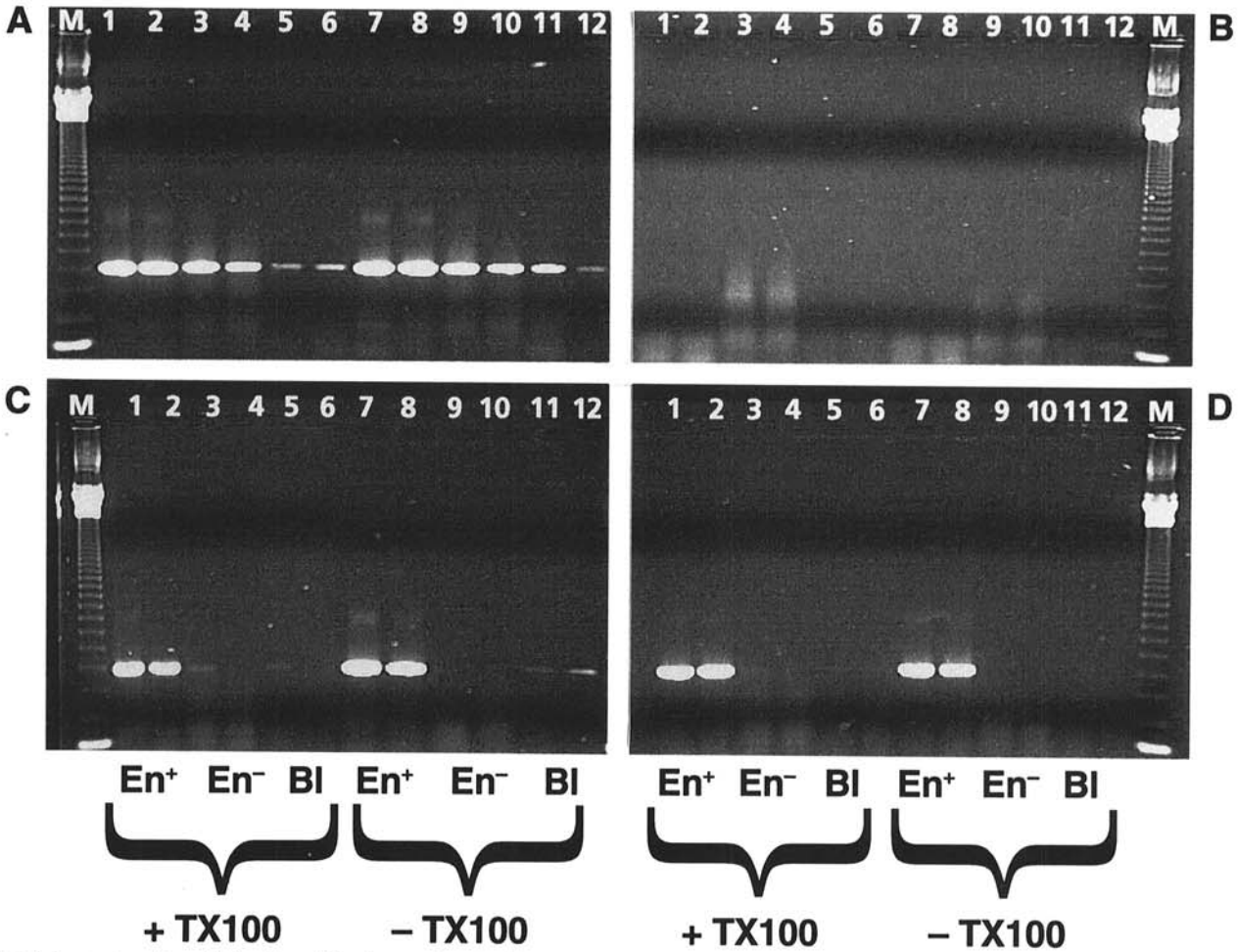

Fig. 3. Gel electrophoretic analysis of amplification product (486 bp) after immunomagnetic capture, reverse-transcription (RT) polymerase chain reaction of potato leafroll virus (PLRV) from uninfected or primarily infected dormant potato tubers. Optimization conditions tested were: addition of 1% of both cellulase and macerozyme to the tuber extracts (En^+) or not (En^-); addition of 0.2% Blotto (nonfat milk cocktail) during the RT step (Bl) or not (En^-); time of incubation of the RT reaction (1 h [1-11] or 2 h [2-12]); or addition of 0.5% Triton X-100 to the tuber extracts. **A,** Lane M, 123-bp DNA ladder; and lanes 1 through 12, PLRV-infected cv. Kennebec tuber. **B,** Lanes 1 through 12, uninfected cv. Bintje tuber; and lane M, 123-bp DNA ladder. **C,** Lane M, 123-bp DNA ladder; and lanes 1 through 12, PLRV-infected cv. Bintje tuber. **D,** Lanes 1 through 12, PLRV-infected cv. Désirée tuber; and lane M, 123-bp DNA ladder.

combination US1-DS1 was employed. A $MgCl_2$ concentration of 2.5 mM yielded the maximum ΔRQ (Fig. 4B). Because the RQ^- values over the different $MgCl_2$ concentrations did not change, the lower ΔRQ values may reflect a less efficient probe cleavage or annealing at concentrations above or below 2.5 mM. Alternatively, the overall PCR performance also may have been affected at different $MgCl_2$ concentrations. The effect of increasing the extension time at 72°C during PCR with 2.5 mM $MgCl_2$ and 35 cycles is shown in Figure 4C. An increase from 10 to 30 s doubled the ΔRQ from 0.6 to 1.2, respectively. A further increase to a 60-s extension yielded a more modest increase in ΔRQ. Figure 5 shows a comparison between the ΔRQ after 35 and 45 cycles. The ΔRQ increased from 0.0 to 2.5 after 35 cycles when the concentration of PLRV varied from 2.5 pg to 25 ng. Over the same concentration range, the ΔRQ after 45 cycles increased from 0.2 to 3.6. This demonstrates the semiquantitative nature of the assay, because the results show a measurable increase in FAM fluorescence concomitant to an increase in the number of cycles. This, in turn, reflects an increase in specific product formed by *Taq* polymerase activity during amplification.

To determine the sensitivity of the system, the threshold ΔRQ was calculated from the data of the experiment shown in Figure 5. Based on *t* distribution values, a sample with a ΔRQ value above the threshold is significantly greater than that of a PCR reaction run without template at a confidence level of 99% and, therefore, is considered positive. The threshold ΔRQ value is normally calculated for each experiment; in this case, it was 0.29. The lowest concentration of PLRV virions in which a positive score could be assigned by this method was 25 pg after 45 cycles. This was also the virion concentration at which a PCR product could still be detected after gel electrophoresis (Fig. 5). In all samples in which ΔRQ values were less than the threshold, no amplicon was detected by gel electrophoresis. Therefore, the calculated yes-no threshold corresponded exactly to the limit of visual scoring after gel electrophoresis.

Detection of PLRV in dormant tubers of potato cultivars by fluorogenic 5′ nuclease RT-PCR assay after IMC. The effectiveness of the fluorogenic 5′ nuclease RT-PCR assay was assessed by applying the complete, optimized procedure to detect PLRV in dormant, primarily infected potato tubers of different cultivars stored for 12 weeks after harvesting. Results obtained were validated by gel electrophoresis and comparison with the current inspection method (Table 4). All uninfected tubers were scored as negative by all assays used (data not shown). All infected tubers were scored as positive by all assays used. ΔRQ values for tubers originating from PLRV-inoculated potato plants ranged from a minimum of 1.06 to a maximum of 3.15. All the ΔRQ values were well above the threshold for a positive score, calculated to be 0.18. A 100% agreement between the fluorogenic assay, visual scoring after gel electrophoresis, and ELISA on leaves of sprouted tubers was found regardless of the potato cultivar. Results for selected positive samples of cultivars Bintje and Désirée obtained both by the fluorogenic assay and gel electrophoresis are shown in Figure 6.

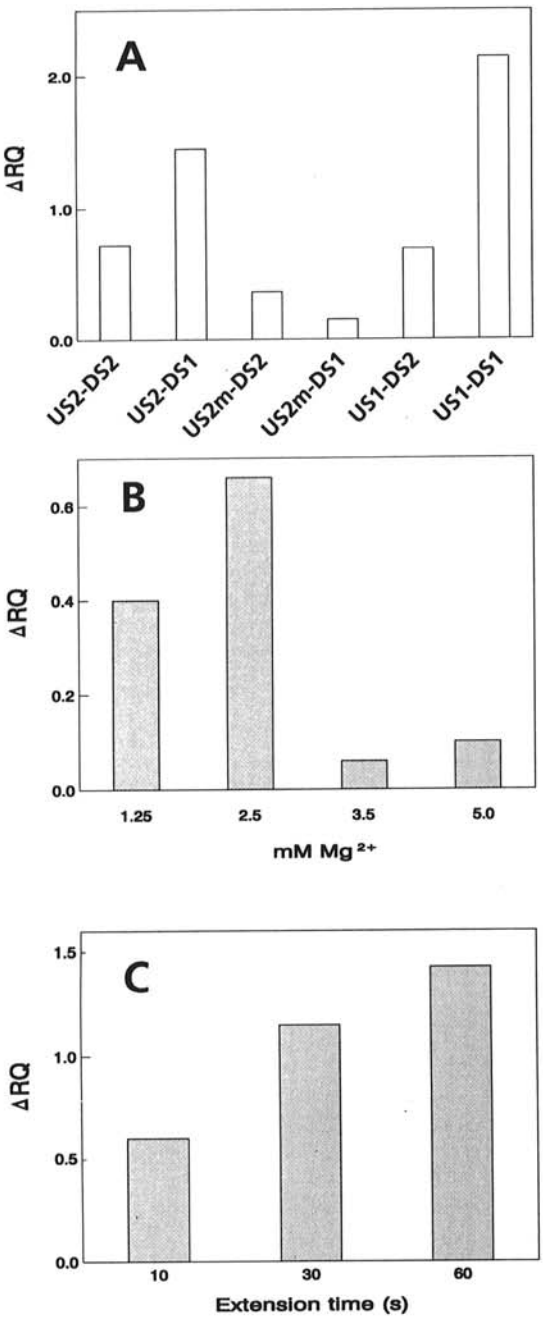

Fig. 4. Optimization of the fluorogenic 5′ nuclease reverse-transcription polymerase chain reaction assay. **A,** Effect of different primers in combination with the TaqMan probe. Amplification conditions: 2.5 mM $MgCl_2$, 35 cycles, 10-s extension time, and 2.5 ng of input potato leafroll virus (PLRV). **B,** Effect of the $MgCl_2$ concentration on ΔRQ. Amplification conditions: 35 cycles, 10-s extension time, and 2.5 ng of input PLRV. **C,** Effect of the extension time on ΔRQ. Amplification conditions: 2.5 mM $MgCl_2$, 35 cycles, and 2.5 ng of input PLRV.

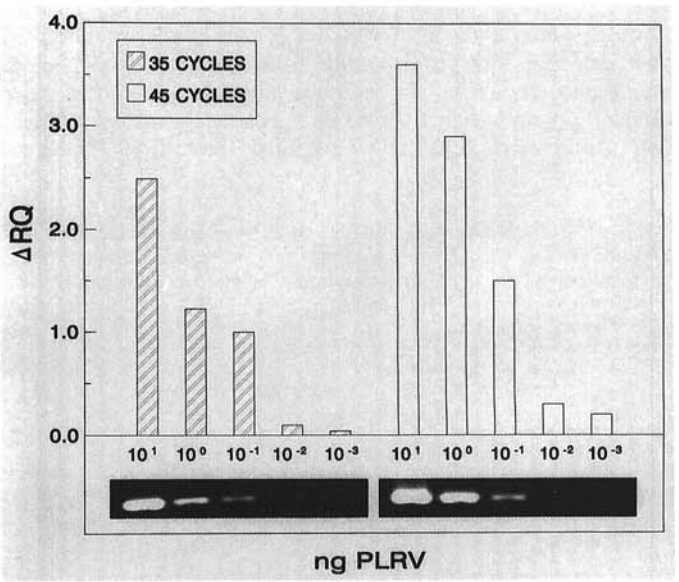

Fig. 5. Comparison of fluorogenic 5′ nuclease reverse-transcription polymerase chain reaction assay (expressed in ΔRQ) and gel electrophoresis (ethidium bromide staining) for detection of potato leafroll virus (PLRV). Effect of the number of cycles. Amplification conditions: 2.5 mM $MgCl_2$, 30-s extension time, and 25, 2.5, 0.25, 0.025 and 0.0025 ng of input PLRV. Amplification product is 486 bp.

DISCUSSION

The current indexing method for PLRV and other viruses in seed potatoes is to perform ELISA on shoots of sprouted tubers. This procedure is time-consuming and labor-intensive but necessary because direct detection of these viruses in tubers is not reliable (5,16). A procedure that can detect PLRV directly in potato tubers would simplify the inspection process and offer a beneficial alternative to the current indexing method for seed potatoes. A new procedure should be robust, reliable, and suitable for high-throughput analyses. RT-PCR–based methods of detecting PLRV have been described (3,15,16) and are rapid, specific, sensitive, and efficient for diagnostic purposes. However, large-scale application of such methods for routine assays is difficult. Problems can be caused by the lack of a simple, pre-PCR nucleic acid extraction procedure and the use of a heterogeneous, post-PCR electrophoretic analysis of amplified material. The results presented in this report show that PLRV can be detected directly in dormant potato tubers by a homogeneous RT-PCR–based assay that has the potential for automation for routine use. The new procedure consists of pre-PCR IMC of virions from tuber extracts by paramagnetic beads carrying a specific antiserum against PLRV; RT of a specific portion of the PLRV coat protein (CP) open reading frame (ORF); and a fluorogenic 5′ nuclease assay (with the TaqMan system).

The specificity of the primers and amplification conditions used in this assay have been documented (3) and were confirmed in this study. The feasibility of an immunomagnetic separation step to isolate PLRV from tuber extracts also was demonstrated. IC of virions to immobilized antibodies in ELISA plates has been described as a method to avoid nucleic acid extraction involving phenol manipulations (13). IC has been improved in our system by use of paramagnetic beads carrying an antiserum against PLRV. IMC is being used increasingly as a method to separate and concentrate target pathogens from water, food, and clinical samples (14). We found that the effectiveness of IMC was about 10 times higher than that of IC.

For reliable detection of PLRV in dormant tubers stored for different periods after harvest, the role of the cell wall-degrading enzymes cellulase and macerozyme during the preparation of tuber sap must be stressed. Addition of these enzymes to the extracts dramatically improved the capability of the IMC/RT-PCR method to detect PLRV in dormant tubers of different cultivars. The improvement due to the action of the enzymes was not observed when detection was performed by ELISA on tuber extracts (data not shown). Therefore, the enzymes probably allow for a more efficient capture of intact virions by the paramagnetic beads rather than making more virus antigen available for ELISA. The sensitivity of a method involving enzyme immunoassay in combination with paramagnetic beads was about 10 ng of PLRV diluted into healthy potato sap (1). By combining IMC with RT-PCR, we found that the detection limit of PLRV in healthy tuber sap was at least 100 times lower. A further advantage of IMC/RT-PCR on ELISA tests can be mentioned. A positive result with the latter method does not prove the presence of viable virus. Instead, when utilizing IMC with subsequent detection of nucleic acid, a positive result is attributable to the presence of intact virus.

The post-PCR detection process is one of the major drawbacks in scaling-up a PCR-based method for high-throughput and automated applications (2). This is due to the heterogeneous nature of the postamplification detection assays most often employed, such as gel electrophoresis or methods involving hybridization or capture techniques (17). The ideal situation for a post-PCR system is that of a homogeneous assay in which the processes of amplification and detection occur simultaneously without the need to separate reaction components afterward.

The fluorogenic 5′ nuclease PCR assay described by Livak et al. (9) approaches such a situation and offers the possibility of detecting the accumulation of specific amplification products. This is made possible by hybridization and cleavage of a double-labeled fluorogenic probe during PCR. We developed a probe (TaqMan) specific for a region of the CP ORF of PLRV and optimized the assay for the detection of this virus. To maximize the ΔRQ, the most important reaction and cycling parameters ($MgCl_2$ concentration, extension time, and number of cycles) affecting the 5′ nuclease PCR assay were optimized. The optimized procedure was applied in conjunction with the IMC step to detect PLRV in dormant tubers under conditions simulating real-world situations. Results were validated by comparison with the current postharvest inspection method in which tested tubers were planted, sprouted, and retested by ELISA on shoots. The presence of PLRV-specific amplification products also was verified by EtBr-stained agarose gels. Post-PCR analysis was performed immediately after thermo-

TABLE 4. Detection of potato leafroll virus (PLRV) in dormant tubers of different potato cultivars after 12 weeks of storage by immunomagnetic capture (IMC) followed by fluorogenic 5′ reverse-transcription polymerase chain reaction (RT-PCR) nuclease assay and verification by enzyme-linked immunosorbent assay (ELISA) after the shoot test

Cultivar	No. of tubers tested	Positive by 5′ nuclease assay	Range of ΔRQ values[a]	Positive by EtBr[b]	Positive by ELISA[c]
Bintje	11	11	1.59–2.57	11	11
Désirée	14	14	1.06–3.11	14	14
Kennebec	8	8	2.06–3.15	8	8
Spunta	4	4	2.45–2.94	4	4
Total	37	37		37	37

[a] ΔRQ values were calculated using the average RQ of the uninoculated tubers (1.25 ± 0.05 SD; $n = 5$). The average RQ for the no-template reactions without tuber extract was 1.28 ± 0.03 SD ($n = 4$).

[b] EtBr = ethidium bromide. Visual scoring of the amplified 486-bp PLRV-specific band after gel electrophoresis was as described in text.

[c] ELISA was done after the shoot test (i.e., on secondarily infected material).

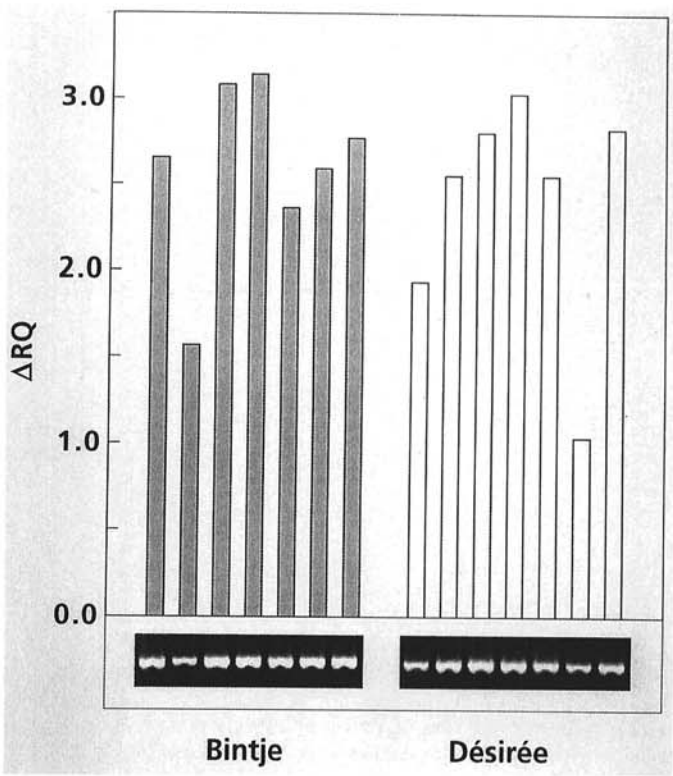

Fig. 6. Comparison of fluorogenic 5′ nuclease reverse-transcription polymerase chain reaction assay (expressed in ΔRQ) and gel electrophoresis (ethidium bromide staining) for detection of potato leafroll virus in dormant potato tubers after immunomagnetic capture. Amplification product is 486 bp.

cycling by transferring the reactions to a microtiter plate and scanning their emission spectra at 512 and 582 nm. The fluorescence intensity values obtained were used to determine the relative change in fluorescence yield (ΔRQ) during amplification. Samples with ΔRQ values above the threshold limit were considered positive for PLRV at a 99% confidence level. Detection of PLRV in dormant tubers of four cultivars showed complete agreement in discriminating between positive and negative samples by the fluorogenic 5′ nuclease RT-PCR assay, gel electrophoresis, and postharvest ELISA of emerged shoots. However, the testing time of seed potatoes was reduced from a minimum of 5 weeks for the current indexing method to 1 day for the fluorogenic IMC/RT-PCR–based assay. In addition, performing direct detection of virus in tubers eliminates the need for a greenhouse, which is required for the shoot test.

The new method of detecting PLRV in dormant potato tubers described here is not only fast and easy to perform but also robust. Therefore, it offers the potential to develop a fully automated system requiring a minimum of laboratory manipulations. Further improvements can be made by decreasing the number of steps. For example, we are investigating the use of different DNA polymerases, such as that from *Thermus thermophilus*, which has both reverse transcriptase and DNA polymerase activity (12). This should allow RT and PCR amplification to be carried out in the same reaction mixture. In addition, the feasibility of IMC/RT-PCR coupled with TaqMan detection demonstrated here should be applicable to many other systems requiring similar improvements in indexing procedures.

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