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Protocol

Optimized approaches for the sequence determination of double-stranded RNA templates

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Double-stranded RNA (dsRNA) is in many cases the only available template for molecular and diagnostic studies of RNA viruses. A novel mycovirus with a five dsRNAs segmented-genome served as a model system for the amplification and cloning of dsRNA segments using several PCR-based methods. Sequences obtained by the classical method; random PCR (rPCR) with a single primer assembled into 4 contigs out of the 5 segments. Moreover, using a modified single primer amplification technique (SPAT) resulted in the amplification of all or part of the dsRNA segments in one RT-PCR. Introducing such modifications into the FLAC method (full-length amplification of cDNA) resulted in amplicons comparable to those of the SPAT method. Full-length PCR products representing the five genomic segments were cloned and sequenced. The optimized conditions for each method are presented and discussed. In another approach, purified dsRNA segments were cloned directly into the blunt end pJET1.2 or the pGEM®-T cloning vectors with low efficiency though. This led to several sequences up to 2.2 kb in length, which could constitute a starting material for other methods like primer walking or as probes for diagnosis.

1. Introduction

Double-stranded RNAs (dsRNAs) are becoming a valuable source for molecular studies, especially for the sequence determination of novel RNA viruses. Besides the ssRNA viruses, which form a dsRNA Replicative Intermediate (RI) during their replication, there are six virus families with dsRNA genomes. These families are *Chrysoviridae*, *Partitiviridae*, *Reoviridae*, *Totiviridae*, *Cystoviridae*, and *Birnaviridae* (Ghabrial and Suzuki, 2009). These viruses infect a broad range of animal, plant, protozoa, bacterial and fungal hosts and in many cases, the infections are lethal (Mertens, 2004). Hence, more sequence information about such viruses would help in developing strategies for their detection and control. The stability of dsRNA compared to ssRNA, and the availability of optimized purification protocols are additional reasons that make dsRNA the template of choice when handling RNA viruses (Morris and Dodds, 1979). Furthermore, dsRNA templates offer two 3′ ends, one of which is complementary to the 5′ end of the plus strand. This property facilitates the determination of terminal sequences because of avoiding the 5′-alterations present in some mRNAs, like cap-structures or VPGs, as might also be the case for some dsRNAs.

Several techniques were published on the amplification and cloning of dsRNA templates (Attoui et al., 2000; Potgieter et al., 2002, 2009; Vreede et al., 1998; Lambden et al., 1992; Imai et al., 1983; Coutts and Livieratos, 2003). One of these methods is the random PCR (rPCR) using a primer with a random hexamer at its 3′ end (Froussard, 1992). This method was established for random amplification of ssRNAs and was applied thereafter for genomic dsRNA viral templates (Márquez et al., 2007). The sensitivity of rPCR was tested for ssRNA templates by Froussard (1992, 1998), who was able to obtain intense amplification products starting from as little template amounts as 1 pg. To our knowledge, the sensitivity of the method for dsRNA templates was not tested. The need for cloning and sequencing of many PCR products, the filling of gaps, the use of other methods such as RACE (Rapid Amplification of cDNA Ends) for the terminal sequence determination and the requirement of highly pure templates to avoid non-specific amplification make the rPCR methods time-consuming, laborious and costly.

A remarkable progress in dsRNA cloning was the establishment of the sequence-independent single-primer amplification technique (SPAT) by Lambden et al. (1992). The method was employed either solely or with modifications to clone dsRNAs of different sizes up to 2.5 kb (Bigot et al., 1995; James et al., 1999; Attoui et al., 2000; Zhang and Rowhani, 2000; Chen et al., 2002). The method was modified further to clone larger dsRNAs (>3 kb) (Vreede et al., 1998; Potgieter et al., 2002, 2009; Mann et al., 2007). These

Abbreviations: SPAT, single-primer amplification technique; FLAC, full-length amplification of cDNA; MMOH, Methyl Mercury Hydroxide.

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modifications include enrichment of the longer dsRNA segments by means of sucrose gradients or purification from electrophoresis gels, labeling and size fractionation of cDNA, ligation of primers with extended lengths, the use of anchor primer, which prime themselves for full-length amplification of cDNAs (FLAC), the use of highly toxic chemicals like MMOH for the efficient denaturation of dsRNA. Although such modifications might be useful for the sequence determination of large dsRNA genomes, many of them might not be available in every laboratory e.g. ultracentrifugation, radioactive labeling of cDNA, MMOH's handling and disposal regulations.

To our knowledge, the direct cloning of viral dsRNAs into dsDNA vectors without any previous transcription and amplification steps was never reported except for one patent application by Skotnicki et al. (1985). Part of this study describes the conditions, results, and evaluation of several attempts to clone genomic dsRNA into DNA vectors for subsequent molecular studies. The modifications of several sequence-independent methods that are based on single-primer amplification and cloning of dsRNA genomes are included. The goal of these optimizations is to set up rapid and reproducible methods for the full-length cloning of viral dsRNA genomes without the need for noxious chemicals or highly expensive equipment.

2. Materials and methods

2.1. dsRNA source, isolation and purification

A novel mycovirus from a *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch] isolate from China was employed in all of the methods studied. The mycovirus has 5 unequally and separately encapsidated genomic dsRNAs. The fungus was grown on PDB medium for one week under shaking (100 rpm) at 25 °C in the dark. The mycelium was collected by filtration through 2 filter papers (Whatman No. 1, Maidstone, England) and frozen at −70 °C until use.

Double-stranded RNA was isolated using the CF-11 fibrous cellulose chromatography (Sigma–Aldrich, Dorset, England) as described by Preisig et al. (1998) with some modifications. Briefly, 5 g of mycelium were put into a 50 mL reaction vessel with one stainless steel grinding ball (25 mm in diameter), frozen in liquid nitrogen, and pulverized in a Mixer Mill MM 400 (Retsch, Haan, Germany) at a frequency of 30 Hz for 30 s. The ground powder was then suspended in 10 mL of 2× STE buffer (0.1 M Tris–HCl, 0.2 M NaCl and 2 mM EDTA, pH 7.0) supplemented with 5 mg/mL Bentonite and 1.5% (w/v) SDS at 60 °C for 5 min. Ten mL of phenol:chloroform:isomylalcohol (5:1:1, pH 4.5) and 100 μL of β-mercaptoethanol were added and the mixture was shaken for 30 min at 37 °C. After centrifugation at 7818 × *g* for 10 min, the supernatant was mixed with 1 g of CF11 and ethanol was added to a final concentration of 17% (v/v). The mixture was shaken for 10 min at 80 rpm and applied into a 15 mL syringe blocked with glass wool. The mixture was pressed into the column and the collected flow through was reapplied to the column. The column was washed with 50 mL of 2× STE containing 17% ethanol (v/v). The bound dsRNA was eluted with 10 mL of 1× STE buffer and precipitated with 1 volume of isopropanol for 1 h at −70 °C. The pellet was collected by centrifugation, washed with 75% (v/v) ethanol, dried, and resuspended in 60–100 μL of distilled water. The extracted dsRNA was treated with DNaseI followed by S1 nuclease (Fermentas, St. Leon-Rot, Germany) for 30 min each as recommended by the supplier. Ten μL of the digested dsRNA were separated on 1% Agarose gel containing 0.5 μg/mL ethidium bromide for 1 h at 120 V in 1× TAE buffer and then visualized under ultraviolet light. The dsRNAs were purified from the agarose gel using the NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany) and were employed as a template in the methods described below.

2.2. Random PCR (rPCR)

Up to 100 ng of a mixture of the 5 dsRNA segments were mixed with 0.25, 0.5, 1 or 2 μM of the primer-dN_6 (5′-CCTGAATTCGGATCCTCCNNNNNN-3′), incubated at 99 °C for 2 min and quenched on ice for 5 min. Two hundred units of RevertAid™ Reverse Transcriptase (Fermentas), 50 mM Tris–HCl (pH 8.3 at 25 °C), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, and 20 units of RiboLock™ RNase Inhibitor (Fermentas) were added. The mixture was incubated at 43 °C for 1 h. At this stage, the cDNA was used either directly in the subsequent PCR or for the synthesis of second strand cDNA as follows. The cDNA was heated at 99 °C for 2 min then quenched on ice for 5 min. Ten units of the Klenow Fragment (Fermentas), 50 mM Tris–HCl (pH 8.0 at 25 °C), 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM dNTPs and distilled water to a final volume of 50 μL were added. The reaction was incubated at 37 °C for 30 min. The dscDNA was purified with the NucleoSpin® Extract II, eluted in 30 μL of distilled water, and stored at −20 °C until use. To test the sensitivity of the rPCR, 10^{-8} to 10^{-1} μg of the dsRNAs were reverse transcribed in the presence of 2 μM of the primer-dN_6 and amplified directly as described above without the Klenow Fragment reaction.

Amplification of the dscDNA took place in a reaction mixture containing; 1 μL of cDNA, 1× Taq Buffer advanced, 1.5 units of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 2 mM $MgCl_2$, 0.25 mM dNTPs, and 1 μM of the primer (5′-CCTGAATTCGGATCCTCC-3′). The thermal cycling was performed in a Biometra T1 Thermocycler (Goettingen, Germany) as follows: 1 cycle at 94 °C for 2 min, 65 °C for 1 min and 72 °C for 1 min, then 35 cycles of 94 °C for 40 s, 52 °C for 30 s and 72 °C for 3 min followed by a final extension step at 72 °C for 8 min.

2.3. Single-primer amplification technique (SPAT)

Primer PC3 (5′-PO4-GGATCCCGGGAATTCGG$(A)_{17}$-NH_2-3′) described by Potgieter et al. (2002) was ligated to the 3′ ends of the dsRNA as follows. About 250 ng of PC3 primer were ligated to 200 ng of purified dsRNAs at a molar ration of >40:1. The ligation mixture included: 50 mM HEPES/NaOH, pH 8.0 (Fermentas), 20 mM $MgCl_2$, 0.01% BSA (Promega, Mannheim, Germany), 1 mM ATP (Fermentas), 3 mM DTT (Roche, Mannheim, Germany), 10% (v/v) DMSO (Sigma–Aldrich), 20% (w/v) PEG_{6000} (Fermentas), 20 units of Ribolock RNase inhibitor (Fermentas), and 30 units of T4 RNA ligase (Fermentas) in a final volume of 30 μL. The ligation components were incubated at 37 °C for 6 h then at 18 °C descending down to 12 °C for overnight. The primer-ligated dsRNAs were purified from excess primer with the NucleoSpin® Extract II and concentrated in the SpeedVac vacuum concentrator for 15 min.

In another treatment, the PEG_{6000}, DMSO, BSA, and Ribolock RNase inhibitor were omitted from the ligation mixture and the reaction was incubated overnight at 16 °C. The reverse transcription, removal of the RNA and annealing of the cDNAs were carried out as described for the FLAC method below with one exception. That is, about 100 ng of the Oligo $(dT)_{18}$ were used to prime the PC3-dsRNA in the cDNA synthesis reaction.

2.4. Full-length amplification of cDNA (FLAC)

About 250 ng of PC3-T7 loop primer (5′-p-GGATCCCGGGAATTCGGTAATACGACTCA CTATATTTTTATAGTGAGTCGTATTA-OH-3′) described by Potgieter et al. (2009) were ligated to 200 ng of purified dsRNAs as described for the SPAT method above.

The purified primer-ligated dsRNAs were denatured at 98 °C for 2 min in the presence of 1 M Betaine and 2.5% (v/v) DMSO then quenched on ice for 5 min. In another treatment, the dsRNA

was denatured with various amount of DMSO (2.5%, 5%, and 15%, v/v) without the implementation of Betaine. The cDNA synthesis reaction contained: 50 mM Tris–HCl (pH 8.3 at 25 °C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 20 units of Ribolock RNase inhibitor, and 400 units of RevertAid™ Premium Reverse Transcriptase (Fermentas). The reaction was incubated at 50 °C for 1 h followed by 15 min at 55 °C. RNA was digested with 0.1 M NaOH at 70 °C for 20 min, followed by the addition of 0.1 M Tris–HCl pH 7.5 and 0.1 M HCl to neutralize the reaction. The cDNA was then incubated at 68 °C for 1 h followed by 1–2 h at 65 °C.

The amplification mixture, adjusted to a final volume of 25 μL, contained 5 μL of cDNA, 320 μM of each dNTP, 2 mM $MgCl_2$ and 1.25 μM of PC2 primer (5′-CCGAATTCCCGGGATCC-3′) and 2.5 units of one of the following DNA polymerases with 1× of their corresponding buffer: Phusion® High-Fidelity DNA Polymerase with Phusion GC Buffer (Finnzyme, Espoo, Finland), Go Taq DNA polymerase with the colorless buffer (Promega), Platinium DNA polymerase (Invitrogen, Darmstadt, Germany), 5 PRIME Taq polymerase with advanced buffer set (5 PRIME), or Long PCR Enzyme Mix with the long PCR buffer (Fermentas). The mixtures were incubated in a Biometra T professional Thermocycler at 72 °C for 2 min followed by 95 °C for 2 min and then subjected to 35 cycles of 95 °C for 25 s with an increment of 1 s per cycle, 65 °C for 30 s and 68 °C or 72 °C (as recommended by the manufacturer) for 5 min followed by a final step of 72 °C for 10 min.

2.5. Direct ligation of dsRNA into pJET1.2 and pGEM®-T vectors

About 200 ng of the dsRNAs were ligated into either the pJET1.2 (Fermentas) or the pGEM®-T (Promega) cloning vector at a molar ratio of about 4:1 (insert: vector). The ligation mixture contained 2.5 Weiss units of T4 DNA ligase and 25 units of T4 RNA ligase (Fermentas), 1 mM ATP, 5% (w/v) PEG_{6000}, 40 mM Tris–HCl, 10 mM $MgCl_2$, and 10 mM DTT in a final volume of 15 μL. The ligation reaction was carried out at 14 °C for 24 h. In a second treatment, the reaction was performed at 14 °C for 24 h followed by 24 h at 4 °C. The ligation mixture was then heat-shock transformed into XL1blue *E. coli* competent cells. The clones obtained were screened by means of either PCR or restriction digestion. Part of the positive clones was also sequenced. The experiment was repeated three times.

2.6. Cloning and sequencing

PCR products were purified from the agarose gel or from the PCR tube with the NucleoSpin® Extract II. Purified products were cloned into pGEM®-T cloning vector or pJET1.2 and transformed into *E. coli* competent cells according to the manufacturer instructions. The sequences were determined using the Sanger sequencing with an ABI 3730XL sequencer (Eurofins MWG Operon, Ebersberg, Germany) and assembled into contigs using the DNA Baser V2.90 RC.

3. Results

3.1. dsRNA purification

Several dsRNA segments were isolated from the fungus *F. graminearum* using the CF-11 chromatography. Digestion with DNaseI and S1 nuclease confirmed the dsRNA nature of the segments (Fig. 1). To assure the viral source of the segments, identical sizes of dsRNAs were isolated from isometric virus-like particles that have been purified from the same fungus (data not shown).

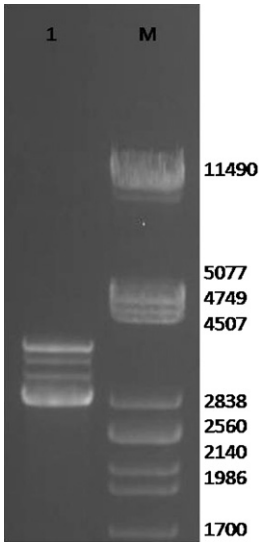

Fig. 1. Agarose gel electrophoresis of dsRNA segments isolated from the fungus *F. graminearum*. The dsRNA was purified by CF-11 chromatography and applied to electrophoresis after digestion with DNase I and S1 nuclease. The dsRNAs were separated on a 1% agarose gel in 1× TAE buffer for 1 h at 120 V and visualized by ethidiumbromide staining. 1: DNase I and S1 nuclease-treated dsRNAs, M: λ-*Pst*I marker.

3.2. Random PCR (rPCR)

The rPCR products created different banding patterns after agarose gel electrophoresis depending on the conditions employed. In the presence of 2 μM of the primer-dN_6, several distinct bands were amplified from 100 pg of dsRNA (Fig. 2, lane 4). When more dsRNA served as a template, the size range and the intensity of the bands increased (Fig. 2, lanes 1–3). A single or no amplification products were obtained when 10 pg or less were applied into the rPCR (Fig. 2, lanes 5–8). The use of the Klenow Fragment for the second strand cDNA synthesis produced a banding profile with

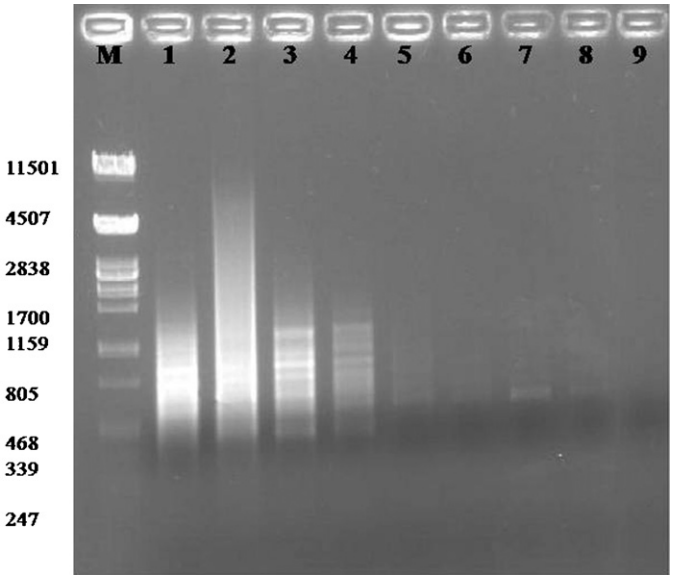

Fig. 2. Agarose gel electrophoresis showing the sensitivity of the rPCR for the amplification of dsRNA templates. The dsRNAs were reverse transcribed with 2 μM of the primer-dN_6 and the cDNA was introduced directly into the PCR without a Klenow Fragment reaction. Different amounts of the dsRNAs were employed: 1: 100 ng, 2: 10 ng, 3: 1 ng, 4: 100 pg, 5: 10 pg, 6: 1 pg, 7: 0.1 pg, 8: 0.01 pg, 9: no dsRNA negative control, M: λ-*Pst*I marker.

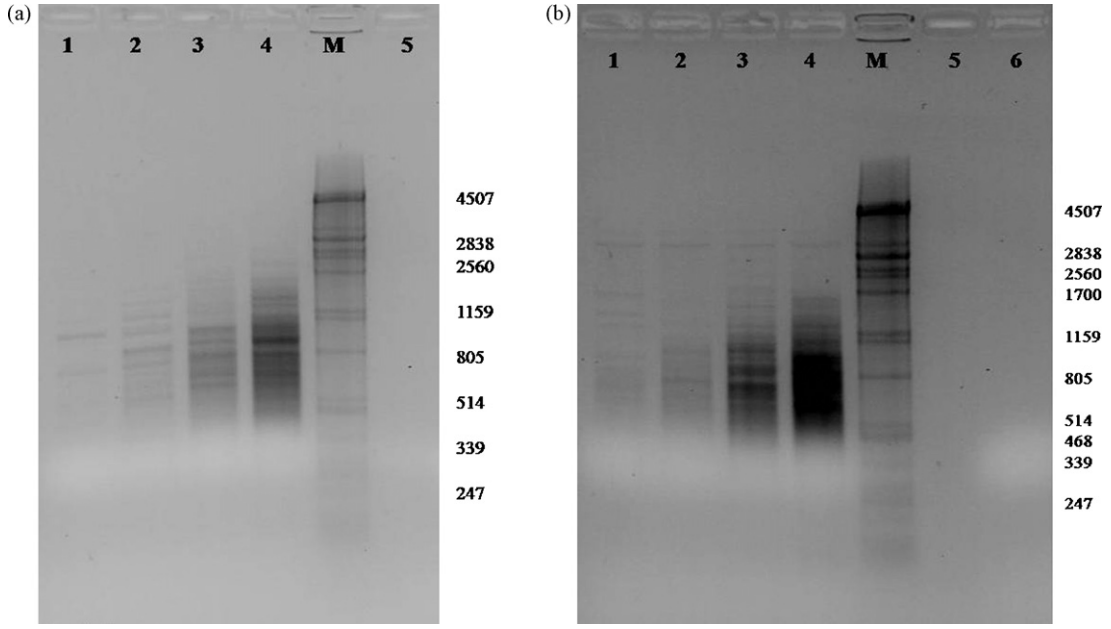

Fig. 3. Agarose gel electrophoresis of rPCR products amplified under different conditions. A mixture of 5 dsRNAs (100 ng) was reverse transcribed into cDNA with different amounts of the primer-dN_6. The products were separated on a 1% agarose gel in 1× TEA buffer for 40 min at 120 V and visualized by ethidiumbromide staining. (A) Second strand cDNA was synthesized using the Klenow Fragment. The concentration of the primer-dN_6 was 1: 0.25 μM, 2: 0.5 μM, 3: 1 μM, 4: 2 μM, M: λ-*Pst*I marker, 5: no dsRNA control. (B) The cDNA was applied directly to the PCR without a Klenow Fragment reaction. The concentration of the primer-dN_6 was 1: 0.25 μM, 2: 0.5 μM, 3: 1 μM, 4: 2 μM, M: λ-*Pst*I marker, 5: empty, 6: no dsRNA negative control.

a size range of 0.4–2.2 kb compared to 0.4–3 kb when the cDNA was applied directly into the PCR (Fig. 3A and B). In the absence of the Klenow Fragment reaction, increasing the concentration of the primer-dN_6 from 0.25 to 2 μM substantially increased the intensity of the bands but not their size range (0.4–3 kb, Fig. 3B). On the other hand, both the size range and the intensity of the bands were reduced with decreasing amounts of the primer, when the Klenow Fragment reaction was included (Fig. 3A). Sequencing of the rPCR products reveal that the 5 segments were not equally amplified (data not shown).

3.3. Single-primer amplification technique (SPAT)

Null to 5 of the dsRNA segments were amplified in full when the ligation of the PC3 primer was performed at 37 °C in the presence of 10% (v/v) DMSO and 20% (w/v) $(PEG)_{6000}$ (Fig. 4, lanes 2, 3, 5, 7 and 9). Furthermore, when the primer-ligated dsRNAs were denatured with DMSO alone, full-length cDNAs were not obtained as was reflected in the PCR results (data not shown). The type of the DNA polymerase employed in the amplification played a key role in the number of full-length products obtained (Fig. 4, lanes 2, 3, 5,

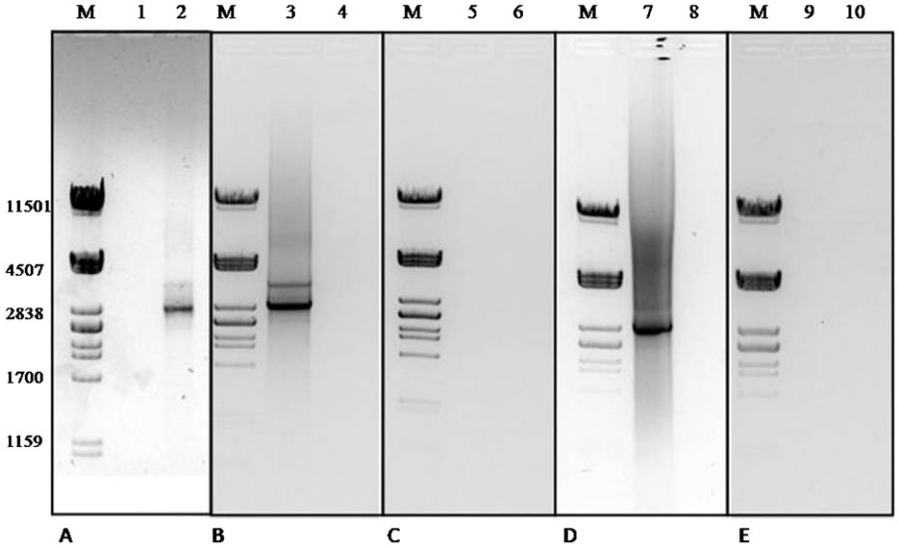

Fig. 4. Agarose gel electrophoresis of RT-PCR products obtained by the single-primer amplification technique. The ligation of the PC3 primer was performed at 37 °C in the presence of 10% (v/v) DMSO and 20% (w/v) $(PEG)_{6000}$. Different DNA polymerases were used in the PCR: A: Finzyme, B: GoTaq, C: Platinium, D: 5 PRIME, E: Long PCR Enzyme Mix. Lanes 2, 3, 5, 7 and 9: amplified products. Lanes 1, 4, 6, 8 and 10: negative control. M: λ-*Pst*I marker. Each polymerase was tested 3 times.

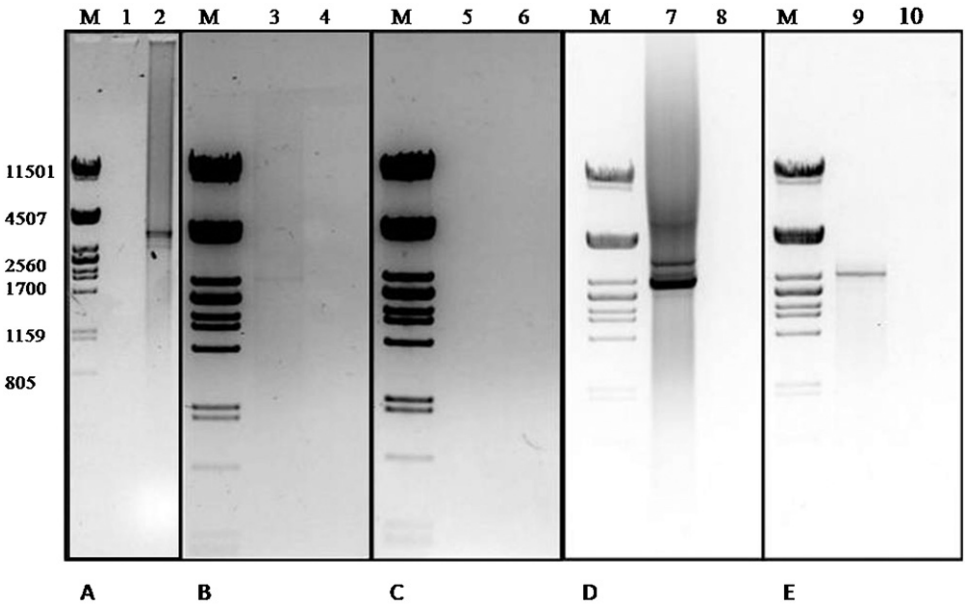

Fig. 5. Agarose gel electrophoresis of RT-PCR products obtained by the FLAC method. The ligation of the PC3-T7 loop primer was performed at 37 °C in the presence of 10% (v/v) DMSO and 20% (w/v) $(PEG)_{6000}$. Different DNA polymerases were used in the PCR: A: Finzyme, B: Platinium, C: GoTaq, D: 5 PRIME, E: Long PCR Enzyme Mix. Lanes 2, 3, 5, 7 and 9: amplified products. Lanes 1, 4, 6, 8 and 10: negative control. M: λ-*Pst*I marker.

7 and 9). For each DNA polymerase, the number of amplified segments is presented in Table 1. When the primer ligation reaction was carried out at 16 °C in the absence of DMSO and $(PEG)_{6000}$, several truncated PCR products with sizes less than 1 kb were obtained (data not shown).

3.4. Full-length amplification of cDNA (FLAC)

When the PC3-T7 loop primer was ligated to the mixture of the dsRNAs at 37 °C in the presence of 10% (v/v) DMSO and 20% (w/v) PEG_{6000} as described above, full-length amplicons representing all or part of the segments were obtained depending on the DNA polymerase employed (Fig. 5). The use of the Phusion® High-Fidelity DNA Polymerase and the supplied Phusion GC Buffer resulted in the amplification of the 5 dsRNAs in one RT-PCR (Fig. 5, lane 2). Similar results were obtained for the DNA polymerase supplied by 5 PRIME (Fig. 5, lane 7). The PCR products per each DNA polymerase are summarized in Table 1. Restriction digestion of the cloned full-length PCR products resulted in 5 different profiles representing all of the segments (data not shown). Clones with different digestion profiles were sequenced and analyzed. The 5 segments have conserved 5′- and 3′-terminal sequences, a known property of most segmented viral genomes (data not shown).

3.5. Direct cloning of dsRNA into DNA vector

The ligation of 200 ng of dsRNA mixture into 50 ng of the pJET1.2 vector produced as few as 5–9 transformants of which 2–4 had an insert longer than 150 bp regardless of the ligation conditions employed. PCR screening of the transformants showed that the longest insert length obtained under the conditions employed is 1 kb (Fig. 6). When the dsRNA was ligated into pGEM®-T vector for 24 h about 7 transformants were obtained 3 of which contain an insert of 150–2100 bp in length. Restriction digestions of the clones with enzymes flanking the insert (NdeI and SacII) produced variable profiles. While in some clones a complete release of the insert was observed (Fig. 7, lanes 2, 5), in other clones the insert was not released indicating that one of the restriction sites might be missing (Fig. 7, lane 3). Although increasing the ligation time to 48 h resulted in improved transformation efficiency (Table 2), the size range of the cloned fragments remains similar to that obtained after 24 h of ligation. The identities of part of the clones were further checked by sequencing (data not shown). The calculated transformation efficiencies for the direct cloning experiment are summarized in Table 2.

Table 1
The efficiency of different DNA polymerases in the SPAT and the FLAC methods.

DNA polymerase	Producer	SPAT[a]	FLAC[a]
Platinium DNA polymerase	Invitrogen	0	0
Taq polymerase	5 PRIME	2–3	4–5
GoTaq	Promega	4–5	1–2
Phusion® High-Fidelity DNA Polymerase	Finnzyme	2–4	5
Long PCR Enzyme Mix	Fermentas	0	1–2

[a] The values represent the numbers of full-length PCR products obtained.

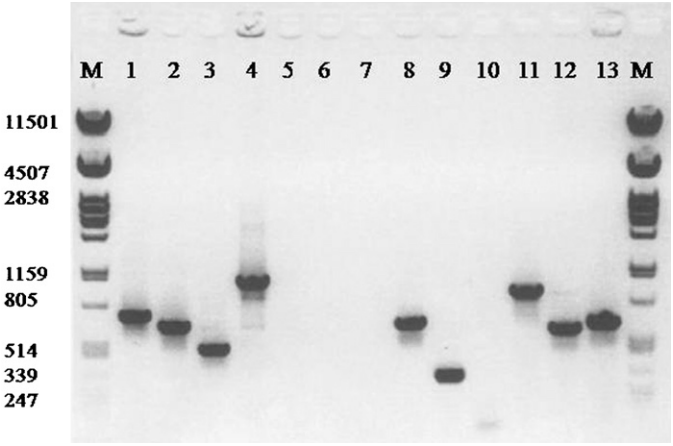

Fig. 6. Agarose gel electrophoresis of the PCR products obtained after direct cloning of dsRNA into pJET1.2 vector. Bacterial colonies were transformed with pJET1.2 vector after ligation with dsRNA. The inserts were amplified with primers flanking the cloning site. M: λ-*Pst*I marker, 1–13: each represents a single colony.

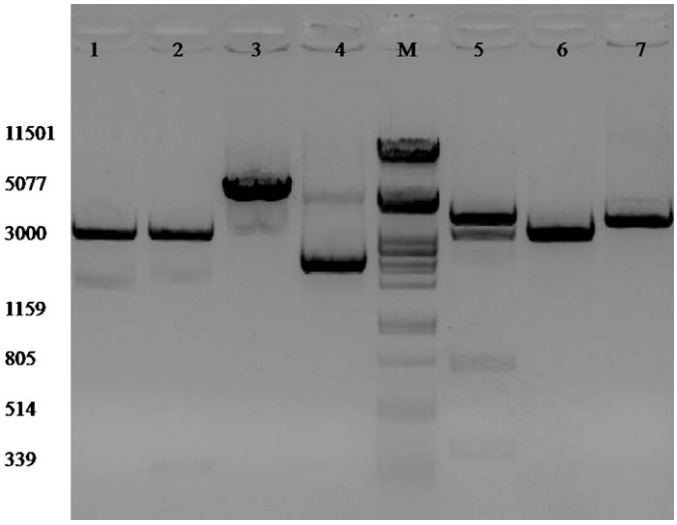

Fig. 7. Restriction digestion profiles of pGEM®-T vectors cloned with dsRNA segments. The ligation reaction was carried out for 24 h. M: λ-*Pst*I marker, 1–7: clones digested with SacII and NdeI.

Table 2
The transformation efficiency of dsRNA-ligated pJET1.2 and pGEM®-T vectors.

Cloning vector	Transformation efficiency[a] and insert size	
	Ligation time 24 h	Ligation time 48 h
pJET1.2	$1.22e^{1}$ (up to 1 kp)	$3.60e^{1}$ (up to 1 kp)
pGEM®-T	$2.80e^{1}$ (up to 2.1 kp)	$1.60e^{2}$ (up to 2.2 kp)

[a] Calculated according to http://www.sciencegateway.org/tools/transform.htm. The results represent the average of 3 independent experiments.

4. Discussion

The isolation of dsRNA from plant tissues has been established by Morris and Dodds (1979) as a tool to detect virus infections. Thereafter, the method, which requires two working days, has been frequently employed to purify dsRNA from plants and fungi as a source for the subsequent molecular biology techniques such as RT-PCR, cloning and sequence determination of viral genomes. The method was optimized further in this study to enable better isolation of intact dsRNA in one day. First, the use of Bentonite in the extraction buffer as an RNase inhibitor helped to protect the dsRNA before and after the phenol treatment. Although dsRNA is more resistant to RNase A than ssRNA, at low salt concentration dsRNA could be degraded partially or fully by this enzyme (Nosek et al., 1993). Also, the ratio of acidic phenol:chloroform:isomylalcohol at 5:1:1 showed to be optimal in term of the amount of dsRNA recovered from the aqueous phase (Sambrook et al., 1989). Acidic phenol mixed with chloroform at the 5:1 ratio probably prevents the loss of RNA in the interphase due to insoluble protein: RNA complexes. Moreover, applying the phenol for 30 min at 37 °C might substitute for the incubation for 1 h at room temperature. Finally, precipitation of the dsRNA in 1 volume of isopropanol for 1 h at −80 °C is equivalent to overnight precipitation with ethanol at −20 °C. Alternatively, the dsRNA could be precipitated with isopropanol by 3 times freeze thawing in liquid nitrogen.

The rPCR method has been established by Froussard (1992) as a tool to amplify whole RNA sequences. Several publications appeared later reporting the use of the method for the random amplification of viral dsRNA genomes (Márquez et al., 2007; Willenborg et al., 2009). In this study, the sensitivity of the method for amplifying a mixture of dsRNA templates is tested and optimized to produce distinct amplicons of considerable sizes. Such optimization would reduce the time and costs of cloning and sequencing of many clones. In the range of 0.1–100 ng of dsRNA, the optimum concentration of the primer-dN_6 for achieving intense products suitable for cloning lie between 1 and 2 μM. The use of the Klenow Fragment for the second strand cDNA synthesis followed by the removal of excess primer-dN_6 has increased the number of smaller amplicons (Fig. 3A). Using dsRNA as a template for reverse transcription would provide dscDNA for the PCR amplification without any need for the Klenow Fragment reaction. Theoretically, the production of a second round of dscDNA would result in shorter DNA templates for the subsequent PCR amplification. Although Froussard (1992) was able to amplify from 1 pg of ssRNA using the rPCR method, only amounts equal to 100 pg or more of the dsRNA templates resulted in considerable amplification products. However, it was possible to obtain few faint bands when 10 pg of dsRNA served as a template for the rPCR. Using a mixture of dsRNAs of 2.5–3.6 kb has resulted in a maximum rPCR product of about 3 kb under the conditions described in this study (Fig. 3B). Probably bigger sized PCR products could be achieved when longer templates are to be used. These results indicate that the rPCR is a powerful and sensitive method for the random amplification of dsRNA templates. The uneven representation of the 5 dsRNAs among the rPCR products as revealed from their sequences, might be mainly due to their unequal abundance in the host.

The SPAT and FLAC methods constitute powerful means for the full-length amplification of long dsRNA templates when performed under optimum conditions. First, incubating the ligation reaction at 37 °C in the presence of 20% PEG_{6000} and 10% DMSO highly improved the ligation of the primer to the 3′ ends of the dsRNA. T4 RNA ligase is in principle an ssRNA ligase but was shown to ligate DNA oligos to dsRNA (Imai et al., 1983) with a lesser efficiency though (Higgins et al., 1979). The ligation reaction can be inhibited by RNA secondary structures and can be enhanced with the addition of 10% DMSO and PEG, where the later acts by macromolecule crowding (Harrison and Zimmerman, 1984). Probably, under the conditions described above, partial denaturation of the dsRNA termini occurs, providing single stranded 3′ ends to the ligation enzyme. Second, a combination of 1 M Betaine and 2.5% DMSO was sufficient to denature the dsRNAs so that the reverse transcription resulted in full-length cDNAs. This treatment might replace the use of the highly toxic chemical MMOH, which is used frequently for such purposes (Potgieter et al., 2009). The employment of DMSO alone at various concentrations for the denaturation of dsRNA was not sufficient for the production of full-length cDNAs. Whereas low DMSO concentrations (up to 15%) might not be enough to denature relatively long dsRNAs, the use of higher concentrations of DMSO might inhibit the reverse transcriptase (Jucà and Aoyama, 1995). Third, the ligations of linkers, which loop back to prime themselves, make the introduction of primers in the reverse transcription step superfluous. This eliminates the chances for initiation of cDNA synthesis from places other than the ends and reduces the production of truncated cDNAs. Fourth, the use of high annealing temperature in the PCR prevents non-specific amplification. Although the calculated melting temperature (T_m) for the primer PC2 is 54–56 °C, going up to 67 °C has not affected the specific annealing of the PC2 primer as revealed by the PCR results (Potgieter et al., 2009). Finally, out of 5 commercial DNA polymerases designed to amplify long DNA templates (4–20 kb), only 2 have produced the complete set of full-length amplifiable products with different intensities, though. The other polymerases produced 0–3 full-length PCR products. It is not easy to interpret these results since the 5 polymerases were applied under optimal conditions using identical number of units, the same cDNA preparations, and the same thermocycler machine. In general, the number of full-length products obtained by the FLAC method was more than that obtained from the SPAT method except for the Go Taq Polymerase. This is probably because more

full-length cDNAs could be synthesized in the absence of floating primers in the reverse transcription step. The higher efficiency of the Go Taq DNA polymerase in the SPAT than in the FLAC method is difficult to interpret.

The attempts to clone viral genomic dsRNA into DNA vectors without prior transcription and amplification steps showed that the principle is valid. Although the method allowed the determination of relatively long sequences (up to 2.2 kb), a clone representing a full-length dsRNA segment was not detected. Surprisingly, the cloning of S1 nuclease-treated dsRNA into the pGEM®-T vector resulted in more transformants and bigger inserts than those obtained from ligation into the blunt end pJET1.2 vector. As far as we know, there is no information in the literature explaining the mechanism by which such direct ligation and transformation take place. Skotnicki et al. (1985) claimed that it is possible to clone, in full or partially, dsRNAs from RF or RI stages of several ssRNA viruses such as AMV, TMV, FDV, BYDV, and BWYV. However, the patent does not state clearly, when it was possible to obtain full-length clones, does not present data on the transformation efficiency, and to our knowledge was not published in any peer-reviewed journal so far. This method should be explored further to understand the underlying molecular mechanisms that might enable further improvements and eventually lead to cloning of full-length dsRNA segments.

In conclusion, the optimization described in this study resulted in reproducible tools for the isolation, transcription, and amplification of full-length products from a mixture of relatively long dsRNA templates without prior need for separation or for the use of noxious chemicals and expensive or time-consuming approaches. Furthermore, the implementation of the direct cloning of dsRNA into DNA vectors as a laboratory protocol requires more molecular investigations and evaluations. Such investigations may help to improve the method for the cloning of full-length RNA molecules and to enhance their transformation efficacy.

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