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Short communication

Development of a general potexvirus detection method

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Abstract

A method was developed for the detection of viruses from the genus *Potexvirus*. Following alignment of full-length RNA sequences and deduced amino acid sequences of 10 different viruses from the genus *Potexvirus*, a number of conserved sequence motifs were identified in the viral replicase-encoding region. Seven different primers based on these motifs were tested for their efficiency as potexvirus group-specific cDNA and/or PCR primer. Several combinations of primers proved capable of generating DNA fragments for each of six different potexviruses tested. One cDNA primer in combination with one PCR primers set proved most successful in reliably generating discrete PCR products. Application of this set to a number of different potexviruses, some for which no sequence data had been published yet, resulted in amplification of virus-specific PCR products for all viruses tested. Sequence analysis of cloned PCR products confirmed their identity. This general potexvirus primer set can be useful for the identification of (unknown) potexvirus infections.

The genus *Potexvirus* currently contains 54 definite and possible species (van Regenmortel et al., 2000) of which many are of considerable agronomic importance. Development of specific detection and identification methods for these viruses is needed to control the problems they cause. Since, for practical purposes precise virus identification at the species level is not always required, there is a growing interest in group-specific or genus-specific detection of plant viruses. With current serological methods, development of such group- or genus-specific methods has not always been successful. As a consequence, the serological detection or identification of new or less well-characterised plant viruses can be problematic when no specific antiserum is available. Molecular methods like reverse transcriptase polymerase chain reaction (RT-PCR) seem to have the potential to help overcome such problems (Langeveld et al., 1991; Tian et al., 1996; Vetten et al., 1997; Winter et al., 1997). Such methods could help in the identification of viruses, as in the recent problems in Europe caused by *Pepino mosaic virus* (PepMV, van der Vlugt et al., 2000) or in solving plant virus taxonomic issues (van der Vlugt et al., 1999; Chen et al., 2001).

Genomes of potexviruses contain five open reading frames (ORFs), are 3′-polyadenylated and contain an m^7Gppp cap structure at their 5′ ends (reviewed by Mentaberry and Orman, 1995). ORF1 encodes a protein involved in virus replication, ORFs 2–4 are generally referred to as the triple gene block (TGB) and encoded gene products are involved in viral cell-to-cell movement. ORF5 encodes the viral coat protein (CP). In an attempt to develop a general potexvirus specific test based on a RT-PCR procedure, sequences of the different coding and non-translated regions from the genomes of different potexviruses were aligned and compared.

Full-length sequences of 10 potexviruses were obtained from different databases: *Bamboo mosaic virus* (BaMV, D26017, 6366 nts), *Cassava common mosaic virus* (CaCMV, U23414, 6376 nts), *Cymbidium mosaic virus* (CymMV, AF016914, 6227 nts), *Foxtail mosaic virus* (FMV, M62730, 6131 nts), *Narcissus mosaic virus* (NamV, D13747, 6955 nts), *Papaya mosaic virus* (PapMV, D13957, 6656 nts), *Plantago asiatica mosaic virus* (PlaMV, Z21647, 6128 nts), *Potato aucuba mosaic*

virus (PAMV, S73580, 7059 nts), *Potato virus X* (PVX, D00344, 6435 nts) and *White clover mosaic virus* (WClMV, X16636, 5846 nts).

The nucleotide and deduced amino acid sequences of all ORFs of these viruses and nucleotide sequences of their non-translated regions (NTR's) were aligned using the Megalign programme from the LaserGene DNA-Star programme package (DNAStar Inc.). The viral replicase-encoding region (ORF1) was too large and too diverse, ranging from 3884 to 4940 nucleotides, to allow simultaneous alignment of the 10 different viral sequences. Therefore, this ORF was split into three overlapping regions (Pol A from nt 1 to 2250, Pol B from nt 1500 to 3750 and Pol C from nt 2700 onwards) which were aligned separately.

Considerable differences in overall lengths and sequences of the non-translated RNA regions and ORFs of the different potexviruses were observed resulting in overall low levels of sequence homology between corresponding regions. Only in the C-terminal region of the replicase, could a number of conserved amino acid sequence motifs be identified. These motifs and their relative positions on the PVX genome (Acc. no. D0034) are presented in Table 1. Motifs Potex-1, -2 and -3 were chosen as possible cDNA priming sites and downstream PCR primer motifs, and motifs Potex-3, -4, -5 and -6 as possible upstream PCR primer sites. For each motif, a degenerated primer was designed and evaluated in the Primer Select programme of the DNAStar programme package (DNAStar Inc.) for possible non-specific priming. Primer sequences are presented in Table 2. cDNA primers Potex-1RC, -2RC and -3RC are the reverse complement (RC) of sequences coding for motifs 1–3. Primers (Life Technologies) were then tested in a RT-PCR procedure on total RNA isolated from potato plants infected with PVX.

Table 1. Conserved amino acid motifs in the C-terminal region of the viral replicase (ORF1) of different potexviruses and their position in the PVX viral replicase protein (D00344)

Motif	Sequence	Position
Potex 1	TFDANTE	1300–1306
Potex 2	QDGAML	1250–1255
Potex 3	GTMARY	1195–1200
Potex 4	KSQWVTK	1162–1168
Potex 5	HQQAKDE	1061–1067
Potex 6	TYAGCQG	921 – 927

To this end, total RNA was isolated from 150 mg leaf discs of infected plants (Verwoerd et al., 1989). The final RNA pellet was dissolved in 20 µl of sterile H_2O. Prior to cDNA synthesis, 3 µl total RNA was annealed to the first strand primer by mixing it with 8 µl 5× first strand buffer (Life Technologies), 1 µl of first strand cDNA primer Potex-1RC, -2RC or -3RC (100 ng μl^{-1}) and 21 µl H_2O, followed by incubation of the tube at 65 °C for 5 min. Thereafter, cDNA was synthesised (van der Vlugt et al., 1999). Five microlitres of cDNA was subsequently amplified by PCR using the different PCR primer combinations (van der Vlugt et al., 1999) at a PCR annealing temperature of 51.5 °C.

In initial experiments the relative efficiency of each of the three cDNA primers (Potex-1RC, -2RC and -3RC) was determined by amplifying each cDNA in combination with each of the upstream primers. For cDNA generated with primer Potex-1RC only the combination with primer Potex-5 yielded a reasonable amount of PCR product of the expected size of 735 bp. cDNA generated with primer Potex-2RC gave a small amount of the expected DNA fragment in combination with primer Potex-4 (276 bp) and a clear product with primer Potex-5 (584 bp). cDNA generated with Potex-3RC gave, both with primers Potex-5 and Potex-6, reasonable amounts of the expected PCR products (419 bp and 839 bp, respectively) (results not shown).

Primers Potex-1RC and Potex-2RC seemed most useful for cDNA synthesis, while the upstream

Table 2. Degenerate Potexvirus cDNA and PCR primers and their positions on the PVX RNA genome (D00344)

Primer	Sequence	Position
Potex 1RC	5′-TCAGTRTTDGCRTCRAARGT-3′	4001–3982
Potex 2RC	5′-AGCATRGCNSCRTCYTG-3′	3848–3832
Potex 3RC	5′-GTATBKNGCCATNGTKCC-3′	3684–3667
Potex 3	5′-GGMACNATGGCNMVATAC-3′	3667–3684
Potex 4	5′-ARTCNCARTGGGTNAMRAA-3′	3569–3587
Potex 5	5′-CAYCARCARGCMAARGAYGA-3′	3265–3284
Potex 6	5′-ACMTAYBGGITGYCARG-3′	2845–2863

PCR primer Potex-5, in combination with either Potex-1RC or Potex-2RC, generated clear amounts of product (results not shown). Primer combination Potex-2RC/Potex-5 occasionally resulted in some a-specific amplification products on cDNA generated with primer Potex-2RC. These were never observed on cDNA generated with primer Potex-1RC.

To test whether cDNA primer Potex-1RC in combination with upstream PCR primers Potex-2RC and Potex-5 was indeed useful for use in a general potexvirus detection method, they were tested on five potexviruses from the plant virus collections of Plant Research International and the Dutch Plant Protection Service, Wageningen. Total RNA was isolated from either $CaCl_2$-dried or glycerol-suspended leaf material infected with either CymMV, *Cactus virus X* (CVX), *Hydrangea ringspot virus* (HRSV), NaMV or *Potato aucuba mosaic virus* (PAMV) and used for cDNA synthesis and PCR as described above. Of both HRSV and CVX no sequence data of the region under investigation have been published. Gel electrophoresis of 10 µl of a 40 µl PCR reaction clearly revealed DNA fragments around 600 bp for the five viruses tested (see Figure 1).

For all five viruses and PVX, the 600 bp PCR fragment was cloned into pGEM-T easy vector (Promega) and transformed to *Escherichia coli* JM109 cells (Promega) according to manufacturers instructions. Purified recombinant plasmid DNA was sequenced on an ABI automatic sequencer using standard M13 forward and reverse primers. Sequence data were compiled and analysed using the Lasergene DNASTAR programme package. All sequences have been deposited at the EMBL nucleotide sequence database and were assigned the following accession numbers: CVX: AJ270985; CymMV: AJ270986; HRSV: AJ270987; NaMV: AJ270988; PAMV: AJ270989 and PVX: AJ270990.

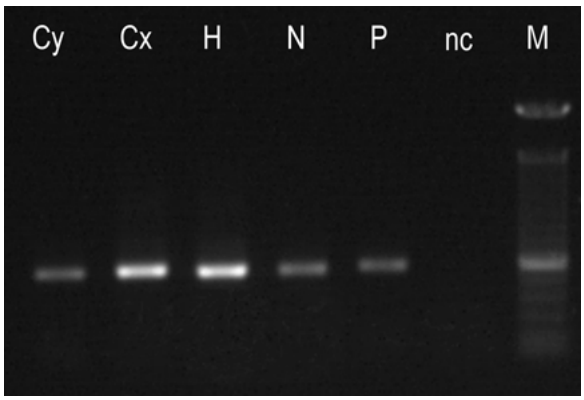

Figure 1. Gel electrophoresis of PCR products of different potexviruses using the general potexvirus RT-PCR primers set. Cy = *Cymbidium mosaic virus*, Cx = *Cactus virus X*, H = *Hydrangea ringspot virus*, N = *Narcissus mosaic virus* and P = *Potato aucuba mosaic virus*. nc = negative control, M = 100 bp marker (Life Technologies). Thick marker band represents 600 bp.

For each of the six viruses, the amplified DNA fragment was 584 bp long. Sequences were aligned to each other and to the corresponding regions from each of the 10 viruses used to identify conserved potexvirus sequence motifs (see above). These alignments revealed overall levels of identity ranging from 48.1% to 68.2% at the nucleotide level and from 53.6% to 76.3% for the derived amino acid sequences. All newly determined sequences, including those from HRSV and CVX for which no sequences have been reported yet, showed the conserved potexvirus sequence motifs Potex-2, -3, -4 and -5. Sequence alignments between homologous sequences for those viruses for which already complete sequence data from their RNA genomes were available in the database (i.e. CymMV, NaMV, PAMV and PVX), showed levels of identity between 96.1% and 98.6%. These results suggest that the DNA fragments amplified by the general potexvirus primer set in this RT-PCR procedure are virus-specific. Potexvirus specificity of the primers was confirmed by a RT-PCR experiment whereby cDNA primer Potex-1RC and PCR primers set Potex-2RC/Potex-5 were tested on RNA from purified carlaviruses *Potato virus S* (PVS) and *Potato virus M* (PVM) and total RNA purified from tomato plants infected with PVX or healthy tomato plants. Only for PVX a DNA-fragment of the expected size of around 600 bp was amplified (see Figure 2).

A phylogenetic analysis of all sequences, including the viral replicase sequence of the carlavirus PVM (Acc. no. X53062), as an outgroup, is presented in Figure 3. This analysis confirmed that both HRSV and CVX clearly group with the other potexviruses.

Universal virusgenus-specific primer sets have proven to be useful in virus detection and sequence analysis of their PCR-fragments helped to identify uncharacterized viruses and allowed insight in taxonomic relationships (Langeveld et al., 1991; Pappu et al., 1993; Liu et al., 1994; Gibbs and Mackenzie, 1997; van der Vlugt et al., 1999 Chen et al., 2001). The results presented above indicate that the set of general potexvirus RT-PCR primers described in this paper, is a useful tool in the confirmation and identification of uncharacterized potexvirus infections. This

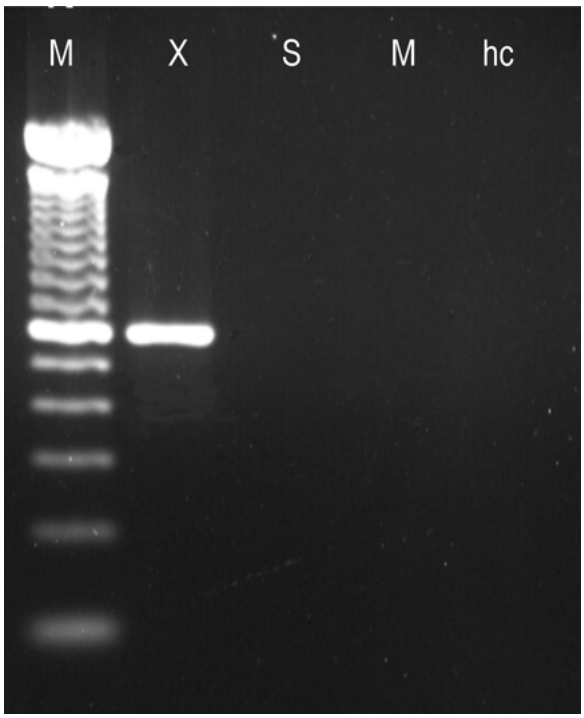

Figure 2. Gel electrophoresis of PCR products of potexvirus PVX and carlaviruses PVS and PVM using the general potexvirus RT-PCR primers set. X = PVX, S = PVS, M = PVM, hc = healthy control, M = 100 bp marker (Life Technologies). Thick marker band represents 600 bp.

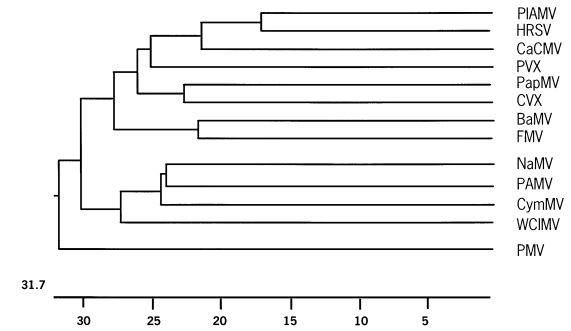

Figure 3. Representation of phylogenetic relations between the sequences of the PCR fragments obtained with the potexvirus primers set and the corresponding regions from the 10 full-length potexviruses used to identify the common motifs. CymMV = *Cymbidium mosaic virus*, CVX = *Cactus virus X*, HRSV = *Hydrangea ringspot virus*, NAMV = *Narcissus mosaic virus*, PVX = *Potato virus X* and PAMV = *Potato aucuba mosaic virus*) (BaMV = *Bamboo mosaic virus*, CaCMV = *Cassava common mosaic virus*, FMV = *Foxtail mosaic virus*, PapMV = *Papaya mosaic virus*, PlaMV = *Plantago asiatica mosaic virus* and WClMV = *White clover mosaic virus*. The carlavirus PVM was included as an outgroup to the tree. Numbers represent nucleotide substitutions (×100).

was also demonstrated by the identification of *Pepino mosaic virus* (PepMV) in protected tomato crops (*Lycopersicon esculentum*) (van der Vlugt et al., 2000). Initial tests suggested the presence of a potexvirus, possibly PepMV, a South American potexvirus which first and only description dates back to 1980 (Jones et al., 1980). RT-PCR with the potexvirus primers set, on total RNA isolated from infected tomato plants and tobacco plants infected with the original pepino isolate of PepMV (Jones et al., 1980), generated DNA fragments of the expected size (±600 bp) for each of the two viruses. Sequence alignments of both cloned fragments showed almost 95% identity, while the identity with PVX, PAMV and other potexviruses was less than 60%. The combination of molecular data, obtained with the primer set described above, with serological and biological data, helped to identify PepMV as the causal agent of the new virus disease in tomato.

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