A new potexvirus associated with strawberry mild yellow edge disease

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A physical map of cDNA clones prepared from dsRNA associated with the MY-18 source of strawberry mild yellow edge (SMYE) was constructed and 854 nucleotides adjacent to the 3’ poly(A) tail were sequenced. The larger open reading frame product of Mr 25,714 showed considerable amino acid homology to the coat protein cistrons of six potexviruses and two carlaviruses. A second product of Mr 11,216 encoded completely within the coat protein cistron, but in a different frame, has similarities to two potexvirus polypeptides. The Mr 25,714 ORF was fused to the Protein A gene in an expression vector and the fusion protein was purified by affinity chromatography and used to immunize a rabbit. The resulting polyclonal antiserum reacted strongly in immunoelectron microscopical tests with filamentous particles resembling those of potexviruses. Such particles were detected in the following SMYE sources: D-74 from Germany, two from the United Kingdom in Fragaria vesca ‘Alpine’ indicator plants and Oregon MY-18 in Rubus rosifolius. Among 27 potexvirus antisera tested for serological reactions none yielded strong decoration. Examination of ultrathin sections of R. rosifolius and F. vesca tissue infected with SMYE revealed aggregates of filamentous particles in phloem parenchyma cells. dsRNA from nine sources of SMYE collected from around the world reacted with the cDNA clones of this potexvirus in Northern hybridizations. It is concluded that the potexvirus is hitherto undescribed and the name strawberry mild yellow edge-associated potexvirus is proposed.

Introduction

Strawberry mild yellow edge (SMYE) is regarded as being caused by a tentative member of the luteovirus group (Matthews, 1982). It is distributed world-wide and one of the most common diseases in cultivated strawberries. SMYE causes severe losses when it occurs in mixed infections with other viruses such as strawberry mottle virus, strawberry crinkle virus or strawberry vein banding virus (Converse et al., 1987). The association of the disease with the luteovirus group is based on the persistent transmission of its agent(s) by the strawberry aphid Chaetosiphon fragaefoli and its symptomatology (Matthews, 1982). Further evidence was given when luteovirus-like particles, limited to phloem cells, were found in thin sections (Yoshikawa et al., 1984) and also in partial purifications (Martin & Converse, 1985). A serological relationship of partially purified preparations of SMYE luteovirus with beet western yellows virus (BWYV) (Spiegel, 1987). Until recently when Rubus rosifolius was inoculated successfully by graft transmission (Martin et al., 1989), Fragaria spp. were the only known hosts of SMYE.

Detection of SMYE is currently being done by graft or aphid transmission to indicator plants. Additionally, dsRNA analysis is used for detection. The production of cDNA clones from dsRNA, purified from infected R. rosifolius, that had been graft-inoculated with the Oregon MY-18 source of SMYE, has been described (Martin et al., 1989; Jelkmann et al., 1989).

Production of antiserum to plant viruses is generally based on the purification of viral particles serving as antigens for the immunization of animals. As an alternative we present the construction of an expression vector containing a viral coat protein gene, and the preparation of a polyclonal antiserum from the polypeptide expressed in vitro. This technique is used widely and was shown to be successful with antibody production to prokaryotic and eukaryotic fusion proteins expressed in vitro (Löwenadler et al., 1986), structural and non-structural viral proteins (Gombart et al., 1989; MacKen-
In this paper we describe partial nucleic acid sequence analysis of one of the previously obtained cDNA clones containing an ORF coding for the coat protein of a potexvirus. Antiserum to this coat protein fused with Protein A allowed detection of potexvirus-like particles from several SMYE sources in immunoelectron microscopic studies. Potexvirus-like particles were also found in ultrathin sections. Hybridization of dsRNA from several sources of SMYE with the cDNA probes is reported. The possible role of this potexvirus as causal agent of the SMYE disease is discussed.

Methods

Virus sources. The virus source, originally used for molecular cloning, was SMYE MY-18 (Martin & Converse, 1985) that had been grafted into R. rosifolius Smith. From these plants dsRNA templates were purified (Martin et al., 1989). For immunoelectron microscopy studies the following sources were used: MY-18, D-74 from Germany maintained in F. vesca L. var. semperflorrens (Duchesne) Seringe cv. Alpine indicator plants by transmission with C. fragaefolii in a greenhouse for 15 years and nos. 21858/9 and 21683/16L from D. Barbara (East Malling, U.K.), purged of any possible non-persistently transmitted viruses by successive aphid transmissions. Sources D-531 from California and MY-18 were used for thin section electron microscopy. For Northern hybridizations of dsRNA purified from SMYE-infected plants, each of the above sources was used except only one from East Malling, plus BC-1 from the Fraser Valley of British Columbia, Canada, 69N from H. Bormans in Belgium, no. 2 from California, QRCR from R. Heath in Australia, and one source from S. Spiegel (Volcani Center, Bet Dagan, Israel).

Analysis of cDNA clones. dsRNA extraction was done as described previously (Martin et al., 1989). dsRNAs were electrophoresed on 1% agarose gels and electroblotted onto Nytran (Schleicher & Schuell). Oligolabelled probes were prepared as per the manufacturer’s directions (BRL). Hybridizations, washings and autoradiography were done as described in Maniatis et al. (1982).

The production of cDNA clones from dsRNA templates has been described previously (Martin et al., 1989; Jelkmann et al., 1989). Several clones that were shown in agarose gel electrophoresis to contain inserts larger than 1 kbp were mapped physically after digestion with various restriction enzymes and agarose gel electrophoresis. Overlapping clones were identified by double digestions and confirmed in Southern blots. DNA analysis and modifications were done as in Maniatis et al. (1982) unless stated otherwise.

Restriction fragments of selected clones were purified after separation in 1% low melting point agarose gels and subcloned into the multiple cloning site of Bluescript M13+ (Stratagene). These plasmids were prepared for sequencing of both cDNA strands by unidirectional deletions with exonuclease III and religation after mung bean nuclease treatment according to Henikoff (1984). dsDNA sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977) was performed using either the Klenow fragment of DNA polymerase I (BRL) or Sequenase DNA Polymerase (USB) (Toneguzzo et al., 1988). Single strand DNA templates were prepared with the helper phage M13-K07 (Pharmacia). Sequence data were collected, assembled and analysed with an IBM AT computer using the Gene-Master software (Bio-Rad). Sequence alignments of the coat proteins of several potexviruses were done with the progressive sequence alignment program of Feng & Doolittle (1987).

Construction of the expression plasmid. The coat protein gene was obtained from cDNA clone p463 by digestion with RsaI and ligated into the Smal site of the fusion vector pRIT2T (Pharmacia). The recombinant plasmid (pCPYE12) was used to transform competent Escherichia coli N4830-1 cells containing the phage lambda c1857 temperature-sensitive repressor.

Production of chimeric coat protein antiserum. An N4830-1 culture containing the pCPYE12 plasmid was grown at 30°C in Luria broth containing ampicillin (100 µg/ml) to an O.D.₆₀₀ of 0.8. Fusion protein synthesis was then induced by a temperature shift to 42°C by adding an equal volume of medium preheated to 54°C. After 90 min incubation at 42°C the cells were cooled and centrifuged at 5000 g for 10 min. The cells were resuspended in 0.2 vol of TTBS (0.05 M-Tris-HCl pH 7.4, 0.15 M-NaCl, 0.05% Tween 20) and disrupted by sonication. Clarification of the homogenate was obtained by centrifugation at 20000 g for 30 min. The chimeric coat protein was then affinity-purified on a rabbit gamma globulin-Sepharose 2B column. After several washes with TTBS the chimeric coat protein was eluted with 0.1 M-acetic acid, lyophilized and stored in aliquots at -20°C. Analysis of expression of the fusion protein and degree of purification was done by running an aliquot on a 10% SDS-PAGE gel (Laemmli, 1970) in the presence of low M₇ standards (Pharmacia) and the Protein A from pRIT2T as control.

A rabbit was immunized by intramuscular injection of 1 mg fusion protein in Freund’s complete adjuvant and two booster injections with 0.5 mg fusion protein at 3-week intervals. Blood was collected weekly from the ear veins.

Electron microscopy. For immunosorbent electron microscopy (ISEM) the crude antiserum was diluted 1:1000 in 0.1 M-sodium/potassium phosphate buffer pH 7.0. Pioloform-carbon-coated nickel grids were floated for 5 min on diluted antiserum, rinsed with 2 ml dilution buffer and transferred to leaf homogenate in the same buffer with 2% polyvinylpyrrolidone 10000, 0.2% sodium sulphite and 0.05% sodium azide and incubated overnight at 20°C. Decoration of particles was done with antiserum diluted 1:50 or 1:5 using 15 to 30 min incubation times. Before staining with five drops of 2% uranyl acetate, grids were rinsed with 4 ml of H₂O. Excess liquid was carefully removed with filter paper, and the grids were examined with a Zeiss EM 10 C electron microscope.

For ultrathin sections, samples were taken from the veins of SMYE-infected R. rosifolius (MY-18) and F. vesca cv. Alpine (D-531) leaves. Tissue was fixed in 4% glutaraldehyde buffered in 0.1 M-cacodylate pH 7.0 (CB), postfixed in 1% osmium tetroxide in CB, dehydrated in a series of ethanol and propylene oxide mixtures, and embedded in Epon. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Hitachi electron microscope.

Results

Analysis and sequencing of cDNA clones

Mapping of selected clones by restriction fragment analyses revealed a physical map of about 6-0 kbp in length (Fig. 1). This indicated that about 97% of the genomic dsRNA, estimated from denaturing methylmercuric hydroxide agarose gels to be 6-2 kbp in size, was represented in the cloned cDNA. cDNA clones p309...
Fig. 1. Restriction endonuclease map of cDNA clones prepared from dsRNA associated with the MY-18 source of SMYE. Agarose gel electrophoresis-based size estimation of inserts revealed a genomic length of 6.0 kb. Abbreviations of enzymes are as follows: A, AciI; B, BamHI; E, EcoRI; H, HindIII; M, MfeI; N, NcoI; P, PstI; R, Rsal; S, ScaI; Sc, ScI; T, Tsp506I; X, XbaI.

The predicted amino acid sequence of the 26K and 11K ORFs are given in single-letter code. The cistron of the larger reading frame that starts either at position 1 or at position 58 (24K) is terminated at nucleotide 727. An indicates the 3' poly(A) tail.

Fig. 2. Nucleotide sequence of the 3' terminus of cDNA clone p463. The predicted amino acid sequence of the 26K and 11K ORFs are given in single-letter code. The cistron of the larger reading frame that starts either at position 1 or at position 58 (24K) is terminated at nucleotide 727. An indicates the 3' poly(A) tail.

Fig. 3. Interviral amino acid homology of six potexvirus coat proteins to the 26K ORF of the potex-like virus associated with SMYE disease, using the single-letter code. Gaps were introduced for best fitting using the Feng & Doolittle (1987) progressive alignment programs. The homologies with the SMYE disease-associated potex-like virus are indicated by boxes.

and p463 hybridized to Northern blots of dsRNAs purified from SMYE MY-18-infected R. rosifolius and dsRNA from an Israeli source of SMYE provided by S. Spiegel (Jelkmann et al., 1989).

All sequence data presented in Fig. 2 were deduced from both strands of cDNA clone p463. A terminal poly(A) segment of 10 bases was detected at the 3' end of this clone and presumed to be the 3' end of the viral genome. Clone p441 contained a poly(A) of 41 residues and was found to be identical with p463 to the termination codon, encoding a protein of Mr 25714 from both strands of cDNA clone p463. A terminal poly(A) residue was found in the 3' end of clone p441.
positions 295 and 614 encoding a protein of \( M_r \) 11216 (11K). The 3' non-coding region consists of 128 nucleotides.

Computer-assisted progressive sequence alignment of the 26K polypeptide revealed strong amino acid homology with the coat proteins of six potexviruses, potato virus X (PVX; Huisman et al., 1988), white clover mosaic (WCIMV; Forster et al., 1988; Harbison et al., 1988), papaya mosaic (PMV; Short et al., 1986), potato aucuba mosaic (PAMV; Bundin et al., 1986), clover yellow mosaic (CYMV; AbouHaidar & Lai, 1989) and narcissus mosaic (NMV; Zuidema et al., 1989) viruses (Fig. 3). Only homologies with SMYE are boxed for ease in seeing homologies with this virus. No homology was found with the 11K protein to the 10K protein of NMV and the 7K protein of WCIMV (Zuidema et al., 1989) both which are located internal to their respective coat proteins. Homologies with the coat proteins of the carlaviruses potato virus S (PVS) (MacKenzie et al., 1989) and potato virus M (PVM) (Rupasov et al., 1989) were also found (not included in Fig. 3). Sequence alignments of these carlaviruses with potexvirus coat proteins are shown in the above publications.

**Purification of pCPYE12 fusion protein**

To raise an antiserum against the proposed potexvirus, a gene fusion consisting of the affinity tail of the Protein A gene from Staphylococcus aureus and the potexvirus coat protein gene was constructed. The expressed fusion protein was purified on affinity columns and separated by SDS-PAGE. A protein of approximate \( M_r \) 52000 (data not shown), of which \( M_r \) 25714 (26K) represents the coat protein gene product was used to immunize a rabbit. The resulting polyclonal antiserum was utilized for the ISEM studies described below.

**Particle morphology and ISEM**

Filamentous particles (Fig. 4a) were trapped in moderate numbers from sap extracts of SMYE-infected plants on grids coated with the antiserum to the fusion protein, whereas on uncoated grids particles could be observed only rarely. On antiserum-coated grids at least a 100-fold increase in particle number could be obtained, compared to preimmune serum-coated grids. The particle numbers trapped from different samples of one plant varied considerably, thus a difference in particle concentrations between the four sources analysed (D-74, MY-18, 21585/9 and 21683/16L) could not be determined. No virus particles could be detected in healthy control plants of *F. vesca* and *R. rosifolius*.

The filamentous particles showed a modal length of 482 nm and a diameter of 13 nm. In decoration tests the particles reacted strongly with the antiserum to the fusion protein (Fig. 4b). In further decoration tests 27 antisera to definitive or tentative potexviruses were tested for serological reactions with the filamentous particles from D-74 (Table 1). Nineteen antisera gave no reaction, but eight yielded a very weak indication of decoration (Table 1). A similar result was obtained with the filamentous particles of MY-18 (data not shown). No decoration was observed with carnation latent carlavirus antiserum that is reported to cross-react with strawberry pseudo-mild yellow edge (SPYME) carlavirus (Yoshikawa & Inouye, 1986). In tests with 11 potexviruses, i.e. Argentine
Table 1. Decoration tests with fusion protein antiserum and 27 antisera to other potexviruses using the D-74 source from Germany of the SMYE-associated potexvirus* in extracts of Fragaria vesca cv. Alpine

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Decoration intensity‡</th>
</tr>
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<tbody>
<tr>
<td>SMYE-associated potex</td>
<td>+++</td>
</tr>
<tr>
<td>Argentine plantago</td>
<td>−</td>
</tr>
<tr>
<td>Boussingaultia mosaic</td>
<td>(+)</td>
</tr>
<tr>
<td>Cactus X</td>
<td>−</td>
</tr>
<tr>
<td>Cassava common mosaic (Brazil)</td>
<td>(+)</td>
</tr>
<tr>
<td>Cassava common mosaic (Columbia)</td>
<td></td>
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<tr>
<td>Cassava common mosaic (WF)</td>
<td>(+)</td>
</tr>
<tr>
<td>Chicory X</td>
<td>(+)</td>
</tr>
<tr>
<td>Clover yellow mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Cymbidium mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Daphne X</td>
<td>−</td>
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<tr>
<td>Hevea</td>
<td>(+)</td>
</tr>
<tr>
<td>Hydrangea ringspot</td>
<td>−</td>
</tr>
<tr>
<td>Lily X</td>
<td>−</td>
</tr>
<tr>
<td>Lychnis potex</td>
<td>−</td>
</tr>
<tr>
<td>Narcissus mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Papaya mosaic</td>
<td>(+)</td>
</tr>
<tr>
<td>Pepino mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Plantago X</td>
<td>−</td>
</tr>
<tr>
<td>Plantago severe mosaic</td>
<td>(+)</td>
</tr>
<tr>
<td>Potato aucuba mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Potato X</td>
<td>−</td>
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<tr>
<td>Potex Sieg</td>
<td>(+)</td>
</tr>
<tr>
<td>Tamus potex</td>
<td>−</td>
</tr>
<tr>
<td>Tulip X</td>
<td>−</td>
</tr>
<tr>
<td>Viola mottle</td>
<td>−</td>
</tr>
<tr>
<td>White clover mosaic</td>
<td>−</td>
</tr>
<tr>
<td>Wineberry latent</td>
<td>(+)</td>
</tr>
</tbody>
</table>

* Virus was trapped with homologous antiserum at 1:1000 dilution.
† Antiserum dilution was 1:50 for homologous, and 1:5 for all heterologous combinations.
‡ −, No decoration; (+), very weak decoration; +++ , strong decoration.

ISEM investigations with antisera to BWYV and potato leafroll virus were conducted to test for the presence of luteovirus particles anticipating a possible heterologous reactivity of these antisera as reported for an antiserum to BWYV by Spiegel et al. (1986). Whereas luteovirus-like particles were reported to be associated with the MY-18 source (Martin & Converse, 1985) no such particles could be observed in our experiments.

Filamentous particles in ultrathin sections

In order to investigate the location of the potexvirus particles, ultrathin sections were examined from leaf vein tissues of R. rosifolius infected with MY-18 and F. vesca c.v. Alpine infected with D-531. Bundles of filamentous particles, which are believed to represent the potexvirus particles, were observed in phloem parenchyma cells with both sources. Fig. 5(a) and (b) show particles from D-531 in F. vesca, while (c) and (d) represent those found in R. rosifolius infected with MY-18.

Northern hybridization analysis

On non-denaturing agarose gels the dsRNAs purified from eight different sources of SMYE from strawberry all comigrated with the dsRNAs obtained from R. rosifolius infected with MY-18, whereas no dsRNA occurred in purifications from healthy strawberry and Rubus. In Northern hybridizations randomly primed MY-18 cDNA probes hybridized to the dsRNAs of each of the nine SMYE sources tested but not to nucleic acids from healthy strawberry (for details see Jelkmann et al., 1989).

Mechanical transmission

Preliminary experiments revealed that a mechanical transmission of the potexvirus particles to Chenopodium quinoa and C. murale is possible. No symptoms were observed on inoculated leaves but low numbers of filamentous particles could be trapped and decorated with the antiserum in tests done 3 weeks after inoculation. Successive mechanical transmission to the above hosts failed.

Discussion

Our results show that the dsRNA found to be associated with SMYE disease of nine different sources does not represent a replicative form of a luteovirus as was originally anticipated. The 3′-terminal nucleotide sequence of cDNA clone p463 derived from the dsRNA of MY-18 has been determined. The sequence contains a poly(A) tail, a non-coding region and a 26K ORF. Sequence alignments of the 26K ORF with published plant viral sequences indicated that this protein has no homology with proteins of luteoviruses (Veidt et al., 1988; Miller et al., 1988). However, significant homologies were found with coat proteins of potexviruses, i.e. PMV (Short et al., 1986), CYMV (AbouHaidar, 1988), PVX (Huisman et al., 1988; Morozov et al., 1987), PAMV (Bundin et al., 1986), WCIMV (Harbison et al., 1988) and NMV (Zuidema et al., 1989), and carlaviruses, i.e. PVS (MacKenzie et al., 1989) and PVM (Rupasov et al., 1989). The genomes of the carlaviruses differ in their organization from those of the potexviruses by the presence of an additional 11K ORF 3′-terminal of the coat protein gene. On the other hand, within the coat protein gene of the potexviruses NMV and WCIMV an
ORF (Zuidema et al., 1989) similar in size and location to the 11K ORF within the coat protein gene of the virus discussed here is also found. In respect of genome organization, therefore, the SMYE-associated virus appears to be a potexvirus.

The capsid protein is either 26K or 24K in size depending on the functional AUG codon. Potexvirus coat proteins range from 18K to 27K in size (Koenig & Lesemann, 1978), and in carlaviruses from 31K to 34K (Koenig, 1982). The particles found in SMYE-diseased strawberries had a modal length of 482 nm, well in the range of 470 to 580 nm known for potexviruses, whereas particles of carlaviruses have modal lengths of 610 to 700 nm (Koenig, 1982). The antiserum made against a fusion protein containing the presumed coat protein reacted strongly with the particles found in all SMYE-affected strawberries studied. These results indicate an association of a potexvirus with the disease.

Strawberries affected by SPMYE contained a carlavirus which had a coat protein of 33K, a particle length of 625 nm, and was serologically related to carnation latent virus (Yoshikawa & Inouye, 1986). This serological relation was not found in decoration tests of our virus with carnation latent virus antiserum. SPMYE virus, therefore, has several properties distinct from those of the potexvirus described here.

Decoration experiments performed with antisera to 27 known potexviruses revealed very weak reactions with some of them, possibly indicating distant serological relatedness. These results suggest that the described strawberry virus is a new member of the potexvirus group. Since this virus obviously was present in the nine
studied sources of SMYE we suggest it be designated SMYE-associated potexvirus (SMYEAV). The aetiology of SMYE disease cannot be understood until a suitable diagnostic tool is available for the proposed luteovirus (Converse et al., 1987). At present we know that the disease was associated with SMYEAV in nine sources, and an association with a luteovirus has been suspected in three reports (Yoshikawa et al., 1984; Martin & Converse, 1985; Spiegel et al., 1986). Both viruses might play a role in the aetiology of the disease. It is still unexplained how SMYEAV as a potexvirus can be associated with a disease known to be aphid-transmitted (see Methods). Generally potexviruses are not thought to be transmitted by aphids (Koenig & Lesemann, 1978). However, a low proportion of aphid transmission has been reported for WCIMV (Goth, 1962). Additionally the aphid transmission of PAMV is known but is dependent on the presence of a helper virus of the potato virus Y group (Kassanis & Govier, 1971).

Since luteoviruses have the ability for heterologous encapsidation of other plant viral RNA genomes as reported for carrot red leaf and carrot mottle viruses (Waterhouse & Murant, 1983), BWYV and lettuce speckles virus (Falk et al., 1979) and for groundnut rosette assistor virus (Casper, 1988) the potexvirus might be transmitted with the help of the SMYE-associated luteovirus reported previously or by some other as yet unknown mechanism.

The results of this paper demonstrate that cloning of dsRNA, identification of the coat protein gene sequence and its expression in fusion protein systems is a suitable technique for the preparation of antisera for recalcitrant viruses whose virions cannot be purified in sufficient purity or concentrations for antiserum production.

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References


similarities to potexviruses in gene arrangement and the encoded amino acid sequences. *Journal of General Virology* 70, 1861–1869.


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