Characterization of potyviruses from tulip and lily which cause flower-breaking

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Five viruses causing colour-breaking of tulip flowers were isolated from tulips and lilies. Tulip-breaking virus (TBV), tulip top-breaking virus (TTBV), tulip band-breaking virus, Rembrandt tulip-breaking virus and lily mottle virus were all characterized as potyviruses by serology and potyvirus-specific PCR. Sequence analysis of amplified DNA fragments spanning a conserved area of the coat protein cistron of potyviruses was performed in order to classify the isolates as distinct viruses or strains. It appears that all tulip-breaking viruses are distinct viruses and TTBV was found to be strain-related to turnip mosaic virus.

Introduction

One of the first literature reports of viral flower colour-breaking in tulips is by Clusius in 1576. He described a variegation of the flower colour in striped and flamed patterns in combination with a weakening of the plants eventually leading to the loss of varieties. These ‘broken tulips’ were highly appreciated in The Netherlands in the 16th and 17th centuries (Van Slogteren & De Bruyn Ouboter, 1941).

McWhorter (1938) distinguished two tulip-breaking viruses in tulips, a colour-removing and a colour-adding virus, causing a light and a dark flower-break, respectively. However, Van Slogteren & De Bruyn Ouboter (1941) found no strict relation between a particular virus isolate and the type of flower-breaking. They suggested a variation in the virulence of the virus strains and also differences in the resistance of plants to explain the various types of flower-breaking in tulips. Since mottle or mosaic viruses from lilies induce flower-breaking in tulips too, Brierley & Smith (1944) compared these viruses with the viruses reported by McWhorter (1938). They considered all virus isolates to be different strains of one virus and grouped the isolates into three subspecies. These differences in opinion about the systematic classification of tulip-breaking viruses were mainly based on host plant reactions. Later on, these viruses were classified as potyviruses (Brandes & Wetter, 1959) with small serological differences between them (Derks et al., 1982; Maeda et al., 1984). Differences in transmissibility to dicotyledonous plants have been reported as well (Alper et al., 1982; Maeda et al., 1984).

Other potyviruses isolated from tulips, but serologically different from tulip-breaking virus, can also induce flower-breaking (Asjes & Segers, 1982, 1985; Mowat, 1985; Hammond & Chastagner, 1988, 1989).

Here we describe five potyviruses from tulip and lily which all induce flower-breaking in tulips. A comparative description of their symptoms in one cultivar of tulip is presented together with serological data and a molecular analysis of a conserved part of the coat protein cistron for the taxonomic classification of these viruses.

Methods

Viruses. Tulip-breaking virus (TBV) was obtained from a single plant of tulip cv. Albury with a dark red break in the flowers and a mild mosaic pattern in the leaves. Tulip top-breaking virus (TTBV) was isolated from tulip cv. Don Quichotte showing necrotic stripes and spots at the leaf tips (Asjes & Segers, 1982) and tulip band-breaking virus (TTBV) from a single plant of tulip cv. Lucky Strike (Asjes & Segers, 1985). Lily mottle virus (LiMV), similar to the Easter lily mottle virus as described by Brierley & Smith (1944), was obtained from Lilium longiflorum cv. Flevo (TBV-lily; Langeveld et al., 1991) with a coarse motiling in the leaves. Rembrandt tulip-breaking virus (ReTBV) originated from a stock of Rembrandt tulip cv. Jack Laan with a deep purple and white flower break and light green bands in the leaves (Derks et al., 1982).

Host plants. Viruses were transferred to tulip cv. Halcro by manual inoculation to investigate differences in symptom expression. Before transfer, TTBV was passaged through Tetragonia expansa and LiMV through Nicotiana benthamiana. The tulips were grown in the field. Herbaceous plants and L. formosanum were grown from seed and tested as hosts by manual inoculation with leaf extracts from infected tulip cvs. Halcro and Jack Laan. The test plants were grown at 20 °C in a greenhouse.

Immunospecific electron microscopy (ISEM). The ISEM procedure was essentially the same as described by Milne & Luisoni (1977).
Table 1. Serological relationships of tulip-breaking viruses

<table>
<thead>
<tr>
<th>Antisera to tulip-breaking viruses and other potyviruses</th>
<th>TBV</th>
<th>TBBV</th>
<th>TTBV</th>
<th>ReTBV</th>
<th>LiMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBV (tulip cv. Texas Flame, RE102)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ReTBV (tulip cv. Jack Laan, RD60)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LiMV (L. longiflorum, UT478)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WaTV (tulip cv. Lincolnshire, PVAS388)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TuMV-L1 (tulip cv. Lincolnshire, PVAS389)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TuMV (7142)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TuMV (PVAS134)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BYMV* (QB 901, 957)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ISMV† (SS216)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PVY*§ (Y271)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PVY§ (Y273)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* BYMV, bean yellow mosaic virus.  
† ISMV, iris severe mosaic virus.  
‡ PVY*, potato virus Y*.  
§ PVY*, potato virus Y*.  

Antiserum were diluted to 1:20 in 67 mM-sodium phosphate buffer pH 7.0, both in the coating and in the decoration steps. Preparations were negatively stained with 2% uranyl acetate in water.

The antisera were prepared at the Bulb Research Centre or were provided by D. Z. Maat, Wageningen, The Netherlands (turnip mosaic virus, TuMV 7142) and J. Hammond, Beltsville, Md., U.S.A. (Washington tulip virus, WaTV PVAS385, TuMV-L1 PVAS389 and TuMV PVAS134). The antisera against tulip-breaking viruses from tulip cvs. Jack Laan (RD60) and Texas Flame (RE102) were described previously (Derks et al., 1982); the LiMV antisemat (UT478) was prepared against LiMV propagated in N. benthamiana.

RNA preparations. For PCR analysis, total RNA was isolated from leaves of infected plants of cvs. Halcro and Jack Laan by phenol–chloroform extraction; 50 to 100 mg of leaf material was ground under liquid nitrogen, mixed with 400 µl of phenol and 400 µl of extraction buffer, containing 1 M-Tris–HCl pH 8.0, 100 mM-NaCl, 10 mM-EDTA and 1% SDS, and incubated for 5 min at 70 °C. After centrifugation the water phase was extracted three times with phenol–chloroform (1:1). RNA was precipitated with ethanol and dissolved at a concentration of 0.5 to 1 µg/µl. The average recovery of total RNA was about 0.5 µg/mg leaf material.

Oligonucleotides. Oligonucleotide primers U341 and D341, spanning a 341 bp fragment in the conserved area of the coat protein cistron of potyviruses, were described by Langeveld et al. (1991).

Reverse transcriptase-polymerase chain reaction. Reverse transcription was performed on 1 to 2 µg of total RNA extracted from leaves in a 20 µl reaction mixture containing 67 mM-Tris–HCl pH 8.8, 16 mM-(NH₄)₂SO₄, 67 mM-MgCl₂, 67 µM-EDTA, 380 µmol of each dNTP, 50 pmol of downstream primer D341, 5 units ribonuclease inhibitor (Gibco BRL) and 60 units Moloney murine leukaemia virus reverse transcriptase (Gibco BRL). Incubation was for 10 min at 50 °C and 60 min at 42 °C. Reverse transcription reaction mixture (10 µl) was added to 40 µl of polymerase reaction mixture containing 10 mM-Tris–HCl pH 8.8, 50 mM-KCl, 1.5 mM-MgCl₂, 20 µg/ml gelatin, 1 unit of Taq polymerase (Gibco BRL) and 25 pmol upstream primer U341. Mixtures were layered with 50 µl of mineral oil and 33 reaction cycles were performed with periods of 3 min for annealing at 52 °C, 1 min for synthesis at 72 °C and 30 s for melting at 94 °C. Then, 10 µl samples of the reaction mixtures were analysed on 1.5% Seakem agarose gels in the presence of 0.5 µg/ml ethidium bromide.

Restriction enzyme digestion of amplified fragments. Part of the amplification reaction mixture (10 µl) was directly digested with 10 units of TagI restriction enzyme (Gibco BRL) after adjustment of the MgCl₂ concentration to 5 mM. Incubation was for 2 h at 65 °C.

Cloning and sequencing of amplified fragments. After electrophoresis, amplified DNA fragments were isolated from agarose gels by freeze–squeezing (Tautz & Renz, 1983), phosphorylated with T4 DNA kinase (Gibco BRL) and concatenated by ligation. Single fragments, obtained by digestion of the concatemers with BamHI and EcoRI, were ligated into M13 vectors. The nucleotide sequences of these fragments were established by the method of Sanger et al. (1977).

Phylogenetic analysis. Sequences were aligned with the CLUSTAL V program (Higgins & Sharp, 1988, 1989). Distance matrices were calculated by pairwise comparison of the 92 amino acid sequences with the DISTANCE program of the GCG package (Devereux et al., 1984) using the matrix of Schwartz & Dayhoff (1979). Output distance matrices of this program were recalculated using the Poisson correction \(d = -100 \ln(D_{ref})\). Cluster dendrograms were constructed with the program PILEUP from the GCG package (Devereux et al., 1984) implementing the unweighted pair group maximum averages (UPGMA) clustering algorithm (Sneath & Sokal, 1973).

Results

Host plant symptoms

Tulips of cv. Halcro were inoculated with leaf extracts containing TBV, TBBV, TTBV, ReTBV or LiMV. Symptoms were observed in the year after infection. TBV-infected plants showed a mild mosaic in the leaves, and a dark red break in the flowers. TTBV induced large yellow–green and grey necrotic ringspots in the leaves and a light yellow colour in the flower, bordered by dark red areas. In contrast to the symptoms observed with TBV, TTBV symptoms developed most severely at the top of the leaves and the flowers. TTBV gave rise to light green bands in the leaves and a yellow–white flower break. The symptoms described above formed the basis
for naming these viruses top-breaking and band-breaking. With LiMV, a dark green stripe-like mosaic pattern was visible in the leaves and a light yellow colour. The leaf extract from tulip cv. Jack Laan (ReTBV) induced symptoms in cv. Halcro varying from yellow-white flowers with red streaks and a light green colour. The leaf extract from tulip cv. Jack Laan (ReTBV) induced symptoms in cv. Halcro varying from yellow-white flowers with red streaks and a light green colour.

Like LiMV, which was originally isolated from lilies, TBV and ReTBV caused a systemic stripe-like mosaic pattern in the leaves of *L. formosanum* seedlings. TTBV did not infect *L. formosanum*.

TTBV could be transmitted by manual inoculation to a range of test plants, including *N. edwardsonii*, *N. tabacum* cv. White Burley, *Chenopodium quinoa* and *Cucumis sativus*, which were all locally infected, and *T. expansa* and *N. clevelandii*, which were both systemically infected.

LiMV infected *C. quinoa* and *T. expansa* locally, and *N. clevelandii* and *N. benthaminana* systemically. TBV, TBBV and ReTBV did not infect any of these test plants.

**ISEM**

Antisera raised against known potyviruses cross-reacted with the tulip-breaking viruses, indicating that the five viruses are indeed potyviruses. Antisera raised against various tulip-breaking and other potyviruses were used to distinguish the five viruses TBV, TBBV, TTBV, ReTBV and LiMV serologically (Table 1). All antisera showed cross-reactivity with one or more tulip-breaking viruses, with the exception of the TuMV-L7 and TuMV 7142 antisera which are highly specific for TTBV, suggesting that these three viruses are identical or closely related. The antisera of ReTBV and TBV showed identical cross-reactivities with other viruses, which means that ReTBV and TBV cannot be distinguished by
their own antisera. The LiMV antiserum, however, exhibits cross-reactivity with ReTBV but not with TBV, which suggests that TBV and ReTBV are serologically different.

**Identification of the viruses with PCR**

The tulip-breaking viruses were also identified as potyviruses by PCR using a potyvirus-specific set of general primers (Langeveld et al., 1991). The primers supported the amplification of a 341 bp DNA fragment in each crude total RNA preparation from plants infected with one of the five tulip-breaking viruses. The fragment represents a conserved region in the coat protein cistron of the potyvirus genome, and its amplification confirms that these viruses are potyviruses. Analysis of the fragment with *TaqI* restriction enzyme digestion (Fig. 1) was performed to reveal possible contamination with other potyviruses. Each of the breaking viruses demonstrated a unique digestion pattern with fragments together totalling 341 bp in length. This excludes the possibility of cross-contamination or contamination of these isolates with other potyviruses. Remarkably, LiMV occurring in lilies has a *TaqI* restriction pattern identical to that of TBBV.

**Phylogeny**

For taxonomic classification of the tulip-breaking viruses, the nucleotide sequences of the amplified fragments of each virus were determined after cloning the fragments into an M13 sequencing vector. From each virus several clones were analysed to reveal possible PCR artefacts. Only in the case of TBV had a C, at position 40 in the last position of a triplet, changed into a T in one of the three clones that were sequenced. This substitution does not change which amino acid is encoded by the triplet.

The sequences were then compared with other known potyvirus sequences, revealing that the sequences of TTBV and TuMV (Tremblay et al., 1990) are almost identical. Both viruses have 14 different nucleotide residues and only one different amino acid residue in this region of the potyvirus genome (Fig. 2). The alignments of sequences in Fig. 2 demonstrate that these tulip-breaking isolates are different viruses with large sections in common, suggesting that closer relationships exist between these viruses than between them and the other potyviruses. In order to quantify the relationships of these viruses with other potyviruses, the amino acid sequences were translated from the nucleotide sequences and aligned with the corresponding sequences of 27 other potyviruses. A matrix of evolutionary distances was computed from this alignment and a dendrogram (Fig. 3) was constructed according to the UPGMA algorithm (Sneath & Sokal, 1973). This tree, based on the 92 amino acids of a conserved area in the coat protein, shows clustering of the tulip-breaking viruses together with the virus isolate from Gloriosa and the plum pox virus (PPV) strains.

In order to classify the isolates into distinct viruses and strains, a frequency distribution was computed of pairwise evolutionary distances between the same 92 amino acid region of 32 different potyviruses. The frequency plot (Fig. 4) shows a bimodal distribution of evolutionary distances between distinct viruses and strains. The imaginary line separating the distance distributions of strains and viruses shown in Fig. 4 can also be drawn in Fig. 3. Of the tulip-breaking viruses, only the distance between TTBV and TuMV appears at the peak representing strains of the same virus, suggesting that only TTBV and TuMV are strain-related and that the other tulip-breaking viruses should be
considered distinct potyviruses. All other viruses known to be strains show distances of less than 4-5 units, except for MDMV-B and SCMV-SC (see Fig. 4 for abbreviations) with a distance of 6-7 units. The distances between TBV and TTBV (4-4 units) and between TBV and TuMV (4-5 units) occur in the area dividing strains from viruses.

Discussion

Five potyviruses associated with flower-breaking in tulips and lilies were identified and considered as separate entities on the basis of both symptom expression in tulips and host range. The transfer of other isolates of TBV and LiMV to the same tulip cultivar (Halcro), however, revealed that TBV, ReTBV and LiMV are difficult to differentiate on the basis of flower and leaf symptoms. In inoculation experiments TBV did not infect a range of lily cultivars, whereas some of them were infected by ReTBV. LiMV is the most important virus in lilies. We propose that the virus be named the lily mottle virus, according to Brierley & Smith (1944) in the first description of this virus, instead of TBV-lily (Maeda et al., 1984; Langeveld et al., 1991).

The serological data obtained in this study were conclusive about the nature of the tulip-breaking viruses as potyviruses. Only TTBV reacted specifically with TuMV and TuMV-L2 antisera, suggesting a close relationship with these viruses. Host plant reactions and serology both suggest that TTBV and TuMV-L2 may be synonymous (Hammond & Chastagner, 1988, 1989). Also, the nucleotide sequences of the 341 bp fragment revealed a strain relationship between TTBV and TuMV. Tulip chlorotic blotch virus (TCBV), another potyvirus inducing colour-breaking in tulips but not included in this study, is not serologically related to TBV (Mowat, 1985). It is, however, closely related to TuMV, according to serological and nucleic acid hybridization data (Hammond & Chastagner, 1988), and may therefore be strain-related to TTBV as well. We will refer to TTBV as a strain of TuMV by naming it TuMV-TTB in future reports. TBBV is serologically related to WaTV (Hammond & Chastagner, 1988, 1989), which implies that these two viruses may also be synonymous. The ISEM technique, employed in this study to reveal serological relationships between the viruses, is prone to the detection of virus-specific epitopes by polyclonal antisera but is nevertheless fully dependent on the specificity of the antisera used. Raising antibodies that are specific to a particular potyvirus can be hampered by the unstable variable N-terminal domain of the coat protein (Shukla & Ward, 1989). Our serological data might, therefore, be inadequate for discriminating between close and distant relationships in the case of tulip-breaking viruses. To allow further taxonomic classification of these viruses it was necessary to obtain viral RNA sequence data. General primer RT-PCR was performed and the amplified DNA fragments were subjected to sequence analysis. The PCR primers were made complementary to two highly conserved regions in the coat protein cistron 277 bp apart from each other. All tulip-breaking viruses supported amplification of the expected 341 bp fragment, which was also found in other potyviruses (Langeveld et al., 1991). The observed digestion patterns of the amplified fragments obtained with the TaqI restriction enzyme were in agreement with the determined sequences of each tulip-breaking virus indicating that the clones which were sequenced are true representatives of the amplification products.

Shukla & Ward (1989) have shown that a frequency
distribution of similarity between the complete coat protein sequences of 10 different potyviruses is bimodal for strains and distinct viruses. When a frequency distribution of distances between the tulip-breaking viruses and 27 other known potyviruses is computed, using the amino acid sequence of the coat protein region between the PCR primers, the distribution is also bimodal but there is a continuum between strains and distinct viruses. The distance between TBV and TTBV (4-4 units) appeared at this continuum and therefore in this case a conclusion about relationship is not possible on the basis of this short sequence of the potyvirus genome alone. As a strain relationship between TBV and TTBV is not evident from the serological data because the TBV antiserum does not cross-react with TTBV, we will therefore consider TBV and TTBV as distinct potyviruses. From the frequency distribution of distances it appeared that all other tulip-breaking viruses are distinct viruses. The distance between LiMV and TTBV (9-1 units) also strongly suggests that these are distinct viruses. The dendrogram constructed from the pairwise distances demonstrates all tulip-breaking viruses clustering into one group with PPV and with the virus isolated from Gloriosa by Dr D.-E. Lesemann, Braunschweig, Germany (Langeveld et al., 1991), also suggesting that the tulip-breaking viruses are related but distinct potyviruses.

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References


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