TYLCSV DNA, but not infectivity, can be transovarially inherited by the progeny of the whitefly vector *Bemisia tabaci* (Gennadius)

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**Abstract**

The transovarial transmission of two species of begomovirus, *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV), through generations of *Bemisia tabaci* of the B and Q biotypes has been investigated. Different life stages of the progeny of viruliferous female whiteflies have been analysed by PCR detection of viral DNA and infectivity tests. Our results indicate that TYLCSV DNA can be detected in eggs and nymphs, and to a lesser extent adults, of the first-generation progeny. Infectivity tests using a large number of adult progeny of the first, second, and third generation indicate that even when viral DNA is inherited, infectivity is not. For TYLCV, neither viral DNA nor infectivity were associated with the progeny of viruliferous female whiteflies. Because the inherited viral DNA is unable to give rise to infections, the transovarial transmission of TYLCSV DNA appears to have no epidemiological relevance.

**Keywords:** *Bemisia tabaci; TYLCV; TYLCSV; Geminiviridae; transovarial transmission**
females to their progeny (Ghanim et al., 1998). Recently, TYLCV–B. tabaci relationships have been reviewed by Czosnek et al. (2002).

Transovarial passage of plant viruses in their vectors can have dramatic consequences on virus epidemiology because the source of inoculum can be maintained in the vector even in the absence of plants hosting the virus. Transovarial transmission is rare among insect-transmitted plant viruses and has been associated with replication of the virus in its vector (reviewed in Hull, 2002). Begomoviruses are regarded as circulative in the whitefly because they must be actively transported across vector membranes and they must survive inside the vector during circulation until they are inoculated. The coat protein (CP) plays a fundamental role in the acquisition–circulation process of geminiviruses (Azzam et al., 1994; Briddon et al., 1990; Höfer et al., 1997, Noris et al., 1998), and it is likely that receptors, binding to the viral capsid, may be present in the B. tabaci midgut, an ideal site for viral endocytosis (Czosnek et al., 2002). Circulation of virions within the insect body is aided by the presence of GroEL, a chaperone produced by an endosymbiont, which binds to TYLCV CP protecting it from degradation (Morin et al., 2000). Nevertheless, while CP is essential for virus translocation within insect body, it could be unnecessary for long-term storage of viral DNA. Virions not interacting with insect receptors (i.e. when receptors are saturated) could invade cells and tissues where they disassemble, the CP is progressively destroyed, and the viral DNA binds to proteins that protect it from degradation (Czosnek et al., 2002). If transovarial transmission occurs, then the inheritance of intact functional virions should make the insect viruliferous while the inheritance of uncoated viral DNA should not.

In the present study, we have investigated whether transovarial transmission can occur in vector–begomovirus combinations found in tomato-growing areas of the western Mediterranean basin. For this purpose, TYLCSV and TYLCV together with B and Q biotypes of B. tabaci were used.

Results

All the data on virus detection in egg, nymph, and adult whiteflies refer to samples which tested positive in PCR using ketose reductase (KR)-specific primers, thus proving that

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Fig. 1. PCR analysis (ethidium bromide-stained agarose gels) of B. tabaci progeny. Size of the amplified fragment is indicated on the right. (a) Amplification of a partial sequence of the ketose reductase gene with KR(207+) and KR(578–) primers from single eggs, nymphs, and adults of B. tabaci. Samples producing negative results were not further analysed. −: no template control; M, 100-bp ladder (Invitrogen). (b–c) Amplification of a TYLCSV fragment of the capsid protein gene with TY1(+) and TY2(–) primers (Accotto et al., 2000) from eggs (b), nymphs and adults (c) of the first-generation progeny of infective B. tabaci females. −: no template control; M, 1-kb ladder (Invitrogen); P+, TYLCSV-infected plant; W+, TYLCSV-infected whitefly.
DNA suitable for PCR was contained in the insect preparations. Fig. 1a shows the KR-specific 372-bp amplified fragment obtained from whitefly eggs, nymphs, and adults.

Progeny of the B biotype: detection of TYLCSV DNA and infectivity tests

Out of 107 females singly tested on tomato plants, 61 were able to transmit TYLCSV as demonstrated by symptom development 20–25 days after inoculation. Due to the short life span of adult whiteflies, we could not wait for symptoms to develop in plants before the analysis of insects. Therefore, we analysed tomato plants by PCR after 4 days of inoculation access period (IAP) to select the parental viruliferous females at an early stage. All the PCR-positive plants developed disease symptoms.

The progeny of 48 infective females was studied. PCR analyses of their eggs, third-instar nymphs, and adults obtained on cucumber plants revealed the presence of the virus-specific 580-bp fragment in 10 out of 110 eggs, 32 out of 110 nymphs, and 5 out of 250 adults (Figs. 1b and c; Table 1). These fragments were never obtained from eggs, nymphs, and adults of control insects. PCR analyses of the second- and third-generation progeny adults did not reveal the presence of TYLCSV DNA in 50 samples of each of the two tested generations (Table 1). The amplification signals obtained from eggs, nymphs, and adult progeny were weaker than those obtained from insects fed on infected plants and used as positive controls (Figs. 1b and c).

The adult progeny that developed from eggs laid on cucumber by viruliferous whiteflies were tested for their ability to transmit TYLCSV. None of 84 tomato plants, each exposed to five first-generation progeny adults (420 adults), developed symptoms of TYLCSV infection or gave a hybridisation signal when blotted with the specific TYLCSV probe (not shown). Similarly, none of 20 plants used to test infectivity of second- and third-generation adults developed TYLCSV infection (Table 2).

Progeny of the B biotype: detection of TYLCV-[PT] DNA and infectivity tests

Out of 90 females singly tested on tomato plants, 48 were able to transmit TYLCV-[PT], an isolate from Portugal (Navas-Castillo et al., 2000), as demonstrated by symptom development 20–25 days after inoculation. We analysed the progeny of 32 viruliferous females of the B biotype, early selected by PCR as described above. All the PCR-positive plants developed disease symptoms. PCR analyses of 100 eggs, 100 third-instar nymphs, and 125 adults obtained on cucumber plants never revealed the presence of TYLCV-[PT] DNA. PCR analyses of the second- and third-generation progeny adults also failed to reveal the presence of TYLCV-[PT] DNA in 60 and 40 samples, respectively.

Adult progeny that developed from eggs laid on cucumber by viruliferous whiteflies were tested for their ability to transmit TYLCV-[PT]. None of 82 tomato plants, each exposed to five first-generation progeny adults (410 adults), developed symptoms of TYLCV-[PT] infection or gave a hybridisation signal when blotted with the specific TYLCV-[PT] probe (not shown). Similarly, none of 20 plants used to test infectivity of second- and third-generation adults was infected by TYLCV-[PT] (Table 2).

Progeny of the Q biotype: infectivity tests

Since no transmission was recorded in the progeny of the B biotype, we decided to perform similar infectivity tests with whiteflies of the Q biotype, which is also widely present in the Mediterranean area. For these assays, viruliferous females that had transmitted the virus were selected as described above.

Infectivity tests using first-, second-, and third-generation progeny of 15 viruliferous females provided no evidence of TYLCSV transmission: neither symptoms nor hybridisation signals were detected from 34, 16, and 16 tomato test plants exposed to adults of the first-, second-, and third-generation progeny, respectively (Table 2).

As with TYLCSV, when we analysed the infectivity of the progeny of 14 viruliferous females carrying TYLCV-[PT], we did not detect any symptom or hybridisation signal from the 24, 16, and 16 tomato test plants exposed to adults of the first-, second-, and third-generation progeny, respectively (Table 2).

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Table 1

<table>
<thead>
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<th>Life stage</th>
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<tr>
<td>Eggs (1st generation)</td>
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<tr>
<td>Nymphs (1st generation)</td>
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<tr>
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<tr>
<td>Third-generation adults</td>
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*Samples were analysed by PCR followed by agarose gel electrophoresis and ethidium bromide staining.

Table 2

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<th>No. insects</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>third</td>
<td>100</td>
<td>0/20</td>
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</tr>
<tr>
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<td>0/34</td>
<td></td>
</tr>
<tr>
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<tr>
<td></td>
<td>third</td>
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<tr>
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<td>80</td>
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</table>
Control experiment

TYLCSV was not detected by PCR in the 15 adults fed on the cucumber leaves that had been exposed to viruliferous males. Similarly, we failed to detect the virus in 15 eggs, third-instar nymphs, and adults of the offspring generation (not shown). The cucumber leaves exposed to viruliferous males did not reveal the presence of TYLCSV in PCR analysis (not shown), indicating that the viral DNA detected in the first-generation progeny was not transmitted via the plant but was inherited transovarially. Consistent with this, none of 12 tomato seedlings exposed to 60 offspring adults developed TYLCSV infection.

Discussion

TYLCSV DNA was detected by PCR analysis of the progeny of viruliferous whiteflies, showing for the first time in the case of this virus the transovarial passage of viral DNA from females to their first-generation progeny. Eggs and nymphs appeared more frequently positive for TYLCSV DNA compared to adults (10–30% versus 2%). This can be explained either by progressive viral DNA degradation over time in the insect or as a consequence of some enzymatic degradation of tissues hosting viral DNA (or virions). On gels, specific TYLSV bands obtained from the progeny of viruliferous whiteflies were always weaker than those obtained from whiteflies fed on infected plants. By contrast, TYLCV-[PT] DNA was never detected in the progeny (eggs, nymphs, or adults) of viruliferous whiteflies, even in more than 300 samples.

The experimental design and the control experiment carried out on TYLCV-immune plants (cucumber) rule out any possibility that mechanisms other than transmission through the egg can explain vertical transmission.

Transovarial inheritance of TYLCV DNA has been reported by Ghanim et al. (1998) to occur with high frequency. These authors detected the Israeli strain of TYLCV in 80% of the eggs and in more than 50% of the offspring adults. We cannot explain the reason for such different results, and we can only make some hypotheses about fine differences between the viruses or the insect colonies used. TYLCV-[PT] is very closely related (97% sequence identity) to a “mild” isolate of TYLCV also present in Israel (Antignus and Cohen, 1994), and to a lesser extent (91% sequence identity) to the “severe” isolate (Navot et al., 1991) used by Ghanim et al. (1998). Therefore, we cannot exclude that a “severe” isolate may perform differently when transovarial transmission is analysed. Transovarial transmission may not be a common feature of these begomoviruses, even within a species. Concerning the insect populations, B-biotype whiteflies were used both in our experiments and in those of Ghanim et al. (1998). Nevertheless, differences in the vector colony could explain the different results.

Czosnek et al. (2002), reviewing the work of Ghanim et al. (1998), noticed that, using a different B. tabaci colony and an almost identical TYLCV isolate, viral DNA was detected in eggs and up to the third-instar nymphs, but not in the adult progeny of viruliferous insects. The genetic variability of B. tabaci has been inferred from the analysis of mitochondrial (COI) (Frolich et al., 1999) and of ribosomal (ITS) DNA (De Barro et al., 2000), but information on genetic and biological variability within B biotype populations is not yet available. Finally, one technical difference is worth noting when comparing our transovarial transmission data with those of Ghanim et al.: when analysing single insects, they performed hybridisations following PCR while we preferred to avoid the hybridisation step since it certainly boosts the signal but is more prone to even minor contamination.

We do not know how and in which form (naked DNA or virions) TYLCSV is passed on to the whitefly progeny, but during the first phase of acquisition, begomoviruses circulate in B. tabaci as encapsidated virions, as shown for TYLCV by Ghanim et al. (2001) and for the two bipartite geminiviruses Tomato mottle virus and Cabbage leaf curl virus (ToMoV and CaLCV) by Hunter et al. (1998). Some days after acquisition, however, only viral DNA is detectable in the whitefly vector (Rubinstein and Czosnek, 1997). In our case, we detected the viral DNA in whitefly stages that developed several days after acquisition by the parental females, and therefore, TYLCSV is likely inherited by the progeny in the form of viral DNA. Functional virions would be likely to make the insect viruliferous, while the mere presence of viral DNA may not be sufficient to make the whitefly infective. This hypothesis is consistent with the findings of Rubinstein and Czosnek (1997) who demonstrated that the disappearance of the virus CP was associated with a rapid decrease in the ability of the whitefly to transmit the virus.

When working with very small samples, like whitefly eggs (the body weight of a female laying such eggs does not exceed 50 µg), it is important to avoid false negative results. The ketose reductase (KR) gene control indicated that attempts to extract DNA from very small samples, like individual eggs, provided inconsistent results, so that a simple boiling procedure is to be preferred for such material. This is in accordance with observations of Ghanim et al. (2001) who obtained better PCR results using untreated dissected whitefly organs compared to the same organs subjected to a DNA extraction procedure. The PCR protocol, based on the amplification of part of the KR gene, proved to be both very specific and robust, and therefore might be useful in future research on B. tabaci. Preliminary real-time PCR experiments targeting KR gene-specific sequences suggest that this gene is present in a single copy or in very few copies in the insect genome (unpublished). As a consequence, if a DNA preparation is suitable for the amplification of such a gene, even relatively few copies of a target virus sequence should also be detectable.
The transmission experiments we performed involved large numbers of offspring adults of both B and Q biotypes, of the first, second, and third generation, and clearly show that, even if viral DNA was inherited, infectivity was not. For TYLCSV, we checked the infectivity of 590 first-generation adults, while for TYLCV-[PT] we checked the infectivity of 530 first-generation adults. All these adults developed from eggs laid by females that had been selected as viruliferous. Ghanim et al. (1998) reported that some adults of the progeny were able to transmit TYLCV to healthy tomatoes. Our results are clearly different and indicate that, in the absence of virus host plants, B. tabaci does not represent a source of inoculum.

Failure to obtain vertical transmission of infectivity indicates that infected tomato crops, as well as bean and pepper, are the main, if not the exclusive sources of infection for TYLCV (Navas-Castillo et al., 1999; Reina et al., 1999). Because TYLCV and TYLCSV, and the B and Q biotypes of B. tabaci, are widespread in the western Mediterranean basin, we can conclude that in this area the progeny of viruliferous whiteflies do not have per se any ability to transmit these viruses.

Only weak amplification of viral DNA fragments, but no infectivity, was detected in the progeny of viruliferous female whiteflies, suggesting that probably, only traces of viral DNA, and not the whole virions, are inherited. Our results suggest that the pathways of begomovirus circulation in the body of B. tabaci, as described by different authors (Ghanim et al., 2001; Harrison, 1985; Hunter et al., 1998), seem to be restricted to virus particles introduced into the alimentary canal when whiteflies feed on infected plants.

The difference in results of transovarial transmission between TYLCSV and TYLCV suggests that these viruses, sharing a relatively low genome similarity (approximately 75% sequence identity) and differing both in host range and transmission characteristics, may have different relationships with the vector.

Our results confirm that transovarial inheritance of viral DNA in the vector can occur in begomoviruses although virus transmission through the egg is not a common feature, even within a species. Further studies on other begomovirus–B. tabaci associations may reveal to what extent traces of viral DNA can be found in whitefly progeny. In any case, these biological observations appear to have no epidemiological relevance because the inherited viral DNA is unable to give rise to infections. However, the inheritance of viral DNA via the egg is intriguing, especially if we consider that such transmission represents a dead end path for begomoviruses. The presence of contaminant begomovirus DNA in ovaries and eggs, as well as reduction in longevity and fecundity in whitefly hosts (Rubinstein and Czosnek, 1997), is reminiscent of a viral insect pathogen.

**Material and methods**

**Maintenance of viruses and whiteflies**

Tomato plants (Lycopersicon esculentum cv Marmande) were infected with TYLCSV, originally isolated in Sardinia (Kheyr-Pour et al., 1991; GenBank acc. no. X61153) and TYLCV-[PT], originally isolated in Portugal (Navas-Castillo et al., 2000; acc. no. AF105975), by agroinoculation and maintained in growth chambers under strict containment.

B. tabaci colonies of the B and Q biotypes (Simón et al., 2003), collected in Liguria and Sicily (Italy), respectively, were maintained in growth chambers at 25 °C, with a photoperiod of 16 h light– 8 h dark, on cucumber plants (Cucumis sativus cv Marketer) in nylon and Plexiglas cages. The biotype status was determined by esterase, RAPD-PCR, and Squash Silver Leaf assays.

**Transmission assays**

Adults of B. tabaci, B biotype, were allowed to acquire TYLCSV for 48 h on an infected tomato, 30–40 cm high, in a growth chamber at 25 °C. The females were then transferred singly onto healthy tomato (cv Marmande) seedlings at the six-leaf stage inside a glass cylinder cage for the IAP. Four days later, a small portion of apical leaf tissue was sampled from the plants with a living female and analysed for virus infection by PCR (see below). The following day, all the females which proved to be infective (i.e. whose tomato test plant tested positive in PCR) were transferred onto a cucumber plant (cv Marketer), immune to TYLCSV (Crespi et al., 1991), to lay eggs and kept for 1 week. The surviving females were then removed. All PCR-positive tomato plants were kept and developed disease symptoms in about 3 weeks from inoculation. Eight days after the start of oviposition, eggs of the first-generation progeny (laid by infective females) were sampled from two to three leaves, singly detached from the leaf tissue using sterile needles and transferred to a microplate well under a stereomicroscope. Only yellow eggs were sampled, avoiding the transparent ones that were freshly laid and whose embryonic development was still in the very early phase. Each egg was then crushed with the needle in 20 μl of 0.5 × TE buffer for subsequent DNA extraction (see below). Fifteen days after the start of oviposition, third-instar nymphs were sampled from two to three leaves and treated as described for the eggs. Twenty days after the start of oviposition, adults of the new generation began to emerge. Some of them were sampled for PCR analysis but most were transferred onto healthy test tomato seedlings at the six-leaf stage, in groups of five, to feed for 4 days. Thirty adults of the first-generation progeny were transferred onto a new cucumber plant to lay eggs for 1 week and to allow development of the second-generation progeny. As soon as the second-generation adults emerged, some of them were sampled while most
of them were transferred onto healthy test tomato seedlings at the six-leaf stage, in groups of five, to feed for 4 days. Thirty adults of the second-generation progeny were transferred onto a new cucumber plant to oviposit for 1 week and to allow development of the third-generation progeny. Similarly, some of the newly emerged adults of the third generation were sampled and the others were transferred onto healthy test tomato seedlings at the six-leaf stage, in groups of five, to feed for 4 days. The whole experiment was repeated using TYLCV-[PT] and the same B. tabaci B-biotype colony.

Two similar experiments were carried out using the Q biotype in combination with TYLCV or TYLCV-[PT]. In these experiments, we only checked the infectivity of the adults of first-, second-, and third-generation progeny, as described above, without sampling eggs, nymphs, and adults for presence of viral DNA.

Controls

The cucumber plants used are immune to TYLCSV, but a control experiment was done to exclude the possibility that nymphs and adults of the new generation might acquire the virus by feeding on the same cucumber plant where viruliferous females fed before them, probably injecting some virions.

Two hundred adult whiteflies were allowed to acquire TYLCSV for 48 h on an infected tomato plant. Two groups of 30 males were then transferred to two healthy tomato seedlings for a 24-h inoculation period to check their infectivity; they were then isolated, in a clip-cage of 10 cm in diameter, on two cucumber leaves. After 4 days, the males were removed and virus-free adults were introduced to the same clip-cages (50 insects per cage) on the same cucumber leaves. Two days later, 15 adults were sampled. One week later, the surviving adults were eliminated and 15 eggs were sampled. After another 2 weeks, 15 nymphs were sampled. Finally, when the new adults emerged, 15 of them were sampled, together with the leaf portion inside the clip-cage. The remaining 60 adults were transferred, in groups of five, onto 12 healthy tomato seedlings for 4 days, and plants were observed for virus symptoms for 30–40 days. All sampled insects and plant material were analysed for viral DNA by PCR as described below.

Preparation of insect and plant DNA

DNA was extracted from adult whiteflies and test plants essentially as described by Accotto et al. (2000), but reducing the volume of solutions for the insects. Briefly, single adults were crushed in Eppendorf tubes with 100 μl of extraction buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% SDS, 10 mM β-mercaptoethanol) and sterile sand. Leaf samples (100–150 mg) were similarly crushed with 500 μl of extraction buffer. The suspension was incubated at 65 °C for 5 min, then 0.3 volume of 5 M potassium acetate was added, the tubes were kept on ice for 10 min and centrifuged for 10 min. The supernatant was transferred to a new tube and 0.7 volume of cold isopropanol was added. Tubes were centrifuged for 10 min at 4 °C, the pellets were washed in 70% ethanol, briefly vacuum-dried, and resuspended in 0.5× TE buffer (20 μl for adult whiteflies, 500 μl for plant samples).

A different procedure was followed for eggs and nymphs. DNA was extracted by simply crushing single eggs and nymphs with a sterile needle in 20 μl of 0.5× TE buffer in a microplate well. The suspensions were homogenised by a 10-min treatment in an ultrasonic bath, transferred to 0.5 ml Eppendorf tubes, and boiled for 3 min. Following brief centrifugation, these extracts were used immediately for PCR or stored at −20 °C. One microliter of the extracts was used in 25-μl PCR reactions.

Detection of insect and virus DNA

All DNAs extracted from insects were first analysed for the presence of whitefly DNA because single eggs and nymphs are samples of particularly small size. Two primers were designed on the B. tabaci ketose reductase (KR) gene (GenBank acc. no. AF067126), KR(207+) (5'-GGCA-TACGTTGGAATTTGTGG-3') and KR(578–) (5'-TGGA-CACCGACGGACAAAG-3'). In a limited number of extracts from eggs and nymphs, the 372-bp whitefly-specific amplicon could not be detected. Those samples were not further analysed.

Primers TY1(+) and TY2(−) were used to detect TYLCV and TYLCV-[PT]. These degenerate primers amplify a 580-bp fragment of the capsid protein gene of several begomoviruses (Accotto et al., 2000).

All PCR reactions were performed in a Hybaid PCR Sprint thermal cycler, using a reaction mix containing 2 mM MgCl₂, 200 μM each dNTPs, 400 nM each primer, and 0.02 U/μl Taq polymerase (Polytaq, Polymed, Italy). The amplification protocol for viral DNA consisted of 45 cycles (30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C) followed by a final extension step of 7 min at 72°C. For insect DNA amplification, a “touch-down” protocol was used to improve specificity. Forty cycles (30 s at 95 °C, 30 s at 60 °C for the first cycle—then decreasing the temperature by 1 °C at each cycle until reaching 52 °C—and 30 s at 72 °C) followed by a final extension step of 7 min at 72 °C were carried out. Aliquots of reaction products were analysed on 1.2% agarose gels.

Detection of virus DNA in plants by squash-blot hybridisation

Tomato test plants exposed to the whitefly adults of the first, second, and third generation derived from infective females were checked for the presence of TYLCSV and TYLCV-[PT] by squash-blot hybridisation. One month after inoculation, the apical part of each plant was squashed on
positively charged nylon membranes (Roche Applied Science, Basel, Switzerland): UV light was used to fix nucleic acids on the membranes, which were then prehybridised and hybridised with TYLCSV- or TYLCV-specific probes. These probes were obtained by incorporating digoxigenin-dUTP during PCR reactions performed with virus-specific primers (Accotto et al., 1998). The probes were used at a final concentration of 1 ng/ml of hybridisation buffer, and the washing steps following hybridisation were at high stringency (0.1× SSC, 0.1% SDS at 65°C). Detection was performed with the chemiluminescent substrate CDP-Star (Roche) according to manufacturer’s instruction.

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References


