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# PCR-amplification of tomato yellow leaf curl virus (TYLCV) DNA from squashes of plants and whitefly vectors: Application to the study of TYLCV acquisition and transmission

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**Abstract**

DNA of tomato yellow leaf curl virus (TYLCV), a geminivirus transmitted by the whitefly *Bemisia tabaci*, was amplified from squashes of infected tomato plants and of viruliferous vectors using the polymerase chain reaction (PCR). Samples of infected tissues as small as 1 $mm^2$ were squashed onto a nylon membrane. A 1 $\times$ 2 mm strip containing the squash was introduced into a 25 $\mu$l PCR reaction mix. The reaction products were subjected to gel electrophoresis, blotted and hybridized with a radiolabeled virus-specific DNA probe. TYLCV DNA was amplified from squashes of leaves, roots, and stem of infected tomato and from individual viruliferous whiteflies. The same squash could be used several times to amplify different virus DNA fragments with various sets of primers. Thus plant and insect squashes can be used as templates for the amplification of geminiviral DNA with no need to prepare tissue extracts or purify nucleic acids. The squash-PCR procedure was applied to study whitefly transmission of TYLCV. Tomato plants were inoculated by placing a single viruliferous insect in the center of a young leaflet. In some plants TYLCV DNA was detected at the site of inoculation as early as 5 min after the beginning of the access feeding and in all plants after 30 min. The squash-PCR procedure also was applied to the study of TYLCV acquisition by the insect vector. TYLCV DNA was detected in the head of whiteflies as early as 5 min after the beginning of the access feeding on infected tomato plants. Viral DNA was detected in the thorax after 10 min and in the abdomen after 25 min.

## Introduction

The polymerase chain reaction (PCR) is widely used in plant pathology for the diagnosis of plant diseases, allowing the detection of very small amounts of the disease agent in the infected plant, and also the cloning of genomic fragments of the pathogen (Henson and French, 1993). The PCR usually requires the purification of the target DNA although it has been demonstrated that plant DNA can be amplified from crude extracts of leaves (Klimyuk et al., 1993), from aqueous extracts of tissues squashed on a membrane (Langridge et al., 1992) and even from leaf and root pieces (Berthomieu and Meyer, 1991). We have shown previously that TYLCV DNA can be detected in tomato tissues and in whiteflies squashed on a membrane, following hybridization with a radiolabeled (Navot et al., 1989) or with a chromogenic DNA probe (Zilberstein et al., 1989). We also have demonstrated that TYLCV DNA can be amplified from nucleic acids isolated from tomato plants and from individual whiteflies by PCR (Navot et al., 1992). In this communication we have combined both methods and we have demonstrated that TYLCV DNA can be amplified by PCR, using as template plant and insect tissues squashed on a membrane. The squash-PCR procedure was applied to study the acquisition of TYLCV by the whitefly vector and the transmission of the virus to tomato plants.

## Materials and methods

*Maintenance of virus cultures, whiteflies and plants*

Cultures of the Israeli isolate of TYLCV (Navot et al., 1991) were maintained in tomato plants (*Lycopersicon esculentum* Mill., cv. FA144). *Bemisia tabaci* Genn. of the B type (Cohen, 1993) were reared on cotton plants (*Gossypium hirsutum* L., cv. Akala) grown in insect-proof wooden cages as previously described (Zeidan et al., 1991).

*Inoculation of tomato plants*

One-month old seedlings were caged with viruliferous insects (insects that fed for 24 h on infected tomato plants). After 48 h, the insects were removed and the plants were reared in insect-proof cages.

*Sampling of plant and insect tissues*

Plant and insect tissues were squashed on a nylon membrane (Qiabrane, Biolabs) as previously described (Navot et al., 1989). The samples were used as is; exposure to heating or to UV light was unnecessary. Samples could be stored in a dry state for several weeks without using their capacity to serve as template for PCR. A strip of 1 × 2 mm was cut and immersed into the 25 $\mu$l PCR mix contained in a 0.5 ml Eppendorf-type vial prior to initiation of amplification.

*Amplification of TYLCV DNA using the polymerase chain reaction*

TYLCV DNA fragments were amplified using the following pair of primers (0.2 mM each) deduced from the nucleotide (nt) sequence of the genome of TYLCV from Israel (Navot et al., 1991): V61 (nt 61-80, viral strand, 5′ATACTTGGACACCTAATGG3′) and C473 (nt 473-457, complementary strand, 5′AGTCACGGGCCCTTACAA3′), V781 (nt 781-800, viral strand, 5′CTCACAGAGTGGGTAAGAGG3′) and C1256 (nt C1256-1229, complementary strand, 5′TTAATTTGATATTGAATCATAGAAATAG3′), V1769 (nt 1769-1790, viral strand, 5′GCGAACAGTGGCTCGTAGAGGG3′) and C2120 (nt 2120-2097, complementary strand, 5′CAGGCAAAACAATGTGGGCCAGG3′).

Oligonucleotides were purchased from Biotechnology General, Rehovot, Israel. The cycling protocol was as follows: initial denaturation for 10 min at 94 °C, annealing of primers for 10 sec at 56 °C, addition of 1 $\mu$ of *Taq*I polymerase, extension for 80 sec at 72 °C and denaturation for 50 sec at 94 °C; subsequent cycles were: 55 sec at 56 °C, 80 sec at 72 °C and 50 sec at 94 °C; after 30 cycles using a Techne PHC-2 thermocycler, the reaction was terminated by a 7 min incubation at 72 °C (Navot et al., 1992).

*Analysis of PCR products*

The PCR products were collected, subjected to electrophoresis in a 1% agarose gel and photographed. The amplified virus DNA was identified after blotting and hybridization with radiolabeled plasmid pTYH19 containing a full-length copy of the TYLCV genome (Navot et al., 1991). Autoradiography was for 1 to 5 h.

## Results

*Amplification of TYLCV DNA from squashes of viruliferous whiteflies and of infected tomato plants*

TYLCV DNA was amplified from squashes of viruliferous whiteflies and of infected tomato plants. A single whitefly that was caged for 24 h with an TYLCV-infected plant was squashed on a nylon membrane. Leaf number 3 (numbered from the youngest true leaf), the stem between leaves number 3 and 4 and roots from the virus source tomato plant were also squashed on a membrane. Similarly, non-viruliferous insects and leaf number 3 of a non infected plant were squashed on the membrane. In parallel, one ng DNA of plasmid pTYH19 was spotted on a membrane. All samples were subjected to the PCR reaction using primers V61 and C473. The products of the reaction were collected, subjected to electrophoresis, blotted and hybridized with the virus-specific DNA probe to confirm the identity of the amplified viral DNA fragment. Figure 1 shows that a 410 bp DNA fragment was amplified from tissue squashes of viruliferous insect and of infected plant.

*Amplification of several TYLCV DNA fragments from a single squash of infected tomato leaf*

Figure 2 shows that a single squash of infected tomato leaf can serve several times as template, allowing amplification of a different viral DNA fragment each time. A ~ 410 bp fragment (encompassing most of the TYLCV intergenic region and the V2 gene) was amplified with primers V61 and C473 (see genetic map of

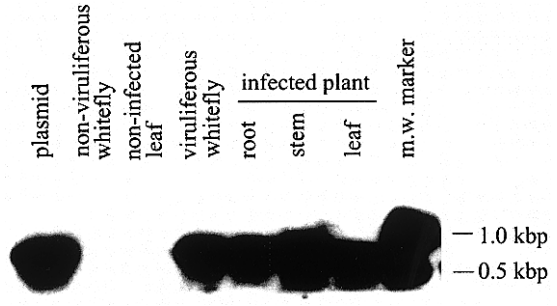


*Figure 1.* PCR-amplification of TYLCV DNA from squashes of infected tomato plants and of viruliferous whiteflies. True leaf number 3, the stem and roots of an infected plant, together with a viruliferous whitefly, were squashed on a membrane. A leaf from a non-infected plant and a non-viruliferous insect were squashed also. One ng DNA of plasmid pTYH19 was spotted on the membrane. Strips of 1 × 2 mm containing the samples were introduced into the PCR reaction mixtures and subjected to amplification using primers V61 and C473. The aqueous phase was subjected to agarose gel electrophoresis, blotted and hybridized. Molecular weight (m.w.) represent the DNA fragments of the 1 kb ladder hybridizing with the pTZ18R moiety of plasmid pTYH19.

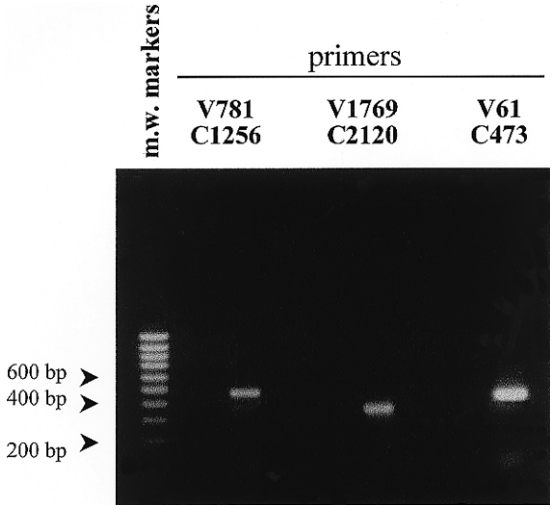


*Figure 2.* Sequential amplification of three different TYLCV DNA fragments using the same squash of infected tomato leaf. A viral DNA fragment was first amplified using primers V61 and C473. Then the squash was washed with sterile water and subjected to a second round of PCR using primers V1769 and C2120. The squash was washed again and submitted to a third round of amplification using primers V781 and C1256. The aqueous phase was subjected to agarose gel electrophoresis and stained with ethidium bromide.

TYLCV in Navot et al., 1991). Then the squash was washed twice in 500 $\mu$l sterile water in an Eppendorf tube, blotted dry and subjected to a second round of PCR. A ~ 350 bp DNA fragment (encompassing the C-terminus of the Rep protein gene) was amplified using primers V1769 and C2120. The squash was washed again and submitted to a third round of amplification using primers V781 and C1256. This time a ~ 480 bp (encompassing the C-terminus of the capsid protein gene) fragment was amplified.

*Inoculation of tomato plants by a single viruliferous whitefly*

Virus transmission by viruliferous insects was determined by assaying the plant tissue at the site of inoculation for the presence of viral DNA, as a function of the length of the access feeding period. Insects were picked randomly from the whitefly colony and caged for 24 h with an infected tomato plant. The viruliferous insects were then placed on the center of tomato leaf number 3 (one insect per plant). After 5, 10, 15, 20 and 30 min, the insects were discarded and their position on the inoculated leaflet was marked. Plants were analyzed only if the insect did not move for the entire inoculation feeding period. The tissue at the inoculation site was squashed on a membrane and assayed for the presence of the virus using primers V61 and C473 as shown in Figure 3A. Twelve plants were assayed for each inoculation time point. Examination of the sex of the inoculating insects indicated that all were females. Figure 3B shows that TYLCV DNA was detected in 2 of the 12 plants following a 5 min inoculation feeding period. The virus was detected in 7 of the 12 plants after 10 min and in all plants after 30 min of inoculation feeding. It is possible that in some cases, negative results were obtained because the localization of the inoculation site was not accurate.

*Acquisition and spread of TYLCV in the whitefly* Bemisia tabaci

Acquisition and spread of TYLCV in the insect during acquisition feeding on infected tomato plants was studied by assaying for the presence of TYLCV in the insect head, thorax and abdomen as a function of the length of the feeding period. Insects randomly picked from the insect colony had access to leaf number 3 of infected tomato plants for 5, 10, 15, 25 and 35–40 min. Fourteen insects were collected randomly at each time point and were immediately frozen. The sex of the insects was noted. The head, thorax and abdomen of each individual insect were severed, squashed on a membrane and subjected to PCR using primers V61 and C473, as illustrated in Figure 4. TYLCV was detected in the

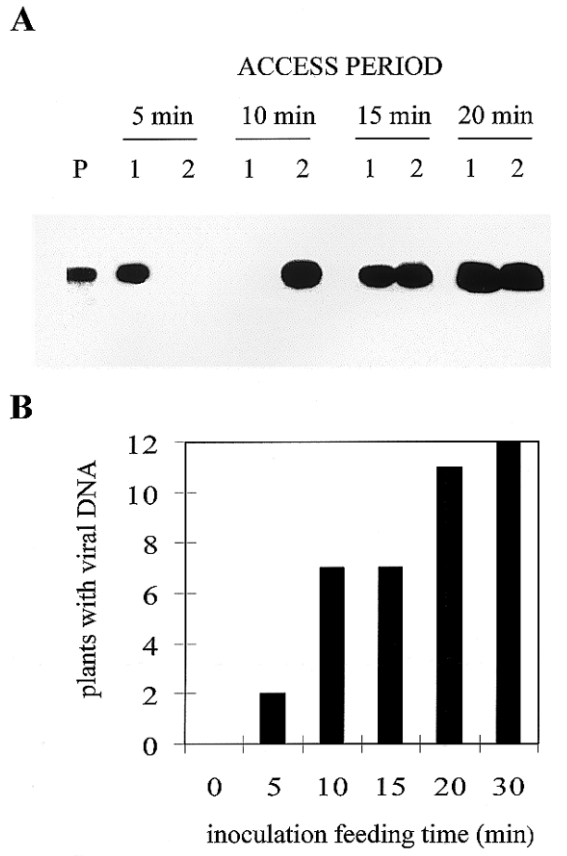


*Figure 3.* Inoculation of TYLCV by a single whitefly estimated by PCR-amplification of TYLCV at the site of inoculation. After viruliferous insects had access to tomato plants (one insect per plant) for the periods of time indicated, the tissue at the site of inoculation was squashed and processed as described in Figure 1. For each time point, twelve plants were inoculated (*A*) amplification of TYLCV DNA from two plants (1, 2) and from plasmid pTYH19 (P) using primers V61 and C473, followed by hybridization with the virus DNA probe. (*B*) number of plants containing detectable viral DNA as a function of the inoculation feeding time.

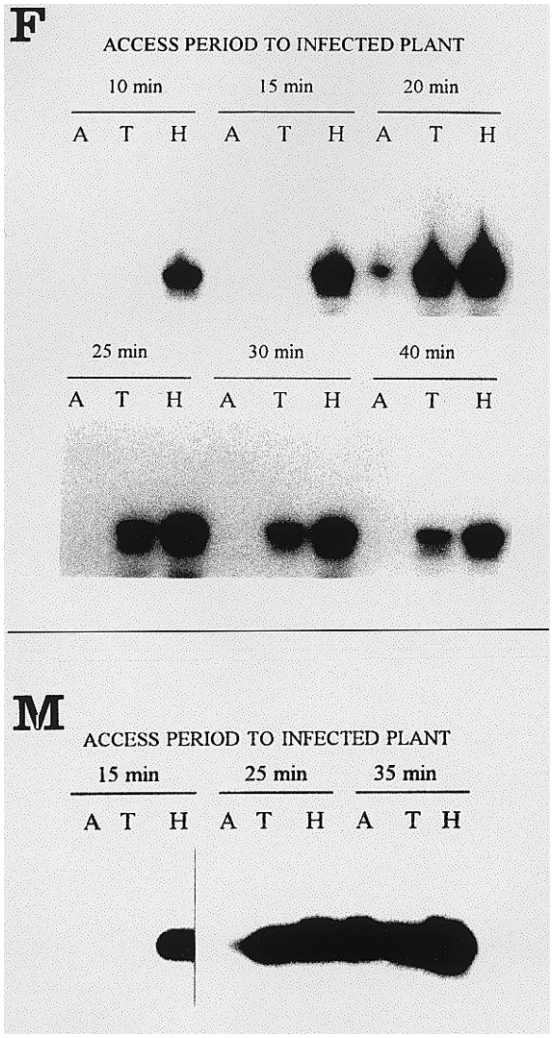


*Figure 4.* Acquisition and spread of TYLCV in individual whiteflies. Insects that had access to infected tomato plants for the periods of time indicated were collected. Head (H), thorax (T) and abdomen (A) of individual female (F) and male (M) whiteflies were squashed on membranes and subjected to PCR using primers V61 and C473. The reaction products were subjected to gel electrophoresis and hybridized with the virus DNA probe.

head of 3 insects (3 of the 12 females) as early as 5 min after access to the plant. Five minutes later, the virus was detected in the head of all insects, in the thorax of two females and in that of the only male. TYLCV was detected after 15 min in the head of all insects, in the thorax of 7 of the 11 females and in that of 1 of the 3 males. In addition to the head of all insects, the virus was detected in the abdomen of 2 of the 3 males after 25 min, not in that of the females. After 40 min, the virus was present in the head and thorax of all insects; it was detected in the abdomen of the 2 males but only in that of 2 of the 12 females. After 24 h of access feeding, the virus was present in the head, thorax and abdomen of all insects analyzed, females and males.

## Discussion

In this communication we have shown that TYLCV DNA can be amplified from infected leaves squashed onto pieces of nylon membrane of about 1 $mm^2$. Squashes of individual viruliferous whiteflies have also

served as template in the PCR reaction. The squash-PCR procedure is easy and fast, the membrane can be used as is and results are highly reproducible. The purification of DNA from quantities of tissues similar to those squashed would be uncertain and extremely laborious at best. The squashes on the membrane can be kept at ambient temperature for many weeks without losing their template qualities in the PCR reaction. The same squash can serve several times as template allowing, for example, the amplification of different DNA fragments. Squashes of minute amounts of tissues such as scratches, stabs, anatomical sections could be used as template. The procedure should suit such goals as the screening, identification and cloning of geminiviruses using universal primers (e.g. Rojas et al., 1993; Rybicki and Hughes, 1990; Wyatt and Brown, 1996), and probably of other pathogens.

The squash-PCR procedure was applied to study the acquisition and transmission of TYLCV by its insect vector. Tomato plants can be inoculated by a single viruliferous whitefly. The virus was detected at the site of inoculation by the insect in 2 of the 12 plants assayed as early as 5 min after access feeding started, in more than half of the plants after 15 min and in all plants after 30 min. These data indicate that the time taken by each insect to probe the leaf tissues before injecting the virus is variable. Although viral DNA was amplified from some plants as early as 5 min after the start of inoculation feeding, the amounts of viral DNA inoculated were not sufficient to induce yellow leaf curl disease. The inoculated plants used as template for TYLCV DNA amplification were not followed up because the site of inoculation was excised, thereby eliminating (most of) the inoculum. Instead, a similar inoculation experiment was conducted but without excising the site of inoculation. The presence of disease symptoms was monitored 6 weeks after inoculation. None of the plants inoculated by a single viruliferous insect for a period of less than 30 min developed disease symptoms. Up to 20% of the plants were diseased following a 35–45 min inoculation period, 20–40% were diseased after 50–60 min and up to 60% after 60 min of inoculation feeding. Hence the squash-PCR procedures could detect viral DNA amounts during inoculation feedings that were insufficient to induce symptoms. In earlier experiments, Cohen and Nitzany (1966) used 3 insects per plants for a 48 h inoculation period and obtained about 80% transmission with TYLCV from Israel (as measured by the number of infected plants/total plants). Mehta et al. (1994) used one insect per plant and obtained 20% of diseased plants with TYLCV from Egypt, but after a 3 day inoculation feeding period.

Five minutes of whitefly feeding were sufficient to detect viral DNA in squashes of inoculated plants. Five minutes were also sufficient to detect viral DNA by PCR in squashes of the head of some of the insects feeding on infected plants. The sensitivity of the method can be compared with previous results of ours where viral DNA was detected by Southern blot hybridization of DNA from individual insects after a minimum of 30 min of access-feeding (Zeidan and Czosnek, 1991). Since it is postulated that the insect stylet has to reach the phloem to pick up virus (or inoculate it), the velocity of stylet penetration can be much faster than previously thought (Pollard, 1955). Leaf penetration has been studied by Walker and Perring (1994) who have analyzed the electronic waveforms produced during feeding of *B. tabaci* on Lima bean (*Phaseolus lunatus*). Their results have indicated that it took an average of 16 min (as early as 10 min for some of the insects, as late as 45 min for others) from initiation of leaf penetration to phloem ingestion. The difference between these and our results may reflect differences in the sensitivity of the tests or in the anatomy (accessibility of the phloem in the leaf) of the source plant. The amount of viral DNA acquired during the first minutes of access feeding may not be sufficient to induce the TYLCV disease once transmitted to test plants. It has been reported that the minimum time of feeding on infected plant for effective inoculation of TYLCV from Israel was 15–30 min (Cohen and Harpaz, 1964). It was 1 h for the TYLCV from Jordan (Mansour and Al-Musa, 1992) and from Italy (Sardinia) (Caciagli et al., 1995).

We have also followed the spread of TYLCV in its insect vector by assaying the presence of viral DNA in the head, thorax and abdomen of insects as function of the length of the acquisition feeding period. The path followed by whitefly-transmitted geminiviruses in their vector is not well known (Cicero et al., 1994; Harris et al., 1995). Whiteflies appear to transmit geminiviruses in a circulative manner. During the latent period (from 8 h to 24 h), virus is taken up with phloem sap through the stylets and passes from the maxillary food canal in the feeding apparatus (acquisition). Then it enters the esophagus and filter chamber. In the digestive system, virions penetrate the foregut to directly enter the hemocoel in close proximity to the salivary glands. From there they find their way to the ducts of the salivary system (inoculation). Our study provides preliminary data on the velocity of virus translocation in the insect, results that should be confirmed and refined

by other techniques such as immunolocalization or *in situ* hybridization. Our data indicate that TYLCV can be detected in the head of whiteflies 5 min after the acquisition feeding period started. The virus started to be detected in the thorax of some of the insects after 10 min and in the abdomen after 25 min. The difference observed in the distribution of TYLCV between males and females (present in a ratio of approximately 1 : 4.5) was explained by the fact that in some females, the entire midgut, including the portion within the filter chamber, is pushed into the thorax by the developing ovaries, leaving only the hindgut in the abdomen (Cicero et al., 1994; Harris, personal communication). Therefore, the hindgut seems to contain little, if any, virus after a short acquisition access feeding period on infected tomato. Since it has been demonstrated that DNA can be amplified from tissue sections (Chiu et al., 1992) and from individual cells (Nuovo et al., 1992), it should be possible to refine the localization of TYLCV in the insect by using anatomical dissections of organs as substrate for the squash-PCR reaction.

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