IMMUNOLOCALIZATION OF TOMATO YELLOW LEAF CURL SARDINIA VIRUS IN NATURAL HOST PLANTS AND ITS VECTOR BEMISIA TABACI

V. Medina1, M.S. Pinner2, I.D. Bedford2, M.A. Achon1, C. Gemeno1 and P.G. Markham2

1 Departament Producció Vegetal i Ciència Forestal, Universitat de Lleida, Avda. A. Rossell Roura 191, 25198 Lleida, Spain
2 Department of Disease and Stress Biology, John Innes Centre, Colney Lane NR4 7UH, Norwich, United Kingdom

SUMMARY

To increase our understanding of the mechanisms of insect-transmission of begomoviruses we studied the distribution of coat protein (CP) and DNA of Tomato yellow leaf curl Sardinia virus (TYLCSV) in its insect vector (Bemisia tabaci), and in tomato plants (Lycopersicon esculentum). The study was extended to other begomoviruses and a potential overwintering host plant for TYLCSV, black nightshade (Solanum nigrum). Immunogold labelling (IGL) showed that a polyclonal antiserum against the coat protein of African cassava mosaic virus (ACMV) (ACMV-CP-As) cross-absorbed with healthy plant and insect tissue, cross-reacted specifically with both homologous (ACMV, non-transmissible isolate), and heterologous [(TYLCSV and Asystasia golden mosaic virus, (AGMV)] viruses. ACMV-CP-As revealed that TYLCSV is located in mature and immature sieve elements, the nuclei of the companion cells, and the cytoplasm of the phloem parenchyma cells of L. esculentum and S. nigrum, with a similar pattern of distribution in both species. In situ DNA-hybridization (ISH) using a specific TYLCSV-DNA-probe showed that the TYLCSV-DNA occurred mainly in the vascular tissues of stems and roots of S. nigrum. In some leaves the infection spread to the palisade parenchyma, confirming that the DNA of the virus is not phloem-restricted in this host.

IGL of viruliferous B. tabaci detected both TYLCSV and AGMV in cells of the filter chamber, whereas the non-transmissible isolate of ACMV was detected only in the lumen of the insect gut. No labelling was obtained in non-viruliferous individuals of B. tabaci. The antiserum labelled, for the first time, the primary glands of the salivary gland system of TYLCSV-viruliferous B. tabaci, suggesting that the circulative pathway of TYLCSV can be completed as intact virions.

Key words: TYLCSV, begomovirus, immunogold labelling, in situ hybridization, Bemisia tabaci.

INTRODUCTION

Viruses in the genus Begomovirus (Family Geminiviridae) infect many important agricultural plants worldwide including bean, cassava, cotton, melon, pepper, potato, squash, tobacco, tomato and watermelon. Begomovirus species related to the tomato yellow leaf curl disease (TYLCD) occur worldwide in tropical and subtropical regions causing important yield losses in tomato (Lycopersicum esculentum L.) (Czosnek and Laterror, 1997), and a few other vegetable crops (Navas-Castillo et al., 1999). In the Mediterranean region two begomovirus species, Tomato yellow leaf curl Sardinian virus (TYLCSV) and Tomato yellow leaf curl virus (TYLCV) cause important diseases of tomato (Accotto et al., 2003). Previously they were named TYLCV-Sar and TYLCV-Is, respectively, and were considered as strains of TYLCV (Fauquet et al., 2003). Both species are present in southern Spain, Italy and the Canary Islands (Spain), whereas in Portugal only one species has been reported (Louro et al., 1996; Monci et al., 2000; Accotto et al., 2000; 2003). Natural recombination between the two virus species has been reported resulting in new species, for instance the Tomato yellow leaf curl Malaga virus (TYLCMav) (Navas-Castillo et al., 2000), now prevalent in Spanish populations (Monci et al., 2002). Currently, most authors consider the TYLCD-related begomoviruses as a viral complex.

In southern Europe, TYLCD-related begomovirus species are transmitted by their only known insect vector, the tobacco whitefly, Bemisia tabaci. This whitefly is an important plant pest in itself and is the vector of viruses in several other virus families (Wisler et al., 1997). The B. tabaci species comprises several biotypes (Perring et al., 1993; Bedford et al., 1994; Brown et al., 1995; Frohlich et al., 1999), the number of which continues to rise because molecular techniques provide increasingly finer levels of genotypic resolution (Cervera et al., 2000). For this reason most authors refer to this species as the B. tabaci complex.

Some of the B. tabaci biotypes have been shown to exhibit different levels of transmission efficiency based on their abilities to adapt to different host plants (Bedford et al., 1994). Biotype B, also named by some au-
Fig. 1. Immunogold labelling localization of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in tomato (A-B) and *African cassava mosaic virus* (ACMV) in *Nicotiana benthamiana* (C-E). A: general view of vascular zone of TYLCSV-infected tomato. B (detail of A, arrow): labelled nucleus of a companion cell. Notice the absence of background and specific labelling of electron-dense structures inside this nucleus. C: Minor vein of ACMV-infected *N. benthamiana* leaf. D (detail of C, left arrow): typical fibrillar ring inside nucleus induced by begomoviral infection of a bundle sheath cell. E (detail of C, right arrow): electron-dense material also labelled by ACMV antiserum in necrosed cell. (BS: bundle sheath cell, CC: companion cell, PhP: phloem parenchyma cell, SE: sieve element; bars: A = 5,3 µm; C = 2 µm, B, D & E = 0,4 µm; gold particle diameter: 15 nm).
thors B. argentifolii, the silverleaf whitefly (Perring et al., 1993; Wisler et al., 1998), is polyphagous and highly fecund, so even when individually it has a low transmission efficiency, it is highly effective as a population for spreading disease (Bedford et al., 1994).

Acquisition and transmission of begomoviruses by B. tabaci is persistent and circulative (Cohen and Nitzany, 1966, Ghanim et al, 2001a). The long-term association between TYLCSV and its vector seems to affect the transmission efficiency, longevity and fecundity of B. tabaci (Rubinstein and Czosnek, 1997) and can result in transovarial transmission (Ghanim et al., 1998). The close association between this virus and vector suggests it may replicate within the vector (Goldman and Czosnek, 2002). Recent studies suggest that the host plant species may also interfere in the genetic activity of the virus (Sinisterra et al., 2005).

TYLCSV has been characterized as phloem-restricted and non-mechanically transmissible (Mehta et al., 1994, Morilla et al., 2004). Therefore its spread depends on the presence of B. tabaci. Winter temperatures in central and northern Europe are not high enough to maintain overwintering populations of B. tabaci, however TYLCV and TYLCSV are present throughout the year in southern Europe (Bedford et al., 1994). Perpetuation of these viruses between cropping seasons could be explained if they have alternative hosts that maintain a reservoir of the inoculum, or if viruliferous whiteflies enter a susceptible crop each year. TYLCSV also infects Datura stramonium, the black nightshade (Solanum nigrum), Mercurialis ambigua and Solanum luteum (Sanchez-Campos et al., 2000), which are common weeds in south eastern Spain and could serve as overwintering or intercropping virus reservoirs (Bedford et al., 1998).

The main objective of this study was to determine the distribution of both coat protein (CP) and DNA of TYLCSV in plants and in the insect vector, as a contribution to the model for begomovirus transmission. We used a combination of molecular and microscopy techniques (immunogold labelling [IGL] combined with electron microscopy, and in situ DNA hybridization [ISH]) to determine the distribution of TYLCSV in tomato and S. nigrum plants and B. tabaci. The IGL study in B. tabaci was extended to other begomoviruses, African cassava mosaic virus (ACMV, a non transmissible isolate), and Asystasia golden mosaic virus (AGMV, a transmissible isolate).

**MATERIALS AND METHODS**

*Virus isolates, insect cultures and transmission tests.*

The Spanish isolate of TYLCSV was maintained in tomato (cv. Kondine Red) and Nicotiana benthamiana plants in quarantined greenhouses under L:D 16:8 h at 25°C at the John Innes Centre (JIC, UK). The transmissible isolate of AGMV and the non-transmissible isolate of ACMV originated from Benin and Kenya, respectively (see Bedford et al. 1994 and Liu et al., 1997), and were maintained in N. benthamiana and Asystasia gangetica, respectively under the same glasshouse conditions. The colony of B. tabaci originated from Israel (biotype B), and was cultured on cotton plants within the JIC’s quarantined insectary.

The transmission tests were undertaken by placing non-viruliferous insects from the cotton colony on infected plants for a 24 h acquisition period after which they were transferred to healthy plants (25 insects per plant) for a 24 h inoculation period. After inoculation some insects were processed and the rest killed with a bifenthrin-based insecticide.

**Localization of the coat protein: immunogold labelling (IGL).** The plant and insect materials were: healthy tomato, TYLCSV-infected tomato and S. nigrum, healthy N. benthamiana, ACMV (non-transmissible isolate)-infected N. benthamiana, non-viruliferous B. tabaci, and B. tabaci that had acquired TYLCSV, AGMV or the non-transmissible strain of ACMV. Plant tissue was collected and immediately placed into fixative (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) and trimmed into small pieces (1 × 10 mm). The insect specimens were collected, anaesthetised with CO2 and immersed in fixative after removing wings and legs. Both plant tissue and insects were then transferred to new fixative, dehydrated in an ethanol series and cold embedded in LR white, medium-grade resin (London resin, Woking, Surrey, UK) (Wells, 1985).

IGL followed the protocol of Pinner et al. (1993) on ultra-thin sections (70 nm) mounted on carbon-coated gold grids. A polyclonal antiserum against ACMV-CP expressed in Escherichia coli (Liu et al., 1997) was diluted 1:100 in blocking buffer (200 mM tris-HCl buffer, pH 7.4 containing 1% v/v Tween 20, 0.1% gelatin and 1% w/v bovine serum albumin). Ultra-thin sections of plant and insect tissue on grids were incubated overnight at 4°C in diluted antiserum, then washed for 1 min in distilled water and incubated for 1 h at room temperature in a 1:20 dilution of 15 nm gold-conjugated goat-antirabbit IgG antiserum (Janssen Auroofpe, Janssen Pharmaceuticals, Oxford, UK). Finally, the ultra-thin sections were stained with uranyl acetate (15 min) and lead citrate (2 min) and then examined with Jeol 1200 EXK or Phillips 120 electron microscopes. A minimum of two grids per block and a total of six blocks of each sample were analysed. A total of 5 samples (plant or insect) of each origin were analyzed.

**Localization of the DNA: in situ hybridization (ISH).** For localisation of the DNA we performed ISH and optical microscopy on healthy and TYLCSV-infected leaves of S. nigrum following the protocol of Jackson...
Fig. 2. Immunogold labelling localization of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in *Solanum nigrum*. **A**: vascular (up) and mesophyll (down) areas. **B** (detail of **A**): strong labelling of nucleus of a companion cell and lumen of sieve elements. **C** (detail of **B**, lower square): labelling of sieve element. **D** (detail of **B**, upper square): labelling of electron-dense material of a necrosed cell. **E**: vascular zone of a minor vein. **F**: higher magnification of **E** (CW: cell wall, N: nucleus, SE: sieve element; bars: A & E = 2.5 μm, B = 0.5 μm, C, D & F = 0.3 μm; gold particle diameter: 15 nm).
(1991). Plants were collected from the field in south eastern Spain during summer and winter in order to compare virus distributions in the two seasons. Five samples were analysed for leaf, stem or root. A total of 5 plants per season were processed. Samples were fixed with 4% paraformaldehyde in 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 (PBS) for 12-16 h at 4°C, washed (PBS 3 × 10 min), and treated with a 0.85% NaCl solution (30 min, 4°C) to avoid carbonate precipitation. After dehydration in increasing concentrations of ethanol, infiltration was done in two steps replacing ethanol by a clarifying agent (Histoclear, J.T. Baker, S.A., Spain) and replacing this by paraffin at 50°C (Paraffin 52/54, Probus S.A., Spain). Blocks were kept at 4°C until sectioning. Sections were prepared and ISH processed using a hydrolyzed RNA probe of TYLCV labelled with digoxigenine (DIG) (Liu et al., 1997).

Nomenclature of different parts of the gut followed Rosell et al. (1995) and Ghanim et al. (2001b).

RESULTS AND DISCUSSION

Localization of the viruses in host plants. The IGL-electron microscopy assay confirmed cross-reactivity of ACMV-CP-As against AGMV and TYLCSV, as well as the phloem-limitation or “phloem tropism” of most begomovirus virions (Sudarshana et al., 1999). Cross-reactivity of ACMV-antisera with several begomoviruses has already been reported using other serological methods (Macintosh et al., 1992). A heterologous antisera was used instead of a homologous one because results obtained in previous experiments had shown non-specific labelling by our homologous antisera even after a second purification and cross-adsorption. In contrast, labelling with ACMV-CP-As produced a very specific signal with no or very little background noise.

Using ACMV-CP-As, the label was mostly found inside the mature and immature sieve elements, and the nuclei of companion and phloem parenchyma cells (Figs. 1 and 2) of TYLCSV-infected leaves in a pattern similar to that described by Rojas et al. (2001) for TYLCV. No differences were found among the distributions of IGL between TYLCSV-infected tomato, TYLCSV-infected S. nigrum, and ACMV (non-transmissible strain)-infected N. benthamiana were found using ACMV-CP-As. In the companion cells, the label consisted of electron-dense structures in the nucleus (Figs. 1 A, B and 2B). Electron-dense structures (Figs. 1E and 2D) from disorganised vascular cells, and the inside of the phloem tubes (Figs. 2C), were also labelled. In general, the characteristic morphology of geminivirus particles was not clearly distinguished. We did not find gold labelling of palisade and spongy parenchyma cells of infected leaves, so it seems that TYLCSV, as a virion, is phloem-limited.

ISH has proved a good technique to localise the DNA of TYLCSV in fresh sections at low magnification. No signal was observed in the tissues of healthy S. nigrum (Fig. 3A), whereas the infected samples showed ISH in the phloem, mostly in the vascular tissue (Fig. 3B-E), and occasionally in the palisade parenchyma (Fig. 3D-E), but never in the epidermal cells. This suggests that in S. nigrum the DNA of TYLCSV can move out of the phloem. Movement of begomoviral DNA out of the phloem has been reported before by Michelson et al. (1997) for TYLCV in susceptible and tolerant isogenic tomato lines. Morilla et al. (2004) demonstrated that TYLCV-DNA is confined to phloem in N. benthamiana and tomato plants in both single and mixed infections with TYLCV. The lack of phloem-limitation of TYLCV-DNA in S. nigrum but not in N. benthamiana suggest the existence of host-specific factors involved in virus movement, as has been pointed out by other authors for begomoviruses (Harries and Robinson, 1999; Rojas et al., 2005).

Although most of the ISH signal was observed in the leaves of the summer samples, it was also found in root samples collected in the summer and winter (Fig. 3F), which confirms that TYLCSV can overwinter in S. nigrum, as previously suggested by Bedford et al. (1998).

Immunolocalisation of the viruses in the insect vectors. In our transmission experiments, except those involving healthy plants, or plants infected with the non-transmissible isolate of ACMV, insects transmitted the virus after a 24 h-acquisition period. This agrees with the minimal range of acquisition and latent periods reported previously for other begomoviruses (Cohen and Nitzany, 1966; Ghanim and Czosneck, 2000; Ghanim et al., 2001a).

Microscope observations on B. tabaci focused on the beginning of abdomen and prothorax, where the filter chamber and salivary glands are located, respectively (Ghanim et al., 2001b). Structures similar to thylacoids and disrupted chloroplasts were found in the lumen of the anterior midgut of viruliferous (all viruses) and non-viruliferous B. tabaci (Fig. 4A). In the individuals that had acquired from plants infected with TYLCV or AGMV, the gut cells were labelled (Figs. 4 and 5 D, E, respectively). In the individuals that had acquired the non-transmissible isolate of ACMV, only structures in the lumen of the gut were labelled (Fig. 5 A-C). This suggests that the gut cell wall is a barrier for the non-transmissible strain. Labelling in the ACMV-non transmissible strain appeared scattered, with no aggregates, which could suggest digestion of the virus by gut enzymes. Most of our results for ACMV (non-transmissible strain) are comparable to those obtained with TYLCV and Trialeurodes vaporariorum, a non-vector of TYLCV, by Czosnek et al. (2002), supporting the idea that the gut cells can be a selective barrier for begomoviruses.

IGL was also found in the primary salivary glands of some TYLCSV-viruliferous insects (Fig. 6). Only struc-
Fig. 3. *In situ* hybridization (ISH) of *Tomato yellow leaf Sardinia curl virus* (TYLCSV)–DNA on thin sections of *S. nigrum*. 
A: healthy leaf. B-E: TYLCV-infected leaves (B and D: transverse sections of veins, C and E: longitudinal sections of veins). 
F: TYLCV-infected root (arrows indicate vascular tissues).
Fig. 4. Immunogold labelling of *Tomato yellow leaf curl Sardinia virus* (TYLCSV)-viruliferous *Bemisia tabaci*. A: transport vesicles in the gut cells between the lumen of the gut and the haemocoel containing material (probably aggregates of virions) labelled by the ACMV-CP antiserum. B (detail of A). Notice in A the crystalline-like structures (arrow) in the lumen of the gut (bars: A = 5.7 µm; B = 0.3 µm; gold particle size 15 nm).

Fig. 5. Immunogold labelling of *African cassava mosaic virus* (ACMV, non-transmissible isolate) (A-C) and *Asystasia golden mosaic virus* (AGMV, transmissible isolate) (D-E) at filter chamber level of viruliferous *Bemisia tabaci* specimens. B and C are details of A (right and left arrow). Notice in A that only structures in the lumen of the gut microvilli are labelled (Bars: A = 1.9 µm; B, C and E = 0.2 µm; D = 1 µm; gold particle size = 15 nm).
Fig. 6. Immunogold labelling of Tomato yellow leaf curl Sardinia virus (TYLCSV) at salivary glands level of B. tabaci. A: Longitudinal section of head and thorax of B. tabaci with indication of the anatomy of mouth parts. B (detail of the area indicated in A): prothorax area of B. tabaci showing the situation of salivary glands (ASG: accessory salivary glands, PSG: primary salivary glands). C (detail of B): strong IGL of saliva drops in saliva secretory cells of PSG. D and E (details of C, left and right arrows, respectively). Notice the absence of background and specific labelling of saliva drops (B: brain, Cpr: cibarial pump retractor muscles, Cvr: cibarial valve retractor muscles, Ec: epicibarial gustatory organ, Hc: hypocibarial gustatory organ, Lr: labrum, Ph: pharynx, Pr: piston retractor muscle, Rt: rostrum). (Bars: A = 45 µm, B = 13.5 µm, C = 2 µm, D & E = 0.4 µm; gold particle size: 15 nm). A: optical micrograph, B-E: electron micrographs.
tures resembling saliva drops were labelled (Fig. 6 C-E). This is the first time that a TYLCSV has been detected as intact virions in the salivary glands. Previously, 

**Tomato mottle virus** (ToMoV) and 

**Cabbage leaf curl virus** (CaLCV), also begomoviruses, were detected in the salivary glands of the vector, *B. tabaci* (Hunter et al., 1998), but the type of salivary gland involved was not specified. As has been previously suggested, based in its localization in vector tissues, TYLCSV seems to follow a transmission pattern closer to that of leafhoppers (Markham et al., 1994). Brown and Czosnek (2002), demonstrated by ISH the presence of TYLCSV-DNA in the nucleus of 3 of the 14 cells that compose the primary salivary glands of *B. tabaci*. This study has, for the first time, shown the presence of TYLCSV-CP in these glands demonstrating that TYLCSV can circulate as virions in *B. tabaci*.

However, not all the whiteflies that were dissected for this study showed gold label in the salivary glands. The same has been reported by other authors (Ghanim et al., 2001a). This could be explained by variation in the amounts of virus among plants and/or variation in feeding activity among the whiteflies (Czosnek et al., 2002). We have also observed differences among the bacterial populations within the whiteflies that were studied (data not shown), which could explain the variability of transmission rates reported in the literature (Czosnek et al., 2001) and in our studies.

It has been suggested that endosymbiotic bacteria inside *B. tabaci* can prevent the destruction of TYLCV in the haemolymph of their vector. The virus would interact with GroEL homologues produced by insect endosymbiotic bacteria, as many plant circulative viruses do (Akad et al., 2004). Future work on these endosymbionts could explain the sometimes erratic presence of TYLCSV in insects. In order to process samples more efficiently, insect parts removed for microscopic analysis could be analyzed by PCR to determine if the insect is viruliferous or not before proceeding with the preparation (Noris, E., personal communication).

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