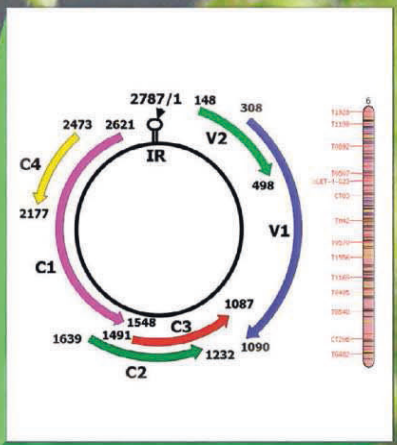
Henryk Czosnek
Editor

Tomato Yellow Leaf Curl Virus Disease

Management, Molecular Biology, Breeding for Resistance



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TOMATO YELLOW LEAF CURL VIRUS DISEASE

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edited by

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CONTENTS

PART I

WORLDWIDE EXPANSION OF TYLCV

CHAPTER 1

APPEARANCE AND EXPANSION OF TYLCV: A HISTORICAL POINT OF VIEW

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1. INTRODUCTION

In 1959, the Israeli Ministry of Agriculture urged farmers in the Jordan Valley to replace the tasty but soft tomato "Marmande" with the long–shelf life variety "Money Maker," which was more suitable for export. A month after transplanting (August), most of the tomato plants in the region were affected by a disease of unknown etiology. Symptoms included severe stunting of plant growth, erect shoots, and markedly smaller and misshaped leaflets. The leaflets that appeared immediately after infection were cupped down and inward, and subsequently developing leaves were strikingly chlorotic and showed an upward curling of the leaflet margins. When young plants were infected, they barely produced any marketable fruits (Cohen & Nitzany, 1960). The growers' first reaction was to blame the change in tomato variety and they demanded compensation from the Ministry of Agriculture. Dr. F. E. Nitzany, head of the Virology Laboratory at the Volcani Center, Agricultural Research Organization (ARO), Israel, was asked to determine the causal agent of the disease and find solutions to the problem. A field survey revealed that most of the tomato plots in the area had been completely destroyed, and that the disease was accompanied by large populations of whiteflies. The whitefly population had built up in the nearby cotton fields, a crop which was being grown on a commercial scale for the first time in Israel. Soon enough, the suspicion that the whiteflies were the vector of the disease was confirmed, following controlled transmission experiments in the laboratory. Moreover, the "Marmande" tomato was found to be as susceptible as "Money Maker" to the disease, which was found to be viral in nature (Cohen & Nitzany, 1960). The virus was named *Tomato yellow leaf curl virus* (TYLCV) by the late Professor I. Harpaz of the Hebrew University (Cohen & Harpaz, 1964). Interestingly, similar disease symptoms had first been

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observed on tomatoes grown in the Jordan Valley as early as 1929, as well as in subsequent years (Avidov, 1944). The outbreaks of TYLCV disease were always accompanied by large populations of whiteflies (Cohen & Berlinger, 1986). However, the geminate shape of the viral capsid was first observed in 1980 (Russo et al., 1980), and it was only in 1988 that the virus was isolated (Czosnek et al., 1988). It took another 3 years to clone and sequence the virus, and to demonstrate that the genome of TYLCV is composed of only one single-stranded (ss) DNA molecule (Navot et al., 1991).

The first evidence of economic damage to vegetable crops caused by the whitefly *Bemisia tabaci* (Gennadius) in Israel was recorded in 1931 (Avidov, 1944). Since 1935, it has been a permanent pest, mainly in the Jordan Valley. Avidov concluded that the *Bemisia* whitefly can raise as many as 15 generations per year in the Jordan Valley, due to the favorable climate in the area (Avidov, 1944). The silvering of squashes caused by *Bemisia*, which was observed as early as 1963 (Baery & Kapoller, 1963), and the very wide host range of this insect indicate that the B (or silverleaf) biotype has been present in this region for a long time.

2. VIRUS–VECTOR INTERACTIONS

2.1. Acquisition and transmission

In 1960, the first steps were taken toward controlling the TYLCV epidemic. The virus–vector relationship was studied by testing the transmission efficiency of TYLCV by whiteflies. Following 48 h of acquisition access feeding on infected tomato, only 5% of the male whiteflies transmitted the virus by transmission feeding of a single insect per test plant. However, female whiteflies were able to transmit the virus with 32% efficiency, sixfold better than their male counterparts. Transmission feeding with 1, 3, 5, 10, and 15 viruliferous female whiteflies per plant yielded transmission rates of 32%, 83%, 84%, 86%, and 100%, respectively (Cohen & Nitzany, 1966).

It was found that the virus is circulative and persistent in the insect (Cohen & Nitzany, 1966). Once the whitefly vector feeds on an infected host plant and acquires the virus, viral transmission can occur within hours, and may continue for the life span of the vector. Acquisition and transmission thresholds were found to be between 15 and 30 min. However, at least 4 h were required to obtain high infection rates. The latent period was found to be from 21 to 24 h. In tests carried out with whiteflies having a life span of 20–50 days, following 48 h of acquisition feeding, only 2 out of 39 female whiteflies retained the virus for 20 days. Shorter acquisition feedings resulted in shorter virus-retention periods. TYLCV transmission efficiency by its vector declines with time; most of the females failed to transmit the virus for more than 10 days after acquisition (Cohen & Nitzany, 1966). Besides acquisition by adults, it was found that the virus is also acquired by the whitefly larval stages. Following feeding on an

infected plant, 28% of the emerging adults were able to transmit the virus (Cohen & Nitzany, 1966).

To test for virus transmission from viruliferous females to their progeny (transovarial transmission), viruliferous whiteflies were allowed to lay eggs on cotton plants, which are immune to TYLCV. Upon emergence from the pupal stage, the adult offspring were immediately transferred to TYLCV-susceptible plants for a 48 h transmission feeding. Out of 360 female offspring tested, none was found to transmit the virus. Thus it was concluded that TYLCV is not transmitted to the whitefly progeny.

The issue of whether TYLCV is transmitted transovarially to the whitefly progeny came up again for debate 30 years later, when different findings were published. Using molecular tools as well as PCR amplification (which were unavailable back in the 1960s), it was demonstrated that TYLCV DNA is transmitted transovarially to the progeny of viruliferous whiteflies (Ghanim et al., 1998). This was confirmed in an independent study by Polston et al. (2001) who also found that progeny of viruliferous whiteflies indeed contain TYLCV DNA. In another study, Bosco et al. (2004) demonstrated that DNA of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) is transmitted to the whitefly progeny, whereas DNA of TYLCV is not. However, while according to one study (Ghanim et al., 1998), the TYLCV-carrying whitefly progeny were able to transmit the virus to test plants, in other studies (Polston et al., 2001; Bosco et al., 2004), the whitefly progeny, although containing TYLCV DNA, were unable to transmit the virus, supporting the original results obtained in the late 1960s (Cohen & Nitzany, 1966).

2.2. Periodic acquisition

While studying virus–vector interactions, a unique phenomenon, which was termed "periodic acquisition," was observed (Cohen & Harpaz, 1964). It was found that following TYLCV acquisition, viruliferous whiteflies progressively lose infectivity and about 10 days after completion of the acquisition feeding period, most of the insects are no longer able to transmit the virus. However, during that period, the vector is unable to compensate for its steadily decreasing viral-transmission capacity by reacquiring the virus from the infected source plant. That is, another cycle of acquisition feeding, while the vector can still transmit the virus (albeit at a decreasing efficiency), does not restore the transmission capability to its original efficiency. The vector must first completely lose its transmission ability before it can reacquire the virus (Cohen & Harpaz, 1964). A proteinaceous factor which appeared to be related to the phenomenon was found in homogenates of insects, and was termed periodic acquisition-related factor (PARF). This factor, via membrane feeding to nonviruliferous whiteflies, inhibited acquisition, transmission, and retention of TYLCV by the whiteflies (Cohen, 1967, 1969; Marco et al., 1972). Unfortunately, research into the mechanism underlying this phenomenon was never completed. Therefore,

whether this is an active antiviral mechanism or a temporary blockage of the salivary glands by degradation products of the viral capsid protein remains a mystery.

The long latent period of 21 h, the phenomenon of periodic acquisition, and the relatively long and efficient inoculation period of about 4 h suggest that the use of a fast-killing insecticide could effectively control the spread of TYLCV. Indeed, soon after the epidemics broke out, it was demonstrated that spraying with the cyclodiane "Andrin" solved the problem (Cohen et al., 1963). However, the whiteflies soon developed resistance to the insecticide and research shifted to cultural crop management and sanitation.

3. THE USE OF YELLOW MULCH TO PROTECT CROPS

In 1940, while working in the Jordan Valley, a researcher named K. M. Mendel observed that mulching of summer tomato nurseries with sawdust accelerates seedling growth (Avidov, 1944). This growth acceleration was attributed to the finding that the soil temperature under the mulch was cooler by 8–10°C than the temperature of bare soil. However, it was also noticed that the whitefly population on the mulched seedlings was much lower than on nonmulched seedlings (Avidov, 1944). Avidov first thought that the smell of the resin secreted from the sawdust repelled the insects. However, the same controlling effect was achieved by mulching the seedlings with straw and the scent-effect theory was rejected. Avidov also found that during the day, the temperature immediately above the sawdust mulch sometimes reached 47–51°C (temperatures that were later found to be lethal to whiteflies in a dry climate). He therefore concluded that the repelling effect of the sawdust mulch occurs by creating "an atmosphere of death" on its surface which repels the whiteflies (Avidov, 1944).

In an attempt to better understand the effect of straw mulching on whiteflies, the possible effect of whitewashing seedbed soil on whiteflies was also studied (Avidov, 1944). It was found that, 8 days after sprouting, the average number of whitefly eggs per seedling for whitewashed soil was 18.5, compared to 60 whitefly eggs per seedling in nonmulched soil. The same maximum soil surface temperature was recorded for the whitewashed soil (44°C) and the nonmulched control plot (45°C). These findings suggested that soil surface temperature is not the only factor involved in the mulch-based whitefly-controlling mechanism (Avidov, 1944).

3.1. How does it work?

Following Avidov's observations, Nitzany et al. (1964) demonstrated that, indeed, straw mulch can reduce the spread of another whitefly-borne virus, the semipersistent *Cucumber vein yellowing virus* (CVYV). Mulching cucumber seedlings with straw markedly reduced the whitefly population and, as a consequence, delayed CVYV spread for about 10 days. The straw mulch also increased

yield and vegetative development of the cucumber plants (Nitzany et al., 1964). Subsequent to Nitzany's work from 1964, straw mulch was used to control the spread of TYLCV (Cohen et al., 1974). The mulch was very effective in preventing the spread of the virus and the whitefly populations for the first 18 days following germination. However, it was important to extend the duration of the mulch's controlling effect beyond the first 18 days after germination, and the described putative mechanism underlying this effect was therefore reevaluated. In 1962, Mound demonstrated that yellow color attracts whiteflies (Mound, 1962). It was suggested that yellow radiation, which induces vegetative behavior, may be a component of the insect host–selection mechanism (Mound, 1962). This raised the possibility that yellow color also contributes to the controlling effect of the mulch. Thus, using an aphid flight chamber, the effect of straw on whitefly dispersal was studied (Cohen et al., 1974). It was found that nearly three times more whiteflies were attracted to sticky cardboard plates covered with straw compared to those covered with tomato leaves. Moreover, the number of whiteflies attracted to fresh straw was double the number of whiteflies attracted to old straw which had first been exposed to field conditions for 25 days (Cohen et al., 1974). It should be noted that the yellow color of fresh straw is much more intense than that of old straw, the latter fading with exposure to intense solar radiation.

The correlation between the mulch controlling effect and its attractiveness to whiteflies was demonstrated by testing the effects of four different-colored mulches on whiteflies: straw, and three different-colored polyethylene sheets – yellow, silver, and blue (Cohen & Melamed-Madjar, 1978). All four mulches reduced the spread of TYLCV compared to the nonmulched control, with the yellow mulch being the most effective. Moreover, the yellow mulch was the most attractive to whiteflies, in both an aphid flight chamber and the field. In the latter experiments, sticky traps consisting of Petri dishes covered with different-colored polyethylene sheets or with cropped straw were used. The traps were placed on same-colored mulch treatments. Indeed, 77 whiteflies were trapped on the yellow mulch, while only 39 whiteflies (nearly half) were trapped on the silver mulch, 23 whiteflies were trapped on the blue mulch, and 11 whiteflies were trapped on the straw mulch (Cohen & Melamed-Madjar, 1978). Once again, these results clearly demonstrated that the whiteflies were attracted to the yellow color of the mulch.

3.2. Effect of temperature

To study the role of temperature in the controlling ability of the yellow mulch the following experiments were carried out. Four temperature-controlled heating plates (each 10 cm in diameter) were attached to the floor of a flight chamber, 20 cm apart (Cohen, 1982). Yellow-painted Petri dishes covered with glue on the upper side were placed on the heating plates. The temperature of two opposing plates was set to 25°C, and that of the other two to 50°C. In each

experiment, 200 whiteflies were introduced into the flight chamber from the top; the number of insects adhering to the traps was counted 1 h later. After seven repeats, no significant differences were found in the attraction of the whiteflies to yellow traps heated to 50°C (total of 559 whiteflies) or to 25°C (total of 538 whiteflies) (Cohen, 1982). This indicated that high temperature does not repel the whiteflies, as it had been previously suggested (Avidov, 1944).

In another experiment, the combined effect of color and heat was studied. A similar experimental design was used except that, in this case, the yellow traps were not covered with glue, so the attracted whiteflies that landed on the traps could then fly away. The number of dead whiteflies found on each yellow trap was recorded 1 h after their release into the chamber. This time, the results showed significant differences between the treatments; significantly more dead whiteflies were found in the high-temperature plates. Thus, following a total of seven different experiments, no dead whiteflies were found on the plates heated to 25°C, compared with 203 dead whiteflies found on the plates heated to 35°C (Cohen, 1982). These results also contradicted the earlier hypothesis that whiteflies are repelled by high temperature. The controlling effect of yellow mulch therefore appears to be due to a combination of the whitefly attraction to the yellow color of the mulch and its consequent death due to dehydration induced by the high temperature of the mulch. It should be noted that the typical Israeli climate is semiarid – high temperature and low humidity. Moreover, in the tomato-growing regions, soil temperatures exceeding 30°C are quite common. Thus, the use of yellow plastic mulch to protect vegetable crops from whiteflies and whitefly-borne viruses has become common practice in Israeli agriculture (Zaks, 1997).

4. TYLCV EPIDEMIOLOGY

4.1. Wild hosts

In a series of studies aimed at finding ways to control viral spread, a search for the virus inoculum sources in the hot valleys of Israel was performed (Cohen et al., 1988). The surveys were carried out by collecting seeds or cuttings of plants and weeds (mainly the perennials) common to the Jordan Valley region. The samples (seedlings or cuttings) were inoculated with TYLCV to determine which species is susceptible to the virus and which could serve as a potential host. Plants that were found to be susceptible to the virus were tested again for the presence of TYLCV in another set of samples brought from the field. *Cynanchum acutum* was found to be the only natural perennial host of TYLCV. This weed is concentrated along the western bank of the Jordan River (where it covers large areas), a few kilometers east of the main tomato production region at the time. During the winter months (December–February), only the subterranean parts of the plant survive. The plants start growing again in the spring, reaching full vegetation in August–September, concomitant with the

increase in the whitefly population and the tomato-transplanting period. Since this host was concentrated at some distance from the tomato-growing areas, it was important to determine whether whiteflies could cross this distance. Therefore, an area of about 100 m^2, fully covered with *C. acutum* plants and a large population of whiteflies, was dusted with "Fire Orange," a daylight-fluorescent dust, using a mechanical hand duster (Cohen et al., 1988). This dust persisted on the whiteflies for at least 9 days. Whitefly movement was recorded by positioning yellow sticky traps at various distances from the dusted plants, and these traps were monitored weekly for the appearance of fluorescent whiteflies. Indeed, 1 week after the release, fluorescent whiteflies were found in the tomato fields, at a distance of 7 km from the dusting site.

4.2. Viruliferous whiteflies

Most interesting results were obtained when the percentage of viruliferous whiteflies in the general whitefly population was studied during the peak population period (September–November in our case), at which time the infection rate of nonprotected tomato plants reaches 90–100% (Cohen et al., 1988). Whiteflies were collected in the field from different hosts using a cordless rechargeable vacuum cleaner adapted to collect insects into a plastic cylinder (Cohen et al., 1989). The insects were released into a cage with a glass top and were then collected in groups of 20 into small clip cages. The clip cages were placed on the leaves of healthy tomato test plants (one clip cage per plant) and the whiteflies were allowed to feed for 48 h. Following this inoculation access period (IAP), the clip cages were removed, and the test plants were sprayed and monitored for the development of disease symptoms. Only 5.4% of the whitefly population collected on *C. acutum* was viruliferous, compared with 3.2% of the whiteflies collected from a tomato field. One explanation for the relatively low percentage of viruliferous whiteflies within this field population may be the aforementioned periodic acquisition effect.

4.3. Crop-free period

The Arava region of Israel is a 200 km long, 5–10 km wide arid region extending from the Dead Sea to the Red Sea. The climatic conditions during the winter, and moderate temperatures combined with intense solar radiation due to lack of clouds, make this region ideal for growing vegetable crops. The lack of water in the region is overcome by a pipeline from the north and the use of local wells. In 1982–1986, severe viral epidemics occurred in the Arava, threatening the future of vegetable crop cultivation in the region. The major viruses were found to be *Zucchini yellow mosaic virus* (ZYMV) and *Cucumber mosaic virus* (CMV) in cucurbits, *Potato virus Y* (PVY) in pepper, and TYLCV in tomato.

In Israel, TYLCV is widespread mainly in the late summer and autumn, due to the peaking whitefly population during that period (September–November).

The tomato season in the Arava region begins in mid-August. At that time, no infected wild hosts of TYLCV, such as the annual *Malva parviflora* or the perennial *C. acutum*, are found in the region. To determine whether the virus is already present at the beginning of the tomato season in the Arava region, tomato trap plants were distributed in the fields of the Arava and left for a week. Then the plants were collected, sprayed, and kept in an insect-proof greenhouse where the appearance of TYLCV-induced symptoms was monitored. No virus was found in the tomato trap plants dispersed weekly from June to the beginning of the tomato season in August. These results indicated that TYLCV is not endemic to the Arava region, but rather wasa being introduced every year by an influx of whiteflies from the western parts of Israel. Unfortunately, there is no direct evidence for this hypothesis. However, whiteflies have been trapped in mid-August in the northern, desert part of the Arava at a distance of approximately 20 km from the nearest cultivated fields, which may indicate that the whiteflies are dispersed over great distances.

During June and July, local vector populations were found to be relatively low and the natural sources of TYLCV were scarce. Cultivated fields were found to be the major source of whiteflies in this region. Therefore, in order to reduce whitefly-transmitted viral epidemics (such as TYLCV), a vegetable crop-free period for those months was suggested. Indeed, following the implementation of a 2-month crop-free period in 1986, 20 years ago, there has been no TYLCV or any other vegetable virus epidemic in the Arava region (Ucko et al., 1998).

5. BREEDING FOR TYLCV RESISTANCE

Genetic resistance in the host plant is an ideal defense against whitefly-transmitted (as well as other) viruses, since it requires no chemical input and/or plant seclusion and can potentially be stable and long-lasting. Thus, the best way to reduce TYLCV spread is by breeding tomatoes that are resistant or tolerant to the virus. Since all cultivars of tomato (*Solanum lycopersicum*) are extremely susceptible to TYLCV, wild tomato species have been screened for their response to the virus (Lapidot & Friedmann, 2002). The first attempts at breeding for TYLCV-resistant tomato plants were made in the early 1970s using *S. pimpinellifolium* accession LA 121 as the resistant source (Pilowsky & Cohen, 1974). After a few years of repeated tries to introgress the resistance into the domesticated tomato (*S. lycopersicum*), the resistance level of LA 121 was found to be insufficient and efforts were shifted to accessions of *S. peruvianum*, which was found to express a higher level of TYLCV resistance. Indeed, in 1986, the first commercial TYLCV-resistant tomato hybrid TY20 was released (Pilowsky & Cohen, 1990; Pilowsky et al., 1989). The breeding efforts continued, and led to the development of highly TYLCV-resistant lines which do not exhibit symptoms following inoculation with TYLCV (Friedmann et al., 1998; Lapidot et al., 1997). Moreover, it was demonstrated that tomato lines expressing a high level of TYLCV resistance serve as a poor inoculum source for the virus

(Lapidot et al., 2001). Today, due to the continuous breeding efforts of a number of research groups, including the Volcani group, elite commercial TYLCV-resistant tomato hybrids are available (Lapidot & Friedmann, 2002).

6. CONCLUDING REMARKS

TYLCV spread very rapidly from its origin in the Jordan Valley to other parts of Israel and neighboring countries in the eastern Mediterranean, such as Cyprus, Egypt, Jordan, Lebanon, Syria, and Turkey. However, over the last decade, the geographic range of TYLCV has greatly expanded to include the western Mediterranean, Japan, the Caribbean, and the southeastern United States (Polston and Anderson, 1997; Polston et al., 1999; Moriones & Navas-Castillo, 2000). Today, TYLCV is a limiting factor in tomato cultivation worldwide. The reasons for its vast spread and its establishment as a worldwide menace are discussed later in this book.

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CHAPTER 2

AN INSULAR ENVIRONMENT BEFORE AND AFTER TYLCV INTRODUCTION

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1. OVERVIEW

Tomato yellow leaf curl virus (TYLCV, genus *Begomovirus*, family *Geminiviradae*), vectored by the whitefly *Bemisia tabaci*, is one of the tomato infecting viruses which is inducing the most obvious symptoms. The severe growth reduction of the plants and the typical yellowing and curling of the leaves due to TYLCV infection is easily detected by farmers, even not being familiar with those symptoms. Therefore, it is expected that the introduction of TYLCV in a new environment is detected soon after the first infection of tomato plants. This was the case in 1997, when TYLCV was detected for the first time in Reunion, an island of the Indian Ocean at about 700 km east of Madagascar (Peterschmitt et al., 1999). One more reason for which it is thought that the delay between introduction and detection was short is that the local Plant Protection Services were aware of the TYLCV risk.

Subsequently to the first detection of TYLCV, the sampling of infected tomato plants and the collection of *B. tabaci* vectors over time gave us a unique opportunity to monitor the emergence and installation of a virus and its vector in an insular environment.

Firstly, we describe the situation before the arrival of TYLCV in Reunion and in the close environment of the South West Islands of the Indian Ocean (SWIO). Indigenous populations of *B. tabaci* were detected in all the islands whereas indigenous begomoviruses infecting tomato were detected in all of them but not in Reunion and Mauritius. Secondly, we describe the outbreak of TYLCV in 1997 in Reunion and the identification of the so-called cosmopolitan biotype B of *B. tabaci*. Thirdly, we describe the spread of TYLCV to the tomato production area within 2 years, and the evolution of TYLCV

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populations and the distribution of *B. tabaci* populations after 1997. Finally, we discuss the risk of the simultaneous presence in the SWIO of the threatening TYLCV and the polyphagous biotype B.

2. THE SOUTH WEST ISLANDS OF THE INDIAN OCEAN BEFORE THE INTRODUCTION OF TYLCV

Originating from South America, tomato is now produced and consummated in all the tropical and subtropical regions. Interestingly, in most of these regions including the SWIO, tomato plants have revealed the presence of indigenous begomoviruses infecting the introduced tomato. Although indigenous populations of *B. tabaci* were detected in most of these tropical regions including SWIO, some of theses biotypes fed and reproduced on tomato to only a limited extent, minimizing transmission of begomoviruses to and from tomato plants (Polston & Anderson, 1997). In the New World where the indigenous biotype A populations did not readily feed on tomato, most of the tomato infecting begomoviruses were detected on tomato following the introduction of the polyphagous biotype B (Polston & Anderson, 1997; Ribeiro et al., 2003). In SWIO where indigenous populations of *B. tabaci* were identified, indigenous begomoviruses were transmitted to tomato by theses populations in natural conditions (Delatte et al., 2002).

Three species of begomoviruses indigenous of the SWIO were identified from tomato, one from Madagascar, *Tomato leaf curl Madagascar virus* (ToLCMGV), one from Mayotte, *Tomato leaf curl Mayotte virus* (ToLCYTV), and one from Seychelles, *Tomato leaf curl Seychelles virus* (ToLCSCV) (Delatte et al., 2005b: Lett et al., 2004). The symptoms induced on tomato by these viruses are similar to those induced by TYLCV but without yellowing. Sequence analysis revealed that these viruses had genome organizations of monopartite begomoviruses and that ToLCMGV, ToLCYTV, and ToLCSCV belong to the African begomoviruses but represent a distinct monophyletic group that we have tentatively named SWIO (Figure 1). All of the SWIO isolates examined were apparently complex recombinants. None of the sequences within the recombinant regions closely resembled that of any known non-SWIO begomovirus, suggesting an isolation of these virus populations. This is consistent with the geological history of this region where Madagascar and Seychelles, the continental derived islands, drifted away from the Gondwana about 130 million years ago (Figure 2). It is supposed that the progressive decrease of gene flow resulted in the differentiation between the populations of SWIO and those of the continents. Interestingly, no indigenous begomoviruses were detected on tomato in the two most eastern islands of the SWIO, namely Reunion and Mauritius (Mascarenes Islands). This may be explained by the recent volcanic origin of these islands which emerged from the Indian Ocean within the last 10 million years but also by the relatively important distance from Madagascar and the eastern dominant winds which both limited the possibility of viruliferous

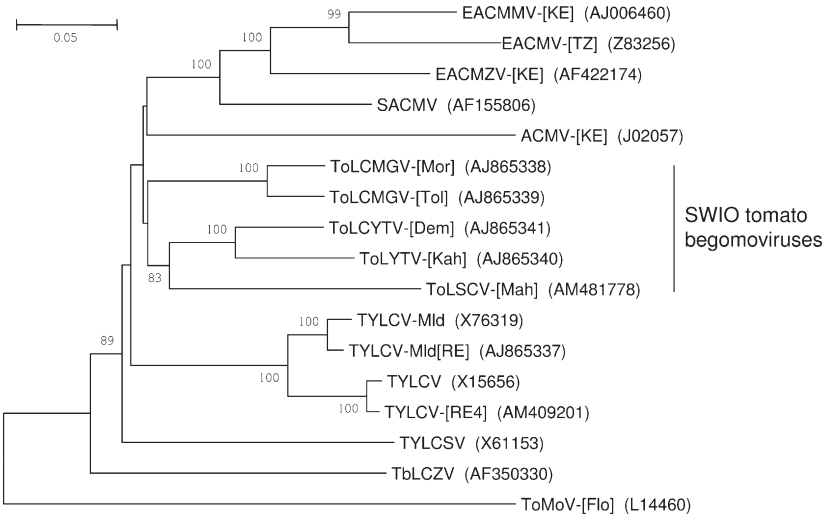

Figure 1. Neighbour joining tree indicating the relationships between the full-length DNA A sequences of tomato begomovirus isolates from the South West islands of the Indian Ocean and those of representative sampling of publicly available African and Mediterranean begomoviruses. The tree was constructed using Jukes–Cantor distances and rooted using ToMoV-[FL] as an outlyer. Numbers associated with the nodes indicate the percentage support for those nodes in 1,000 bootstrap replicates. Horizontal distances represent genetic distances, as indicated by the scale bar, whereas vertical distances are arbitrary. Nucleotide sequence database accession numbers of sequences used in this study: African cassava mosaic virus – [Kenya] (ACMV-[KE]), East African cassava mosaic virus – [Tanzania] (EACMV-[TZ]), East African cassava mosaic Malawi virus – [Kenya] (EACMMV-[KE]), East African cassava mosaic Zanzibar virus – [Kenya] (EACMZV-[KE]), South African cassava mosaic virus (SACMV), Tobacco leaf curl Zimbabwe virus (TbLCZV), Tomato leaf curl Madagascar virus – [Morondava] (ToLCMGV-[Mor]), ToLCMGV-[Toliary] (ToLCMGV-[Tol]), Tomato leaf curl Mayotte virus – [Dembeni] (ToLCYTV-[Dem]), ToLCYTV-[Kahani] (ToLCYTV-[Kah]), Tomato leaf curl Seychelles virus – [Mahé] (ToLCSCV-[Mah]), Tomato yellow leaf curl virus (TYLCV), Tomato yellow leaf curl virus – Mild (TYLCV-Mld), TYLCV– Mild[Reunion] (TYLCV-Mld[RE]), TYLCV-[Reunion4] (TYLCV-[RE4]), Tomato yellow leaf curl Sardinia virus (TYLCSV) and Tomato mottle virus – [Florida] (ToMoV-[FL]).

vectors to reach the Mascarenes. The risk of introduction due to human activities was also limited because of the distance and the relatively recent settings of permanent settlements in these islands, about 400 years ago. On the contrary, although the volcanic islands of Comoros emerged in the same period as the Mascarenes, it is apparently the shorter distance to Madagascar (300 km), the earlier permanent settlements and the dominant winds that have permitted the introduction of SWIO begomoviruses, either naturally through viruliferous vectors and/or through human activities.

Although no SWIO begomoviruses could be detected in the Mascarenes, *B. tabaci* was reported from Reunion on cassava as early as 1938 (Bouriquet,

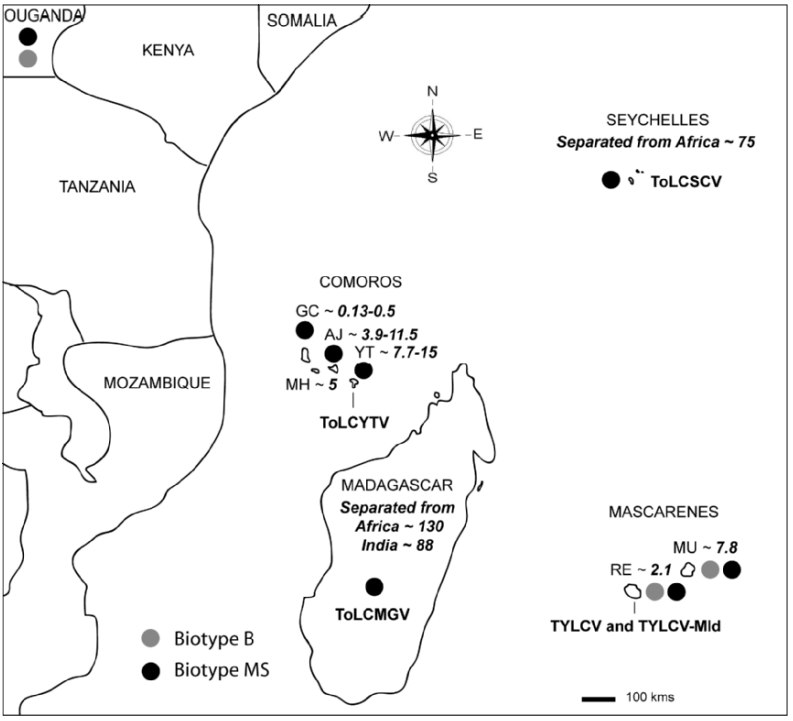

Figure 2. Map of the South West islands of the Indian Ocean showing their geological age indicated in million years (Warren et al. 2003). Besides Madagascar and Seychelles, which are continental-derived islands, the other islands are of volcanic origin: Grande Comore (GC), Mohéli (MH), Anjouan (AJ), Mayotte (YT), Reunion (RE), and Mauritius (MU). Distribution of tomato begomoviruses are indicated: Tomato leaf curl Madagascar virus (ToLCMGV), Tomato leaf curl Mayotte virus (ToLCYTV), Tomato leaf curl Seychelles virus (ToLCSCV), Tomato yellow leaf curl virus (TYLCV), and the mild strain of TYLCV (TYLCV-Mld). Distribution of *Bemisia tabaci* biotypes are also indicated: the indigenous biotype Ms and the exotic biotype B.

1938) and later in 1953 (Luziau, 1953). However there was no further report or detection of *B. tabaci* in Reunion before the outbreak of TYLCV in 1997. The suspicion of the existence of indigenous populations of *B. tabaci* in Reunion and in the SWIO was confirmed using cytochrome oxidase 1 (CO1) sequencing (Figure 3) (Delatte et al., 2005a). The SWIO populations formed a new distinct genetic group that is sister to two other groups, the B and Q biotypes. It was named Ms after the Mascarenes Archipelago. The Ms biotype was thought to be indigenous to the region as it was detected in all the SWIO. Ms populations of *B. tabaci* induced silverleaf symptoms on Cucurbita sp., and were able to acquire and transmit TYLCV. Adult individuals of the Ms biotype were detected on several families of plants, e.g., Convolvulaceae, Euphorbiaceae, Solanaceae, Fabaceae, Verbenaceae, Brassicaceae, Cucurbitacae, suggesting that

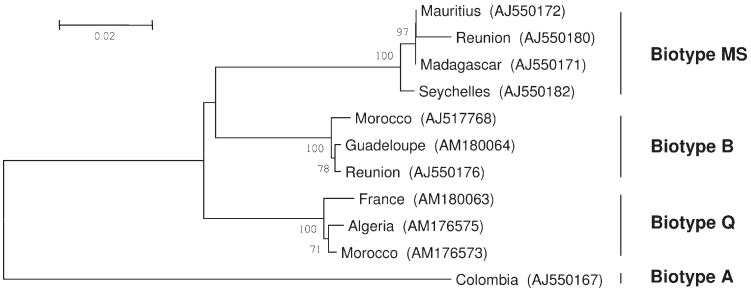

Figure 3. Rooted neighbour-joining tree showing the genetic distance among 816 nt cytochrome oxydase I fragments of *Bemisia tabaci*. Sequences are identified with their geographic origin followed by their Genbank accession number. The scale measures the Jukes–Cantor distance between sequences. Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes.

it is a polyphagous biotype. It has been estimated (on the basis of mitochondrial CO1 markers) that Ms biotype diverged from B and Q about 3 (±0.3) million years ago (Delatte et al., 2005b), much after the time of the continental separation of Madagascar from the African continent (about 130 million years). The expected African origin was confirmed by the detection of a polyphagous populations of *B. tabaci* from Uganda (genotype cluster Ug7) closely related to biotype Ms according to CO1 (98–99% identity) (Sseruwagi et al., 2005). The detection of seven other genotype clusters in Uganda beside the Ug7 populations, whereas only biotype Ms was detected in the SWIO, suggests that the *B. tabaci* populations of SWIO have originated from Africa following a founder effect.

3. THE OUTBREAK OF TYLCV IN REUNION IN 1997 AND THE DETECTION OF THE BIOTYPE B OF *B. TABACI*

In September 1997, typical TYLCV symptoms, namely, stunting, reduced leaf size, leaf curling, and yellow margins, were observed on tomato plants on a farm of the South of Reunion near Saint Pierre (Peterschmitt et al., 1999) (Figure 4). Diseased plants gave positive reactions by TAS-ELISA and an expected size product was obtained by PCR with degenerate primers designed to amplify a region of the A component of begomoviruses. The sequencing of this cloned PCR product and later of the complete cloned genome showed that plants were infected with a member of the Mild strain of TYLCV (TYLCV-Mld) (Delatte et al., 2005b). The alignment of complete genomes showed that the highest nucleotide identity was obtained with members of the TYLCV-Mld strain that were isolated elsewhere shortly before the 1997 outbreak in Reunion: TYLCV-Mld[JR:Shz] (99.1%) isolated after its first detection in 1996 in Japan, TYLCV-Mld[PT] (98.8%) isolated after its first detection in 1995 in Portugal,

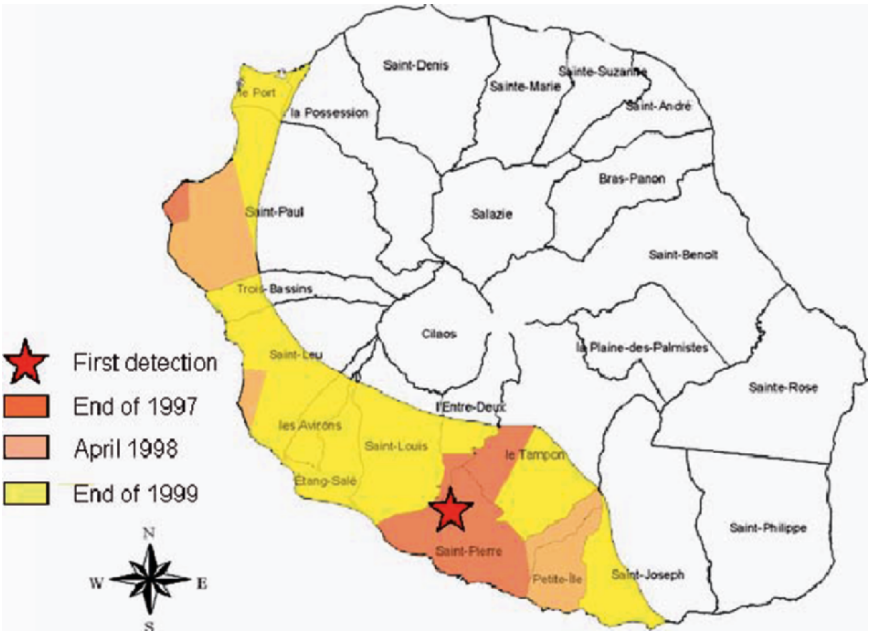

Figure 4. Map of Reunion Island showing the location of the first farm in which TYLCV was detected and its progressive spread to the whole tomato-growing area between 1997 and 1999.

and TYLCV-Mld[ES:72:97] (98.8%) isolated after its first detection in 1997 in Spain. The nucleotide identity was the lowest with TYLCV-Mld[IL] (97.8%), the type member of the strain, isolated before 1994 in Israel. It seems that closely related isolates were simultaneously spread to different regions of the world in the mid-1990s including Reunion. About 3 months after the first detection in September 1997, TYLCV was detected in 13 farms in the region of Saint Pierre and also in a small area near Saint Paul in the West of the island (Figure 4). Severe economic losses were observed, up to 85% in outdoor and/or protected tomato crops. Tomato is grown year round in Reunion and is the most grown vegetable crop. Farmer (Know You Seed), the most popular tomato cultivar grown in Reunion, was found to be highly susceptible to TYLCV.

As stated above, *B. tabaci* were not reported in Reunion between its second report in 1953 and the outbreak of TYLCV in 1997. It was only at the time of the TYLCV outbreak that *B. tabaci* has been observed on tomato crops, but population levels were low compared with those of the whitefly *Trialeurodes vaporariorum* Westwood. During the first 6 months of 1998, *B. tabaci* was also detected in plants occurring near infected crops: *Euphorbia heterophylla* L., *Lantana camara* Mold., *Solanum melongena* L., *S. nigrum* L., *Phaseolus vulgaris* L. *B. tabaci* individuals collected from these plant species and sequenced

in their CO1 gene were found to belong to two distinct biotypes. Some of them were of the indigenous biotype Ms but others clustered with individuals of the biotype B (Figure 3). The B biotype individuals of Reunion are not thought to be indigenous because, beside Mauritius where biotype B was detected in 1998 (Ganeshan & Abeeluck, 2000), biotype B individuals were not detected in the SWIO. We suppose that these biotype B individuals were recently introduced, maybe from Mediterranean countries together with the Mediterranean TYLCV-Mld.

4. THE EVOLUTION AFTER 1997

Although the number of farms in which infected tomato plants were detected outdoors and indoors had increased from 13 by the end of 1997, to 29 in April 1998 (Figure 4), TYLCV had apparently not spread to the whole tomato growing area (mainly on the Western leeward coast; the inner mountain areas and the Eastern coast are not convenient for tomato production). By the end of 1998 to the beginning of 1999, a survey showed that almost the whole tomato-growing area was infected with TYLCV, from Le Port in the North to Saint Joseph in the South and towards the inner island up to 900 m altitude (Figure 4). It was only in 2003 that TYLCV symptoms were observed in the eastern part of Reunion, near the Southeastern town of Saint Rose.

Prior to 1997, begomovirus-induced symptoms were never reported in Reunion. As the first tomato samples infected with TYLCV were most probably collected shortly after its introduction (see above), a unique opportunity was provided to analyse the evolution of TYLCV population almost from the initial inoculum in an isolated agroecosystem, apparently free of any other tomato-infecting begomovirus. A total of 111 samples were obtained from surveys conducted from 1997 to 2004 in the main tomato growing areas in the western part of Reunion. Genetic variation of TYLCV-Mld[RE] was monitored (Delatte et al., 2007). The very low diversity of the isolates observed in 1997 did not provide any evidence of multiple TYLCV introductions in Reunion. In addition, no other *Begomovirus* species or strains were detected during the studied period. The very low initial diversity was followed by a quasi-linear increase in genetic diversity across years. Analysis of population effective size indicated that TYLCV-Mld[RE] in Reunion was in expansion which is consistent with a founder effect due to the introduction of a small virus population in an insular environment. Surprisingly, one nucleotide substitution introducing a premature stop codon in the C4 ORF was observed in an increasing number of isolates in the population of TYLCV-Mld[RE] over time, contrasting with the other substitutions which were observed at lower frequencies. This substitution which shortens the C4 protein by four amino acids may have been selected during TYLCV-Mld[RE] evolution.

The 8-year sampling for the evolution studies was stopped in April 2004 when an isolate of the so-called recombinant TYLCV strain was detected in Saint

Gilles in the northwest region of Reunion (Delatte et al., 2005a, b). This new strain caused more severe yellow leaf curl symptoms than those usually observed with the Mild strain. Intraspecific competition between the two strains is under investigation. This new introduction illustrates how difficult it is to protect an environment from begomovirus infection even in an isolated island. We have recently shown that not only plants and whitefly vectors can be a mean of introduction but also the tomato fruit itself (Delatte et al., 2003).

Evolution studies of the vector populations showed evidence of introgression of the indigenous Ms population into the introduced B population. A multiple sampling survey conducted on the *B. tabaci* biotypes during 2 years (2001–2002) with microsatellite markers in Reunion revealed that biotype B was predominant on the island, with however proportions of the two biotypes varying according to geographic or ecological factors (Figure 5) (Delatte et al., 2006). The B biotype was found predominantly in the north, west, and south part on crops, corresponding to the tomato growing area and leeward dry coast. While, the biotype Ms predominated on weeds in the windward and humid coast, B and Ms biotypes coexist in sympatry throughout most of their geographical ranges. Interestingly, the genetic study revealed a third group of whiteflies genotype, intermediate between B and Ms biotypes (Figure 6). This

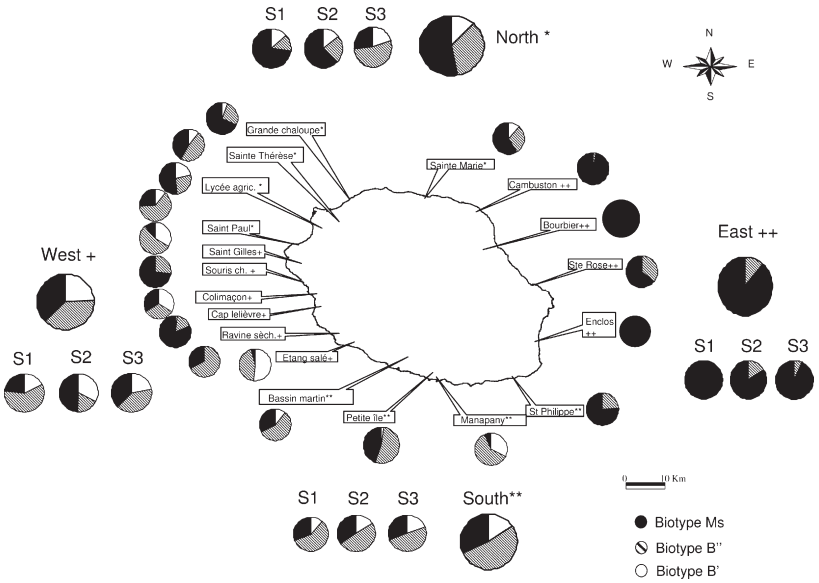

Figure 5. Map of Reunion Island with whitefly biotype B and Ms global repartition in absolute numbers per sector and sampling period (S1, February–March 2001; S2: September–October 2001; S3: February–March 2002). The different sites are represented individually with years grouped, for biotype Ms, groups B′ (pure biotype B) and B″ supposed to be a B form introgressed with Ms alleles (see Figure 6). The sampled sites are represented, and the symbols refer to the sectors they belong to.

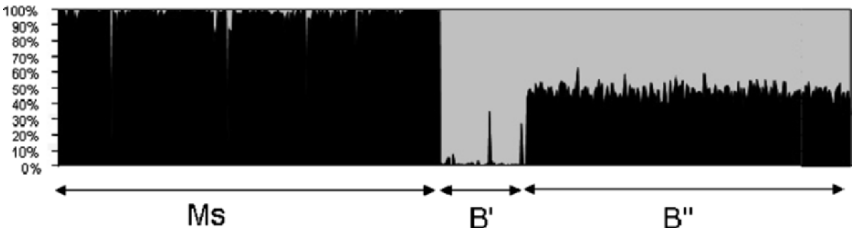

Figure 6. Genetic structure of *Bemisia tabaci* sampled over Reunion Island. Summary plot of estimates of Q (the estimated membership coefficient for each individual in each cluster) given by the software Structure v2.1 with the admixture option (Pritchard et al. 2000). Each of the 567 individuals is represented by a single vertical line broken into K populations (K = 2 in this case), with length proportional to the inferred proportion of B ancestry; individuals from the two subgroups B′ and B″ have been represented in different sections of the graph to emphasize their genetic differences.

group had asymmetrical and locus-specific introgressions between both B and Ms biotypes, especially detected within syntopic populations. This group was therefore proposed as being a hybrid group between B and Ms populations. However, there is no clinal geographical structure typical of classical hybrid zones. The biotypes situation on Reunion appears as a novel strategy of invasion, which does not refer to displacement of a population, to competition by interference for food, or to a complete eradication of one biotype, but rather to the introgression of one population into another. This might lead to the complete disappearance of the parental biotypes and the appearance of a fitter hybrid group of whitefly, or the coexistence of the three groups. More evolutionary time is needed to confirm the extent of the different populations, and know the long-term outcome of introgression in the field.

5. RISK ASSESSMENT FOR THE SOUTH WEST ISLANDS OF THE INDIAN OCEAN

The introduction of exotic begomoviruses into Reunion and exotic *B. tabaci* populations into Reunion and Mauritius is generating new risks for the SWIO that need to be assessed (Figure 2). On the vector side, there is a risk of spread of biotype B populations to the other SWIO where indigenous begomoviruses are infecting tomato. As biotype B was found to be dominant on vegetable crops compared to biotypes Ms (Delatte et al., 2006), the introduction of biotype B in these islands may increase the transmission of these viruses to and within tomato with an increased impact on tomato production. Introduction of biotype B may even induce emergence of so far weed infecting begomoviruses in cultivated crops. On the virus side, there is a risk of overlapping between the distribution areas of the indigenous begomoviruses and the exotic TYLCV either by the introduction of the indigenous begomoviruses into Reunion or the introduction of TYLCV into the islands infested with the indigenous begomoviruses.

Knowing the propensity of begomoviruses to recombine (Fauquet et al., 2005), emergence of new recombinant begomoviruses, possibly with increased virulence and modified host range, is expected. The natural recombinant detected between TYLCV and TYLCSV in Spain (Monci et al., 2002) demonstrated that the probability of such an occurrence is high, especially as the genetic distance between TYLCV and the SWIO indigenous ToLCVs is similar to the distance between TYLCV and TYLCSV (Figure 1).

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CHAPTER 3

THE *BEMISIA TABACI* COMPLEX: GENETIC AND PHENOTYPIC VARIATION AND RELEVANCE TO TYLCV–VECTOR INTERACTIONS

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1. OVERVIEW

The purpose of this review is to present an overview of "the biotype concept" in relation to the whitefly *Bemisia tabaci* (Gennadius) vector of *Tomato yellow leaf curl virus* (TYLCV), the plant virus, which is the topic of this volume. It seems an unlikely coincidence that this single species of whitefly, itself widely variable and plastic, is the arthropod vector of a widespread, dynamic suite of closely related viruses species that also diversify rapidly and adapt to human activities. This chapter will contextualize current scientific knowledge, and raise questions where understanding is lacking – or not yet congealed to reach a satisfactory conclusion. This will involve delineating the characteristics, processes, and concepts that unite or set apart the *B. tabaci* complex from other whitefly species, and other vector–virus complexes. Also discussed will be the characteristics that uniquely delimit variants or "biological types" of *B. tabaci* – recognizable both in terms of biological and/or genetic variability, which yield distinct consequences in agroecosystems – that would not prevail if such variability were absent or irrelevant. The review will also address how knowledge of different and shared characters among biotypes and less well-studied haplotypes (phenotypic variants), could assist in predicting whether a variant could become an invasive, or successful vector. And, how greater than expected genetic variation, together with phenotypic plasticity, influence virus–vector competency, virus dispersion, and virus host adaptation or host range shifts, and support diversification or emergence of begomoviral species. The unprecedented invasiveness of this insect pest and plant virus vector has contributed widely to the intrigue that has fostered the recent interest in this ancient whitefly species. As well, so do its fascinating biology, unresolved taxonomy, unprecedented (apparent)

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interspecific variability, and extent of reproductive isolation. This review will present a historical perspective of the biotype concept, and describe the attributes of the *B. tabaci* complex relevant to its role as a vector and pest in agriculture. It also will provide examples of the best-studied biological types of *B. tabaci* and their significance to begomovirus disease outbreaks. A generalized sequence of events outlining the "history of the biotype concept" and the contributions of many to its legacy is provided in Table 1. It is particularly important to credit our many colleagues whom over the years have generously contributed whitefly and virus collections for molecular analysis. Without them, much of the work described here would not have been possible. It is regrettable that space limitations do not allow inclusion of a comprehensive chronology citing all that have made important contributions to this new field of study. Even so, every effort has been made to highlight key events and the contributions of as many as possible. This chapter is dedicated to Dr. Julio Bird, *Emeritus*, University of Puerto Rico, a priceless mentor and friend who continues mostly unknowingly through his insights and keen observations, to inspire "students of *B. tabaci*" around the world.

Table 1. Chronological history of the "biotype concept"

1889	P. Gennadius described *B. tabaci* (*Aleyrodes tabaci*)
1914	Quaintance and Baker established *Bemisia* as a genus (*inconspicua*)
1936	H. H. Storey reports outbreaks of virus-like disease in cassava in Africa; Takahashi synonomized *B. hibisci* with *B. tabaci*
1957–1977	In Puerto Rico J. Bird provides the first evidence for polyphagous (Sida race) and monophagous (Jatropha race) *B. tabaci*
1957	L. Russell synonymized nine additional species/two genera into the *B. tabaci* taxon (following the decisions to lump instead of split the species by two systematists before her)
1975	Costa and Russell (1975) reported that *B. tabaci* did not colonize cassava where it was native in Brazil, but noted that it readily colonized cassava plant in Africa
1978	Mound and Halsey further synonymized the species (total 23 species, 2 genera)
1980	Outbreak of the A biotype in the southwestern US deserts and NW Mexico; previously undescribed begomoviruses and criniviruses (Brown, 1990, 1994)
1980–1982	Geminiviruses are recognized as a new group of plant viruses containing ssDNA (Goodman, 1981; Hamilton et al., 1982)
1980–1981	First "suspect B" biotype documented, Hawaii (R. Gill, personal communication/Bernar Kumashiro, Bishop Museum, Honolulu)
1985–1990	Ornamentals in continental USA and Europe colonized by *B. tabaci* instead of *T. vaporariorum,* "the norm" (Alderman, 1987; Lindquist and Tayama, 1987)
1986–1987	Silverleaf and irregular ripening observed in Florida for first time (Schuster et al., 1990, 1991; Yokomi et al., 1990). Invasive B biotype not yet recognized

(continued)

Table 1. (continued)

1987–1988	Poinsettias infested with *B. tabaci* found in Arizona for the first time (W. Miller, D. Bryne-UAZ); Miller provides colony to Brown laboratory hypothesizing the "lab vector colony" was contaminating his greenhouse poinsettia plants (Costa and Brown, 1990)
1988–1989	Costa and Brown carry out experiments to differentiate the poinsettia *B. tabaci* from AZ native
1988–1989	Unprecedented outbreaks occur in vegetables and cotton in the Dominican Republic, Texas, and Guatemala (Brown, 1988, unpublished); B biotype sample collected in Cancun hotel (report by Costa et al., 1993)
1990	Immature [and adult] forms of the whitefly are shown to cause the silverleaf disorder by Yokomi et al. 1990; [Costa and Brown, 1990]
1990	Costa and Brown (1990) present poster and abstract identifying polymorphisms between the poinsettia and lab colonies (The Entomological Society of America) and providing preliminary evidence of viable female offspring from A × B crosses and B biotype associated squash silvering
1990	Field populations in Arizona are 70% B biotype and 30% A biotype (Costa, Brown, Butler, unpublished). Resistance to several chemistries is reported for the B biotype 1990–1995 (numerous authors)
1990	Severe cassava mosaic virus epidemic begins in Uganda (reviewed in Legg and Fauquet, 2004), associated with severe disease and unprecedented whitefly outbreaks (numerous reports). Samples tested in AZ laboratory indicate the B biotype is not responsible (Brown J. K., unpublished data)
1990	Dominican Republic imports tomato seedlings infected with TYLCV (Bird & Brown, unpublished USAID report); B biotype identified using esterases; noted colonizing cassava (Brown, 1990, unpublished)
1990–1991	Unprecedented outbreaks occur in vegetables and cotton in the USA desert southwest (Brown, 1990; Bird and Brown, 1992); USDA initiates Five Year Plan and annual workshops that are attended by international scientists
1991	First description of the A and B type esterase polymorphisms, host range and life history differences; first association of squash silverleaf symptoms with colonization by the B biotype; suggested that SSL was a phytotoxic disorder, not caused by a transmissible agent (Costa and Brown, 1991). Assignment of "A" and "B" biotype designations
1992–1994	Host associated biotypes described in cassava and okra in Ivory Coast (Burban et al., 1992), suggesting *B. tabaci* associated with cassava were host-restricted. Legg et al. (1994) demonstrated cassava-restricted and polyphagous (sweet potato host) *B. tabaci* in Uganda
1991–1994	Esterase morphotypes reveal extraordinary variability in general esterase patterns in worldwide populations. Assemble suite of colonies at John Innes Centre for study. Alphabetical designations assigned to morphotypes.Erroneously designates polymorphic *B. tabaci* as biotypes. Many remained uncharacterized (Brown et al., 1995; Costa et al., 1993a; Bedford et al. 1994)
1993	Costa et al. (1993) report the rapid spread of the B biotype into the American Tropics using esterase morphotypes and SSL as indicators; insecticide resistance also associated with the B biotype while A is controllable. Central American teams hold workshop in Honduras to develop Action Plan modeled after US plan

(continued)

Table 1. (continued)

1993–1995	Perring et al. (1993) and Bellows et al. (1994) propose the name *B. argentifolii* to replace the B biotype designation and erect a separate species. Ultimately, the name is not widely accepted as evidence is provided demonstrating widespread polymorphisms with the species. It is proposed that *B. tabaci* is better described as a complex or a group of sibling species (Brown et al., 1995)
1993	*Tomato yellow leaf curl virus* introduced into the USA (Polston et al., 1994) and elsewhere in the Caribbean
1994	The B biotype reported in Australia (Gunning et al., 1995)
1994–1997	Report from Bedford et al (1994) via collaboration with Brown laboratory characterized in biological and biochemical terms representative *B. tabaci*; populations reared at John Innes Centre. Rosell and Bedford carry out morphological study on populations (Rosell et al., 1997). Bedford reports a monophagous *B. tabaci* from *Aystasia*. in Benin (E esterase type)
1994–1996	In Puerto Rico, the B biotype displaced the Jatropha and Sida biotypes (Bird and Brown, unpublished data)
1994–present	Neonicotinoids widely used to control the B biotype successfully (Dennehy et al., 1996; Horowitz et al., 1998, 2005)
1996	Arizona, USA laboratory evaluates mt16S sequence as a molecular marker. Results point to an Eastern Hemisphere origin for the B biotype [Middle East/Africa], providing assistance to natural enemy (Brown et al., 1995; Frohlich et al., 1994; Kirk et al., 2000)
1996	The Q biotype recognized as important, native *B. tabaci* vector and pest in Mediterranean Basin (Guirao et al., 1997).
1996–2000	Severe cassava mosaic disease is caused by a recombinant (Zhou et al., 1997) and transmitted by an invasive *B. tabaci*, likely from western Africa (Legg et al., 2002)
1998	TYLCV introduced into the east coast states and Yucatan Peninsula of Mexico (Ascencio-Ibáñez et al., 1999)
1999	*Tomato yellow leaf curl Sardinia virus* displaced by TYLCV (Sanchez-Campos et al., 1999)
2000	TYLCV introduced into Puerto Rico on seedlings from Florida and TYLCV was introduced (Bird et al., 2001). Soon TYLCV will be introduced into a number of countries in Asia
1997–2003	Development and validation of the mtCOI as an informative marker and systematically assess representative collections, worldwide (Brown et al., 1995; Frohlich et al., 1999). Numerous labs present comparative results. Recognition of divergent phylogeographic groups and haplotypes. The B biotype will continue to be reported in new locations, worldwide
2000	The B biotype is widespread in South America, including Argentina, Brazil, Paraguay (Brown, 2000, personal observation). Studies lead to the report by Viscarret et al. (2003) of a native *B. tabaci* (ARG) that is somewhat divergent from the A biotype (South American group), and sympatric with the B biotype
2001–2002	The local Spanish "Q" biotype displaces the introduced B biotype in Spain (Early rumors of B biotype resistance to neonicotinoids in Spain and Morocco (Moya et al., 2001)

(continued)

Table 1. (continued)

2002–2005	A native biotype that is a close relative to the Spanish Q biotype is identified in Israeli cotton fields. First evidence for development of resistance to neonicotinoids under laboratory selection in Israel (Horowitz et al., 2003, 2005; Nauen et al., 2002)
2003	The monophagous T biotype is reported from *Euphorbia characias* in Italy (Simon et al., 2003b)
2003–2005	Sserwagi et al., discover that a non-B like *B. tabaci* from Uganda can induce foliar silvering (2005) and that not all *B. tabaci* that colonize cassava are host-restricted (2006)
2004–2005	TYLCV introduced into Mexico's Pacific Coast where tomatoes are grown for fresh market export (Brown & Idris, 2006)
2005–2006	Introduction of the Q biotype in the USA; introductions reported in China, Japan, and Mexico (Brown et al., 2005; Chudong et al., 2006; Dennehy et al., 2005; Martinez & Brown, 2007; Ueda & Brown, 2006; Zhang et al., 2005). TYLCV-mild now widespread in Asia
2005–2006	Severe cassava mosaic virus epidemic continues to spread westward and southward in Africa. Brown, French, and Legg (2002) demonstrate involvement of an invasive biotype and possible hybridization between the "Invader" and "Local" *B. tabaci*
2006	The Spanish "S" biotype is reported more widely distributed than first thought. In addition to Spain, the S biotype occurs in Africa (Rua et al., 2006)
2005–2006	In the USA the Spanish Q biotype and closely related variants introduced on ornamental plants has not been reported in field crops and appears to be restricted to greenhouse grown plants; Q Biotype Task Force Website reporting US distribution of the Q biotype (http://www.mrec.ifas.ufl.edu/LSO/bemisia/bemisia.htm)
2006	TYLCV introduced into Arizona and Texas on tomato transplants (Idris et al., 2007 (in press); Isakeit et al., 2006)

2. BACKGROUND

2.1. *Bemisia tabaci* (*Gennadius*) is an emergent whitefly pest and vector

Whiteflies are classified in the family Aleyrodidae (Sternorrhyncha: Hemiptera/s.o. Homoptera) (Campbell et al., 1994, 1995; Gill, 1990; Martin, 2003; Mound & Halsey, 1978). Whiteflies are unique among insects in that they employ a "modified" paurometabolus metamorphosis, and they are haplo-diploid insects reproducing by arrhenotokyous parthenogenesis (Blackman & Cahill, 1998; Byrne & Bellows, 1991). The closest whitefly relatives are aphids, mealybugs, psyllids, and scales, all of which have piercing and sucking mouthparts specialized for feeding in plant phloem (Byrne & Bellows, 1991; Gill, 1990). This suborder is well known for harboring obligate, primary endosymbionts that coevolve with their host (Campbell, 1993; Thao et al., 2004), and synthesize amino acids that are in short supply in phloem sap (Buchner, 1965).

Among whiteflies, *B. tabaci* represents an unusual example of extreme polyphagy in that as a species, it colonizes several hundred herbaceous eudicots. This is in contrast to most other whitefly species, which colonize flowering woody perennials. *B. tabaci* is primarily adapted to the subtropics/tropics and is competent across a range of ecological zones and in climates that span arid deserts, dry-subtropical, and Mediterranean conditions. The taxonomy of *B. tabaci* has long confounded systematists leaving the status unsatisfactorily unresolved (Gill, 1990).

The unusual biotic features of this whitefly contribute to its apparent ease in adapting to changing environmental conditions, and also to its growing economic importance as a pest and vector of plant viruses in agriculture (Byrne & Bellows, 1991; Gerling, 1990). *B. tabaci* is a pest and virus vector on all continents where agriculture is practiced, where it colonizes agronomic, fruit, and vegetables crops. Also *B. tabaci* has adapted to protected environment production facilities, in which ornamentals and vegetables are produced in temperate and more recently, in subtropical climates. Certain *B. tabaci* biotypes (characterized in biotic terms) or haplotypes (uncharacterized, genetically distinct variants) are no longer restricted to their native habitat, having extended their geographic and host range beyond once endemic boundaries. This has occurred only recently through introductions of *B. tabaci* transported on plants by international trade.

Increased monoculture cropping and year-round production practices, together with cultivation of genetically uniform crop varieties, are among the most important factors that have contributed to recent whitefly outbreaks and subsequent virus epidemics. Year-round production in tropical climates has eliminated or shortened host-free periods, facilitating unprecedented population increases and the adaptation of this whitefly to monoculture cropping practices. Consequently, once a colonizer of native, uncultivated eudicots in marginal habitats or the dry-subtropical understory species, the *B. tabaci* complex has become an "invasive species." As well, *B. tabaci* is the most important arthropod vector of several groups of emerging plant viruses that cause damaging diseases primarily in vegetable and fiber crops. The most prevalent and widespread is the genus, *Begomovirus* (family, *Geminiviridae*), to which all TYLCV strains and species are assigned.

2.2. Whitefly-transmitted geminiviruses

Begomoviruses (genus, *Begomovirus*; family *Geminiviridae*) comprise a group of circular, single-stranded DNA plant viruses (Lazarowitz, 1992). They are unusual among plant viruses because most have RNA genomes. Although several hundred species are now recognized, when the group was established fewer than 20 viruses had been assigned a name, and for most etiology had not been demonstrated. For perspective, it is important to realize that the *Geminiviridae* was established only in 1978 (Goodman, 1981; Rybicki, 1994). Only during the

last 30 years begomoviruses have become widely considered as emergent viral pathogens in food, fiber, and ornamental crops in much of the world where food is produced. Begomoviruses are pathogenic to field crops in subtropical/tropical world regions, and are also problematic in controlled environment production systems in most temperate regions (Brown, 1990, 1994; Brown & Bird, 1992). Several, including TYLCV (Polston et al., 1999) and *Squash leaf curl virus* (Idris et al., 2006) have been introduced to nonendemic areas, which at least in one case, has resulted in displacement of an endemic begomovirus species (Sanchez-Campos et al., 1999).

Whitefly-transmitted viruses (prior to the discovery and classification of the genus *Begomovirus*) were referred to as "rugaceous" viruses (Bird & Maramorosch, 1978). They are recognized in nature by the characteristic leaf curling, mosaic, and bright yellow or yellow-green symptoms they cause in endemic hosts and in cultivated plant species. It was not until the 1970s when the first electron micrographs were produced, was their novel "geminate" particle morphology revealed (Goodman, 1981). The subsequent discovery that that begomoviruses contained a ssDNA genome (Goodman, 1977) fueled the interest of many, and the development of recombinant DNA technologies soon facilitated cloning of the first begomoviral genomes. By 1981 the DNA sequence had been determined for only several of these viruses, all of which contained a bipartite genome (Haber et al., 1981; Hamilton et al., 1982), which came to be referred to as DNA A and DNA B. The first monopartite begomoviral genome was discovered in what is now the type species of TYLCV from Israel (Navot et al., 1991), and the topic of this book. Since then, many more monopartite begomoviral species have been described. The unexpected upsurge of *B. tabaci* in cropping systems, beginning in 1976, has had such a profound effect on the emergence and diversification of new begomovirus pathogens in agricultural systems worldwide, it would not have been possible to predict that by 2006 a complete genome sequence would be determined for several hundred viral species, with more than 350 GenBank records of prospective or confirmed species (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.029.htm).

3. TRANSMISSION SPECIFICITY AND COADAPTATION OF *B. TABACI*–BEGOMOVIRUS COMPLEXES

3.1. Virus–vector specificity and transmission competency

Transmission specificity is a highly conserved feature of begomovirus–*B. tabaci* vector complexes. This is borne out by the knowledge that all members of the genus, *Begomovirus*, have a single whitefly species, *B. tabaci*, as their arthropod vector. Virus–vector specificity can be corroborated biochemically based on evidence residing in the viral coat protein, the only viral-encoded protein required for whitefly-mediated transmission (Briddon et al., 1990). The coat

protein maintains a high degree of conservation at the amino sequence level (Harrison & Robinson, 1999). Such conservation is imposed by a number of important multifunctional constraints, including particle assembly (encapsidation) and particle stability. It is also involved directly in interactions with the host plant where it interacts with other viral proteins that facilitate cell-to-cell movement, and possibly systemic infection. Finally, the coat protein in its role as a transmission determinant interacts with as yet unidentified whitefly proteins at the gut and salivary gland membranes, (and with at least one protein that is encoded by the primary endosymbiont), to facilitate vector-mediated transmission. Another notable multi-trophic feature of *B. tabaci* is that it harbors an obligate endosymbiont, *Candidatus* Portiera aleyrodidarum, with which it has a mutualistic relationship (Costa et al., 1993b, 1995; Thao & Baumann, 2004; Zchori-Fein & Brown, 2002). In addition to synthesizing certain amino acids used by the whitefly host, the primary endosymbiont encodes a 60S heat shock protein (HSP60) that interacts molecularly with the begomovirus nucleocapsid as virions circulate in the vector haemolymph (Morin et al., 1999, 2000) toward the salivary glands. This HSP60 is thought to bind to virus particles and promote particle stability. It also seems likely that this prokaryotic chaperone affords further protection by masking the virion surface, thereby limiting or delaying the innate immune responses of the whitefly (Brown & Czosnek, 2002).

Compelling evidence for cellular-based specificity has been provided at the level of "transmission efficiency," which has been demonstrated for certain begomovirus–vector combinations originating from the same geographical locale, and/or in some cases through a long-term interaction with a limited suite of hosts, and so probably it is not surprising that they have coevolved to some extent. How this is manifest at the molecular or cellular levels, is not entirely known. Recall that three particular coat protein amino acid residues located at conserved positions in the capsid monomer are involved in *B. tabaci*-mediated begomovirus transmission, and that the integrity of the amino and carboxyl termini are essential for the assembly of stable virions (Briddon et al., 1990; see refs in Brown & Czosnek, 2002; Hallan & Gafni, 2001; Kirth & Savithri, 2003; Noris et al., 1998) collective.

These collective observations are supported by a positive correlation between the genetic structures of vector genotypes and the viral coat protein at local, regional, and global scales (Brown & Idris, 2005; Simon et al., 2003a). One study has examined virus–vector complexes in the Near East/Asia, while another provided a reconstructed, global phylogeny for representative haplotypes/biotypes – both analyses employed the *B. tabaci* mtCOI (unrelated to transmission) and the viral coat protein gene (directly involved in transmission) to assess the congruence of phylogeographic relationships. In both, the whitefly mtCOI and viral CP were phylogenetically concordant, suggesting coevolutionary implications. As such, it may be possible to postulate a role for the whitefly vector in viral coat protein evolution. The whitefly vector exhibits a range of coadapted phenotypes that could contribute directly or indirectly to virus transmission efficiency and disease spread (Brown & Czosnek, 2002; Brown & Idris, 2005; Czosnek et al., 2001; Simon et al., 2003a).

Having said that begomovirus–vector specificity is conserved among all members of the *B. tabaci* complex, it is likely that subtle selection influences or modulates the transmission processes at coevolving virus–vector interfaces that could vary among different biotypes (variants). Such differences could give rise to variation in transmission competency, which has been documented for different virus–vector combinations (Idris et al., 2001; McGrath & Harrison, 1995). Begomoviruses exhibit varying degrees of transmission competency through molecular interactions with their respective endemic vector. The capsid protein is thought to interact with (putative) virus receptors, and possibly with other vector-encoded proteins during hemolymph-mediated transport and entry into the salivary glands, and possibly when exiting in the saliva. However, such specific attributes that might confer differing degrees of competency have not been identified (see refs in Brown & Czosnek, 2002). Once ingested, "transmission competency determinants" would seem possibly to reside at the level of the midgut-filter chamber membrane barriers, and/or, after virions have crossed the gut barrier to the hemolymph, a successful interaction with the salivary gland receptors is essential. Evidence from TYLCV serial transmission studies (Q and B biotypes) suggested that a large virus load may accumulate more rapidly in the whitefly gut before virus crosses the gut membrane barrier, compared to rate of translocation from the haemolymph into the salivary glands, revealing a pattern also reported for the New World *Squash leaf curl virus* in the A biotype vector (Caciagli et al., 1995; Cohen et al., 1989; see refs in Czosnek et al., 2001; Rosell et al., 1999). This may be due to slow or variable rate of release of the virions from the gut to the hemolymph, to the differential binding of virions by HSP60 molecules, and/or to variable interactions (affinity, avidity) with salivary gland receptors, all which could yield a phenotype of differential rate of salivary gland entry (based on transmission phenotype). Though such dynamics can be postulated at the cellular and molecular level, they are poorly understood. Likewise, it is not known whether virus–vector interactions are advantageous, detrimental, or neutral to *B. tabaci* fitness (Czosnek et al., 2001). The ability to perturb or neutralize whitefly-mediated begomovirus transmission as a viable disease control strategy rests upon an understanding of these fundamental cellular and molecular interactions.

3.2. Coadaptation in Begomovirus–vector–host complexes

Genetic evidence has demonstrated DNA sequence-level variability and the widespread employment of intermolecular recombination (Bisaro, 1994; Padidam et al., 1999) in begomovirus populations. Such virus-related factors can result in differences in rates of viral replication/accumulation and movement, in virus and vector (feeding) phloem tissue tropisms, altered host range, increased pathogenicity, susceptibility in cultivars of the same species (Polston et al., 2006), and virus survival, emergence, or displacement under different vector biotype and host plant pressures. Other resultant factors important to virus

prevalence or displacement owing particularly to vector-mediated transmission (in the context of virus–host interactions), are differential accumulation of virus in host tissues, depending on the stage of plant at time of infection, or differences in tolerance or susceptibility of the host, from which virus is ingested. Vector-related genetic factors that drive coadaptation include vector host range, fecundity, developmental rate, dispersal characteristics, natural enemy attack, degree of polyphagy, insecticide resistance, and probably endosymbiont composition, all which can vary subtly or dramatically, depending on the particular biotype. Such indirect factors may influence the dispersal/transmission of viruses that have little bearing on subtle differences in competency.

B. tabaci exhibits variation in host range and host preference, both, which may directly influence the subsequent exposure (or not) of begomoviruses to new hosts. In some instances begomoviruses are host-restricted, as is the cases of cassava-infecting viruses (Brown et al., 2004a, b; Legg & Fauquet, 2004; Legg et al., 2002), *Bean golden yellow mosaic virus* (BGYMV) from the Caribbean, and *Jatropha mosaic virus* (JMV) in Puerto Rico (Bird, 1957; Brown, 2001). For JMV however, the host range of the vector was found to limit the natural spread of the virus (Brown & Bird, 1996), whereas, for BGYMV the virus host range is limited, while for cassava-infecting viruses in Africa, both vector and virus may be limited by host range.

For other begomoviruses, such as the numerous TYLCV strains and species, encountering new prospective hosts through the intervention of polyphagous vector variants has apparently contributed to a number of viral species having a broad host range. Among those polyphagous biotypes recognized so far are the New World A and Sida biotypes, the Old World B and Q (and relatives) biotypes, and haplotypes that are endemic to Asia (China, India, and Japan). A notable example involving a single difference in an already-limited natural virus host range has been illustrated in Spain when TYLCV introduced from Israel displaced the endemic *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in tomato (Sanchez-Campos et al., 1999). Because TYLCV infects bean while TYLCSV does not, when bean crops prevailed in the interim between tomato crops, TYLCV overseasoned in the bean crop, making it possible for TYLCV to survive and rapidly displace the endemic TYLCSV.

Collectively, the *B. tabaci* species complex embodies a plethora of interacting phenotypic, biochemical, molecular, and cellular attributes that contribute to the ability of this unique insect to respond rapidly to environmental changes, while also facilitating begomovirus transmission to suitable hosts. The adaptability of *B. tabaci* and begomoviral pathogens to ever-changing environments, including monoculture crop systems, suggests that begomovirus–*B. tabaci* complexes have coevolved in marginal, subtropical habitats (Brown et al., 2004a, b). That *B. tabaci* does not develop or display corresponding morphological features that signify such adaptation, suggests that such astute responsiveness may be attributable to differential gene expression instead of permanent physical or chemical structure evolution, as seen in many other organisms. Hence, these

vector–pathogen complexes are capable of exploiting monoculture agriculture, particularly when year-round cropping is practiced. Monoculture cropping systems thereby provide more abundant, longer-term food supplies that promote the development of large vector populations, ample opportunity for the virus and vector complex to adapt to cultivated hosts, and the selection of new and emergent viruses that are most sustainable in monoculture settings. This result of a phenomenon is the primary reason for the writing of this book.

3.3. Case studies: vector–virus–host interactions

Given the nearly universal specificity of begomovirus–*B. tabaci* vector interactions, and a theoretical "single vector" threshold for transmission, differences in host preference or transmission competency may not be as relevant as population size when the vector is abundant. For example, transmission studies for the New World virus of tomato, *Chino del tomate virus* (CdTV), and the New and Old World A and B biotypes, respectively, revealed that both biotypes ingested virus 93% of the time, based on PCR detection. However, CdTV was more efficiently transmitted by the endemic A biotype (SW US–NW Mexico) at 50%, compared to the Old World B biotype (Idris et al., 2001) at 27%. These results implicated passage of virions across the midgut barrier, or the acquisition–transmission stages of the transmission pathway. Nonetheless, when the B biotype became widely established and then displaced the A biotype in west coast Mexico tomato crops, CdTV disappeared from tomato fields, even though CdTV can still be found in local weeds (Brown, 2006, unpublished).

In a study involving two Old World tomato leaf curl isolates from Yemen, the Old World *Watermelon curly stunt virus* (Yemen), and the New World *Squash leaf curl and Bean calico mosaic viruses*, all of these viruses were efficiently transmitted by the AZ–B biotype, irrespective of geographical origin, host, or monopartite/bipartite genome. Transmission frequencies in all combinations were nearly 100% with ten viruliferous adults, compared to 70–80% for five adults (Bedford et al., 1994). These results suggest that transmission frequency is not correlated significantly with extant origin of virus or vector. Perhaps the extreme polyphagy and high fecundity (in the field) of the B biotype contribute atypically to the observed results.

Transmission studies for TYLCSV (from Spain), TYLCV (Israel and Jordan) with the B and local bio/haplotype (Q and Q-like) vectors (Caciagli et al., 1995; Cohen & Nitzany, 1966; Mansour & Musa, 1992), indicated that the Q and B biotypes transmitted TYLCSV with similar efficiency, but that the retention time for both biotypes and TYLCSV was less than previously reported for TYLCV (Caciagli et al., 1995). In a later study in which the basis for field displacement of TYLCSV by TYLCV was investigated, no discernable selective advantage could be demonstrated for TYLCV or TYLCSV in coinfected tomato plants, suggesting that displacement of one virus by the other

would be unlikely. However, in contrast to the previous study, the Q biotype was a more efficient vector for both viruses, compared to the B biotype, e.g., TYLCSV was less efficiently transmitted by the B than by the Q by a factor of two. Initially, it was predicted that because the native Spanish Q biotype was the more efficient vector, the endemic TYLCSV would prevail together with TYLCV as long as the Q continued to predominate. This result would be expected if transmission competency alone were responsible for prevalence of one virus over the other. However, examination of the host range of the two viruses, revealed that at least one cultivated species (bean) was a crucial overseasoning virus reservoir for TYLCV when tomato crops were unavailable, whereas, TYLCSV did not infect bean. Thus, the absence of a significant reservoir host for TYLCSV when tomato was not a viable host appears to have contributed more powerfully to virus selection, than (putative) coevolved virus–vector transmission determinants (Sanchez-Campos et al., 1999). The Q biotype and its closest relatives in the Middle East are native to the Mediterranean–Middle East–North Africa region (Brown, 2000), but the exact origin of the B biotype is still unknown. Even so, these results suggest that the Spanish Q and its close relative from Israel (Horowitz et al., 2003), both which belong to the North African–Mediterranean–Middle Eastern clade, are coevolved with the Mediterranean–Middle Eastern viruses (e.g., TYLCV from Israel and with TYLCSV from Spain). Results further suggest that the B biotype did not originate in this immediate region. Thus, the discovery of the origin of the B biotype will allow further exploration of this hypothesis for its endemic begomoviruses.

Virus–vector studies have also illustrated a feasible, direct correlation between the transmission efficiency of three phylogeographically divergent virus–vector combinations from different regions in Africa. Results indicated that each particular virus–vector pair that was native to the respective endemic virus proved under laboratory conditions the most efficient combination (McGrath & Harrison, 1995). However, the transmission efficiency for each respective combination was not studied under field conditions, and such a comparison could have been highly corroborative.

Finally, it is possible to postulate (and continue to test) the generalized hypothesis that if a *B. tabaci* haplotype can feed (and in most cases, breed) on a begomovirus-infected host, it will likely transmit the virus, all else being equal (Bedford et al., 1994). For begomovirus–vector complexes in monoculture systems, the vector population size, host range of the vector and virus, and differential susceptibilities of the vector to insecticides, likely override most molecular determinants of transmission competency or efficiency. Although this observation appears to apply most readily to monoculture settings, and may not necessarily hold true for host-restricted *B. tabaci*–virus complexes, or for those present in less disturbed locales, or for example where polyculture is implemented, or for endemic virus–vector combinations that utilize native, uncultivated plant hosts.

3.4. TYLCV species and strains, and endemic or exotic biotype transmission

The B and Q-like biotypes are considered the most important extant begomovirus vectors in tomato crops for much of the world, contributing both to disease spread and to the emergence of new species and recombinants. Each of these biotypes/close relatives has coevolved with about an equal number of TYLCV species recognized as emergent pathogens. Among them, TYLCV and TYLCV-mild, both originating in the Middle East have been introduced to the American Tropics/US Sunbelt States, and/or to Europe–Mediterranean regions, Asia, and Australia, respectively. These introductions occurred either following or coincident with the widespread introduction of the B biotype.

The TYLCV species in Africa and Australia are only recently under study but it is expected that the African viruses have likewise coevolved with their native vectors, and that TYLCV in Australia will be readily transmitted by the B biotype, which invaded there in 1994 (Gunning et al., 1997). In Asian-Pacific agricultural systems, the two local biotypes WAN and EAN are probably competent vectors of endemic *Tomato leaf curl virus* (TLCV-Aus), the begomovirus described there from tomato in 1971, but given the introduction of the exotic B (1994) together with TYLCV (2006), it is not possible to predict how these recent disruptions will affect the outcomes there.

In Japan, TYLCV-mild has become established and both the B and Q biotypes likewise have been introduced in recent years (Ueda & Brown, 2006; Ueda et al., 2005). In India (Rekha et al., 2005) and China (Chu et al., 2006; Zhang et al., 2005), clearly the B and/or Q biotypes threaten to displace the local Asian haplotypes and begomoviral species, but it is premature to predict the outcomes of the invasive biotype–virus complexes on disease spread or the dynamics of endemic biotypes and viruses.

4. BIOLOGICAL AND GENETIC VARIATION – A CRYPTIC SPECIES

4.1. Phenotypic, taxonomic, and genetic conundrums

4.1.1. Bemisia tabaci is "rare" among whiteflies owing to its polyphagous phenotype

The natural hosts of *B. tabaci* are annual or perennial eudicots native to the dry subtropical understory and/or desert riverbanks and washes throughout the subtropics/tropics, and mild climate Mediterranean/desert locales where prolonged freezing temperatures are rare or nonexistent. This usually polyphagous species is known to colonize over 500 plant species including its native hosts, as well as a large number of cultivated fiber, vegetable, and ornamental plants (Cock, 1986, 1993). It is likely that the majority of *B. tabaci* are moderately to highly polyphagous, though many variants remain uncharacterized. The host range of certain highly polyphagous *B. tabaci* such as the A, B, Q biotypes probably comprise at least 100 or more species (Bayhan et al., 2006; Bethke et al., 1991; Butler et al., 1983;

Cock, 1986, 1993), but it is unlikely that any biotype or haplotype is capable of equally colonizing all hosts. There is good evidence that exposure over time allows adaptation and that certain hosts are preferred, possibly owing to the "exposure" history of the particular population. Finally, it seems highly likely that polyphagous *B. tabaci* will far outweigh the importance of host-restricted or monophagous biotypes (Bedford et al., 1994; Burban et al., 1992; Legg et al., 1994; Maruthi et al., 2002; Simon et al., 2003b) as vectors of TYLCV.

4.1.2. Cryptic nature

The *B. tabaci* complex is an excellent example of a "cryptic species," for which genetic variation is evident, but no morphological characters are demonstrable for pupae or adults. In fact, the external morphology for the species has apparently remained status for some time (Gill, 1990; Martin, 2003; Rosell et al., 1997; others). However, *B. tabaci* immature forms respond to plant surface topologies by altering size and shape of setae, hairs, pores, and waxy protrusions, making it visibly adaptive (Mohanty & Basu, 1986; Gill, 1990; Martin, 2003; Mound, 1963; Mound & Halsey, 1978). These traits have confounded the taxonomy of *B. tabaci* and may in part explain its often polyphagous habits, even though certain *B. tabaci* engage in monophagy. The capacity for polyphagy in *B. tabaci* differs from most other whitefly genera and species, which are characteristically host-specific (Martin, 2003). Interestingly, the greenhouse whitefly *Trialeurodes vaporariorum* (West.), another polyphagous species, exhibits no external morphological variation of the pupal case in response to host surface or other environmentally induced features. Likewise, it does not exhibit monophagy, as occurs for *B. tabaci*. Consequently, *B. tabaci* is unusual among the Aleyrodidae and highly adaptable to varied conditions, particularly those imposed by human activities.

Variants for which biological (phenotypic) differences are recognized have most recently been referred to as "biotypes," and previously, as races (Bird & Sanchez, 1971; Bird & Maramorosch, 1978). Fewer than a dozen biotypes have been definitively characterized, and about ten additional variants are incompletely studied. It is likely that the majority of biological variants that occur throughout the world are unstudied. Among the most evident phenotypic differences among *B. tabaci* biotypes are polyphagy or host-specialization, host range for polyphagous haplotypes, fecundity (less than 50 to greater than 300 offspring/female lifetime), dispersal behavior (long- or short-distance flight), propensity to develop resistance to different classes of insecticides, plant virus transmission competency, and the composition of secondary endosymbionts.

Certain biotypes exhibit significant genetic variability and corresponding phenotypic variation (Frohlich et al., 1999; Brown, 2001; Viscarret et al., 2003; De Barro et al., 2000), but there are a number of exceptions for which genetic and phenotypic variability do not go hand in hand. One example involves the endemic *B. tabaci* that colonize cassava (monophagous) compared to non-cassava (polyphagous) colonizers in eastern sub-Saharan Africa (Sseruwagi et al., 2005),

which vary minimally at 2–5% (mtCOI) but have a dramatically different host preference. In contrast, cassava colonizers from East and West Africa also are restricted to cassava, but diverge at 8% or greater. These examples suggest that the adaptation to cassava has occurred more than once in Africa. In another case, the monophagous Jatropha biotype from Puerto Rico is minimal divergent at ~2% (mtCOI) with respect to its polyphagous counterpart, the "Sida" biotype with which it is sympatric. Also, the Jatropha biotype is moderately divergent at (2–3%) from the polyphagous A biotype native to the southwestern USA, the latter being polyphagous, while the former is monophagous. Finally, the B and Q biotypes diverge by about 8% (mtCOI), but they are thought to have a similar host range and generally to be competent TYLCV vectors.

4.2. The biotype concept and the increased importance of *B. tabaci*

Dr. Julio Bird first recognized phenotypic variation in *B. tabaci* on the island of Puerto Rico during studies carried out from 1953 to the present. Bird designated the polyphagous variant, the "Sida race," and the monophagous variant, "the Jatropha race," based on the preferred host plant of each (Bird, 1957; Bird & Sanchez, 1971; Bird & Maramorosch, 1978; Brown & Bird, 1992). In 1975, Russell (USA) and Costa (Brazil) took notice of Bird's discovery, pointing out that in Brazil *B. tabaci* was never observed colonizing cassava (Costa & Russell, 1975), which is native to South America. In contrast, *B. tabaci* throughout Africa widely colonized cassava plants after cassava was introduced there as a staple crop by European colonists (Storey, 1936).

The scientific literature from 1928–1970 is replete with reports of virus disease outbreaks and associated with *B. tabaci* infestations in cassava and other food and fiber crops. And, from the turn of the century to 1957, the literature contains original descriptions of ~23 whitefly species (2 genera), which were eventually grouped under the single taxon *B. tabaci* (Russell, 1957). Following the synonymization of the species variable behaviors began to be noted for this whitefly, which suggested that the species comprised a number of "races" (Bird, 1957; Bird & Sanchez, 1971; Costa & Russell, 1975), later, termed "biotypes" (Costa & Brown, 1991).

During the mid-1930 to the 1960s increasingly more frequent outbreaks of *B. tabaci* were reported worldwide. Infestations and/or virus-like diseases occurred in cotton in Sudan, affected vegetable crops in India and cassava in Africa, caused yield losses in soybean in Brazil, and severe leaf crumpling symptoms in cotton crops in Arizona and California, USA (see refs in Brown, 1990, 1994; Brown & Bird, 1992). Literature from this era revealed that much attention was centered on pesticide use to reduce crop damage caused by whitefly feeding and virus-like diseases. However, several studies addressed the biology of this whitefly and its emerging importance as a pest and vector of plant viruses (Bird, 1957; Bird & Maramorosch, 1975, 1978; Bird & Sanchez, 1971; see refs in Brown, 1990, 1994; Brown & Bird, 1992; see refs in Byrne, 1990;

Byrne & Bellows, 1991; Costa, 1976; see refs in Gerling, 1990; Muniyappa, 1980; Varma, 1963).

The accidental introduction of the B biotype in the USA and Caribbean during 1986–1990 brought with it the first widespread awareness of *B. tabaci* as an invasive pest and vector. Its establishment was marked by outbreaks of phytotoxic symptoms in tomato (irregular ripening) and cucurbits (squash silverleaf disorder, SSL) (Schuster et al., 1990, 1991; Yokomi et al., 1990), which were thought possibly to be of viral etiology. Soon thereafter, similar phytotoxic-like disorders were soon observed widespread in cucurbits, cole crops (Brown et al., 1991), and tomato plantings in the western USA, Mexico, and the Caribbean region. Eventually the symptoms became diagnostic for the presence of the B biotype, with which the disorder was associated (Bedford et al., 1994; Brown et al., 1991, 1995a; Costa & Brown, 1991; Costa et al., 1993a). Costa and Brown (1991) demonstrated that SSL was associated with B biotype feeding and that a transmissible agent was not involved in the symptomatology. Coincident with the rapid invasion of the B biotype in the Tropical Americas was the emergence of plant viruses, soon identified as begomoviruses. Particularly notable were diseases of cabbage, cucurbits, and tomato, which were not preferred hosts of native *B. tabaci*.

During 1991 B biotype populations exploded, reaching unprecedented levels in irrigated cropping systems of the southwestern USA, the Caribbean region, and the American Tropics, reaching South America in 1994. Australia, China, Egypt, Europe-Mediterranean region, Israel, Japan, Pakistan, and Turkey reported B biotype outbreaks next, making it the first *B. tabaci* biotype to become a cosmopolitan pest and vector. As rapidly, its propensity to develop insecticide resistance became apparent, and so efforts were undertaken to monitor resistance in whitefly management programs (Anthony et al., 1995; Costa et al., 1993a, 1994; Dittrick et al., 1989; Denholm et al., 1996; Horowitz et al., 1998, 2005; Nauen et al., 2002; Yassin et al., 1990).

The polyphagous B and Q biotypes now predominate in agricultural systems in subtropics–tropics and temperate locales. It is expected that the continued unrestricted movement of plants infested with *B. tabaci* could result in introductions of additional, damaging biotypes. The B and Q biotypes have an overlapping host range, but less is known about the host preference for the Q than the B biotype. Both colonize widely grown fiber and vegetable crops, including bean, cotton, cucurbits, eggplant, pepper, okra, and tomato. The B biotype also colonizes cole crops, *Lantana*, soybean, sesame, and a number of ornamental species. The Q also colonizes certain ornamentals, including poinsettia. Protected tomato production in controlled environment facilities is a rapidly growing industry in the USA, Mexico, and Central America. Further, the majority of ornamentals, bedding plants, vegetable seedlings, and some nursery stock are now produced in subtropical and Mediterranean locales for export, making these plants important vehicles for redistribution of the whitefly and

plant viruses, including TYLCV. If the Q becomes established in cotton and vegetables, it is likely that producers will be unable to control it using insecticides effective against the B biotype.

The haplotype, referred to as the Spanish Q biotype (Guirao et al., 1997), was previously known as the polyphagous haplotype native to southern Spain (and, the Q esterase pattern). Phylogenetic analysis places it into a clade sister to the B biotype subclade in the major N. African–Mediterranean–Middle Eastern clade (Brown, 2000). Close relatives include *B. tabaci* from Turkey (M or TC), Sudan (SC), populations from Morocco, Israel, Egypt (Berry et al., 2004; Horowitz et al., 2005; Sseruwagi et al., 2005, 2006). The Q and its close relatives are indigenous to the Mediterranean region and cucurbits, tomatoes, and peppers are reported as preferred hosts. Members of this clade are moderately (and possibly widely) polyphagous, and are likely the endemic vector haplotypes of TYLCV species and strains from the Middle East, Spain, Sardinia, Sicily, and North Africa, including Sudan, and Egypt (Brown, 2001; Brown, 2006; Brown et al., 2004a, b; Sseruwagi et al., 2006). The eventual reestablishment of the Q and Q-relative in Israel, and displacement of the B biotype in southern Spain, are attributed to differential insecticide resistances of the Q and B biotypes (Horowitz et al., 2005).

Because the Q biotype also has a broad host range (I. D. Bedford, personal communication, 2006) that includes cultivated and uncultivated species, and it is resistant or tolerant to insecticides that control the B biotype, it poses a new threat. During 2005, it became prevalent on ornamentals exported to China, Japan, Mexico, and at least 22 states in the USA (Brown et al., 2006; Chu et al., 2006; Martinez & Brown, 2007; Ueda and Brown, 2006), with the first report being in Arizona, USA on poinsettia plants by Dennehy et al. (2005). Had molecular genetics diagnostics tools been implemented at the ports of entry, neither the B or Q biotypes would have become so quickly widespread and the introduction of the Q biotype likely could have been avoided altogether.

The B and Q biotypes continue to be imported from offshore ornamentals nurseries, and federal regulations do not prohibit the importation. This could have further significance to US-exported plant products if other countries decide to regulate this insect. Biotypes of *B. tabaci* are not regulated in the USA because not only are *Bemisia* species and biotypes/haplotypes difficult to differentiate by morphological traits, so are certain genera, some of which co-colonize *B. tabaci* hosts.

Discerning whitefly species and biotypes of *B. tabaci* requires molecular-based diagnostics. Such tools have recently been developed and widely implemented to track the distribution and dispersal of *B. tabaci* worldwide. Although molecular markers have not been identified that are sufficiently informative to predict the evolutionary origin and histories of the *B. tabaci* complex, it is possible to ascertain certain biogeographical relationships using the range of genetics-based approaches available at this time.

5. GENETIC VARIATION, BIOGEOGRAPHICAL RELATIONSHIPS, AND MOLECULAR TRACKING OF BIOTYPES

5.1. General esterase and isozyme polymorphisms

Costa and Brown (1990, 1991) coined the *A* and *B* biotype nomenclature based on the observation that two laboratory populations yielded distinct general esterase patterns and had different host preferences. It was known that *B. tabaci* native to the southwestern USA could not colonize poinsettia (Brown, unpublished). Hence when *B. tabaci* infestations became problematic on poinsettia (Alderman, 1987; Linquist & Tayama, 1987) that a distinct variant might be involved. Esterase analysis of *B. tabaci* from the poinsettia plants and the laboratory colony (from cotton in Arizona, 1981) (Butler et al., 1983) reared on pumpkin and employed in virus–vector studies (Brown & Nelson, 1986) produced distinctive characteristic esterase patterns, which were referred to as patterns A (AZ endemic *B. tabaci*) and B (poinsettia colony) (Costa & Brown, 1990). The esterase approach for investigating biochemical polymorphisms was taken from the work of Prabhaker et al. (1987), which demonstrated that *B. tabaci* could be distinguished from two other whitefly species. Concurrent studies with the A and B colonies of *B. tabaci* revealed that they also differed in host range, fecundity and that the B but not the A biotype induced silvering in pumpkin plants (Costa & Brown, 1991). The two were thereafter referred to as the A and B biotypes (Costa & Brown, 1991).

The esterase method was subsequently applied to track the spread of the B biotype in the USA, Caribbean, and tropical Americas, and then in *B. tabaci* populations from the Eastern Hemisphere. Such extensive sampling revealed an unexpected high frequency of polymorphisms for the species. Each unique morphotype was thus assigned an alphabetical designation (A–Q). Selected populations (based on genetic polymorphisms) were established in culture and analyzed for host range, fecundity, morphological variation, mating, virus transmission efficiency, and SSL induction. The results revealed a broad range of distinctive phenotypes with no definitive morphological differences (Bedford et al., 1994; Rosell et al., 1997), leading to the recognition that *B. tabaci* was a highly variable species. When selected colonies were subjected to insecticide resistance evaluation, they were found to be highly polymorphic as well (Anthony et al., 1995; Coats et al., 1994; Costa et al., 1993a). These results underscored a new importance of developing DNA–based methods to assess genetic variation in this polymorphic species.

5.2. Genetic polymorphisms, molecular markers, and phylogenetic relationships

Several groups have examined genetic variation using general esterases and isozymes, while others have relied upon Random Amplified Polymorphic DNAs

(RAPDs)–PCR (Ariyo et al., 2005; Burban et al., 1992; Byrne et al., 1995; Gawel & Bartlett, 1993; Guirao et al., 1997; Gunning et al., 1997; Moya et al., 2001; Ryckewaert & Alauzet, 2001; Wool et al., 1991; Zanic et al., 2005). The greatest drawback to esterase and RAPDs analyses for biotype identification has been the inattention to suitable, internal reference populations, making a number of data sets only minimally interpretable. Another problem has been the "reproducibility" between laboratories in some instances. It is of historical interest to note that data from RAPDs and isozyme analysis of the A and B biotype, together with mating studies which showed that they were reproductively isolated (Perring et al., 1993), served as the basis for erecting the species *B. argentifolii* for the B biotype by Bellows et al. (1994).

Subsequently, isozyme analysis (Brown et al., 2000) for esterase typed colonies, revealed further unexpected genetic variability and borderline genetic distances for A and B comparisons, calling into question the *B. argentifolii* designation. Later, phylogenetic analysis of the mitochondria16S and then mtCOI for the (well-studied) *B. tabaci* colonies and additional worldwide collections, provided evidence for as much as 15% nucleotide divergence in the mtCOI sequence (Brown et al., 1995b; Frohlich et al., 1994, 1999). From 16S and COI analyses, it was possible to predict that the B biotype was of Old World origin, possibly Africa. Information on the probable origin of the B biotypes (Frohlich et al., 1999) allowed prospectors to delimit the most optimal locales in which natural enemies might be found (Kirk et al., 2000).

Based on these (and recent) analyses it is difficult to draw any other conclusion except that the B biotype is one of many variants in the larger complex (Qiu et al., 2007; Brown et al., 1995, 2004; Berry et al., 2004; DeBarro et al., 2005; Rua et al., 2006; Sseruwagi et al., 2005, 2006). Hence, the suggestion that *B. tabaci* is best described as a group of strain, subspecies, or perhaps, sibling species (Brown et al., 1995; Frohlich et al., 1999). Finally, the common name, silverleaf whitefly, also has become questionable, owing to the discovery of non-B variants from eastern and coastal Africa based on mtCOI analysis (see below), even though the variants induced silvering in cucurbit species (De Latte, et al., 2006; Sseruwagi et al., 2005) per Costa & Brown (1991).

Among several molecular markers examined, the mtCOI sequence reveals the most variability for the *B. tabaci* complex. Subsequent analysis of the mtCOI for representative collections worldwide has demonstrated that this coding region is highly informative, and capable of differentiating *B. tabaci* at the level of "subspecies" (strain, or sibling species) which can be grouped phylogeographically into *major clades and sister clades* within each major clade. Four major clades can be resolved and include the North African/Mediterranean/Middle Eastern, sub-Saharan Africa, Asian-Pacific, and the American Tropics clades (Figure 1). Each major clade embodies a number of sister clades and closely related outliers, depending on the type of analysis (French and Brown, in preparation). Within the major clades, it is presently possible to identify 9–10 major "subspecies." These divergent groups also have been referred to as "races"

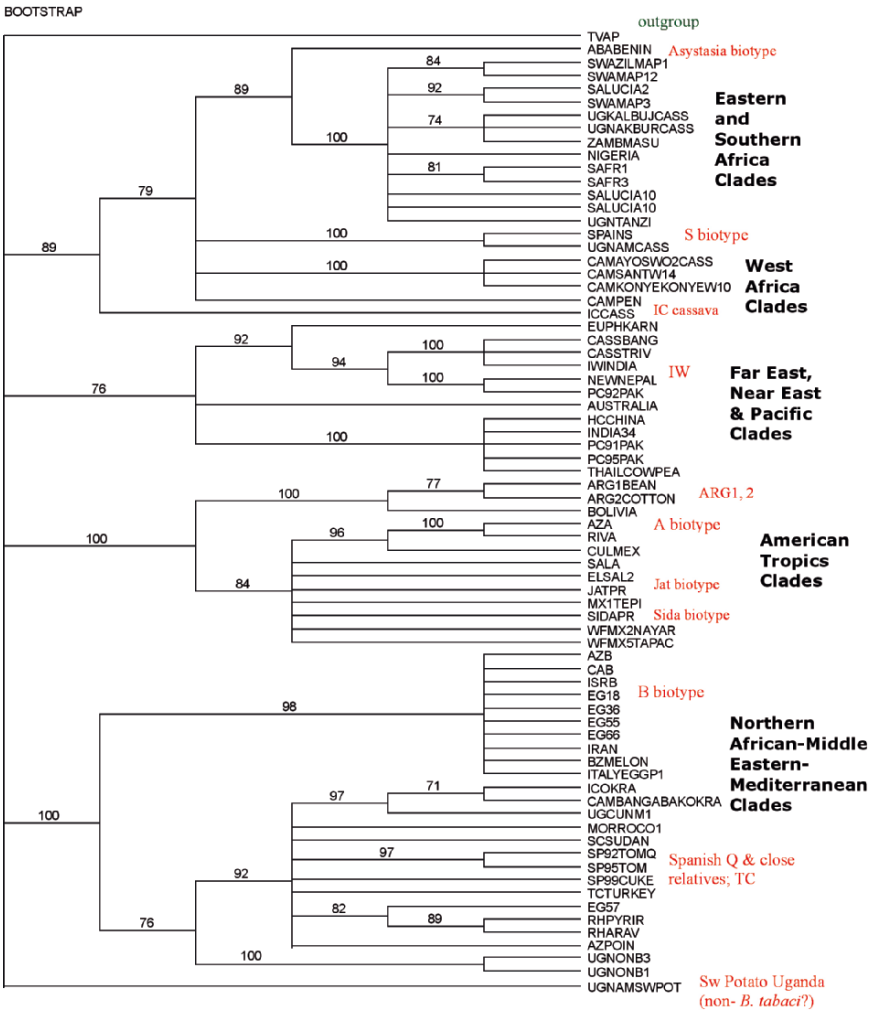

Figure 1. Major clades (bold black) and phylogeographic relationships of B. tabaci for selected haplotypes and biotypes (red) worldwide based on the mitochondria cytochrome oxidase I sequence (780 bp).

(De Barro et al., 2005). This designation, applied by Bird (Bird & Maramorosch, 1978; Bird & Sanchez, 1971) to distinguish the Sida and Jatropha variants (biological types), does not seem applicable because these two races diverge by only ~2% (mtCOI) and belong to the same major clade, otherwise making them biotypes that group under the same strain or "subspecies." The term "race"

therefore is more consistently a synonym for "biological type." Further, because the term "biotype" has been in usage in recent literature, the wisdom in an adjustment in terminology is questionable. In any case, it seems prudent, in the absence of corroborative, molecular genetic and population studies, to retain the term "*B. tabaci* complex" to foster consistent communications.

Nucleotide divergence (%) between the major mtCOI clades reveals similar interclade divergence, making it impossible to root the tree with an evolutionarily basal group. However, intraclade variation provides interesting clues about *B. tabaci* evolution. The mtCOI reveals that the greatest genetic diversity in the *B. tabaci* complex occurs within the African continent, while the second most is found in Asia (Qui et al., 2007; Berry et al., 2004; Brown and French, in preparation; Brown et al., 2004; Kirk et al., 2000; Legg et al., 2002; Viscarret et al., 2003). Within-clade variation is lowest at ~5–8% for the Tropical Americas and Caribbean region, and greatest at ~16–26% for African haplotypes, with the sub-Saharan Africa haplotypes being most divergent. Asian-Pacific *B. tabaci* diverge at ~12–20% and a hot-spot of diversity occurs in southern Asia (Qui et al., 2007) The suggested African origin, with a second site of diversification in Asia seem justifiable owing to the broad climatic variation and geographic/physical barriers that occur in southern Asia and across Africa. These are similar to the patterns predicted for humans and *Drosophila* based on molecular genetics and population studies.

Other molecular markers (16S mtCOI; ITS-1) and population studies using RAPDs and microsatellites (STRs) have provided corroborative evidence for genetic variability in Africa and Asia as well (De Barro et al., 2000; De Latte et al., 2006; Gawel & Bartlett, 1993; Moya et al., 2001; Rekha et al., 2005; Tsagkarakou & Roditakis, 2003).

The higher-than-expected intraspecies variation revealed by protein polymorphisms (general esterases and isozymes) are generally corroborated (to date) by DNA marker analyses and population studies, support congruent phylogeographical patterns of distribution, and reveal substantial genetic variation within Eastern vs. Western Hemisphere populations. The recognition that the *B. tabaci* complex groups with a basis in phylogeography is exciting in and of itself, because for the first time a molecular tool permits the tracking of particular biotypes and other less well-studied genetic variants employing comparative mtCOI analysis and substantial publicly available DNA sequence database. Thus, the mtCOI marker is readily applicable as a DNA-based tool both for establishing phylogenetic relationships, and to identity variants (biotypes or haplotypes) for which reference sequences and/or key biotic traits have been validated.

Even so, the ancient evolutionary history of *B. tabaci* has not been satisfactorily resolved, making additional evolutionary inferences necessary. Achieving this new goal will require the validation of additional, robust molecular markers, and population genetics studies through the development of new and expanded experimental approaches.

5.3. Distribution and tracking the worldwide movement of biotypes and TYLCV

The distributions of selected biotypes and TYLCV strains and species are shown in Figure 2. TYLCV strains and species occur primarily in their endemic areas in the Middle East, but also have been introduced throughout parts of Asia, the Mediterranean region, and the Tropical Americas. In the eastern hemisphere TYLCV-mild (Antignus & Cohen, 1994) has become established throughout the Mediterranean region and in much of Asia (Ueda et al., 2005). Thus, TYLCV-mild and TYLCV will likely displace a number of endemic begomoviruses where it has been introduced. In the scenarios to date the B or the Q biotypes have been important as the primary vector involved. In contrast, TYLCV alone thus far prevails in the Neotropics and the B biotype is the predominant vector. Most recently, during 2004–2007 TYLCV, which occurred in the USA only in the southeastern states, and in the eastern Caribbean and east coast of Mexico, has spread westward in the USA to include Texas (Isakeit et al., 2006) and Arizona (Idris et al., 2007). During 2004–2005 TYLCV also spread to the Pacific Coast states of Mexico, where a pandemic occurred in the Fall 2005 to Spring 2006 in Sinaloa (Brown & Idris, 2006). During the Fall 2006 the virus was also found in tomato plants as far north as Hermosillo, Sonora (Idris et al., 2007), only a few hundred miles from the southern-most tomato-growing

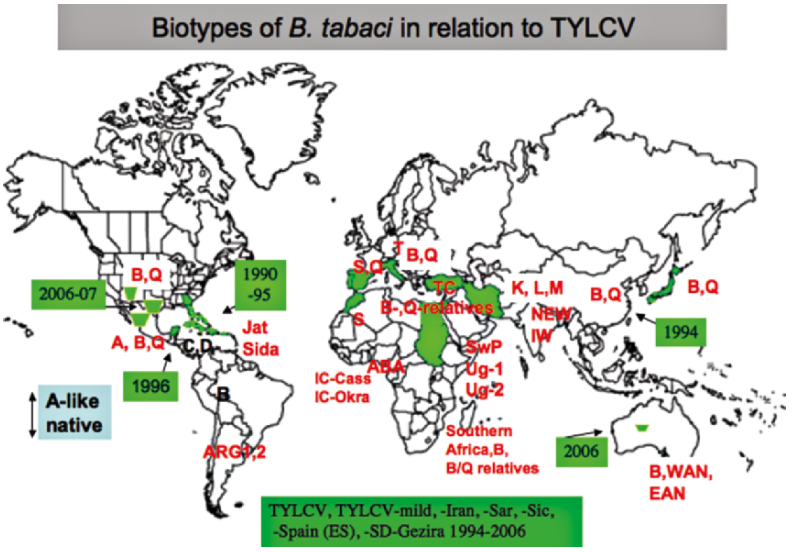

Figure 2. Map illustrating the distribution of well-studied biotypes or haplotypes (red) and major phylogeographical clade associations, in relation to TYLCV (green letters; green-filled).

region in California. Thus far TYLCV in the tropical Americas appears mostly homogeneous, with the exception of a deletion mutant identified in transplants in the Arizona and Texas outbreak. Thus the isolates in the USA and Mexico appear to be distinct, suggesting multiple introductions and/or the emergence of a variant from the introduced isolate.

During 2004–2006 the Q biotype was introduced into the USA on ornamentals from the Mediterranean region (Dennehy et al., 2005), and during 2006 it was identified for the first time in the state of Sonora, Mexico (Martinez & Brown, 2007). It is also present in Guatemala (Brown, 2004, unpublished data). Thus, two exotic (Old World) B and Q biotypes are now problematic in greenhouses at least 22 US states, as well as in several states in Mexico and in Guatemala (Brown, 2004–2005, unpublished).

Of particular concern is that the B and Q colonize at least some of the same hosts, and in many instances coexist on the same plant. This underscores the need to know the composition of *B. tabaci* populations in the fields and in greenhouses and fields, so that relevant chemical control measures can be implemented to maximize control, while minimizing development of highly resistant Q populations. This is essential because biotypes often have distinct insecticide resistance profiles, probably owing in part to selection in production systems that use different suites of chemistries. Until the degree of risk of the Q biotype to US agriculture becomes clear, it is essential that monitoring be undertaken to ascertain the distribution and composition of *B. tabaci* that can negatively affect cotton, vegetable crops, and ornamentals. These three industries intersect more and more frequently as vegetable, fiber, and horticultural production in the USA have become spatially concentrated. Hence, production practices including the widespread importation of ornamentals from overseas-grown mother stocks, and pesticide use, among others, influence and can in certain instances directly cause pest and disease problems affecting one or more of these industries. Certainly the spread of TYLCV in the southern USA and Puerto Rico have been attributed to the interstate transport of TYLCV-infected vegetable seedlings (Bird et al., 2001; Isakeit et al., 2006; Polston et al., 1994, 1999).

In general, whether or not vector haplotypes are well characterized, TYLCV species and strains, and endemic begomoviruses of tomato are expected to be transmissible by haplotypes that colonize tomato/solanaceous hosts, or by those that otherwise exhibit polphagy. Field and laboratory data indicate that the better studied polyphagous *B. tabaci* from the Eastern and Western Hemispheres have similar or overlapping host ranges that include bean, pepper, tobacco, and tomato. Hence such variants could be expected to effectively transmit TYLCV, if not out-competed by the B or Q biotypes. In addition to the B and Q biotypes, some additional prospective TYLCV vectors are known (Figure 2): A- and Sida-like biotypes in the tropical Americas, and local haplotypes ARG from Argentina, D from Nicaragua (A-like), G from Guatemala (A-like), IC-okra (Ivory Coast), haplotype J (Nigeria, cotton), K (Pakistan, cotton), L (Sudan, cotton), M or TC from Turkey (also in the Q-clade), and other members of the B, non-B, and Q-like

clades from the Mediterranean, Middle East, and North Africa) (Sseruwagi et al., 2006). Any of these haplotypes could feasibly support rapid spread and establishment of TYLCV, with potential to displace the more benign begomoviruses endemic to the New World, Asia, and Africa. With this could follow a reduction in begomovirus diversity and altered diversification patterns.

The narrow time frame since the first introductions of TYLCV during 1990, 1996 into the American Tropics and USA and of the highly polyphagous, exotic biotypes B, Q to multiple world locations in both hemispheres should already have provided sufficient warning; however, it seems likely that additional invasions are imminent.

6. CONCLUSIONS

It is hoped that this brief history and comparative treatment of the topic has inspired fundamental and application-based questions that will spawn new directions in research. Much remains to be learned about *B. tabaci* biology, population genetics, evolutionary history, and the basis underlying "coadaptation" between *B. tabaci* and begomovirus–plant complexes. Well-known biotypes of *B. tabaci* are now readily distinguished using molecular diagnostics tools, which can place the origin of a haplotype (or biotype) more or less accurately within the extant phylogeographic origin. The most definitive approach available utilizes PCR amplification and DNA sequencing of a 850 base pair fragment of the mtCOI. Subsequent mtCOI sequence (780 bp) comparisons using an extensive collection of reference (public and unpublished) sequences, permits discrimination not only between biotypes of *B. tabaci*, but also makes possible identification of other whitefly species that colonize the same hosts and may inadvertently be collected with *B. tabaci*. Although a relatively large number of phenotypic variants of *B. tabaci* have revealed an intriguing range of monophagous to polphagous behaviors, it is accurate to state that the majority are poorly understood in terms relevant to (1) the dynamics of virus disease spread and diversification, (2) how host range and host-adaptation influence plant virus pathogen evolution and emergence, (3) specificity and transmission competency, (4) life history trait expression and upsurgence (or not), (5) the propensity to develop insecticide resistance (or not), and (5) the roles of endosymbionts in host range and fitness-directing phenotypes. Even so, the ability to distinguish common biological types has not prompted the establishment of quarantines against the most tenacious *B. tabaci* variants, because morphologically based identification remains the most practical means for general identification of insects at ports of entry. Indeed, another shortcoming in terms of advancing the biotype concept is the paucity of biological data available for the growing number of well-defined variants. This is due primarily to the lack of quarantine-level insectaries in which whitefly colonies can be maintained in isolation and used to carry out rigorous life history, host range, mating compatibility, and virus–vector studies, among others. Soon genomics-based technologies make possible the linking of phenotypic and genetic variability to

gene expression patterns and genomics-based cloning of genes. Identifying genome regions of cross-kingdom conservation will enable identification of orthologous arthropod genes with functionalities in adaptive life history traits, reproductive isolation mechanisms, competitiveness or invasiveness vs. benign colonization, and cellular and molecular coadaptation that confer virus–vector specificity and influence transmission competency, all presently unidentified. Such approaches are expected to enable predictions of volatile or benign biotype invasions, development of more directed, environmentally sound "pest" and "vector" management strategies, and fueling of scientific inquiry that seeks to unravel to the next level the underpinnings of this minute phloem-feeder that for more than 120–140 million years (Czosnek et al., 2001) has continued to perfect its ability to exploit inter-kingdom interactions with resounding success.

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CHAPTER 4

SURVIVAL OF WHITEFLIES DURING LONG-DISTANCE TRANSPORTATION OF AGRICULTURAL PRODUCTS AND PLANTS

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1. OVERVIEW

Whiteflies (Hemiptera: Aleyrodidae) are a major pest of tropical and subtropical crops and of protected crops in temperate regions. Worries about their possible movements to new areas, therefore, are quite justified.

There are clear examples of invasion of new areas through natural spread of insects, such as the colonization of southern California by the giant whitefly, *Aleurodicus dugesii* Cockerell, coming from the south (Bellows & Meisenbacher, 2000), or the spread of *Aleurocybotus indicus* David and Subramaniam, from Senegal, where it was first reported on rice in 1977, to Mauritania, Burkina Faso, Nigeria, and Niger (Alam, 1989), possibly through Mali. In general, whiteflies are poor fliers and their long-distance movements are likely assisted by humans (Byrne & Bellows, 1991). *Dialeurodes citri* (Ashmead) and *D. citrifolii* (Ashmead), for example, were accidentally introduced into the British Isles in 1974 from Florida and successfully eradicated by 1978 (Bowman & Bartlett, 1978). On the contrary, *B. afer* (Priesner & Hosny) is already naturalized in the UK (Malumphy et al., 2004). *Parabemisia myricae* (Kuwana) was introduced into California in 1978, and was already widespread over the state by 1981 (Rose et al., 1981). The ash whitefly, *Siphoninus phillyrae* (Haliday), found for the first time in New Zealand in 1995 (Charles & Froud, 1996), was certainly there because of human activity; so was *A. dispersus* Russell, the spiraling whitefly, found in 1998 at Cairns, northeastern Australia, (Fay, 2001). The same whitefly has been carried to Cape Verde Islands likely by man long ago, so that during a survey made in 2003 and 2004 it was present on more than 205 species belonging to 64 botanical families (Monteiro et al., 2005), just considering the main hosts!

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 57–63.

If these whiteflies cause concern to farmers and growers all over the world, *Bemisia tabaci* (Gennadius) is even more frightening, because of its biological properties, including the ability to vector many plant viruses. The documented transportation of *B. tabaci* is already a long story. Biotype A was taken to Brazil in 1928 (Oliveira et al., 2005), and biotype B in the early 1990s (Oliveira et al., 2005). Biotype B, later described as a new species (Perring et al., 1993) was imported to the USA sometimes before 1986 and then invaded the southern states (Culotta, 1991). *B. tabaci*, most probably the same biotype B, since it was on poinsettia, was detected in East Germany in 1988 (Braasch & Nussbaum, 1992) and never eradicated, in spite of control measures. Being aware of the danger, Polish inspectors were able to intercept *B. tabaci* between 1993 and 1998 (Karnkowski, 1999). Awareness was not sufficient to prevent the appearance of *B. argentifolii* Bellows and Perring (*B. tabaci* B biotype) on the Mauritius Island in 1996 (Ganeshan & Abeeluck, 2000).

The high risk of moving pests from place to place by human activity is one of the few agreed reasons for imposing quarantine restrictions (Kahan, 1982). Hence, it is very important to know the conditions that permit survival of the pest in its different stages, and how long the pest can survive under these conditions.

2. SURVIVAL OF WHITEFLIES

2.1. Abiotic factors influencing survival

Among the abiotic factors influencing survival of whiteflies, in the different stages of their life, we can certainly include temperature and relative humidity (RH). Here we are only interested in the extreme values. RH is only limiting at the low end (perhaps <30%). Temperature can be considered extreme when it is well below the limit of development of each species (either experimentally determined or calculated as lowest temperature threshold) or above the highest survival temperature. The third parameter is survival time, as an answer to the question "for how long at a given temperature and a given relative humidity." Unfortunately, studies aimed at determining the extreme values of these factors are not very common.

2.1.1. Relative humidity

We do not have much information on the influence of RH on whitefly survival, except for a case where *B. tabaci* adults have been subjected to extreme humidity within a range of temperatures (Berlinger et al., 1996). The authors concluded that whitefly survival decreased from 90% to less than 2% upon increasing exposure time (from 2 to 6 h), increasing temperature (from 25°C to 41°C) and decreasing RH (from 100% to 20%). These data and the model proposed by the authors can be useful in determining the limits of autonomous movement of adult whiteflies, but it does not inform us about survival, for example, at high RH and close-to-freezing temperature for longer times, as in freight traveling for 2 days across Europe in winter or in cooled freight in any season.

2.1.2. Temperature

There are two temperature limits for the survival of whiteflies however the upper limit is hardly compatible with transportation of fresh vegetable products. If we consider the possibility of transportation by chance with goods that do not require cooling or controlled conditions (as in a closed car or in a lorry), then we can reasonably presume that critical temperatures can be easily reached and for times long enough to cause the death of adult whiteflies. Referring again to Berlinger et al. (1996), we can assume that the likelihood of *B. tabaci* adult survival is very poor after (only) 6 h at 30–40°C, independently of the RH.

As for the lower limit, adults are the least resistant form of most species, besides being the most easily detected, but they can anyway survive temperatures down to 6°C for at least 4 days (Bosco & Caciagli, 1998). This is one of the lowest temperatures recommended by the USDA for the transportation of many potted ornamental plants and close to the upper limit suggested for florist green materials, just to give an example (Welby & McGregor, 2004).

Other stages, like eggs and nymphs, are much more resistant to cold. Nymphs of B-biotype *B. tabaci* can survive at least 8 days at 4°C (Bosco & Caciagli, 1998), and red-eyed nymphs of *B. argentifolii* show more than 10% eclosion after 9 days at 5°C (Lacey et al., 1999). Eggs of B-biotype *B. tabaci* are still able to hatch after 8 days at 6°C (Bosco & Caciagli, 1998), or after 9 days at 5°C although in very low numbers (Lacey et al., 1999). Eggs of *Trialeurodes vaporariorum* (Westwood) are even more cold resistant; more than 50% of eggs hatch after 20 days at 6°C, and around 50% after 6 days at –3°C or 7 days at 0°C (Stenseth, 1983). Within these ranges of temperature and time you can move almost everything everywhere (2004; Welby & McGregor, 2004).

And there is worse! Eggs laid by *B. tabaci* infected with a severe strain of *Tomato yellow leaf curl virus* (TYLCV) can give birth to infective insects (Ghanim et al., 1998). Transovarial transmission does not occur for *Tomato yellow leaf curl Sardinia virus* (TYLVSV) (Bosco et al., 2004) and has not been proved for a mild strain of TYLCV by the same authors (Bosco et al., 2004), but, nevertheless, if there is one virus transovarially transmitted, others probably exist. So, we could be moving plant pathogenic viruses around together with whitefly eggs.

2.1.3. Biotic factors influencing the survival of whiteflies

Among the many biotic factors that can influence whitefly survival, only the osmotic pressure of the host leaves seems to have a direct bearing. Variations in host leaf water content, and its associated soluble sugar concentration, just outside a relatively narrow range, strongly influence egg survival of *T. vaporariorum* (Castañé & Savé, 1993), but leaf desiccation has a completely different effect on fourth instar nymphs. During the last nymphal stadium, after apolysis has occurred, during a substage called somewhat improperly pupae, whiteflies do not feed (Byrne & Bellows, 1991), and so they can survive desiccation and emerge

after a few days. The excision of "pupae" with a small piece of the leaf they are attached to, which rapidly desiccates, is a common practice for obtaining virgin females and males for crossing experiments (see, e.g., Demichelis et al., 2005).

3. IDENTIFICATION

Although the actual enforcing of regulations is an autonomous decision of each country, a number of whiteflies have received the attention of international organizations of plant protection: *Aleyrodes proletella* Linnaeus, the brassica whitefly, has received attention from the North America Plant Protection Organization (NAPPO, 2001; NAPPO-PAS, 2006), and so have *Aleurocanthus woglumi* Ashby (EPPO/CABI, 1997b), *A. spiniferus* (Quaintance) (EPPO/CABI), and *B. tabaci* with its B biotype (EPPO/CABI; OEPP/EPPO, 2004) by the European and Mediterranean Plant Protection Organization (EPPO).

We do have methods for identifying these undesirable pests. An identification guide to the whitefly fauna of Europe and the Mediterranean region and other quarantine risk species has been prepared in the frame of the European Whitefly Studies Network (Martin, 2000; Martin et al., 2000). Diagnostic protocols have been described for *A. woglumi* and *A. spiniferus* (OEPP/EPPO, 2002a, b). An ELISA test has been set up for rapid identification of adult *T. vaporariourum* and *B. tabaci* (Symondson et al., 1999). Distinction between *B. tabaci* and *B. afer*, and between *T. vaporariorum* and *T. ricini* (Misra) has been the object of attention by Malumphy et al., who have identified stages when morphological identification is more reliable, but have gone further by preparing two multiplex real-time PCR (TaqMan) assays to complement morphological studies (Malumphy et al., 2004). As for *B. tabaci*, we have a number of biochemical and molecular methods for identifying the biotypes, as morphology does not help. If sequencing of whitefly genes (Brown, 2000; De Barro et al., 2005; Frohlich et al., 1999) is hardly feasible in quarantine protocols, Random Amplified Polymorphic DNAs (RAPD)-PCR (De Barro & Driver, 1997) and AFLP (Cervera et al., 2000) can be used to distinguish *B. tabaci* B biotype from other biotypes, with results quite comparable to those obtained by analysis of esterase patterns (Guirao et al., 1997). PCR–RFLP can be used to discriminate monophagous *B. tabaci* populations from polyphagous populations (Abdullahi et al., 2004) and to identify biotypes in the Mediterranean basin (Bosco et al., 2006). The analysis of biotypes/races can be even more detailed by microsatellite markers (De Barro et al., 2005; Delatte et al., 2006).

4. CONTROL MEASURES

So, generally speaking, we have the instruments. Some methods will need to be set up for specific pests, some need standardization, other may need fine tuning for particular conditions (e.g., Can we do RAPD–PCR or PCR–RFLP tests on eggs washed from plant material?), but we could do it.

Other more general instruments for reducing the risk of exporting pests are available or being developed, like area-wide management programs and systems approaches, that achieve quarantine security from multiple control components (Follet & Neven, 2006). All these in view of exclusion of pests. If exclusion fails, we can still use our knowledge to confine, and hopefully eradicate, the undesired "guest." In both cases, agriculture operators and international travelers should be made aware of the existence of destructive, exotic (plant) pests threatening to enter a country in which they are not known to occur, because it is even too clear that the success of plant quarantine programs greatly depend upon public cooperation with quarantine legislation (Berg, 1991).

However, whether quarantine regulations should be applied, and when, is not a decision for scientists. As Kahan (1982) expressed it many years ago.
Yet, in spite of the recognition of quarantines as a control measure, their effectiveness is controversial. The argument stems from the fact that we cannot measure how many pests and pathogens would have entered a new region had quarantine measures not been in effect.

Biologists should provide the rationale behind decisions of this type, not only the basic knowledge of pests (biology, taxonomy, detection, and identification), but also risk analysis (Bartlett, 2004) in order to establish the priorities for political decisions and for future research.

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PART II

THE TYLCV GENOME

CHAPTER 1

THE TOMATO YELLOW LEAF CURL VIRUS GENOME AND FUNCTION OF ITS PROTEINS

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1. OVERVIEW

Tomato yellow leaf curl as a whitefly-transmitted viral disease was first described in 1964 and 1966 (Cohen & Harpaz, 1964; Cohen & Nitzany, 1966), and proven to be caused by a geminivirus in 1988 (Czosnek et al., 1988). But it was not until 1990 (Rochester et al., 1990) and 1991 that the first molecular data on the genome of geminiviruses that cause the tomato yellow leaf curl disease became available (Kheyr-Pour et al., 1991; Navot et al., 1991).

Ever since, an increasing number of tomato (yellow) leaf curl virus genomes became molecularly characterised and were sequenced. For an updated compilation of the relevant tomato-infecting virus species and isolates or strains see Stanley et al. (2005) and Part III, Chapter 2.

In the following, an overview on the genome organisation of tomato (yellow) leaf curl viruses will be presented. Also, a brief description of the biological functions of the viral proteins will be given. The nomenclature including acronyms for some TYLCV species was changed since their first description, in the following the ICTV-approved designations given in Stanley et al. (2005) are used. Most data referred to are derived from studies with *Tomato yellow leaf curl virus* (TYLCV) [GenBank acc. no. X15656] (Navot et al., 1991), *Tomato yellow leaf curl Sardinia virus* (TYLCSV) [X61153] (Kheyr-Pour et al., 1991), *Tomato leaf curl virus* (ToLCV) [S53251] (Dry et al., 1993), and *Tomato leaf curl New Delhi virus* (ToLCNDV) [U15015, U15017] (Padidam et al., 1995), a bipartite TYLCV species.

Tomato (yellow) leaf curl viruses belong to the genus *Begomovirus* within the family *Geminiviridae*. Most begomovirus species have a bipartite genome of two circular single-stranded (ss)DNA molecules, DNA-A (2.6–2.8 kbases)

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and DNA-B (2.5–2.8 kbases). Begomoviruses are transmitted by the whitefly *Bemisia tabaci* in a circulative and persistent manner. TYLCV and TYLCSV were the first begomoviruses proven to possess a single genomic DNA. Consequently, essential viral functions, otherwise encoded by DNA-B, have to be provided by proteins encoded by the single DNA of TYLCV, TYLCSV, ToLCV, and all other true monopartite tomato (yellow) leaf curl viruses.

The molecular biology of geminiviruses has been extensively reviewed, with particular emphasis on replication (Gutierrez, 1999), interaction of the viruses with the plant host in general (Gutierrez, 2000; Hanley-Bowdoin et al., 2000; Hanley-Bowdoin et al., 2004), and in view of their potential to counteract the host defence by silencing suppression (Bisaro, 2006). The reader is referred to these excellent reviews for further details on specific aspects of geminivirus biology. In addition, some earlier reviews on geminiviruses focusing on cytology and pathology still merit attention (Goodman, 1981; Harrison, 1985).

Protein functions were often initially determined for other related begomoviruses, for instance *Tomato golden mosaic virus* (TGMV). Therefore, reference will be given also to these studies in case a particular protein function was better characterised of another begomovirus rather than TYLCV.

After delivery by the insect vector into the phloem of susceptible host plants, geminivirus particles find their way into permissive cells and subsequently into the nucleus of these cells; for well-illustrated overviews see Stanley et al. (2005) or Vanitharani et al. (2005). The molecular details of both processes remain still unresolved for any geminivirus, although nuclear entry has been extensively studied in the case of TYLCV. Once in the nucleus of a permissive cell, the ssDNA of the virion is converted in a double-stranded (ds) replicative intermediate DNA. For mastreviruses it has been shown that small virion-associated DNAs serve as primers for the complementary-strand synthesis. How this step in the multiplication cycle of begomoviruses including TYLCV is triggered remains as yet unknown. The dsDNA associates with histones and can be visualised as "minichromosomes" that serve as templates for transcription and subsequent rolling circle replication yielding multiple copies of plus-strand ssDNA. The ssDNA is either copied again into dsDNA or becomes associated with capsid/nuclear shuttle protein (NSP) for nuclear export. With the aid of movement proteins the DNA–protein complexes invade neighbouring cells. Ultimately, ssDNA-containing virions enter the vascular tissue and spread systemically throughout the host plant. In contrast to a lot of other begomoviruses, TYLCV is phloem-limited. Probably as a consequence of this restricted tissue tropism the monopartite TYLCV species are not mechanically transmitted. Therefore, their reverse genetics relies on plant inoculation using cloned

DNA inserted in *Agrobacterium tumefaciens* T-DNA vectors or biolistic DNA delivery techniques.

2. THE TYLCV GENOME

2.1. The intergenic region

2.1.1. Promoters and transcription

A graphic representation of the monopartite TYLCV genome is shown in Figure 1. The intergenic region of about 200 nucleotides contains the promoters for transcription of the viral-sense genes (V2 and V1) and the complementary-sense genes C1 and C4. Most of the transcript data on begomoviruses stem from analyses using TGMV (Hanley-Bowdoin et al., 1988; Sunter et al., 1989), *African cassava mosaic virus* (ACMV) (Zhan et al., 1991), *Abutilon mosaic virus*

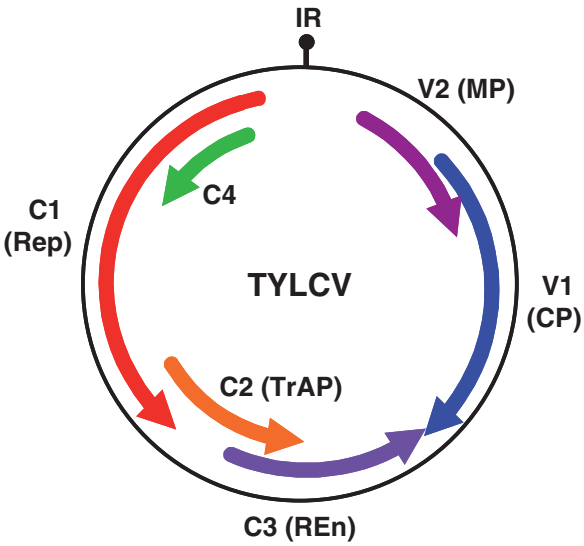

Figure 1. Genome organisation of a typical monopartite *Tomato yellow leaf curl virus*. The single-stranded virion DNA comprises between 2.7–2.8 kb. Genes (ORFs) of virion-sense (V) or complementary-sense strand polarity are designated (V) or (C), respectively. IR: intergenic region. The conserved inverted repeat flanking the invariant nonanucleotide sequence TAATATTAC is symbolised by a stem-loop. V1 encodes the capsid protein (CP), V2 a movement protein, C1 the replication initiator protein (Rep), C2 a transcriptional activator protein (TrAP), C3 a replication enhancer protein (REn), and C4 a symptom and movement determinant.

(AbMV) (Frischmuth et al., 1991) or ToLCV (Mullineaux et al., 1993). There are no transcript data on TYLCV, and the RNA-mapping data on TYLCSV remain of limited access (Bendahmane, 1994). The complementary-sense genes C2 and C3 are transcribed from a promoter located within the C1 gene (Mullineaux et al., 1993), or AC1 gene in case of bipartite begomoviruses (Sunter et al., 1989).

2.1.2. Replication origin and associated cis-acting DNA sequences

For a detailed review on geminivirus replication see Guterriez (1999). The most conspicuous sequence motif in the intergenic region are two inverted repeat sequences of 10–12 conserved bases flanking a 12–14 bases sequence containing the invariant nonanucleotide TAATATT|AC (Lazarowitz et al., 1992). The latter is the target sequence for cleavage by the Rep protein at the position marked by (|) (Laufs et al., 1995; Stanley, 1995).

Rep then remains covalently linked to the 5′ phosphate of the adenosine and the 3′ OH of the thymidine is thought to serve as the primer for virion-strand DNA synthesis by cellular DNA polymerase(s). Upon passing again the inverted repeat sequences after one "round of replication" a second subunit of the Rep multimer, probably associated with the replisome complex, cleaves at the T|A-junction and the free 3′ OH is thought to attack the phosphotyrosyl bond of the Rep-linked adenosine and by transesterification forms a phosphodiester bond with that adenosine creating a circular ssDNA (Laufs et al., 1995). For this transesterification-associated process the formation of a stem-loop structure is required (Orozco & Hanley-Bowdoin, 1996), however not for the initial Rep cleavage that triggers the start of virion-strand DNA synthesis (Heyraud et al., 1993). The inverted repeat/nonanucleotide sequence is frequently referred to or drawn as "stem-loop", the hallmark of a geminivirus replication origin. A lot of the biochemical details of these processes were resolved using TYLCSV DNA and TYLCSV Rep protein (Heyraud-Nitschke et al., 1995; Laufs et al., 1995).

2.1.3. Other origin-associated sequences

In the vicinity, mostly but not exclusively 5′ of the inverted repeat/nonanucleotide sequence, short (8–12 nucleotides) direct repeat sequences, "iteron sequences", are found (Argüello-Astorga et al., 1994). These are recognised and bound by Rep protein, and are assumed to act as specificity determinants for interaction of a given Rep protein with its cognate coding DNA (Eagle et al., 1994; Fontes et al., 1994a, b). Additional evidence for such a sequence-specific origin recognition was also derived by using the two species TYLCV and TYLCSV (Jupin et al., 1995), and have since led to an elaborated model for specificity of geminivirus Rep-origin recognition in general (Argüello-Astorga & Ruiz-Medrano, 2001). However, biochemical data for the various TYLCV species on the direct binding of Rep to such sequences remain limited (Akbar Behjatnia et al., 1998; Chatterji et al., 1999, 2000).

An interesting new twist in the potential importance of intergenic region sequences for virus-host interactions is illustrated by the recent finding that these sequences in an as yet unexplained fashion may contribute to silencing of geminivirus gene expression (Pooggin et al., 2003).

3. TYLCV PROTEIN FUNCTIONS

In the following a brief summary on the functions of the different proteins encoded by the viral genes (ORFs) will be given. Emphasis will be on experiments involving the proteins of TYLCV, TYLCSV, ToLCV, and ToLCNDV, complemented by analyses of other begomoviruses where appropriate.

3.1. The capsid protein

The capsid protein (CP) of TYLCV has a size of about 30 kDa and is encoded by ORF V1. Note that in some early publications the nomenclature of the viral-strand polarity genes was inverted between V2 and V1. This has been changed by the convention that V1 is now used for the CP coding ORF and V2 for the "precoat" or movement protein-encoding ORF.

Contrary to some bipartite begomoviruses, the CP of TYLCSV is essential for infectivity (Wartig et al., 1997). The CP binds and serves to package the ssDNA (Palanichelvam et al., 1998), it localises to the nucleus of infected cells and is often found associated with the nucleolus (Kunik et al., 1998; Rojas et al., 2001). Whether encapsidation of the viral ssDNA occurs within the nucleus, or whether ssDNA–CP complexes move out of the nucleus and final encapsidation takes place in the cytoplasm remains open. Contrary to mastreviruses, where virions are obvious within the nuclei of infected cells, begomovirus particles are only visible in the nuclei of young and early infected cells (Kim et al., 1978). The same is true for TYLCV. Nevertheless, the TYLCV CP is karyophilic and bipartite nuclear localisation sequences essential for CP function and virus infectivity were mapped in the amino-terminus of the protein (Kunik et al., 1998; Palanichelvam et al., 1998). Also, determinants for CP subunit assembly have been mapped to the N- and C-terminal parts of the protein (Hallan & Gafni, 2001). As TYLCV is monopartite and none of the encoded proteins share extensive similarity with the NSP and movement protein (MP) encoded by ORFs BV1 and BC1 on DNA-B of bipartite begomoviruses, these functions have to be assured by proteins encoded by the single DNA of TYLCV. Experiments demonstrating the capacity of the TYLCV CP to facilitate export of both ssDNA and dsDNA are consistent with this assumption (Rojas et al., 2001). Hence, the CPs of TYLCV and of other monopartite begomoviruses are to be considered as the functional homologues of the NSP of bipartite begomoviruses, an assumption also supported by a potential common origin in evolution (Kikuno et al., 1984).

In addition to its role to assure systemic movement of virions throughout the vascular system of an infected plant, the geminivirus CP determines

the transmission specificity by the insect vector (Briddon et al., 1990). For TYLCSV, a west Mediterranean TYLCV species, amino acids of the CP essential for transmission by *B. tabaci* were mapped (Noris et al., 1998). These analyses were further supported by the characterisation of whitefly non-transmissible mutants of *Watermelon chlorotic stunt virus* (WmCSV) (Kheyr-Pour et al., 2000) and AbMV (Höhnle et al., 2001). Meanwhile the three-dimensional structures of the *Maize streak virus* (MSV) and ACMV CP and capsomers have been modelled based upon cryo-electronmicroscopy (Böttcher et al., 2004; Zhang et al., 2001). Interestingly, amino acids critical for whitefly transmission, are located in an exposed loop, which may be part of a protein structure that interacts with insect proteins or other components required for transmission.

In the haemolymph of *B. tabaci*, TYLCV interacts via the CP also with GroEL proteins of the insect's endosymbiont(s) (Morin et al., 1999, 2000). Interfering with the CP-GroEL interaction blocks TYLCV transmission. The molecular details of this interaction and its biological significance are further elaborated upon in Part III, Chapter 2.

3.2. The movement protein (MP)

Movement, a function assured for bipartite begomoviruses by the MP encoded by BV1 on DNA-B, the second genome component, in the case of TYLCV involves three distinct proteins: the CP, the protein encoded by ORF V2, and also the protein encoded by ORF C4.

The V2-encoded (movement) protein has a size of around 13 kDa, and an equivalent ORF is found in Old World begomoviruses as well as in *Beet curly top virus* (BCTV). Mutations in this gene lead to a disturbance of the ssDNA/dsDNA ratio and affect symptom expression in the plant (Rojas et al., 2001; Wartig et al., 1997). The latter may vary between different TYLCV species: TYLCSV V2 mutants are symptomless in tomato and the virus does not move, whereas a ToLCV V2 mutant systemically moved in tomato, but remained equally symptomless (Rigden et al., 1993). Interestingly, V2 mutants of the bipartite ToLCNDV still led to mild disease symptoms but also had much reduced DNA levels, whereas expression of both the MP as well as the NSP encoded by DNA-B was essential for infectivity (Padidam et al., 1996). This indicates that, despite the presence of a DNA-B-encoded movement protein, the V2 equivalent AV2 of ToLCNDV somehow influences the amount of viral DNA to be "moved" by the other viral movement factors.

As most analyses concerning the DNA-B-encoded proteins BV1 (movement protein) and BC1 (nuclear shuttle protein) employed New World begomoviruses like TGMV or *Squash leaf curl virus* (SqCLV), more distant relatives of Old World begomoviruses, in particular the monopartite ones, the reader is kindly referred to the original literature on these bipartite begomoviruses (Sanderfoot & Lazarowitz, 1996). For an excellent review of the subject see also (Lazarowitz & Beachy, 1999).

3.3. The replication initiator protein (Rep)

The Rep protein is the only geminivirus-encoded protein indispensable for their genome replication (Elmer et al., 1988), and the TYLCVs are no exception to the rule. Rep is a 40–41 kDa protein encoded by ORF C1 of complementary sense polarity. In bipartite begomoviruses it is referred to as AC1 or AL1 protein. The Rep proteins of TYLCSV and TGMV are the biochemically best-characterised examples of geminivirus replication initiators (Hanley-Bowdoin et al., 2000; Laufs et al., 1995), and the TYLCSV Rep protein is the only geminivirus protein for which structural data of true atomic resolution are available (Campos-Olivas et al., 2002).

The Rep protein has a modular domain structure and forms oligomers of distinct classes or higher-order multimers (Orozco et al., 1997, 2000). Its prime function for viral DNA replication is the recognition of origin sequences located in the intergenic region, the conserved inverted repeat flanking the invariant nonanucleotide (see above), followed by a sequence-specific DNA cleavage. It then recruits (components of) the replisome and triggers initiation of viral-strand DNA synthesis by a cellular polymerase (Gutierrez, 1999). Which polymerase is used remains unknown. Rep is not only able to specifically recognise and cleave DNA sequences, it also is a nucleotidyl transferase and catalyses the formation of ssDNA circles as end products of rolling circle replication. The origin cleavage and joining activity of Rep resides in its amino-terminal domain of about 120 amino acids (Campos-Olivas et al., 2002; Heyraud-Nitschke et al., 1995). For further details, the reader is referred to (Gutierrez, 1999; Hanley-Bowdoin et al., 2000; Laufs et al., 1995).

Besides this catalytic activity of Rep, the very same amino-terminal domain also mediates a sequence-specific double-stranded DNA binding by Rep. This way it is assured that only genome components cognate to a given Rep are multiplied. The recognition targets are the so-called iteron sequences located in the vicinity of the inverted repeat (stem-loop) motif (Akbar Behjatnia et al., 1998; Argüello-Astorga et al., 1994). So are the amino-terminal Rep domains or the cognate intergenic region sequences harbouring the iterons not exchangeable between the otherwise close relatives TYLCV and TYLCSV (Jupin et al., 1995). Similar findings were reported for a variety of other begomoviruses and BCTV (Choi & Stenger, 1995). This specific dsDNA binding is essential for virus multiplication. Whether it only serves to autoregulate Rep expression or whether it is also essential for DNA replication as such is not entirely clear. In that respect it is curious that the satellite DNA associated with ToLCV from Australia does not have any iteron or iteron-like sequences, yet is perfectly replicated along with the viral genome (Lin et al., 2003). The same is true for the beta-DNAs, additional DNA components required for the disease symptoms of various monopartite Asian and African begomoviruses (Briddon & Stanley, 2006).

An interesting potential alternative to regulate Rep activity at the origin of replication has been reported for *Mung bean yellow mosaic India virus*

(MYMIV) CP that binds to and inhibits the catalytic functions of Rep *in vitro* (Malik et al., 2005); whether this has also some *in vivo*-significance remains to be determined.

Another biochemical activity of Rep is its capacity to hydrolyse nucleoside triphosphates, and mutants of TYLCSV Rep impaired in this function are replication deficient (Desbiez et al., 1995). Based upon sequence comparisons, it was suggested that geminivirus Rep proteins are members of a superfamily (SFIII) of helicases (Iyer et al., 2004; Koonin, 1993). Biochemical proof for such a helicase activity of TYLCSV Rep has been achieved only very recently (Clérot & Bernardi, 2006).

The three-dimensional structure of the amino-terminal catalytic domain of TYLCSV Rep revealed an unexpected structural similarity with a variety of RNA-binding proteins as eukaryotic poly-A tract-binding protein or splicing factor U1A and with the dsDNA-binding domains of key mammalian tumour virus proteins like large T-antigen of Simian Virus 40 or E1A protein of papilloma viruses (Campos-Olivas et al., 2002). This similarity placed the geminivirus Rep proteins within a large family of structurally related rolling circle replication initiator proteins and provided novel insights into the evolution of proteins of quite different origin that act in nucleic acid metabolism (Dyda & Hickman, 2003; Hickman et al., 2002).

Apart from these activities of Rep, which are more or less directly linked to its function as a rolling circle replication initiator and its auto-interaction to form oligomers, Rep interacts also with a variety of other proteins. Among these is a second geminivirus protein, the replication enhancer protein REn, encoded by ORF C3 (or AC3/AL3, respectively) (Castillo et al., 2003; Settlage et al., 2005). Contrary to Rep, the REn protein is not essential for viral DNA replication (see below).

A further and important activity of the Rep protein is its binding to cellular proteins, in particular the retinoblastoma protein pRB (Kong et al., 2000). In fact, the existence of RB-related proteins in plants was first proven using geminivirus Rep proteins (Xie et al., 1996). In the current model of geminivirus DNA replication, it is thought that via Rep RB-related protein interaction the virus triggers extra G0/G1 S-phase cell cycle transitions in infected cells. This way, enzymes required for DNA replication and accessory factors like proliferating cell nuclear antigen (PCNA) become available and are recruited for multiplication of the viral DNA (Gutierrez, 2000; Hanley-Bowdoin et al., 2000). Indeed, TYLCSV Rep has been shown to directly interact with PCNA, possibly to recruit this "sliding clamp" to the viral origin and the replisome (Castillo et al., 2003). In that context it is interesting that also the Rep of mastreviruses directly interacts with replication factor C (RF–C), the "clamp loader", possibly again to ultimately recruit PCNA to the origin and the replisome (Luque et al., 2002).

Yet another cellular protein with which TYLCSV Rep interacts with is the E2-SUMO-conjugating enzyme NbSCE1 (Castillo et al., 2004). Here, an interesting connection of TYLCV replication with the sumoylation pathway has

been uncovered. Whether Rep itself is a target of sumoylation or what other significance such an interaction for begomovirus biology may have remains, however, to be determined. Nevertheless, the authors showed that replication of TGMV was reduced in transgenic plants expressing SUMO sense or antisense transcripts, an observation that further supports the relevance of the sumoylation pathway for begomovirus multiplication.

3.4. The replication enhancer protein (REn)

The second protein of TYLCV and other begomo- and curtoviruses, which is more or less directly involved in viral DNA replication, is the about 16 kDa replication enhancer protein REn encoded by ORF C3 (or AC3/AL3, respectively). It is not essential for viral DNA replication as such, yet it boosts the amount of viral ds- and ssDNA that accumulates during infection and thus indirectly influences the extent of symptom expression (Gutierrez, 1999; Settlage et al., 2005; Sunter et al., 1990). REn of TGMV was shown to interact with itself (oligomerisation), with Rep and with pRB. This implies the existence of a complex network of interactions between Rep, REn, and pRB. Also TYLCSV REn has been shown to not only interact with Rep but also with PCNA (Castillo et al., 2003), the sliding clamp of the replisome. Hence, a coherent picture appears to emerge where Rep, REn, and accessory factors like PCNA of the replisome act in a balanced and concerted way to assure efficient geminivirus DNA replication.

To even further enlarge and complicate the network of interacting proteins, ToLCV REn was found to bind to a NAC-domain protein of tomato (SlNAC1), a class of proteins that are often induced upon pathogen infection (Selth et al., 2005). Yet, the exact way in which the REn/SlNAC1 connection contributes to the dramatic increase of DNA replication as a consequence of REn action remains to be elucidated.

3.5. The transcriptional activator protein (TrAP)

Encoded by ORF C2 (AC2/AL2), begomo- and curtoviruses express an about 15 kDa protein with a positively charged N-terminus including a nuclear localisation sequence (van Wezel et al., 2001), a central core with a zinc finger-like region (Noris et al., 1996), and a distinct acidic C-terminal activation domain (Hartitz et al., 1999). For a recent review of the multiple TrAP functions see (Bisaro, 2006). Together with the C3-encoded REn TrAP is synthesised from a bi-cistronic messenger expressed from a strong promoter located upstream C3 in the Rep coding sequence C1 (Sunter et al., 1989; Townsend et al., 1985). TrAP enhances transcription of the virion-sense promoter of DNA-A and the BV1 and BC1 promoters of DNA-B in bipartite begomoviruses (Haley et al., 1992; Sunter & Bisaro, 1992), hence its name. TrAP is not essential for DNA replication *per se*, and in case of TYLCSV it is even

dispensable for infectivity in *N. benthamiana*, but required for infection of tomato (Wartig et al., 1997). TYLCSV C2 protein binds both ss- and dsDNA in a sequence non-specific manner, and its central core domain is sufficient to do so (Noris et al., 1996). It therefore may be required to direct host-specific cellular transcription factors indirectly to responsive promoters, similarly as the herpesvirus VP 16. Consistent with its role to activate transcription is the nuclear localisation of TrAP, which appears to be regulated by its phosphorylation status (Wang et al., 2003).

Further to their role as transcription activators, the AC2/C2 proteins of several begomoviruses including *Tomato yellow leaf curl China virus* (TYLCCNV) have been shown to act as silencing suppressors (Dong et al., 2003; Trinks et al., 2005; van Wezel et al., 2002; Vanitharani et al., 2005; Voinnet et al., 1999). The detailed molecular mechanism of this silencing suppression activity by AC2/C2 proteins remains to be elucidated (Chellappan et al., 2004). Direct binding and sequestration of siRNAs or miRNAs, similar to the mode of action of the p19 silencing suppressor (Vargason et al., 2003), appears not to be the case (Chellappan et al., 2005). Rather, the induction of cellular factors involved in the negative regulation of the host's silencing machinery may be the reason for the suppressor activity.

Interestingly, also transactivation-independent silencing suppression by BCTV C2 and a TGMV C2 mutant has been demonstrated, probably due to inhibition of a cellular adenosine kinase (ADK) that becomes activated upon virus infection (Wang et al., 2005). A link between C3-triggered silencing suppression and the host's methyl cycle illuminates yet another facet of the multitude of ways by which (gemini)viruses counteract the host's defence.

The results by Wartig et al. (1997) on host-specific requirement of the TYLCSV C2 protein for elicitation of disease – required in tomato but dispensable in *N. benthamiana* – may provide further clues on the mechanism of silencing suppression, if this were the reason for such a host-dependent infectivity phenotype.

3.6. The C4 protein

Embedded within the C1 (Rep) gene but in a different reading frame, an ORF with a coding capacity for an about 12 kDa protein is found on the DNA (A) of begomoviruses and curtoviruses. Its amino acid sequence is the least conserved one among all geminivirus proteins. Although a C4 ORF is present in ACMV, the first geminivirus genome ever sequenced, its importance had escaped notice for a while. Early experiments with TGMV had shown that C4 is not essential for infectivity (Elmer et al., 1988), but mutagenesis experiments with TYLCSV and ToLCV proved the essential nature of the C4 protein and its biological relevance as a pathogenicity factor (Jupin et al., 1994; Rigden et al., 1994). Moreover, for TYLCSV the C4 protein contributes also to the spread of the virus (movement) throughout the plant. By contrast, further experiments with TGMV and *Potato yellow mosaic virus* (PYMV) confirmed that C4 is non-essential, (Pooma & Petty, 1996; Sung & Coutts, 1995). However, in the case of

TGMV C4 protein apparently regulated expression of Rep (Gröning et al., 1994). Hence, the C4-encoded protein turned out to have clearly different functions in the monopartite TYLCVs and curtoviruses (Stanley & Latham, 1992) as opposed to begomoviruses from the New World.

Meanwhile, the AC4 proteins have gained considerable interest after it was demonstrated that they also act as silencing suppressors (Chellappan et al., 2005; Vanitharani et al., 2004). In fact, different begomoviruses employ complementary strategies to counteract the host's silencing defence: for instance, the AC4 proteins of ACMV-[CM] and *Sri Lankan cassava mosaic virus* (SLCMV) are silencing suppressors, whereas in *East African cassava mosaic virus* (EACMV) and *Indian cassava mosaic virus* (ICMV) the respective AC2 proteins rather than the AC4 proteins have silencing suppressor activity. As AC4 and AC2 proteins target different steps in the siRNA/miRNA silencing pathways, the spectacularly enhanced pathogenicity observed in double infections by ACMV-[CM] (silencing suppression by C4) and EACMV (silencing suppression by C2) is readily explained. Concerning TYLCV, the reversal of TYLCSV-derived transgene silencing observed upon superinfection of transgenic plants with TYLCSV has not yet allowed the determination whether this was due to action of C2, C4, or yet another protein (Lucioli et al., 2003; Noris et al., 2004). Hence, the interesting question whether the C4 proteins of the various different TYLCV or ToLCV species act also as silencing suppressors remains in suspense.

4. DNA BETA AND THE βC1 PROTEIN

During the last years, a peculiar class of DNA molecules has been found associated with certain Old World begomoviruses; for a review see Briddon and Stanley (2006). These viruses have been classed as monopartite as their DNA-A readily infected the experimental host *N. benthamiana*, but when introduced into their original host plants only symptomless infections resulted. The search for potentially missing DNA components led to the discovery of an additional circular ssDNA molecule of about 1,350 bases, named DNA-β. It shares a stretch of sequence similarity with the 682 bases long satellite DNA of ToLCV (Dry et al., 1997), the "satellite-conserved region", but differs from the sat-DNA in size and by bearing an ORF βC1 that encodes an about 14 kDa protein. DNA-β contains the conserved inverted repeat sequences that flank the invariant nonanucleotide, the origin of geminivirus replication hallmark and is replicated along with the DNA of the virus it associates with. DNA-β does not possess any iteron or iteron-related sequences and is packaged into virus particles that are transmitted by the insect specified by the viral capsid protein.

DNA-β molecules were first described for *Ageratum yellow vein virus* (AYVV) (Saunders et al., 2000) and *Cotton leaf curl virus* (CLCuV) species (Briddon et al., 2001), where they are required for infection of the hosts *Ageratum conyzoides* or cotton, respectively. Since their first description, an ever-increasing number of cases in which a DNA-β is associated with a monopartite Old World begomovirus

were reported (Briddon & Stanley, 2006). Expression of the βC1 protein results in a considerable increase in symptom severity of the respective begomovirus (Saeed et al., 2005; Saunders et al., 2004). This is also true for the TYLCVs, where β DNAs accompany *Tomato leaf curl China virus* (ToLCCNV) (Zhou et al., 2003) and *Tomato yellow leaf curl Thailand virus* (TYLCTHV) (Li et al., 2004). In some cases, the DNA-A by itself is infectious in the original host (Yin et al., 2001), whereas in others the presence of DNA-β is required (Li et al., 2004). Such behaviour as a host-specific pathogenicity determinant suggested that the βC1 protein might act as a silencing suppressor, comparable to other viral pathogenicity factors. Indeed, this was recently shown for the βC1 protein of ToLCCNV (Cui et al., 2005).

Intimately connected to the discovery of the DNA-β satellite-like molecules, yet another class of small DNAs associated with certain Old World monopartite begomoviruses was found, the so-called DNA-1 molecules (Mansoor et al., 1999). They share an A-rich sequence with DNA-β and encode a nanovirus Rep-related protein. Despite the increasing number of DNA-1 molecules described nothing at all is currently known about their function for begomovirus biology (Briddon et al., 2004).

5. OUTLOOK

Although the begomoviruses causing the tomato (yellow) leaf curl disease represent the geminiviruses with the smallest genomes, just 2.7–2.8 kb, they encode a sufficiently complex array of proteins, many of which are multifunctional, to warrant efficient multiplication, spread, and dissemination of the viruses. We only begin to understand the intricacies of the virus-host interplay, which bears many diverse elements of mutual attack and defence. The discovery and analysis during recent years of novel DNA components often associated with the "canonical" monopartite genomes of some TYLCV and ToLCV species has further complicated the picture. The fact that the tomato (yellow) leaf curl viruses in some cases require more than just one DNA may well reflect their unique position in the evolution of the geminivirus genome from monopartite to bipartite. Many questions related to the serious TYLCV-caused tomato disease still wait to be answered, and there may be more surprises "just around the corner".

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CHAPTER 2

MOLECULAR BIODIVERSITY, TAXONOMY, AND NOMENCLATURE OF TOMATO YELLOW LEAF CURL-LIKE VIRUSES

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1. OVERVIEW

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating plant diseases in the world and is spreading fast, covering more than 20 countries across the globe. This disease is caused by several viruses belonging to different species which altogether are referred to as "Tomato yellow leaf curl viruses" (TYLCV). Taxonomically they all belong to at least six species and 15 strains of viruses. This chapter has multiple implications such as taxonomic, nomenclatural, evolutionary, and practical, and its purpose is to provide a clear vision on the status of knowledge of molecular diversity of TYLCV-like viruses, to offer an up-to-date list of virus names and their abbreviations with their corresponding GenBank accession numbers. This study also provides a sense of geminivirus evolution in a short span of time as well as on a long timescale. Human interference is being considered as the major factor for the recent spread of these viruses, thereby promoting and selecting new recombinants, and it is probably only the beginning of what we can envisage for many other geminiviruses on the planet earth. However, it is becoming apparent that TYLCV-like viruses have a better biological fitness to compete with locally present viruses in new ecological niches and thus it would be interesting to unravel and understand more about these biological characteristics in the near future to better appreciate future emergences of geminiviruses in the world.

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 85–118.

2. INTRODUCTION

The tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases of tomato (*Solanum lycopersicum*) crop, causing enhanced damage and encroaching new areas every year (Czosnek & Laterrot, 1997). TYLCD was first identified in Israel in 1930 and since the 1960s, has become the most notorious disease in the Mediterranean region, sub-Saharan Africa, Caribbean islands, Australia, and in several Asian countries like China, India, and Japan. In the 1990s this disease was reported from several US states like Florida, Georgia, and Louisiana (Czosnek et al., 1990; Cohen & Antignus, 1994; Czosnek & Laterrot, 1997; Nakhla & Maxwell, 1998; Polston et al., 1999). A complex of several different geminivirus species belonging to the genus *Begomovirus*, has been associated with TYLCD, also called TYLCV-like viruses, all of which have a monopartite ssDNA genome and are naturally transmitted by the whitefly (*Bemisia tabaci*; Hemiptera: Aleyrodidae) (Moriones & Navas-Castillo, 2000).

The first identified virus TYLCV was named after the disease it causes in Israel (Czosnek & Laterrot, 1997) and by extension and simplification, geminiviruses causing similar symptoms on tomato throughout the world are referred by the same or similar names. Subsequently it was realized that several distinct viruses, recognized by their genomic sequences, and belonging to different virus species were causing similar symptoms on tomato. Thus, new names were created to resolve this situation, despite this it is confusing for many scientists who are not well versed with the taxonomy and nomenclature of viruses. In addition, the frequent recombination occurring between geminiviruses (Padidam et al., 1999), the similar and variable symptomatology on tomato, led to some confusion as far as the exact number of viruses isolated and their pertaining to several virus species. The classification of geminiviruses is now mostly done on the basis of the molecular variability of the DNA-A component and the wealth of virus sequencing in the last decade led to the description of more than 57 species of tomato geminiviruses in the world. This chapter is aimed at shedding some light on this molecular diversity to better appreciate the relationships between closely related viruses to the firstly described TYLCV from Israel. It is also an opportunity to describe the geographical distribution of these viruses and in particular the recent spread of TYLCV across the globe, indicating the huge impact of human interference on the evolution of plant viruses.

Solanaceous crops in general are very good hosts for geminiviruses and tomato in particular is the host for the largest number of geminivirus species. There are about 57 species listed to date, and with more than 50 potential new species yet to be identified, originating from all over the world (Fauquet et al., 2007). Though, TYLCV is neither the most important nor the most devastating tomato geminivirus, it is certainly the one that is fast spreading across the world for which we have documentation. However it is important to realize that there are many other tomato geminivirus diseases in different parts of the world that are equally devastating.

3. GENOME ORGANIZATION

TYLCV and its related virus species are members of the genus *Begomovirus*, family *Geminiviridae*, the genome of which is strictly monopartite (Kheyr-Pour et al., 1991; Navot et al., 1991) and it encodes six open reading frames (ORFs), four on the complementary (–) strand (C1, C2, C3, and C4) and two on the viral (+) strand (V1 and V2) (Navot et al., 1991). So far no satellite DNA molecules associated with TYLCV-like viruses have been identified or isolated (for details see Part II, Chapter 1).

4. TOMATO BEGOMOVIRUS TAXONOMY AND NOMENCLATURE

The taxonomy and nomenclature of geminiviruses have been under consideration by the *Geminiviridae* study group for the last 15 years. This intense activity was triggered by the identification of a huge number of geminiviruses and the increasing difficulty to name and classify them in a reliable and convenient way. The first step was to build a comprehensible system for nomenclature of geminivirus species that would have flexibility to accommodate the large number of known and unknown viruses and therefore offer an unlimited number of combinations (Fauquet et al., 2000). The adopted system essentially involves adding the name of a country, a city, or a location before the word "virus" within the virus name, i.e., *Tomato yellow leaf curl Sardinia virus*. This has been used extensively for tomato geminiviruses as exemplified in Figure 2. The second step was to establish a list of species demarcation criteria for geminiviruses in general and for begomoviruses in particular. A first attempt was made in 1995 (Rybicki et al., 1994) and was further refined in 2003 (Fauquet & Stanley, 2003). This list comprises many different types of criteria including molecular, biological, and serological, but undoubtedly one of the most widely used criterion is the percentage of identity between the sequences of the DNA-A component of two different viruses.Firstly, it was decided that only the A component sequence would be considered for taxonomic consideration as many viruses do not have a B component, and some are mono-bipartite viruses. Secondly, the species demarcation threshold has been fixed to 89%, however this is only one indicator and not an absolute rigorous threshold, in other words there could be exceptions. This criterion has however been used consistently and is the basis for the current classification (Stanley et al., 2005; Fauquet et al., 2007). TYLCV-like viruses are no exception and have followed this rule with some difficulties due to extensive genomic recombinations between some members of different species (see below). Practically, when a new geminivirus species is identified from the complete sequence of its DNA-A component, a new name is created either by the combination of words describing the symptoms not previously used (*Tomato **rugose** mosaic virus*), or by the addition of a location name for virus names already used (*Tomato leaf curl **Gujarat** virus*).

Recently, due to the large number of viruses within a species, the *Geminiviridae* study group recognized the need for a common organization system below the species level (Fauquet & Stanley, 2005). So far it was accepted that all viruses pertaining to a species would be called "isolates" and that we would have at least two different levels; – the strain level corresponding to a stable inherited trait (differential symptom, host range, or transmission, sequence deletion, recombination or variation, etc.), – the variant level corresponding to minor sequence differences probably not stably inherited in the next generations. This has been translated into the names of the viruses, in such a way that one can get the minimal basic information to locate in space and time a virus isolate and can read if the species or strain status has been given to this virus (Fauquet & Stanley, 2005). For example we can list some of the isolates belonging to the species *Tomato yellow leaf curl virus* as follows:

Tomato yellow leaf curl virus		
Tomato yellow leaf curl virus – Gezira		(TYLCV-Gez)
Tomato yellow leaf curl virus – Gezira [Sudan:1996]	AY044138	(TYLCV-Gez [SD:96])
Tomato yellow leaf curl virus – Iran		(TYLCV-IR)
Tomato yellow leaf curl virus – Iran [Iran]	AJ132711	(TYLCV-IR[IR])
Tomato yellow leaf curl virus – Israel		(TYLCV-IL)
Tomato yellow leaf curl virus – Israel [China:Shangai 2:2005]	AM282874	(TYLCV-IL [CN:SH2:05])
Tomato yellow leaf curl virus – Israel [Cuba]	AJ223505	(TYLCV-IL [CU])

The species names are not abbreviated and are written in italics, the strain names are not italicized and can be abbreviated, the isolate names are not italicized and some strain (TYLCV-Iran; TYLCV-Mild; TYLCV-Gezira) and isolate descriptors (TYLCV-[Israel:Rehovot:1986]) are added to the name. The descriptor of the strain level is written before the square brackets, while the isolate descriptors are between brackets and are composed of the country, the location and the year of sampling, when available. The descriptor for the strain can be a symptom type, a location or a country, using the first one described irrespective if all the members of that strain do or do not fit with that descriptor. Variants are by default not described and are represented by isolates within strains (see Tables 1 and 3). Formally species names are not abbreviated, and in most of the publication, species names are written once to indicate the classification of the isolates used in the publication, however in this specific chapter, species names will be used several times and we therefore propose to exceptionally abbreviate the species names as well (i.e., Tomato yellow leaf curl virus, "TYLCV").

Consequently, for geminiviruses there is a perfect equivalence between nomenclature and classification and before a name is given to a new geminivirus isolate, one should enquire about the classification of this new isolate, its classification and taxonomy, which will in turn determine the way of writing its name. This system is also important in order to "read" correctly the virus names in publications and reports, and also for different scientists to "speak" a common language understood by everybody. For example TYLCV-Iran is a strain of the species TYLCV isolated from Iran, but ToLCIRV is a new virus species called Tomato leaf curl Iran virus, and this abbreviation should be completed with the descriptors of that isolate for example: Tomato leaf curl Iran virus – [Iran:Iranshahr], ToLCIRV-[IR:Ira].

5. GEMINIVIRUS DISEASES OF TOMATO

At least 57 different species of geminiviruses (mostly begomoviruses), are reported to be capable of infecting tomato (Table 1). Among the tomato-infecting monopartite geminiviruses, *Tomato pseudocurly top virus* (TPCTV) is the only member of the genus *Topocuvirus* and *Tomato leaf roll virus* (ToLRV) is a member of the genus *Curtovirus* and all other tomato-infecting geminiviruses are begomoviruses. Both monopartite and bipartite geminiviruses infect tomato. Some monopartites, only from the Old World (OW) have been found to be associated with satellite DNAs like DNA-β (Figure 1). A large majority of these viruses are geographically distributed between Asia, India, Africa, and America, there are however a few exceptions to this rule (TYLCIDV, ToLCV, TYLCKaV, and ToLCPV) and there is no obvious explanations for it. TYLCV is part of a so-called TYLCV cluster in the African branch that does comprise 6 species of monopartite begomoviruses. The updated list of geminiviruses infecting tomato has been published by Fauquet et al. (2003), Fauquet and Stanley (2005), and Fauquet et al. (2007).

6. TOMATO BEGOMOVIRUSES IN THE NEW WORLD

A large number of bipartite tomato-infecting geminiviruses has been reported from the New World (NW) mostly from the American continents, including the Caribbean islands. These include Tomato mottle virus (ToMoV) from Florida, Tomato yellow mosaic virus (ToYMV) from Venezuela, Tomato severe leaf crumple virus (ToSLCV) from Central America, Tomato leaf crumple virus (TLCrV), Tomato leaf curl Sinaloa virus (ToLCSinV), Tomato mottle Taino virus (ToMoTV) and Chino del tomate virus (CdTV) from Mexico, Tomato rugose mosaic virus (ToRMV), Tomato severe rugose virus (ToSRV), Tomato chlorotic mottle virus (ToCMoV), Tomato golden mosaic virus (TGMV), Tomato golden mottle virus (ToGMoV), Tomato severe leaf curl virus (ToSLCV), from Brazil, Tomato mosaic Havana virus (ToMHV), Potato yellow mosaic Trinidad virus (PYMTV) in Trinidad, and Potato yellow mosaic virus (PYMV) in Martinique, and Guadeloupe (Fauquet & Stanley, 2005).

Table 1. List of 57 virus isolates (written in black) used in this chapter belonging to 57 different species (written in green). The accession number of the complete A component sequence is indicated in the second column and the abbreviation of the name of the virus isolates is indicated in the third column

Chino del tomate virus		
Chino del tomate virus – [Mexico:Sinaloa:Soybean:2005]	DQ347945	CdTV-[MX:Sin:Soy:05]
Merremia mosaic leaf curl virus		
Merremia mosaic virus – [Puerto Rico:]	AF068636	MeMV-[PR:]
Potato yellow mosaic Panama virus		
Potato yellow mosaic Panama virus – [Panama:Divisa:Tomato]	Y15034	PYMPV-[PA:Div:Tom]
Potato yellow mosaic Trinidad virus		
Potato yellow mosaic Trinidad virus – [Trinidad and Tobago:Tomato]	AF039031	PYMTV-[TT:Tom]
Potato yellow mosaic virus		
Potato yellow mosaic virus – [Venezuela]	D00940	PYMV-[VE]
Tomato chino La Paz virus		
Tomato chino La Paz virus – [Mexico:Sinaloa MM1:2005]	DQ347948	ToChLPV-[MX:SinMM1:05]
Tomato chlorotic mottle virus		
Tomato chlorotic mottle virus – [Brazil:Igarape 1:1996]	DQ336353	ToCMoV-[BR:Iga1:96]
Tomato curly stunt virus		
Tomato curly stunt virus – [South Africa:Onderberg:1998]	AF261885	ToCSV-[ZA:Ond:98]
Tomato golden mosaic virus		
Tomato golden mosaic virus – Yellow vein [Brazil]	K02029	TGMV-YV[BR]
Tomato golden mottle virus		
Tomato golden mottle virus – [Guatemala:R2:1994]	AF132852	ToGMoV-[GT:R2:94]
Tomato leaf curl Arusha virus		
Tomato leaf curl Arusha virus – [Tanzania:Tengelu:2005]	DQ519575	ToLCArV-[TZ:Ten:05]
Tomato leaf curl Bangalore virus		
Tomato leaf curl Bangalore virus – [India:Bangalore 5]	AF295401	ToLCBV-[IN:Ban5]
Tomato leaf curl Bangladesh virus		
Tomato leaf curl Bangladesh virus – [Bangladesh:2]	AF188481	ToLCBDV-[BD:2]
Tomato leaf curl China virus		
Tomato leaf curl China virus – [China:Guangxi 18:2002]	AJ558119	ToLCCNV-[CN:Gx18:02]
Tomato leaf curl Guangdong virus		
Tomato leaf curl Guangdong virus – [China:Guangzhou 2:2003]	AY602165	ToLCGuV-[CN:Gz2:03]

(continued)

Table 1. (continued)

Tomato leaf curl Guangxi virus		
Tomato leaf curl Guangxi virus – [China:Guangxi 1:2003]	AM236784	ToLCGxV-[CN:Gx1:03]
Tomato leaf curl Gujarat virus		
Tomato leaf curl Gujarat virus – [India:Varanasi:2001]	AY190290	ToLCGV-[IN:Var:01]
Tomato leaf curl Hsinchu virus		
Tomato leaf curl Hsinchu virus – [Taiwan:Hsinchu:2005]	DQ866131	ToLCHsV-[TW:THsi:05]
Tomato leaf curl Indonesia virus		
Tomato leaf curl Indonesia virus – [Indonesia:Lembang:2005]	AF198018	ToLCIDV-[ID:Lem:05]
Tomato leaf curl Iran virus		
Tomato leaf curl Iran virus – [Iran:Iranshahr]	AY297924	ToLCIRV-[IR:Ira]
Tomato leaf curl Java virus		
Tomato leaf curl Java virus – [Indonesia:Ageratum]	AB162141	ToLCJV-[ID:Age]
Tomato leaf curl Joydebpur virus		
Tomato leaf curl Joydebpur virus – [Bangladesh]	AJ875159	ToLCJoV-[BD]
Tomato leaf curl Karnataka virus		
Tomato leaf curl Karnataka virus – [India:Bangalore:1993]	U38239	ToLCKV-[IN:Ban:93]
Tomato leaf curl Laos virus		
Tomato leaf curl Laos virus – [Laos]	AF195782	ToLCLV-[LA]
Tomato leaf curl Madagascar virus		
Tomato leaf curl Madagascar virus – [Madagascar:Morondova:2001]	AJ865338	ToLCMGV-[MG:Mor:01]
Tomato leaf curl Malaysia virus		
Tomato leaf curl Malaysia virus – [Malaysia:Klang:1997]	AF327436	ToLCMYV-[MY:Kla:97]
Tomato leaf curl Mali virus		
Tomato leaf curl Mali virus – [Mali]	AY502936	ToLCMLV-[ML]
Tomato leaf curl Mayotte virus		
Tomato leaf curl Mayotte virus – [Mayotte:Dembeni:2003]	AJ865341	ToLCYTV-[YT:Dem:03]
Tomato leaf curl New Delhi virus		
Tomato leaf curl New Delhi virus – [India:New Delhi:2005]	DQ169056	ToLCNDV-[IN:ND:05]
Tomato leaf curl Pakistan virus		
Tomato leaf curl Pakistan virus – [Pakistan:Rahim Yar Khan 1:2004]	AB116884	ToLCPKV-[PK:RYK1:04]
Tomato leaf curl Philippines virus		
Tomato leaf curl Philippines virus – [Philippines:San Leonardo:2005]	DQ092867	ToLCPV-[PH:SLeo:05]

(continued)

Table 1. (continued)

Tomato leaf curl Sinaloa virus		
Tomato leaf curl Sinaloa virus – [Nicaragua:Santa Lucia]	AJ608286	ToLCSinV-[NI:SL]
Tomato leaf curl Sri Lanka virus		
Tomato leaf curl Sri Lanka virus – [Sri Lanka:Bandarawela:1997]	AF274349	ToLCSLV-[LK:Ban:97]
Tomato leaf curl Sudan virus		
Tomato leaf curl Sudan virus – [Sudan:Gezira:1996]	AY044137	ToLCSDV-[SD:Gez:96]
Tomato leaf curl Taiwan virus		
Tomato leaf curl Taiwan virus – [Taiwan]	U88692	ToLCTWV-[TW]
Tomato leaf curl Uganda virus		
Tomato leaf curl Uganda virus – [Uganda:Iganga:2005]	DQ127170	ToLCUV-[UG:Iga:05]
Tomato leaf curl Vietnam virus		
Tomato leaf curl Vietnam virus – [Vietnam:Hanoi:1998]	AF264063	ToLCVV-[VN:Han:98]
Tomato leaf curl virus		
Tomato leaf curl virus – [Australia]	S53251	ToLCV-[AU]
Tomato mild yellow leaf curl Aragua virus		
Tomato mild yellow leaf curl Aragua virus – [Venezuela:10]	AY927277	ToMYLCAV-[VE:10]
Tomato mosaic Havana virus		
Tomato mosaic Havana virus – [Cuba:Quivican]	Y14874	ToMHV-[CU:Qui]
Tomato mottle Taino virus		
Tomato mottle Taino virus – [Cuba]	AF012300	ToMoTV-[CU]
Tomato mottle virus		
Tomato mottle virus – [US:Florida:1989]	L14460	ToMoV-[US:Flo:89]
Tomato rugose mosaic virus		
Tomato rugose mosaic virus – [Brazil:Uberlandia:1996]	AF291705	ToRMV-[BR:Ube:96]
Tomato severe leaf curl virus		
Tomato severe leaf curl virus – [Mexico:Rioverde 2:2005]	DQ347947	ToSLCV-[MX:Rio2:05]
Tomato severe rugose virus		
Tomato severe rugose virus – [Brazil:Minas Gerais:2000]	AY029750	ToSRV-[BR:MG:00]
Tomato yellow leaf curl Axarquia virus		
Tomato yellow leaf curl Axarquia virus – [Spain:Algarrobo:2000]	AY227892	TYLCAxV-[ES:Alg:00]
Tomato yellow leaf curl China virus		
Tomato yellow leaf curl China virus – [China:Guangxi 102:2004]	AM050555	TYLCCNV-[CN:Gx102:04]

(continued)

Table 1. (continued)

Tomato yellow leaf curl Guangdong virus		
Tomato yellow leaf curl Guangdong virus – [China:Guangzhou 3:2003]	AY602166	TYLCGuV-[CN:Gz3:03]
Tomato yellow leaf curl Kanchanaburi virus		
Tomato yellow leaf curl Kanchanaburi virus – [Vietnam:2005]	DQ169054	TYLCKaV-[VN:05]
Tomato yellow leaf curl Malaga virus		
Tomato yellow leaf curl Malaga virus – [Spain:421:1999]	AF271234	TYLCMalV-[ES:421:99]
Tomato yellow leaf curl Mali virus		
Tomato yellow leaf curl Mali virus – [Mali]	AY502934	TYLCMLV-[ML]
Tomato yellow leaf curl Sardinia virus		
Tomato yellow leaf curl Sardinia virus – Sicily [Italy:Sicily]	Z28390	TYLCSV-Sic[IT:Sic]
Tomato yellow leaf curl Thailand virus		
Tomato yellow leaf curl Thailand virus – [Myanmar:Yangon:1999]	AF206674	TYLCTHV-[MM:Yan:99]
Tomato yellow leaf curl Vietnam virus		
Tomato yellow leaf curl Vietnam virus – [Vietnam:Hanoi:2005]	DQ641697	TYLCVNV-[VN:Han:05]
Tomato yellow leaf curl virus		
Tomato yellow leaf curl virus – [Israel:Rehovot:1986]	X15656	TYLCV-[IL:Reo:86]
Tomato yellow margin leaf curl virus		
Tomato yellow margin leaf curl virus – [Venezuela:Merida 57]	AY508993	TYMLCV-[VE:Mer57]
Tomato yellow spot virus		
Tomato yellow spot virus – [Brazil:Bicas 2:1999]	DQ336350	ToYSV-[BR:Bic2:99]

Rojas et al. (2005) reported the presence of a begomovirus complex infecting the tomato crop in Nicaragua. These viruses include: ToLCSinV, ToSLCV, Squash yellow mild mottle virus (SYMMoV), Euphorbia mosaic virus (EuMV), and Pepper golden mosaic virus (PepGMV). In the Caribbean Basin, geminiviruses associated with peppers (*Capsicum* spp.), Pepper huasteco yellow vein virus (PHYVV), and PepGMV are known to infect both tomato and pepper (Roye et al 1999). There are many more viruses in the world that infect tomato and also typical "tomato" viruses that infect other crops, but the objective of this paragraph is only to provide a sense of the large diversity of geminiviruses infecting tomato and not to provide an exhaustive list.

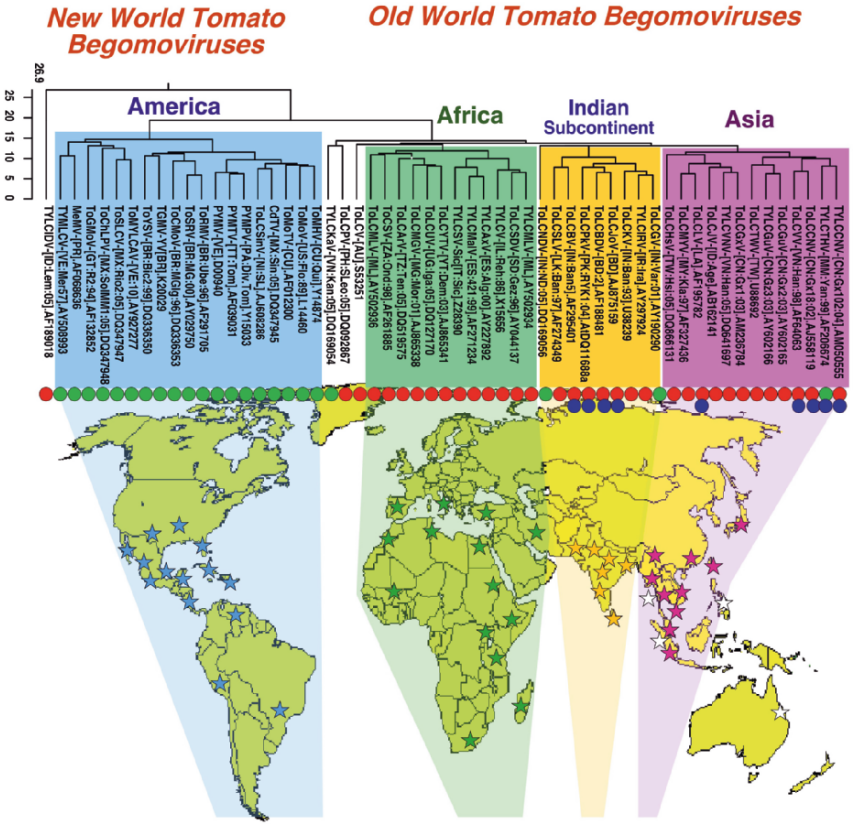

Figure 1. The diagram represents a world map on which each of the 57 members representing 57 begomovirus species as per the ICTV list of 2006. The exact name and accession numbers of the isolates are listed below. The upper part of the diagram shows a phylogenetic tree of these 57 viruses using their complete A component sequence. The Clustal V algorithm of the program MegAlign from DNAStar has been used and distances in percentage difference are indicated on the left. The tree shows a partition in 4 major clusters, America, Africa, the Indian subcontinent and Asia respectively in blue, green, orange, and purple colors, belonging to two branches called, NW Tomato Begomoviruses and OW Tomato Begomoviruses. The individual viruses composing these clusters are positioned on the world map as stars of the same colors. In addition, there are four "outsiders", meaning not fitting with the geographical distribution described above, that have been indicated on the map as white stars and located in Indochina, Indonesia, Philippines, and Australia. Furthermore, we have indicated if the genome of these viruses has two (green dot) or one (red dot) component and if a beta molecule has been found associated to each virus (blue dot). The accession numbers of the virus sequences used in the figure and their complete names can be found in Table 1.

Table 2. List of 59 virus isolates (written in black) used in this chapter belonging to 15 different strains (written in red) and six different species of the *Tomato yellow leaf curl virus cluster* (written in green). The accession numbers of the complete A component sequence is indicated in the second column and the abbreviation of the name of the virus isolates and strains is indicated in the third column

Tomato leaf curl Sudan virus		
Tomato leaf curl Sudan virus – Gezira		**ToLCSDV-Gez**
Tomato leaf curl Sudan virus – Gezira [Sudan:Gezira:1996]	AY044137	ToLCSDV-Gez[SD:Gez:96]
Tomato leaf curl Sudan virus – Shambat		**ToLCSDV-Sha**
Tomato leaf curl Sudan virus – Shambat [Sudan:Shambat:1996]	AY044139	ToLCSDV-Sha[SD:Sha:96]
Tomato leaf curl Sudan virus – Yemen		**ToLCSDV-YE**
Tomato leaf curl Sudan virus – Yemen [Yemen:Tihamah:2006]	EF110890	ToLCSDV-YE[YE:Tih:06]
Tomato yellow leaf curl Axarquia virus		
Tomato yellow leaf curl Axarquia virus – [Spain:Algarrobo:2000]	AY227892	TYLCAxV-[ES:Alg:00]
Tomato yellow leaf curl Malaga virus		
Tomato yellow leaf curl Malaga virus – [Spain:421:1999]	AF271234	TYLCMalV-[ES:421:99]
Tomato yellow leaf curl Mali virus		
Tomato yellow leaf curl Mali virus – Ethiopia		**TYLCMLV-ET**
Tomato yellow leaf curl Mali virus – Ethiopia [Ethiopia:Melkassa:2005]	DQ358913	TYLCMLV-ET[ET:Mel:05]
Tomato yellow leaf curl Mali virus – Mali		**TYLCMLV-ML**
Tomato yellow leaf curl Mali virus – Mali [Mali]	AY502934	TYLCMLV-ML[ML]
Tomato yellow leaf curl Sardinia virus		
Tomato yellow leaf curl Sardinia virus – Italy		**TYLCSV-IT**
Tomato yellow leaf curl Sardinia virus – Italy [Italy:Sardinia:1988]	X61153	TYLCSV-IT[IT:Sar:88]
Tomato yellow leaf curl Sardinia virus – Sicily		**TYLCSV-Sic**
Tomato yellow leaf curl Sardinia virus – Sicily [Israel:Henryk:2005]	DQ845787	TYLCSV-Sic[IL:Hen:05]
Tomato yellow leaf curl Sardinia virus – Sicily [Italy:Sicily]	Z28390	TYLCSV-Sic[IT:Sic]
Tomato yellow leaf curl Sardinia virus – Sicily [Tunisia:Bkalta 3:2002]	AY736854	TYLCSV-Sic[TN:Bk3:02]
Tomato yellow leaf curl Sardinia virus – Spain		**TYLCSV-ES**
Tomato yellow leaf curl Sardinia virus – Spain [Spain:Almeria 2:1992]	L27708	TYLCSV-ES[ES:Alm2:92]
Tomato yellow leaf curl Sardinia virus – Spain [Spain:Canary]	AJ519675	TYLCSV-ES[ES:Can]
Tomato yellow leaf curl Sardinia virus – Spain [Spain:Murcia 1:1992]	Z25751	TYLCSV-ES[ES:Mur1:92]
Tomato yellow leaf curl Sardinia virus – Spain [Morocco:Agadir:2002]	AY702650	TYLCSV-ES[MA:Aga:02]

(continued)

Table 2. (continued)

Tomato yellow leaf curl virus		
Tomato yellow leaf curl virus – Gezira		**TYLCV-Gez**
Tomato yellow leaf curl virus – Gezira [Sudan:1996]	AY044138	TYLCV-Gez[SD:96]
Tomato yellow leaf curl virus – Iran		**TYLCV-IR**
Tomato yellow leaf curl virus – Iran [Iran]	AJ132711	TYLCV-IR[IR]
Tomato yellow leaf curl virus – Israel		**TYLCV-IL**
Tomato yellow leaf curl virus – Israel [Australia:Brisbane:2006]	1934*	TYLCV-IL[AU:Bri:06]
Tomato yellow leaf curl virus – Israel [China:Shangai 2:2005]	AM282874	TYLCV-IL[CN:SH2:05]
Tomato yellow leaf curl virus – Israel [Cuba]	AJ223505	TYLCV-IL[CU]
Tomato yellow leaf curl virus – Israel [Dominican Republic]	AF024715	TYLCV-IL[DO]
Tomato yellow leaf curl virus – Israel [Egypt:Ismaelia]	AY594174	TYLCV-IL[EG:Ism]
Tomato yellow leaf curl virus – Israel [Egypt:Nobaria:1991]	EF107520	TYLCV-IL[EG:Nob:91]
Tomato yellow leaf curl virus – Israel [Israel:Rehovot:1986]	X15656	TYLCV-IL[IL:Reo:86]
Tomato yellow leaf curl virus – Israel [Italy:Sicily:2004]	DQ144621	TYLCV-IL[IT:Sic:04]
Tomato yellow leaf curl virus – Israel [Japan:Haruno:2005]	AB192966	TYLCV-IL[JR:TosH:05]
Tomato yellow leaf curl virus – Israel [Japan:Misumi:Stellaria]	AB116631	TYLCV-IL[JR:Mis:Ste]
Tomato yellow leaf curl virus – Israel [Japan:Miyazaki]	AB116629	TYLCV-IL[JR:Miy]
Tomato yellow leaf curl virus – Israel [Japan:Omura:Eustoma]	AB116630	TYLCV-IL[JR:Omu:Eus]
Tomato yellow leaf curl virus – Israel [Japan:Omura]	AB110217	TYLCV-IL[JR:Omu]
Tomato yellow leaf curl virus – Israel [Japan:Tosa:2005]	AB192965	TYLCV-IL[JR:Tos:05]
Tomato yellow leaf curl virus – Israel [Jordan:Tomato:2005]	EF054893	TYLCV-IL[JO:Tom:05]
Tomato yellow leaf curl virus – Israel [Lebanon:Tomato:2005]	EF051116	TYLCV-IL[LB:Tom:05]
Tomato yellow leaf curl virus – Israel [Mexico:Culiacan:2005]	DQ631892	TYLCV-IL[MX:Cul:05]
Tomato yellow leaf curl virus – Israel [Morocco:Berkane:2005]	EF060196	TYLCV-IL[MO:Ber:05]
Tomato yellow leaf curl virus – Israel [Puerto Rico:2001]	AY134494	TYLCV-IL[PR:01]
Tomato yellow leaf curl virus – Israel [Spain:Almeria:Pepper:1999]	AJ489258	TYLCV-IL[ES:Alm:Pep:99]
Tomato yellow leaf curl virus – Israel [Tunisia:2005]	EF101929	TYLCV-IL[TN:05]

(continued)

Table 2. (continued)

Tomato yellow leaf curl virus – Israel [Turkey:Mersin:2004]	AK812277	TYLCV-IL[TR:Mer:04]
Tomato yellow leaf curl virus – Israel [US:Florida]	AY530931	TYLCV-IL[US:Flo]
Tomato yellow leaf curl virus – Mild		**TYLCV-Mld**
Tomato yellow leaf curl virus – Mild [Israel;1993]	X76319	TYLCV-Mld[IL;93]
Tomato yellow leaf curl virus – Mild [Japan:Aichi]	AB014347	TYLCV-Mld[JR:Aic]
Tomato yellow leaf curl virus – Mild [Japan:Atumi]	AB116633	TYLCV-Mld[JR:Atu]
Tomato yellow leaf curl virus – Mild [Japan:Daito]	AB116635	TYLCV-Mld[JR:Dai]
Tomato yellow leaf curl virus – Mild [Japan:Kisozaki]	AB116634	TYLCV-Mld[JR:Kis]
Tomato yellow leaf curl virus – Mild [Japan:Osuka]	AB116636	TYLCV-Mld[JR:Osu]
Tomato yellow leaf curl virus – Mild [Japan:Shimizu]	AB110218	TYLCV-Mld[JR:Shi]
Tomato yellow leaf curl virus – Mild [Japan:Shizuoka]	AB014346	TYLCV-Mld[JR:Shz]
Tomato yellow leaf curl virus – Mild [Japan:Yaizu]	AB116632	TYLCV-Mld[JR:Yai]
Tomato yellow leaf curl virus – Mild [Jordan:Cucumber:2005]	EF158044	TYLCV-Mld[JO:Cuc:03]
Tomato yellow leaf curl virus – Mild [Jordan:Homra:2003]	AY594175	TYLCV-Mld[JO:Hom03]
Tomato yellow leaf curl virus – Mild [Jordan:Tomato:2005]	EF054894	TYLCV-Mld[JO:Tom:03]
Tomato yellow leaf curl virus – Mild [Lebanon;LBA44:05]	EF185318	TYLCV-Mld[ILB;LBA44:05]
Tomato yellow leaf curl virus – Mild [Portugal:2:1995]	AF105975	TYLCV-Mld[PT:2:95]
Tomato yellow leaf curl virus – Mild [Reunion:2002]	AJ865337	TYLCV-Mld[RE:02]
Tomato yellow leaf curl virus – Mild [Spain:72:1997]	AF071228	TYLCV-Mld[ES:72:97]
Tomato yellow leaf curl virus – Mild [Spain:Almeria:1999]	AJ519441	TYLCV-Mld[ES:Alm:99]

* Australian accession #

7. TOMATO BEGOMOVIRUSES IN THE OLD WORLD

The geminiviruses are grouped into OW(consisting of Asia along with the Indian subcontinent and Africa) or NW(encompassing the whole of the American continent) types based upon their geographical origin and number of

genomic components they possess. This classification is also based on their genome sequences (Figure 1). Several OW tomato-infecting begomoviruses have been reported from Asia and some of them are Tomato leaf curl virus (ToLCV), Tomato leaf curl Laos virus (ToLCLV), Tomato leaf curl Malaysia virus (ToLCMV), Tomato leaf curl Philippines virus (ToLCPV), Tomato yellow leaf curl China virus (TYLCCNV), Tomato leaf curl Taiwan virus (ToLCTWV), Tomato leaf curl Vietnam virus (ToLCVNV), *and* Tomato leaf curl Thailand virus (TYLCTHV). Several such viruses have been also reported from the Indian subcontinent, to mention a few – Tomato leaf curl Bangladesh virus (ToLCBDV), Tomato leaf curl Karnataka virus (ToLCKV), Tomato leaf curl Gujarat virus (ToLCGV), Tomato leaf curl Sri Lanka virus (ToLCSLV), Tomato leaf curl Bangalore virus (ToLCBV), and Tomato leaf curl New Delhi virus (ToLCNdV).

Viruses are also being extensively studied and reported from the African continent such as Tomato leaf curl Sudan virus (ToLCSDV), Tomato yellow leaf curl Mali virus (TYLCMLV), Tomato yellow leaf curl Sardinia virus (TYLCSV), Tomato curly stunt virus (ToCSV) (Fauquet et al., 2003; 2007; Fauquet & Stanley, 2005).

8. TOMATO BEGOMOVIRUSES IN THE MEDITERRANEAN BASIN

Among the begomoviruses infecting tomato, TYLCV is the most notorious in terms of the intensity of the disease and the number of countries it is spread into. TYLCV is highly prevalent in the Mediterranean region (Moriones & Navas-Castillo, 2000). In addition to the tomato leaf curl disease the TYLCV causes the bean crumple disease in common bean, and usually in association with the Tomato yellow leaf curl Malaga virus (TYLCMalV), which is a recombination product of two different tomato infecting begomoviruses belonging to two different species, TYLCV and Tomato yellow leaf curl Sardinia virus (TYLCSV) (Monci et al., 2002). TYLCSV infects the tomato crop, but not the common bean (Noris et al., 1994; Sánchez-Campos et al., 1999). Since 1992 TYLCSV-ES was reported as the causal agent in Spain, and epidemics of TYLCD have caused devastating damage to both field and greenhouse tomato crops (Noris et al., 1994; Fauquet et al., 2000). In 1997, another begomovirus, the mild strain of TYLCV (TYLCV-Mld) was also found associated with isolates of TYLCSV in the TYLCD epidemics (Navas-Castillo et al., 1997, 2000). Both viruses are frequently found in mixed infections in single tomato plants (Sanchez-Campos et al., 1999).

Other than Spain, all the isolates found in South Europe and North Africa belong to the species TYLCV and TYLCSV (Accotto et al., 2000; Chouchane et al., 2006; Tahiri et al., 2006). The eastern part of the Mediterranean basin has representatives of the same strains (Anfoka et al., 2005) with the addition of TYLCV-IR from Iran (Bananej et al., 2004). In the Nile region of eastern Africa, Idris & Brown (2005) detected different tomato begomoviruses including the strain TYLCV-Gez that has a high homology with the TYLCV-Mld sequence. Moreover, Shih et al. (2006) reported new symptoms on cultivated tomatoes in Ethiopia

during 2003, and molecular techniques allowed them to identify TYLCMLV as the causing agent for the severe symptoms and member of a new species.

9. PAIRWISE COMPARISONS OF TYLCV-LIKE VIRUSES

One way to appreciate the geminivirus diversity is to use the pairwise sequence comparison method and compare different subsets of viruses. The method is very simple and consists in comparing geminivirus sequences two by two and after aligning them to calculate a percentage of sequence identity for the pair. The exercise is repeated for all the pairs of viruses in a population set and these numbers are then plotted for their distribution, and subsets of viruses can then be compared for this distribution relative to the general distribution. If all virus sequences were randomly and normally distributed we would obtain a single peak with a bell curve shape, and therefore any other distribution will indicate a nonrandom distribution (Figures 2 and 3).

If all tomato-infecting geminivirus sequences, at the species level, are compared; they form two distinct peaks in their pairwise distribution (Figure 2A). The most divergent one ranges from 45% to 60%, while the second one is from 60% to 90%. The first peak corresponds to comparisons between viruses from the OW and the NW (Figure 2A), while the second peak corresponds to comparisons within each world (Figure 2B, C). This diagram is showing that there is the same molecular diversity in each part of the world and that the TYLCV cluster is typical of the OW geminiviruses.

If only tomato viruses of the Mediterranean basin are considered (Figure 3), there is much less diversity and it ranges from 70% to 100% with several peaks (Figure 3A). The least conserved one from 70% to 90% corresponds to the comparisons between the species of that group (Figure 3B), while the comparison between isolates varies between 85% and 100% (Figure 3C). Within virus isolates, one can easily distinguish two peaks, the first one corresponds to virus strains and varies between 85% and 94% (Figure 3D) and finally comparisons between variants varies from 95% to 100% (Figure3E).

This type of distribution implies that viruses cannot occupy the whole molecular diversity spectrum, but only some specific parts of it, probably due to structural and functional constraints. The pairwise comparison system can in turn be an aid for virus classification and it is effectively highly used for geminivirus classification. This molecular diversity analysis is the basis for the classification of TYLCV-like viruses described in the following paragraphs.

10. GEOGRAPHICAL DISTRIBUTION OF TYLCV-LIKE VIRUSES

TYLCD was challenged nearly a century ago in Europe by carrying out breeding for resistance (Herrmann 1921). The virus causing this disease was first mentioned in the early 1960s in Israel (Cohen & Harpaz 1964) and Sudan (Yassin & Nour 1965). In the 1970s, reports on incidence of TYLCD

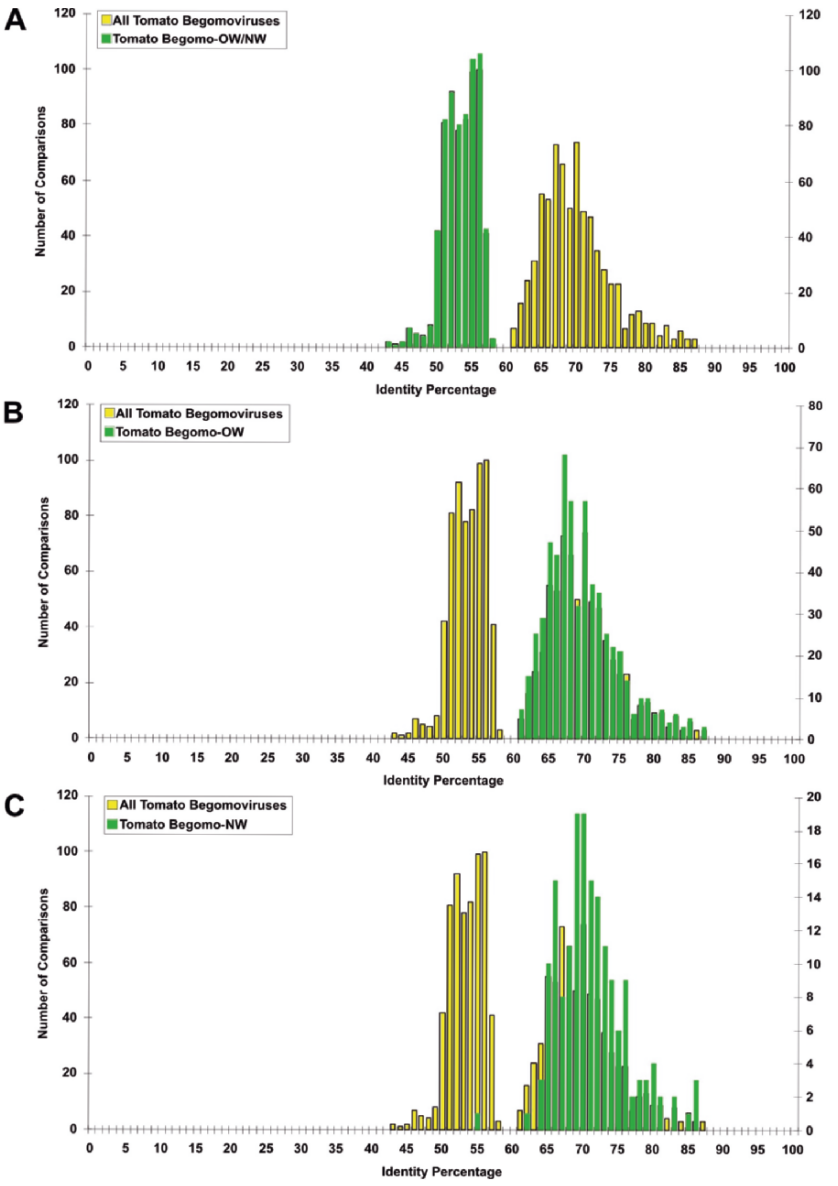

Figure 2. Distribution of 1,596 pairwise identity percentages between 57 tomato begomovirus DNA–A component sequences. The horizontal axes show percentage of pairwise comparisons and the vertical axes show the number of comparisons for each percentage. The yellow bars represent the distribution of the total population of chosen viruses, and the green bars represent specific subgroups of these pairwise comparisons: (A) comparison between NW and OW tomato begomoviruses, (B) comparison between NW and NW tomato begomoviruses, (C) comparison between OW and OW tomato begomoviruses. The comparisons were generated using the Clustal V algorithm of the MegAlign program (DNAstar).

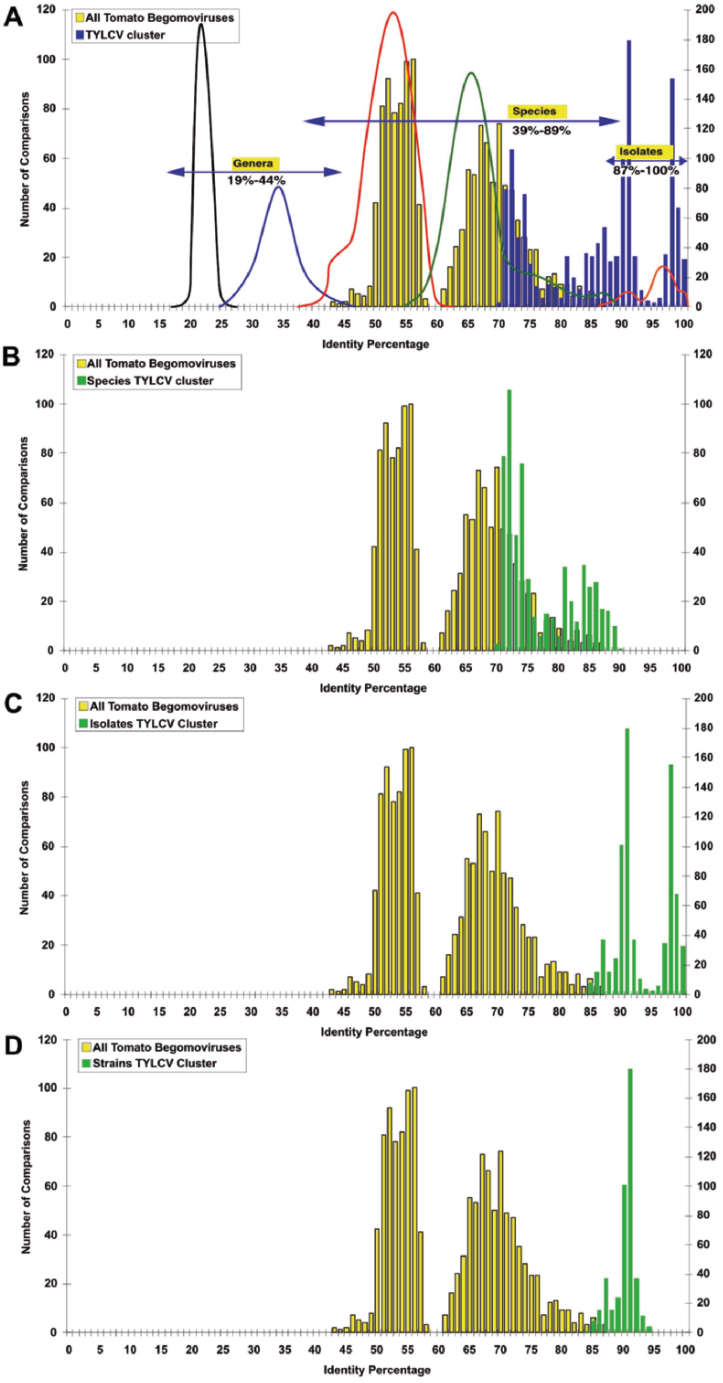

Figure 3. Distribution of 1,710 pairwise identity percentages between 59 tomato begomovirus DNA–A component sequences belonging to the *Tomato yellow leaf curl virus* cluster. The horizontal axes show percentage of pairwise comparisons and the vertical axes show the number of comparisons for each percentage.

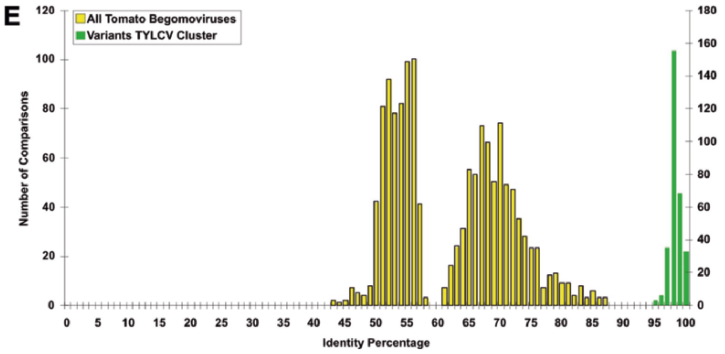

Figure 3. (continued) The yellow bars represent the distribution of the total population of 57 viruses representing 57 species of tomato begomoviruses, and the blue or green bars represent specific subgroups of pairwise comparisons within the TYLCV cluster: (A) comparison between the 59 begomoviruses of the TYLCV cluster at all taxonomic levels; (B) comparison between the 59 begomoviruses of the TYLCV cluster at the species level; (C) comparison between the 59 begomoviruses of the TYLCV cluster at the isolate level; (D) comparison between the 59 begomoviruses of the TYLCV cluster at the strain level, E) comparison between the 59 begomoviruses of the TYLCV cluster at the variant level. The comparisons were generated using the Clustal V algorithm of the MegAlign program (DNAstar). In the panel A, the colored curves indicate the expected position of each of the taxonomic levels.

had suddenly increased in different parts of the world associated with the increased whitefly (*B. tabaci*) population and huge yield loss in tomato production, ranging from 50% to 100%, were recorded (Makkouk et al., 1979). The accepted bipartite nature of begomoviruses had made it difficult to prove that this virus was a monopartite begomovirus, and it is only in the early 1990s that the full length complete sequence of the virus was published and proved to be a monopartite begomovirus (Navot et al., 1991). The studies on whitefly populations, during the early 1990s, indicated that a new biotype of whitefly had emerged with increased capability of acquiring and transmitting geminiviruses in a broader host range (Bedford et al., 1994). In recent years there have been frequent reports of mixed infections of different TYLCV-like viruses (Monci et al., 2002; Accotto et al., 2003; Anfoka et al., 2005) and such mixed infected plants act as the reservoirs for recombination, diversity, and subsequent evolution of these viruses. Furthermore, *Cynanchum acutum* is a natural host of TYLCV that is important in the repeated introduction of the virus into tomatoes in the Jordan Valley (Cohen et al., 1988).

The assessment of the geographical distribution for TYLCV was carried out for the first time in 1990 by hybridization experiments with specific probes (Czosnek et al., 1990), and has been followed by other laboratories since. Nowadays, we can have a better evaluation of its geographical distribution by analyzing the sequences published in GenBank for TYLCV and other closely related viruses.

Interestingly, TYLCV sequences are reported with often an increased aggressiveness and fast spreading in the newly invaded ecosystems. On the world map, TYLCD started to appear first in the Middle East and the Mediterranean basin, particularly in Italy (Khyer-Pour et al., 1991) and Spain (Noris et al., 1994), where initially the new viruses were mistakenly identified as TYLCV isolates. TYLCV was first reported in the NW in the late 1990s (Czosnek & Laterrot, 1997) to progressively invade all Central America and the south of the USA (Polston & McGovern, 1999). It is interesting to note that TYLCV has not been reported in South America so far. Also in the 1990s, there was the first reports of TYLCV in Japan (Kato et al., 1998), followed very recently in China (Wu et al., 2006) and even recently in Australia, despite their very strict quarantine regulations (Tesoriero & Azzopardi 2006).

Table 3 lists the timing of virus distribution of TYLCV-like viruses in the world. Sequence identity is the most important criterion used to study the global distribution of the TYLCV-like viruses and the first TYLCV isolate described from Israel (TYLCV-IL), as well as its mild strain (TYLCV-mild) are the most widely spread across the globe (Figure 4).

There are no definitive explanations for the wide spread of TYLCV, although several hypothesis have been proposed including the physical transfer of infected plantlets from Israel to the Caribbean by commercial companies (Czosnek & Laterrot, 1997), or the fact that the petiole of virus infected exported tomato fruits could serve as inoculum for whitefly transmission (Delatte et al., 2003). In any case, it is apparent that the human trade activities are largely responsible for the uncontrolled worldwide spread of this virus disease, and this could explain the fact that some regions have not yet been invaded such as South America or India (see Part I, Chapter 4). It is also possible that TYLCV isolates enter in competition with local geminiviruses and may not necessarily be successful, and this could account for their absence in some parts of India where extremely aggressive viruses already occupy the tomato niche (Padidam et al., 1995a, b; Chakraborty et al., 2003).

11. MOLECULAR DIVERSITY AND RECOMBINATION AMONG TYLCV-LIKE VIRUSES

Since the late 1970s, reports on incidence and disease symptoms of TYLCD has increased rapidly in different parts of the world, this increase was always associated with the increased population of whitefly (*B. tabaci*) and huge yield losses that ranged from 50% to 100% (Makkouk et al., 1979).

Whiteflies were reported to colonize many crops in Florida (Schuster et al., 1991), California (Perring et al., 1993), Texas, Arizona, Central and South America (Brown, 1994) and in many other parts of the world. The whitefly population reports during the early 1990s indicated that the physiology, behavior, reproduction, and genetics of whiteflies were different from the population that was present before 1989; new biotypes of whitefly had

Table 3. Distribution of *Tomato yellow leaf curl virus* and TYLCV-like isolates in the world; published complete sequences from different countries around the world are indicated in respect of the virus species, strain, and the year of publication. Other complete sequences for the virus are available in GeneBank and are updated regularly

Virus species	Virus strain	Country	Year reported	Reference
TYLCV	**TYLCV-IL**	Israel	1990	Czosnek et al., 1990
		Egypt	1993	Nakhla et al., 1993
		Lebanon	1995	Abou-Jawdah, 1995
		Jamaica	1995	Wernecke et al., 1995
		Portugal	1996	Louro et al., 1996
		Spain	1997	Navas-Castillo et al., 1997
		Japan	1998	Kato et al., 1998
		USA	1999	Polston et al 1999
		Puerto Rico	2001	Bird et al., 2001
		Cuba	2002	Quiñones et al., 2002
		Dominican Rep	2002	Salati et al., 2002
		Italy	2003	Accotto et al., 2003
		Jordan	2005	Anfoka et al., 2005
		China	2006	Wu J. et al., 2006
		Turkey	2006	Köklü et al., 2006
		Mexico	2006	Brown, J.K. & Idris, A.M. 2006
		Australia	2006	Tesoriero, L. & Azzopardi, S. 2006
	TYLCV-Mld	Israel	1994	Antignus, E.Y. & Cohen, S. 1994
		Portugal	2000	Navas-Castillo et al., 2000
		Spain	2000	Navas-Castillo et al., 2000
		Japan	2004	Ueda et al., 2004
		Jordan	2005	Anfoka et al., 2005
		Reunion	2005	Delatte et al., 2005
	TYLCV-IR	Iran	2004	Bananej et al., 2004
TYLCSDV	-	Sudan	2005	Brown, J. & Idris, A. 2005
TYLCMLV	**TYLCMLV-ML**	Mali	1991	Dembele, D. & Noussourou, M. 1991
	TYLCMLV-ET	Ethiopia	2006	Shih et al., 2006
TYLCSV	**TYLCSV-IT**	Italy	1991	Kheyr-Pour et al., 1991
	TYLCV-ES	Spain	1994	Reina et al., 1994
		Jordan	2005	Anfoka et al., 2005
		Morocco	2006	Tahiri et al., 2006
	TYLCSV-Sic	Tunisia	2006	Gharsallah et al., 2006
TYLCMalV	-	Spain	2002	Monci et al., 2002
TYLCAxV	-	Spain	2006	Garcia-Andres et al., 2006

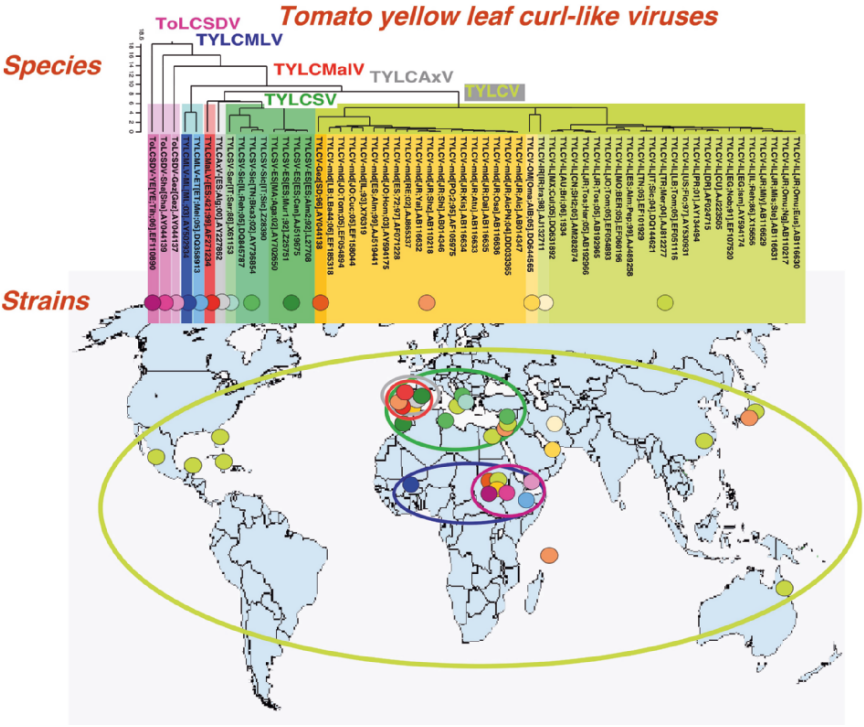

Figure 4. The diagram represents a world map on which each of the 59 members representing 6 begomovirus species of the TYLCV cluster. The exact name, abbreviation, and accession numbers of the isolates are listed in Table 2. The upper part of the diagram shows a phylogenetic tree of these 59 viruses using their complete A component sequence. The Clustal V algorithm of the program MegAlign from DNAStar has been used and distances in percentage difference are indicated on the left. The tree shows a partition in 6 major clusters, one for each of the 6 designated species, TYLCV, TYLCSV, TYLCAxV, TYLCMalV, TYLCMLV, and ToLCSDV, respectively in yellow, green, grey, red, blue, and purple colors. These 6 species constitute the so-called TYLCV cluster of the OW begomoviruses. The individual viruses composing these clusters are positioned on the world map, as dots of various colors representing their pertaining to one of the 15 specific strains of the 6 species, as indicated in the colored boxes at the bottom of the tree. On the world map, the individuals pertaining to the same species are circled with the same color as indicated by the name of the species of the boxes of the on the phylogenetic tree.

emerged with capabilities of acquiring and transmitting geminiviruses more efficiently, also to a broader host range (Bedford et al., 1994) with an outbreak of apparently new geminiviruses (Polston & Anderson 1997). Introducing begomoviruses into the new areas through trading, transportation, and other human activities encourages adaptation and evolution of the invading virus.

Mixed infected host plants with genetically divergent begomoviruses or different species of the same virus (TYLCV) have been reported a number of

times (Ooi et al., 1997; Anfoka et al., 2005), and such mixed infected plants are the reservoirs for recombination, diversity, and evolution of these viruses, which largely depend on recombination for selective advantages (Bonnet et al., 2005).

Increased virulence and disease severity were always reported with the discovery of new recombinants of TYLCV-like viruses (Monci et al., 2002; García-Andrés et al., 2006). Monci et al. (2002) showed how the new recombinant TYLCMalV became prevalent in Spain because of its better acquisition by the whiteflies and its broader host range than both of its parents (Monci et al., 2002; see also Part II, Chapter 3).

12. MECHANISMS FOR MOLECULAR DIVERSITY OF TYLCV-LIKE VIRUSES

Genetic variation within the genome of TYLCV-like viruses could arise either through simple mutations or complicated recombination events. TYLCV-like viruses depend on the replication machinery of the plant host cell and their enzymes for their replication and transcription in the nucleus of infected cell (Hanley-Bowdoin et al., 1999). The replication occurs either through rolling-circle-replication (RCR) or the recombination-dependent-replication (RDR), thus encouraging recombinations (Preiss & Jeske 2003). Begomoviruses escape the repair mechanism of the replication errors, because of the absence of methylation of their replicative forms and thus the lack of mismatch repair, as the mismatch repair normally involves DNA methylation, and this allows the inheritance of the mutations (Roossinck, 1997).

In most cases, the resulting recombinant is naturally selected for a better fitness and/or better acquisition by the whitefly vector (Monci et al., 2002) but most certainly the selected recombinant, with a more aggressive behavior, is not the only recombinant in the recombination pool. Morilla et al. (2004) examined tomato cells infected with both TYLCSV and TYLCV, and they found that the level of mixed infection invades at least one fifth of all the nuclei. This may indicate a huge potential for recombination between TYLCV and TYLCSV, but at least one recombinant could be detected in the mixed infected plants due to its successful replication and movement. Other recombination events might not have the chance to be detected by PCR or hybridization techniques as these recombinants might not have the right combination of sequences to be replicated.

Interaction with the host is another important factor for the selection of recombinants with a better fitness; Fauquet et al. (2005) proposed that the region between AC1–AC4 is subjected to frequent recombinations due to its interaction with the host factors. On the other hand, natural sources of virus resistance and intensive breeding programs can be a major factor in selecting recombinants that can break the resistance (Lapidot & Friedman 2002). Polston et al. (2006) described how the Capsicum species were tamed by TYLCV, which broke the immunity barrier and achieved 100% infection, with the enhanced capability of offering easy whitefly transmission to tomato plants.

Incorporating one strand from a virus into the genome of another virus requires homologous conserved sequences between the donor and the recipient viruses. Such homologous stretches of sequences serve as recombination junctions and are referred as recombination hot spots because of the high frequency of recombination in these sites (Stanley, 1995). The region from 2,600 to 150 nt, encompassing the N-terminus of the Rep and the stem-loop, is an obvious hot spot for recombination (Navas-Castillo et al., 2000; Sanz et al., 1999, 2000; Kirthi et al., 2002; Bananej et al., 2004; Jovel et al., 2004). The inter-cistronic region (ICR), contains *cis*-acting signals that regulate the replication like the iterons and regulatory motifs required for the control of gene expression (Eagle et al., 1994). The ICR also has the highly conserved nonanucleotide sequence TAATATT↓AC containing the nicking/initiation site (↓) for replication (Hanley-Bowdoin et al., 1999). Furthermore, a specific interaction between the N-terminus of the Rep protein and the iteron has been demonstrated, thus the DNA coding for the protein motives and the iterons are functionally associated and are selected together during the recombination events (Chatterji et al., 2000).

Fauquet et al. (2005) predicted another hot spot of recombination in the position around the nucleotide 2000 present within the *Rep* gene, which was based on the study of 19 begomoviruses-infecting tomato with a monopartite genome in the OW (Fauquet et al., 2005). Though the presence of such hot spots were suggested, at the same position for other geminiviruses infecting other hosts (Xie & Zhou, 2003), their precise recombination junction was not mapped. The recently reported recombinant species TYLCMalV (AF271234) and TYLCAxV (AY227892) indicate the presence of another hot spot at the region surrounding the nucleotide 1800, for which both viruses show a recombination event. Sequence alignment of this region, between the recombinants and other viruses; indicates a highly conserved sequence, suggesting that this region may serve as the junction for recombination.

These predicted recombination sites were confirmed by the RDP2 software, by sequence comparisons of representatives of all the species of TYLCV-like viruses and other closely related viruses (Martin et al., 2005). This analysis also identified similar such recombination junctions embedded in small stretches of highly conserved regions located around the nucleotides 500 (CTGAACTTCGACAGCCC), 1100 (CAAATATAATAAA), 1800 (CCTTTAATTTGAATGGG, or AAGATAGTGGGAAT), and nucleotide 2200 (GGAACTTGAT). These sequences are conserved among all TYLCV isolates and other related TYLCV-like viruses, creating a clear linkage point between different species members.

13. RECOMBINATION MAP OF MEMBERS OF THE TYLCV CLUSTER

Figure 5 shows a color-coded diagrammatic representation of the common sequences between virus isolates representing the 6 species and the 15 strains of the TYLCV-cluster. Pairwise comparisons of pairs of sequences were done

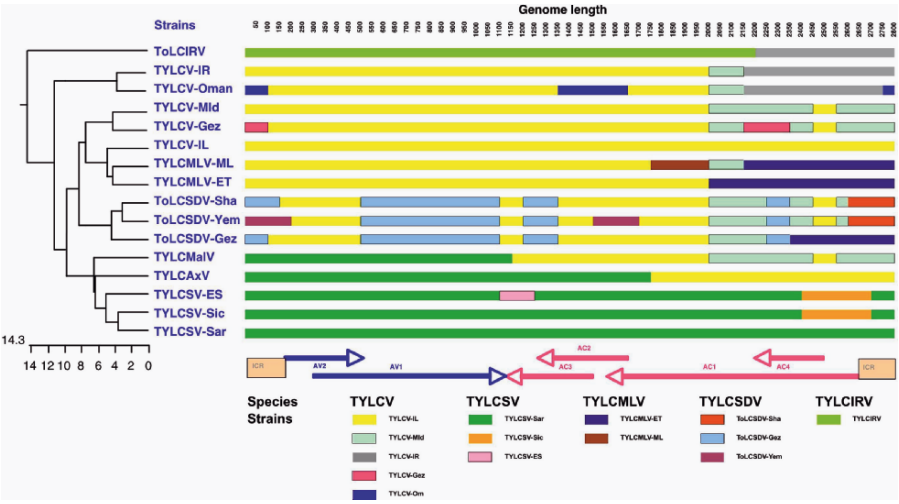

Figure 5. The diagram represents a recombination map of representative members of 15 strains pertaining to the 6 species of the TYLCV cluster. Recombinations were assessed by pairwise comparisons relative to a "species" profile and a "strain" profile (Fauquet et al., 2005). The key for the different colors is indicated at the bottom of the figure while the position of the recombination is indicated by the length of the genomes at the top of the figure and the genetic map of the viruses in the center of the figure. A phylogenetic tree of representatives of the 15 strains of the TYLCV cluster is indicated on the left, and the scale indicates the level of divergence in percentages. The comparisons and the tree were generated using the Clustal V algorithm of the MegAlign program (DNAstar). The scale on the left represents the sequence percentage difference. The abbreviation and accession numbers of the representatives of the 15 strains of the TYLCV cluster are: ToLCSDV-Gez[Gez].AY044137, ToLCSDV-Sha[Sha].AY044139, ToLCSDV-YE[YE:Tih:06].EF110890, TYLCAxV-[ES:Alg:00].AY227862, TYLCMaLV[ES:421:99].AF271234, TYLCMLV-ET[ET:Mel:05]. DQ358913, TYLCMLV-ML[ML:03].AY502934, TYLCSV-ES[ES:Mur1:92].Z25751, TYLCSV-Sar[IT:Sar:88].X61153, TYLCSV-Sic[IT:Sic].Z28390, TYLCV-Gez[SD:96].AY044138, TYLCV-IL[IL:Reh:86].X15656, TYLCV-IR[IR:Ira:98].AJ132711, TYLCV-mld[IL:93].X76319, TYLCV-OM[Oma:AlB:05].DQ644565. The complete names of the viruses can be found in Table 2. In addition a 16th sequence was used (TYLCIRV-[IR:Ira].AY297924) as it is known that TYLCIRV is related to TYLCV-IR via recombination.

between species representatives using a window of 50 nt across the entire length of genome, starting at the nicking site "↓AC" (Fauquet et al., 2005). Common sequences with more than 89% identity were considered similar and the colors indicate sequences of different origins. These common sequences presumably resulted from recombination between different isolates that occurred in the recent or long past. For clarity of the Figure 5, we have ignored sequences that are smaller than 100 nt. In the majority of the cases the real "donor" of sequence is unknown, and we can only point to common sequences between 2 or more viruses. The diagram in Figure 5 is showing some hot spots for recombination as identified in the previous paragraph, i.e., ±100, 500, 1,100, 1,800, 2,200 among others.

14. OPPORTUNITIES FOR RECOMBINATION OF TYLCV-LIKE VIRUSES

The emergence of TYLCV-like viruses is affected by many factors: the global spread of TYLCV encountering other tomato viruses (discussed in this chapter) provides new opportunities for these viruses to invade new ecological niches and to house several viruses within a single plant, thereby promoting recombinations. The frequency of coinfection of viruses belonging to different strains/species from the same region in a single plant is very high for viruses belonging to the TYLCV cluster (Monci et al., 2002; Accotto et al., 2003; Anfoka et al., 2005). For these recombination events, it is currently impossible to precisely distinguish between the old and new recombinants because of their close relationships.

Among all the cases of detectable recombinations in the TYLCV cluster, two belong to the first category, and these are TYLCAxV and TYLCMalV. This can be attested by the fact that despite a high level of surveillance of tomato viruses in Spain it is only recently that they were identified (Monci et al., 2002; Garcia-Andres et al., 2006) and secondly because of the type of recombination having a very high P value (Sawyer et al., 1989) and a very obvious pairwise recombination (Fauquet et al., 2005). It is hypothesized that TYLCV-IL and TYLCV-Mld have been introduced in Spain in the 1990s, allowing recombination with the local viruses, namely TYLCSV-Sac, -Sic, and -ES. These two recombinants are the only two cases where we could identify both parents in the TYLCV-cluster. All the other cases illustrated in Figure 5, presumably result from fairly old recombinations between local viruses known or unknown (marked with a "?" on the figure), with much lower P values for the estimated recombinations (Fauquet et al., 2005).

15. POSSIBLE LINEAGE BETWEEN TYLCV-LIKE VIRUSES

Figure 6, derived from Figure 5, is an attempt to retrace the lineage of TYLCV-like viruses with the geographical perspective. It is to be noted that for clarity, the recent introductions/spread of some TYLCSVs in Morocco, Tunisia, Lebanon, and Israel have been excluded from Figure 6. Primarily, TYLCV and TYLCSV are the two parents of newly created recombinants, namely TYLCAxV and TYLCMalV, resulting from the introduction of at least two strains of TYLCV from the Middle East (TYLCV-IL and TYLCV-mld), and perhaps also from the introduction of TYLCSV from Italy, unless this virus was already present on the Spanish territory (shown by unidirectional blue and yellow arrows on Figure 6). In all other cases it was possible to reconstruct a possible lineage with local viruses and this is compatible with the map and conclusions of Figure 5, supporting again the concept that the involved viruses have been present for a long time in the same region. Until recently the TYLCSV species was confined to Italy–Spain and TYLCV to the Middle East

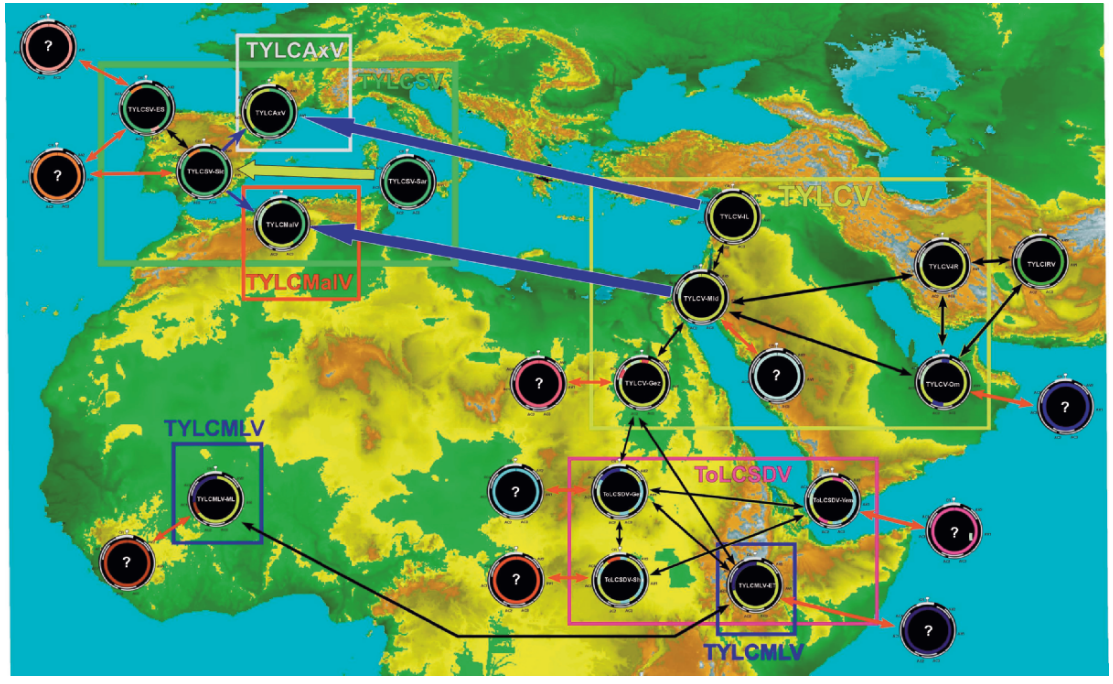

Figure 6. The diagram represents a theoretical lineage of the representative members of 15 strains pertaining to the 6 species of the *Tomato yellow leaf curl virus* cluster. Recombinations were assessed as in Figure 6 and represented as circle ssDNA for each virus with various colors using the same key as in Figure 5. For each virus, representing an average "strain", the genome organization is represented on the outside of the colored ring and the abbreviated name of the virus strain is indicated in the center of the genome organization. In addition a number of unknown recombinant parents have been indicated at the periphery of the diagram, with solid colors as their genome organization and a question mark instead of a name. The full names of the virus strains can be found in Table 2. The blue arrow represents very recent recombinations, the black arrow represents old recombinations and the red arrows represent putative recombinations with putative parents.

extending up to Iran, whereas ToLCSD is confined to the Sudan–Yemen region. The only case that is not highly consistent with the geographical distribution is the TYLCMLV species, with one strain identified from Mali and another in Ethiopia. The Ethiopian strain is related to other isolates belonging to the TYLCV and ToLCSDV species, therefore consistent with the geographical distribution. Trade between Mali and Ethiopia is not very intense and therefore it might not be the only cause for this situation, but it is possible that we are missing viruses between these two regions that could bring intermediates and thereby resolve the question. By the study of its sequence, we can however conclude that the Malian isolate was not a recent introduction of the Ethiopian strain.

The overall geographical distribution of TYLCV-like viruses is fairly consistent with a long term "natural" geographical distribution and a recent "Human" introduction. In addition, we cannot exclude some short distance movement of viruses by human interference. Evidently it is impossible to know what the original donors were, but it is nevertheless possible to retrace the lineage of related viruses.

16. EVOLUTIONARY CONSIDERATION OF BEGOMOVIRUSES AND TYLCV

Emergence of viral diseases can cause considerable damage to the crops (Chua et al., 2000; Hahn et al., 2000; Rybicki & Pietersen, 1999; Schrag & Wiener, 1995). Viruses display much higher variability compared to other organisms and at least two factors are responsible for this; an error-prone replication mechanism in most plant viruses and a high recombination frequency in some plant viruses (geminiviruses). Mutation is the most common viral genomic variation and is estimated to be in the order of 10^{-3}–10^{-5} mutations per base every year. This means that in average and if two mutations were not located twice at the same base, a geminivirus sequence could be completely changed every 3,000 years! The second factor is recombination, which is important among plant viruses and particularly those with single-stranded DNA genomes like geminiviruses. Notably for the members of the family *Geminiviridae*, recombination is extremely common, not only when forced under experimental conditions, but also among naturally occurring isolates (Harrison & Robinson, 1999; Padidam et al., 1999). Geminiviruses are extremely diversified as evaluated by the number of species described so far (more than 200) (Fauquet et al., 2007). Due to the broad feeding habits of the whiteflies, the geminiviruses they carry are introduced in a large number of plant hosts, many of them already containing other geminiviruses and thus their coexistence in the same plant host gives considerable opportunity for recombination to occur. It has been shown that geminiviruses can recombine both at intraspecies and interspecies levels, and even at the intergenera level, and that recombinations are extremely frequent for all types of geminiviruses (Padidam et al., 1999).

A prerequisite for such a recombination is the encounter of different viruses not only in the same host but within the same cell and the same nucleus. Recent

introduction of begomoviruses into new areas provides an ideal *in situ* model to analyze the aspects of genetic adaptation and evolution of an invading virus. The spread of begomoviruses in Spain is now well documented (Sánchez-Campos et al., 1999, 2002). The first reports of infections were made in early 1990s, associated with the presence of TYLCSV. Subsequently introductions of TYLCV isolates were reported, which provided the substrate for interactions, and spread to new host species (Monci et al., 2002; Morilla et al., 2003, 2005; Navas-Castillo et al., 1999). Garcia-Andres et al. (2006) have described a wild reservoir of TYLCD-associated viruses, which helps to get an insight into the bases of begomovirus epidemics and their evolution. They provide evidence for the presence of a novel recombinant begomovirus TYLCAxV for which putative parents, with spatial (geographical area, host), and temporal origin, have been suggested. The colonization of begomoviruses in Spain is an interesting example of successful invasion following multiple introductions (Figure 6). This provides novel sources of variation and conditions for genetic exchange, which helps in local adaptation of the invader begomoviruses (Monci et al., 2002).

The presence of TYLCV has also been reported in pepper plants at rates as high as 100% by Polston et al. (2006), thus demonstrating the ability of pepper to serve as reservoir for the acquisition and transmission of TYLCV. Ambrozevicius et al. (2002) verified a close phylogenetic relationship between begomoviruses infecting tomato and those associated with weeds in tomato fields in the Southeastern region of Brazil, which was studied by direct sequencing of PCR fragments amplified by using universal oligonucleotides for the begomovirus DNA-A, and subsequent computer-aided phylogenetic analysis. This analysis indicated the presence of an additional four possible new species and this high degree of genetic diversity suggested a recent transfer of indigenous begomovirus from wild hosts into tomatoes.

17. CONCLUSION

The analysis of DNA sequences has become the tool of choice to identify geminiviruses and to study their diversity, allowing one to accurately identify the virus and to evaluate its relationship with other isolates (Fauquet & Stanley, 2003; Crespi et al., 1995; Hong & Harrison, 1995; Kheyr-Pour et al., 1991; Noris et al., 1994). Sequence comparisons of geminivirus genomes, genes, intergenic region, and gene products have been used to construct phylogenetic trees (Hong & Harrison, 1995; Howarth & Vandemark, 1989; Padidam et al., 1995a, b). These analyses complemented by pairwise sequence analysis for the complete DNA-A component, have shown that geminiviruses are generally geographically distributed with particularly NW and OW geminiviruses, but also down to much smaller regions (Italy–Spain, Middle-East). Tomato begomoviruses are no exception as exemplified by the very large number of tomato begomovirus species in the world. Among them, TYLCV and closely related viruses form a cluster called TYLCV-like cluster geographically distributed and originally comprising isolates from the Mediterranean basin and Northern part of Africa.

Sequence comparisons have also suggested that new virus species be created to accommodate all TYLCV-like viruses according to the ICTV species demarcation criteria; e.g., *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl Malaga virus* (TYLCMalV), *Tomato yellow leaf curl Axarquia virus* (TYLCAxV), *Tomato yellow leaf curl Malawi virus* (TYLCMLV), and *Tomato yellow leaf curl Sudan virus* (ToLCSDV) (Fauquet et al., 2003; Fauquet & Stanley, 2005). TYLCV and TYLCSV are the two most distinct virus species without recombinations in common, but all the other representative members of species in this cluster do contain various recombinant fragments originating from TYLCV, TYLCSV, and other known or unknown viruses, forming now almost a continuum network between the two species TYLCV and TYLCSV. It thus becomes difficult to clearly demarcate each species, but the current classification is the best one representing the molecular variability in nature. Furthermore these viruses do contain some fragments that are common to other geminiviruses infecting tomato and other plant species, indicating the great capacity of geminiviruses to recombine and the essential role of recombination in their evolution. Novel recombinants like TYLCAxV and TYLCMalV are extremely recent in their origin (less than 12 years?) and this demonstrates the impact of modern agriculture/trade on geminivirus evolution. It remains to be seen if these viruses will have a better fitness and will be able to invade new ecological niches.

A study of the distribution of TYLCV, and TYLCSV in the world indicates that these viruses are rapidly spreading, as a result of human activities like trade, and agribusiness in different parts of the world. However, this also indicates the great capacity of some viruses like TYLCV and TYLCSV to adapt to new niches that they invade very quickly, suppressing the occurrence of local viruses like ToMoV in Florida. So far we have documentation for such worldwide spread only for these viruses that could very well be exceptions, but also could be the beginning of a trend with an increasing global trade. Still many TYLCV recombinants with their local viruses such as ToMoV remain to be identified, as now TYLCV is infecting the same tomato host as local OW and NW geminiviruses. If this was to happen, it could mark the second impact of humans after crop domestication, directly impacting geminivirus evolution by promoting adaptation to newly introduced hosts (domestication) and the creation of hybrids between viruses that have been separated in their natural evolution during several millions of years (international trade).

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CHAPTER 3

RECOMBINATION IN THE TYLCV COMPLEX: A MECHANISM TO INCREASE GENETIC DIVERSITY. IMPLICATIONS FOR PLANT RESISTANCE DEVELOPMENT

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1. RECOMBINATION INCREASES GENETIC DIVERSITY AND DRIVES EVOLUTION OF PLANT VIRUSES

Mutation, reassortment, and recombination are the major sources of genetic variation of plant viruses (García-Arenal et al., 2001; Worobey & Holmes, 1999). During mixed infections, viruses can exchange genetic material through recombination or reassortment of segments (when the parental genomes are fragmented) if present in the same cell context of the host plant. Hybrid progeny viruses might then arise, some of them with novel pathogenic characteristics and well adapted in the population that can cause new emerging diseases. Genetic exchange provides organisms with a tool to combine sequences from different origins which might help them to quickly evolve (Crameri et al., 1998). In many DNA and RNA viruses, genetic exchange is achieved through recombination (Froissart et al., 2005; Martin et al., 2005). As increasing numbers of viral sequences become available, recombinant viruses are recognized to be frequent in nature and clear evidence is found for recombination to play a key role in virus evolution (Awadalla, 2003; Chenault & Melcher, 1994; Moonan et al., 2000; Padidam et al., 1999; Revers et al., 1996; García-Arenal et al., 2001; Moreno et al., 2004). Understanding the role of recombination in generating and eliminating variation in viral sequences is thus essential to understand virus evolution and adaptation to changing environments (de Wispelaere et al., 2005; Vignuzzi et al., 2006; Domingo, 2000; Eigen, 1993).

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Knowledge about the existence and frequency of recombination in a virus population might help understanding the extent at which genes are exchanged and new virus variants arise. This information is essential, for example, to predict durability of genetic resistance because new recombinant variants might be formed with increased fitness in host-resistant genotypes. Determination of the extent and rate at which genetic rearrangement through recombination does occur in natural populations is also crucial if we use genome and genetic-mapping information to locate genes responsible of important phenotypes such as genes associated with virulence, transmission, or breakdown of resistance. Therefore, better estimates of the rate of recombination will facilitate the development of more robust strategies for virus control (Awadalla, 2003).

Recombination appears to be common among members of the family *Geminiviridae*, which have single-stranded DNA genomes (Padidam et al., 1999). In this group of viruses, more notably among members of the genus *Begomovirus*, recombination seems to contribute greatly to the genetic diversification of viral populations (Zhou et al., 1997; Berrie et al., 2001; Pita et al., 2001; Monci et al., 2002; Chatchawankanphanich & Maxwell, 2002; Umaharan et al., 1998; Moffat, 1999; Harrison & Robinson, 1999; Sanz et al., 1999, 2000). Replication of these viruses, in addition to a rolling circle replication (RCR) mechanism (Saunders et al., 1991; Stenger et al., 1991), also involves a recombination-dependent replication (RDR) mechanism (Jeske et al., 2001). RDR provides geminiviruses with a tool by which damaged or incomplete DNA could be recovered for productive infection by homologous recombination and converted into full-size genomic DNA. The existence of this replication mechanism might explain in part the extent at which recombination occurs in geminivirus populations (Jeske et al., 2001; Preiss & Jeske, 2003). Recombination in begomoviruses is found at the strain (Hou & Gilbertson, 1996; Kirthi et al., 2002), species (Zhou et al., 1997; Fondong et al., 2000; Navas-Castillo et al., 2000; Sanz et al., 2000; Martin et al., 2001; Saunders et al., 2002; Monci et al., 2002; García-Andrés et al., 2006), genus (Briddon et al., 1996; Klute et al., 1996), and family (Saunders & Stanley, 1999) levels. The potential of begomoviruses to generate genetic diversity through recombination can be relevant for their ecological fitness, because greater sequence heterogeneity provides a reservoir of virus variants in the population that enables rapid adaptation to changing environmental conditions. Thus, begomoviruses like those in the *Tomato yellow leaf curl virus* (TYLCV) complex exploit gene flow provided by recombination as a mechanism to increase their evolutionary potential and local adaptation.

2. RECOMBINATION HAS PLAYED AN IMPORTANT ROLE IN THE ORIGIN OF VIRUSES OF THE TYLCV COMPLEX

The tomato yellow leaf curl disease (TYLCD) causes severe damage to tomato production in many warm and temperate regions worldwide (Cohen & Antignus, 1994; Moriones & Navas-Castillo, 2000; Varma & Malathi, 2003).

Different virus species and strains of the same virus species have been associated with TYLCD, among them, TYLCV (Moriones and Navas-Castillo et al., 2000; Fauquet et al., 2003; Stanley et al., 2005). In this chapter, TYLCD-associated viruses are referred to as "TYLCV complex". Recombination seems to have played an important role in the origin of viruses of the TYLCV complex. Two case studies are examined here in detail.

2.1. Case study I: The type strain of the species TYLCV is a recombinant virus which shares an ancestral parent with extant Asian begomoviruses

The earliest evidence of naturally occurring recombination within the genus *Begomovirus* was found when the genome of the Mld strain of the monopartite virus *Tomato yellow leaf curl virus* (TYLCV-Mld) (Antignus & Cohen, 1994) was compared with the genome of the type strain of TYLCV (Navot et al., 1991). The nucleotide sequences of the Rep gene and the intergenic region (IR) of the type and Mld strains of TYLCV were only 87% and 78% identical, respectively, whereas the rest of the genome shared 98% nucleotide identity. Harrison & Robinson (1999) suggested that the Rep–IR regions of both genomes were acquired from different parental viruses that could not be identified at that moment. However, when increasing number of begomovirus DNA-A sequences became available, Navas-Castillo et al. (2000) were able to identify the existent viruses more related to such parents. These authors compared the sequences of nine isolates of the TYLCV complex, three of the type strain of TYLCV (the original isolate from Israel, and isolates from the Dominican Republic and Cuba), five of the TYLCV-Mld strain (the original Mld isolate from Israel, and isolates from Spain, Portugal, and Japan), and one TYLCV isolate from Iran (now recognized as the IR strain of TYLCV). When phylogenetic relationships between nucleotide sequences of these isolates were analyzed, changes in the topological position of certain isolates occurred depending on the part of the genome compared (IR and open reading frames – ORFs – V1, V2 and C1–C4). Detailed comparisons throughout the genome using PLOTSIMILARITY diagrams (Wisconsin GCG software package) (Devereux et al., 1984) clearly indicated that four regions (named I–IV in Figure 1) were recognized for which differential distribution of nucleotide identity was observed. Region III comprised about 5′ half of the Rep gene (ORF C1), including the ORF C4 and part of the IR; region I comprised most of the rest of the genome, and regions II and IV were small regions separating region I from region III (Figure 1). In these comparisons, the nucleotide sequences of the TYLCV-Mld isolates from Spain, Portugal, and Japan on the one hand and those of the TYLCV type strain isolates from Israel, Cuba, and Dominican Republic on the other, proved to be almost identical between them throughout the genome. Also, when region I was analyzed, phylogenetic analyses revealed that all TYLCV isolates grouped in a single clade related to *Tomato yellow leaf curl Sardinia virus* (TYLCSV), another species of the TYLCV complex causing the TYLCD.

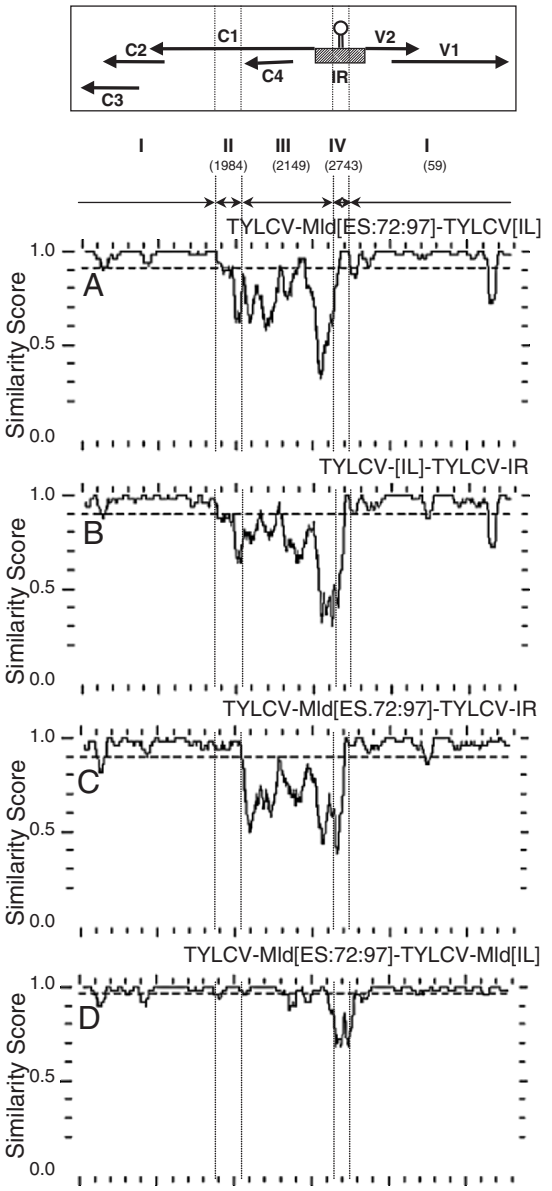

Figure 1. PLOTSIMILARITY diagrams (scanning window = 50) comparing the nucleotide sequences of TYLCV isolates (**A**) of the Mld (isolate ES:72:97) and type (isolate IL) strains, (**B**) of the type (isolate IL) and Iran (isolate IR) strains, (**C**) of the Mld (isolate ES:72:97) and Iran (isolate IR) strains, and (**D**) two isolates of the Mld strain (isolate ES:72:97and IL). Separation between regions I–IV for which differential distribution of nucleotide identity is observed are indicated by vertical dotted lines. In brackets is indicated the first nucleotide of the region (numbers refer to nucleotide positions in the sequence of TYLCV-Mld[ES:72:97]. Positions of the open reading frames (ORFs) and of the intergenic region (IR) are indicated at the top of the figure. Horizontal broken lines are the mean similarity between the sequences compared. GenBank accession number of sequences used for comparison are AF071228 (TYLCV-Mld[ES:72:97]), AJ132711 (TYLCV-IR[IR]), X15656 (TYLCV-[IL:Reo:86]), and X76319 (TYLCV-Mld[IL]). (Adapted from Navas-Castillo et al., 2000.)

However, when comparing sequences in region III, significant changes occurred in the phylogenetic relationships of certain TYLCV isolates. Thus, based on sequences of this region most TYLCV isolates grouped in a single clade related to TYLCSV, but TYLCV (type strain) and TYLCV-IR isolates grouped separately, together with begomovirus isolates Bangalore-2 and Bangalore-4. These two latter viruses were considered at that moment to belong to the begomovirus species, *Tomato leaf curl virus* (ToLCV) (Moriones & Navas-Castillo, 2000), however now they are known to belong to the Asian begomovirus species *Tomato leaf curl Karnataka virus* (ToLCKV) and *Tomato leaf curl Bangalore virus* (ToLCBV), respectively (Stanley et al., 2005). Therefore, the genomes of TYLCV, TYLCV-Mld, and TYLCV-IR begomovirus isolates reflect a modular composition, with genome fragments having diverse phylogenetic origins that were probably put together after successive recombination events.

2.2. Case study II: The type strain of TYLCSV is a recombinant virus which shares an ancestral parent with extant African begomoviruses

Tomato yellow leaf curl Sardinia virus (TYLCSV) is another monopartite begomovirus species of the TYLCV complex that comprises isolates infecting tomato in the Mediterranean Basin, both in southern Europe and northern Africa (Noris et al., 1994; Moriones & Navas-Castillo, 2000). In the TYLCSV clade, at least three different types of sequences can be distinguished, represented by the type strain, originally described from Sardinia (TYLCSV), the Sicily strain (TYLCV-Sic), and the Spain strain (TYLCV-ES). Through comparison of the genome of isolates of the type and Sic strains from Italy following a similar procedure to that described above for TYLCV (i.e., search for topological changes in the phylogenetic trees, and analysis with PLOTSIMILARITY diagrams), evidence was obtained for differences in the phylogenetic origin of the different genomic regions of these isolates probably as a result of recombination events. Two regions could be distinguished when the genomes of these isolates were compared (Figure 2A): region I, in which the percentage of nucleotide identity between TYLCSV and TYLCSV-Sic is 96%, and region II, which includes a shorter fragment that comprises part of the IR, and the 5′ end of ORF C1, in which the percentage of nucleotide identity is significatively lower, about 64%. When nucleotide sequences in region I were phylogenetically analyzed, TYLCSV type strain clustered in the TYLCSV-clade, closely related to TYLCSV-Sic, TYLCSV-ES, and the rest of viruses of the TYLCV complex (Figure 2B). However, after comparison of nucleotide sequences in region II, a significant topological change occurred in the position of the TYLCSV type strain isolate (Figure 2C). Thus in this case, TYLCSV type strain isolate did not group with isolates of the TYLCV complex, but in a clade that comprised all the cassava-infecting begomoviruses from Africa, being the closest related sequence that of an isolate of *South African cassava mosaic virus* (SACMV) (Figure 2C). Therefore, these results strongly suggested that the TYLCSV-type strain resulted

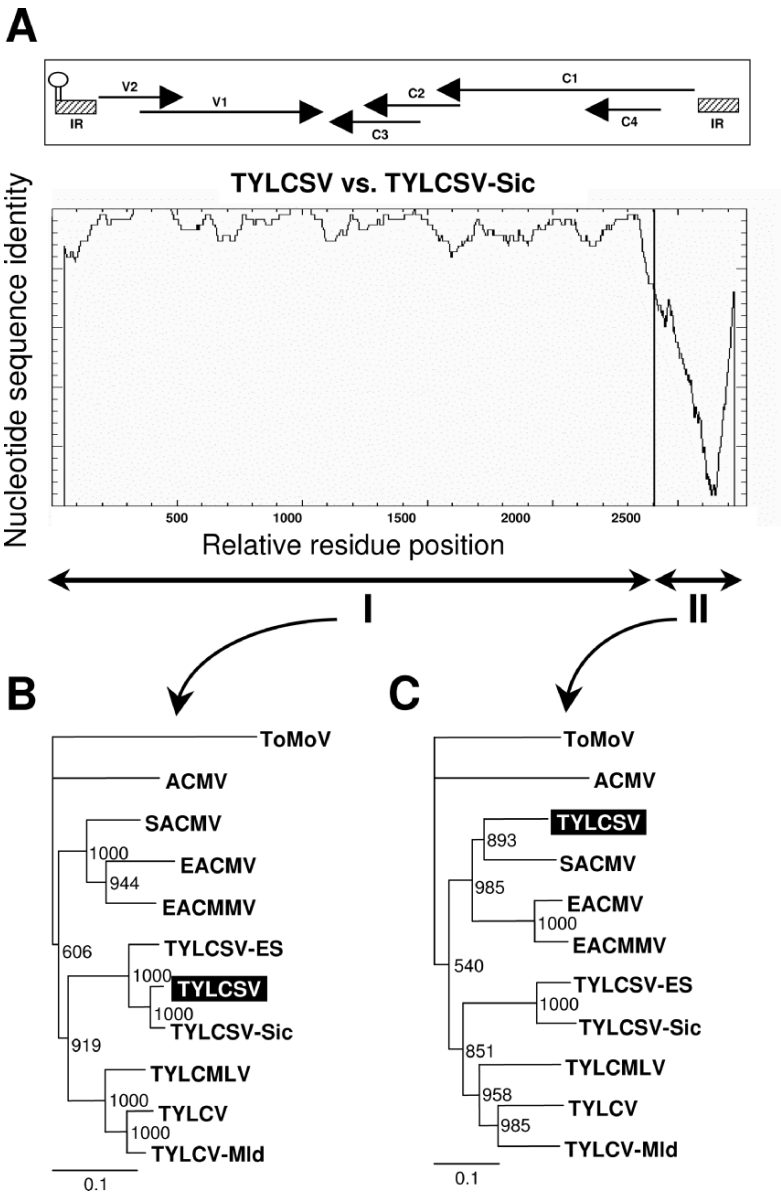

Figure 2. PLOTSIMILARITY diagram comparing the nucleotide sequences of isolates of the type (isolate Sar) and Sic (isolate Sic) strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV). Regions I and II for which differential distribution of nucleotide identity is observed are indicated. Positions of the open reading frames (ORFs) and of the intergenic region (IR) are indicated at the top of the figure (**A**). Phylogenetic relationships for viruses in the TYLCV-complex and the DNA A

from a recombination exchange of genetic material occurred between TYLCSV and African begomovirus ancestors.

2.3. Begomoviruses of the TYLCV complex are evolving through genetic exchange in their travel accross Asia and Africa

The above examples of putative recombinations involving begomoviruses of the TYLCV complex from the Middle East and the Mediterranean Basin seem to reflect exchange of genomic fragments with begomoviruses present in Asia (e.g., India) and Africa. Therefore, it could be hypothesized that an ancestral "TYLCV" evolved and generated new variants (species or strains) by exchanging genetic material through recombination with other begomoviruses in its travel accross different areas of the Old World. Because begomoviruses in the TYLCV complex belong to a clade of begomoviruses of the Old World that affect tomato, and this crop was introduced in this region from America only recently, several scenarios can be suggested. One possibility is that a number of TYLCV-like viruses could have already existed in the wild or cultivated hosts of the Old World before the introduction of tomato. When tomato was grown in different areas, it could have become infected with these preexisting viruses. Alternatively, an ancestor of the begomoviruses of the TYLCV complex infected tomato and, in its travel through the regions of Asia, Africa, and Europe (regions in which tomato has become a major crop), different virus lineages evolved by acquisition of genomic fragments from other begomoviruses by genetic exchange through recombination. Thus, emergence of new begomoviruses could have occurred which shared tomato as common host. As tomato has become a major crop, it could act as a bridge for begomoviruses between other local crops or wild reservoirs, favoring contact between viruses otherwise separated. Spread of *Bemisia tabaci* biotypes highly polyphagous like the biotype B could also have favored exchange of viruses between tomato and other cultivated or wild hosts and thus facilitating recombination to occur. Evidently, it is reasonable that an intermediate situation between the two scenarios proposed is what occurred and probably is occurring. New information about viruses infecting wild and cultivated hosts

Figure 2. (continued) component of representative isolates of viruses infecting cassava from Africa. Relationships were inferred by using the neighbor-joining method on the sequences of the region I (**B**) and II (**C**) deduced from the PLOTSIMILARITY analysis. Support for nodes in a bootstrap analysis with 1,000 replications is shown for figures over 500. Horizontal branch lengths are drawn to scale with the bar indicating 0.1 nucleotide replacements per site; vertical distances are arbitrary. Abbreviations and GenBank accession numbers are as follow: ACMV, *African cassava mosaic virus*, J02057; SACMV, *South African cassava mosaic virus*, AF155806; EACMV, *East African cassava mosaic virus*, Z83257; EACMMV, *East Africa cassava mosaic Malawi virus*, AJ006460; TYLCSV-ES, *Tomato yellow leaf curl Sardinia virus-Spain*, Z25751; TYLCV, *Tomato yellow leaf curl Sardinia virus*, X15655; TYLCSV-Sic, *Tomato yellow leaf curl Sardinia virus-Sicily*, Z28390; TYLCMLV, *Tomato yellow leaf curl Mali virus*, AY502934; TYLCV, *Tomato yellow leaf curl virus*, X15656; TYLCV-Mld, *Tomato yellow leaf curl virus-Mild*, X76319. As outgroup, an isolate of *Tomato mottle virus* (ToMoV) was used (GenBank L14460).

in regions of the Old World could provide some clues about the origin and evolution of this complex of viruses.

In addition to the well-documented examples of recombination shown above, other examples that involved begomoviruses of the TYLCV complex have also been reported in the literature. Thus, an interspecific recombination has been described for begomoviruses infecting tomato in central Sudan (Idris & Brown, 2005). In this case, two recombinant fragments were identified in the genome of the isolate SD:Gez:96 of *Tomato leaf curl Sudan virus* from Gezira (ToLCSDV-[SD:Gez:96]) when compared with the isolate SD:96 from Sudan of the Gezira strain of TYLCV (TYLCV-Gez[SD:96]). Also, Padidam et al. (1999) detected several other putative recombination events also involving viruses of the TYLCV complex by employing a statistical technique for detecting gene conversion based on the program GENECONV. Their analyses, using 64 geminivirus DNA A sequences, identified 420 statistically significant recombinant events, 36 of them being listed and identified. Six of the listed recombination events involved TYLCV or TYLCSV, some of them between strains of the same species (e.g., TYLCSV/TYLCSV-Sic) whereas others had as a partner a non-TYLCV virus from Africa or Asia (e.g., TYLCV/*Chayote yellow mosaic virus*, TYLCSV/*Indian cassava mosaic virus*). Surprisingly, GENECONV identified as recombinant a fragment shared between a Spanish isolate of TYLCSV and *Bean dwarf mosaic virus*, a begomovirus species from the New World.

Rybicki (1994) already pointed out that recombination is probably a powerful tool in the evolution of begomoviruses, not only in the long term but also in the short to medium term. In this sense, Padidam et al. (1999) evaluated the hypothesis that recombination among begomoviruses is contributing to the increasing emergence of new species and suggested that such studies could facilitate understanding of how viruses could evolve in response to changes in the ecosystem. In the next sections of this chapter, we will present data that evidenced the extent at which recombination is contributing to the diversification and adaptation of begomoviruses of the TYLCV complex during their colonization of southern Spain (Western Mediterranean Basin). Emergence and spread of new recombinant viral species belonging to the TYLCV complex is shown from field data. Also evidences from laboratory experiments are provided that support frequent emergence of new recombinant virus variants during single host plant infection cycles, in mixed infections between TYLCV and TYLCSV.

3. RECOMBINATION IS DRIVING POPULATION EVOLUTION OF VIRUSES OF THE TYLCV COMPLEX INVADING NEW GEOGRAPHICAL AREAS: THE CASE OF THE WESTERN MEDITERRANEAN BASIN

The two case studies described in the previous section are examples of ancient, and probably multiple, recombination events that contributed to the emergence of begomoviruses of the TYLCV complex. But if located at the right place and the right time, it could be possible to be witness to the occurrence of such a

recombination event and emergence of the new virus variant originated. This was the case during studies of the epidemics of begomoviruses of the TYLCV complex that recently colonized southern Spain. Following are data that evidence the relevance of recombination in the rapid evolution of such a population for its adaptation to a novel environment.

3.1. Mixed infections: A prerequisite for recombination to occur in a begomovirus population

Mixed infections can be frequent in nature associated with begomovirus epidemics. Thus for example, in a recent survey for viruses associated to TYLCD in epidemics in tomatoes of the Western Mediterranean Basin (Italy and Spain), it was shown the presence of isolates of eight different virus variants of the TYLCV complex occurring simultaneously in the epidemics (Figure 3) (García-Andrés et al., 2007a). Coexistence of isolates corresponding to different virus variants in single fields and even mixed infections in single plants are suggested, which is a prerequisite for recombination to occur. In fact, analysis of begomovirus-related sequences present in single samples demonstrated that several virus strains could coexist (e.g., in sample T570/02:F4:Sic; Figure 4). Therefore, opportunities for genetic exchange are evident, and appearance of novel variants as a result of recombination events can be predicted. As mentioned before, begomovirus replication involves two mechanisms, a RCR (Saunders et al., 1991; Stenger et al., 1991) and a RDR (Jeske et al., 2001; Preiss & Jeske, 2003). Recombinant variants can be produced through the latter mechanism if viruses coexist in the same cell. If viable and competitive, these *de novo* created recombinant viruses can emerge and perpetuate in the population during epidemics.

Recent introduction of begomoviruses into new areas provided an ideal model to analyze aspects of genetic adaptation and evolution of an invading virus population. This was the case of the colonization of southern Spain by begomoviruses of the TYLCV complex associated with TYLCD, which is well documented (Sánchez-Campos et al., 1999, 2002). This case is an interesting example of invasion success following multiple introductions, similar to those reported for animal or plants (Novak & Mack, 2001; Kolbe et al., 2004), in which recombination is providing tools for biological adaptation. Initial colonization with isolates of the ES strain of TYLCSV during the early 1990s, resulted in a relatively stable population in which reduced genetic diversity was observed, a typical result of a population bottleneck upon invasion of a new area (Sánchez-Campos et al., 2002). This could have been detrimental for the success of the invader begomovirus population. However, subsequent introductions of isolates of the type and Mld strains of TYLCV (Navas-Castillo et al., 1999; Morilla et al., 2003) resulted in novel sources of variation, and conditions for recombination to occur, thus providing to the begomovirus population tools to gain potential for local adaptation. This was the case of the novel recombinant variant named *Tomato yellow leaf curl Málaga virus* (TYLCMalV) that

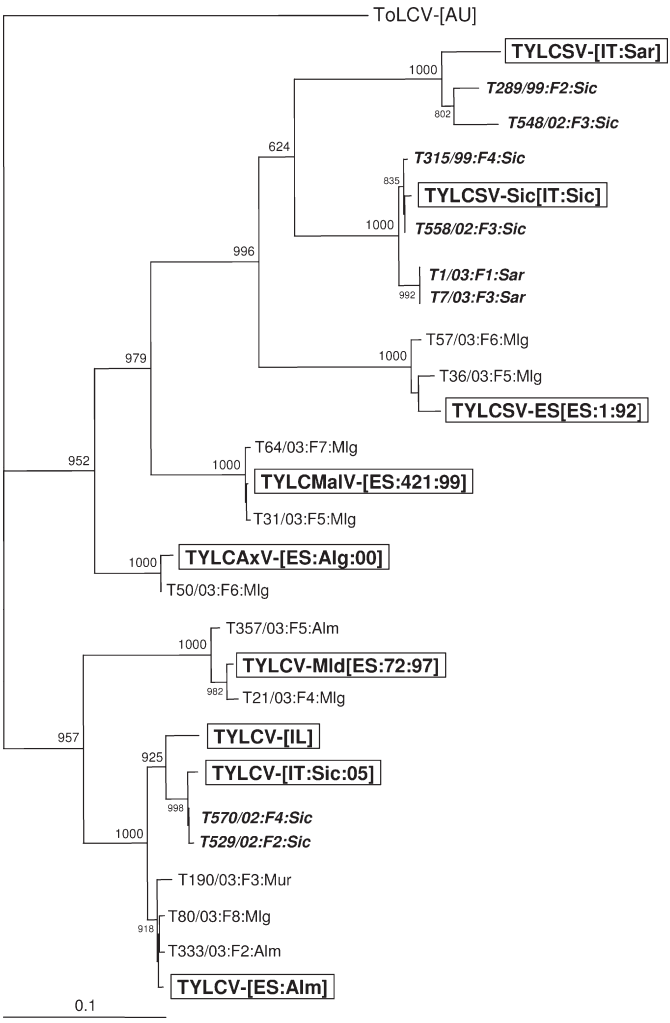

Figure 3. Phylogenetic relationships for *Tomato yellow leaf curl disease* (TYLCD)-associated begomovirus isolates present in tomato samples randomly collected in Italy (italics/bold letters) and Spain (normal letters) between 1999 and 2003. Relationships were inferred based on a sequence of about 300 nucleotides encompassing the intergenic region (IR) by neighbor-joining analysis. Support for nodes in a bootstrap analysis with 1,000 replications is shown for figures over 500. Horizontal branch lengths are drawn to scale with the bar indicating 0.1 nucleotide replacements per site. Vertical distances are arbitrary. Names of isolates refer to host species origin (T, tomato), sample number/year, field (Fi means field i), and sampling region (Sicily, Sic, and Sardinia, Sar, in Italy; Málaga, Mlg, Almería, Alm, and Murcia, Mur, in Spain). Representative isolates are included of begomovirus species associated with TYLCD in the Mediterranean area, the type, Sic and ES strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV), type (isolates from Israel, Spain, and Italy) and Mld strains of *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Málaga virus* (TYLCMalV), and *Tomato yellow leaf curl Axarquía virus* (TYLCAxV) (GenBank accession numbers X61153, Z28390, Z25751, X15656, AJ489258, DQ144621, AF071228, AF271234, and AY227892, respectively) (boxed and bold letters). As outgroup, an isolate of *Tomato leaf curl virus* (ToLCV) was used (GenBank S53251).

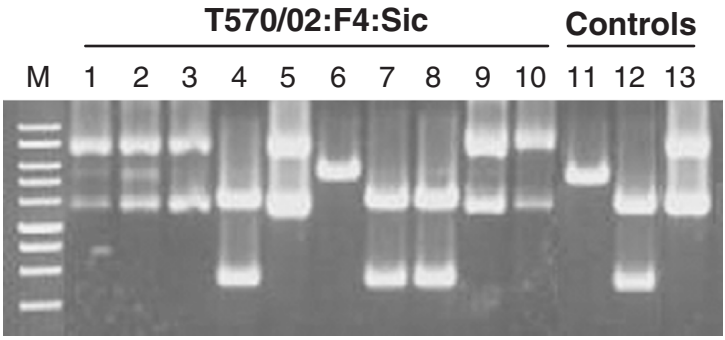

Figure 4. Restriction fragment length polymorphism analysis with the restriction enzyme Bgl II on 10 almost full-length genome clones of *Tomato yellow leaf curl disease*-associated (TYLCD) begomoviruses present in sample T507/02:F4:Sic. The almost full-length genome fragments were PCR-amplified from total nucleic acids obtained from the sample by means of a primer pair designed on nucleotide sequences conserved among all the TYLCD-associated viruses reported from the Western Mediterranean Basin: MA241 (5′-GAATGGGCTTCCCATACTTTGTGTTGC-3′), corresponding to 1739 to 1765 nt of TYLCSV-ES[ES:1:92] (GenBank Z25751), and MA242 (5′-CACTATCTTCCTCTGCAATCCAGG-3′), complementary to 1,719 to 1,696 nt of this same virus. PCR fragments thus obtained were cloned into pGEM-T (Promega Corporation, Madison, USA) to derive the clones analyzed here (lanes 1–10). Equivalent clones obtained from samples infected with known isolates of the type and Sic strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (lanes 11 and 12, respectively) and of the type strain of *Tomato yellow leaf curl virus* (TYLCV) (lane 13) were used as controls. A 1Kb molecular marker (lane M) was included.

emerged as a result of a genetic exchange between isolates of the ES strain of TYLCSV and of the Mld strain of TYLCV. This natural recombinant variant showed to be better adapted ecologically than either parental virus and spread rapidly in the population (Monci et al., 2002). Recently, a novel recombinant between TYLCSV-ES and the type strain of TYLCV was detected in the population, which also seemed to be well adapted ecologically (García-Andrés et al., 2006). Therefore, recombination showed to be an important force driving the evolution of the population of these viruses for adaptation to the novel ecological conditions present in the invaded area.

3.2. Wild hosts: reservoirs of mixed infections for begomovirus epidemics

Native species, acting as reservoirs, can play an important role in the emergence of plant virus epidemics (Hull, 2002). For begomoviruses, studies are available that indicate presence in wild reservoirs (Funayama-Noguchi, 2001; Jovel et al., 2004; Ooi et al., 1997; Roye et al., 1997). To evaluate the possible importance of wild reservoirs as sources of begomovirus genetic diversiy for epidemics, the begomovirus population present in the *Solanum nigrum* L. plant community found in southern Spain was examined. *S. nigrum* is a wild host widely distributed in the Mediterranean area, which can survive for long periods (even for more than 2 years) thanks to the mild climatic conditions present. Infections with

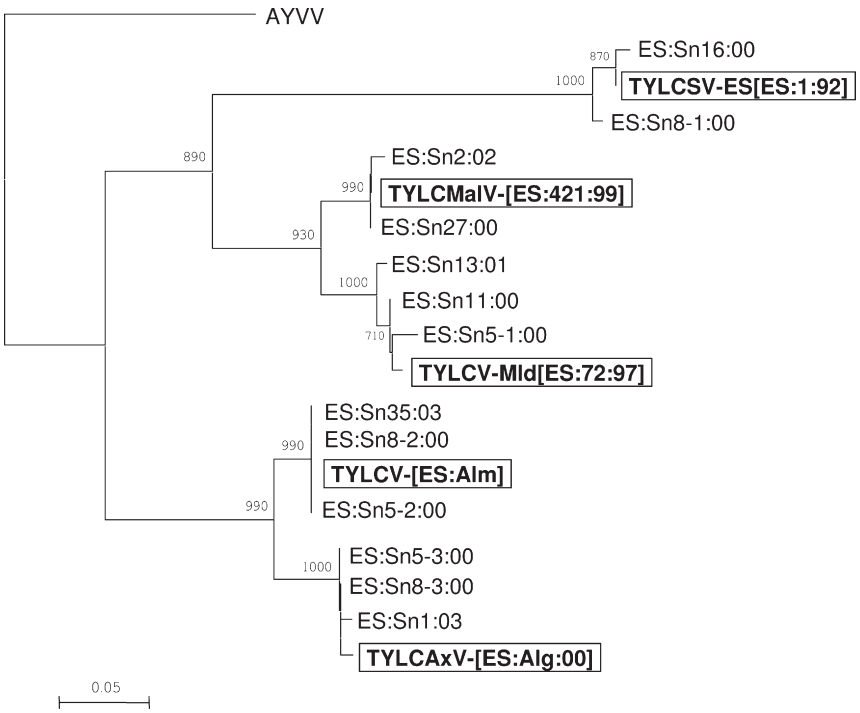

Figure 5. Phylogenetic relationships among begomoviruses detected in plants of the population of the native reservoir *Solanum nigrum* present in Málaga (southern Spain). Relationships were inferred by neighbor-joining analysis of sequences comprising the intergenic region (IR) (about 300 nucleotides). Support for nodes in a bootstrap analysis with 1,000 replicates is shown for figures over 700. Vertical distances are arbitrary and branch lengths are drawn to scale; the bar indicates 0.05 nucleotides substitutions per site. Begomovirus isolates are named according to indications of the Geminiviridae Study Group of the International Committee on Taxonomy of Viruses, giving the code of the country of origin, sample name, and year of collection (e.g., "ES:Sn8-1:00", in which "ES" refers to Spain, "Sn8-1", to isolate 1 derived from the sample number 8 of S. nigrum, and "00" to the year 2000). Representative begomoviruses included (boxed and bold letters) are the same as in Figure 3. As outgroup, an isolate of *Ageratum yellow vein virus* (AYVV) was used (GenBank X74516). (Adapted from García-Andrés et al., 2006.)

TYLCD-associated begomoviruses are known in this plant species (Bedford et al., 1998; Salati et al., 2002; Sánchez-Campos et al., 2000). Our studies indicated that this wild host is an excellent reservoir of variants of viruses of the TYLCV complex for TYLCD epidemics. In fact, phylogenetic reconstruction of sequences of begomoviruses obtained from *S. nigrum* plants sampled in the Málaga region (southern Spain) between 2000 and 2003 demonstrated the presence of isolates of all the TYLCD-associated begomoviruses species and strains reported in Spain (Figure 5). Moreover, mixed infections in single *S. nigrum*

plants were evident, as observed for example in sample Sn8:00, in which TYLCSV-like and TYLCV-like sequences were detected (e.g., isolates ES:Sn8-1:00, ES:Sn8-2:00, respectively, Figure 5). Therefore, *S. nigrum* plants can be an optimal niche for genetic exchanges to give rise to better-adapted recombinant begomoviruses. In fact, we demonstrated the presence in this host species of isolates of a new previously undescribed begomovirus of recombinant nature, named *Tomato yellow leaf curl Axarquía virus*, TYLCAxV (e.g., isolate ES:Sn1:03, Figure 5). This virus variant was demonstrated to be the result from a genetic exchange between isolates of the ES strain of TYLCSV and of the type strain of TYLCV (found coinfecting *S. nigrum* plants, Figure 5). Novel pathogenic properties are demonstrated for TYLCAxV that suggested enhanced ecological adaptation (García-Andrés et al., 2006). We concluded therefore that presence of mixed begomovirus infections in wild reservoirs can be a cause for alarm, because novel recombinants might arise with unpredictable consequences for epidemics of viruses of the TYLCV complex.

4. RECOMBINANTS OCCUR FREQUENTLY IN MIXED INFECTIONS OF VIRUSES OF THE TYLCV COMPLEX

Sequence analyses of field isolates have revealed substantial evidence for widespread occurrence of recombination amongst begomoviruses (Padidam et al., 1999; Sanz et al., 2000; Berrie et al., 2001; Pita et al., 2001; Monci et al., 2002; Chatchawankanphanich & Maxwell, 2002). However, it remains unclear whether recombination represents a frequent phenomenon shaping begomovirus populations during a single host plant infection life cycle. We investigated this aspect for infections with TYLCSV and TYLCV as model system (García-Andrés et al., 2007b). These two viruses coinfect single plants in nature (Sánchez-Campos et al., 1999; Monci et al. 2002; García-Andrés et al., 2006), and even could share single nuclei of an infected plant (Morilla et al., 2004), a prerequisite for recombination to occur. Natural mixed infections were simulated in tomato and the frequency of recombinant genomes was evaluated at several times post coinfection. We found that recombinant-like molecules accumulated in the virus progeny of mixed-infected plants and rapidly constituted a significant proportion of the population (in most cases about 50% of the genomes analyzed, Figure 6A). We also found that parent TYLCSV and recombinant variants generated *de novo* coexisted, suggesting that the latter fit well in the population and were not outcompeted. However, at least in the experimental conditions analyzed, TYLCV was outcompeted, suggesting that it is less adapted to compete *in planta* with either TYLCSV or the recombinants arisen. This was surprising because TYLCV seems to be well adapted to compete during natural epidemics (Sánchez-Campos et al., 1999). Therefore, other factors in addition to competitiveness *in planta* are associated with the success of a begomovirus variant in nature (transmission, host range, etc.). Interestingly, only

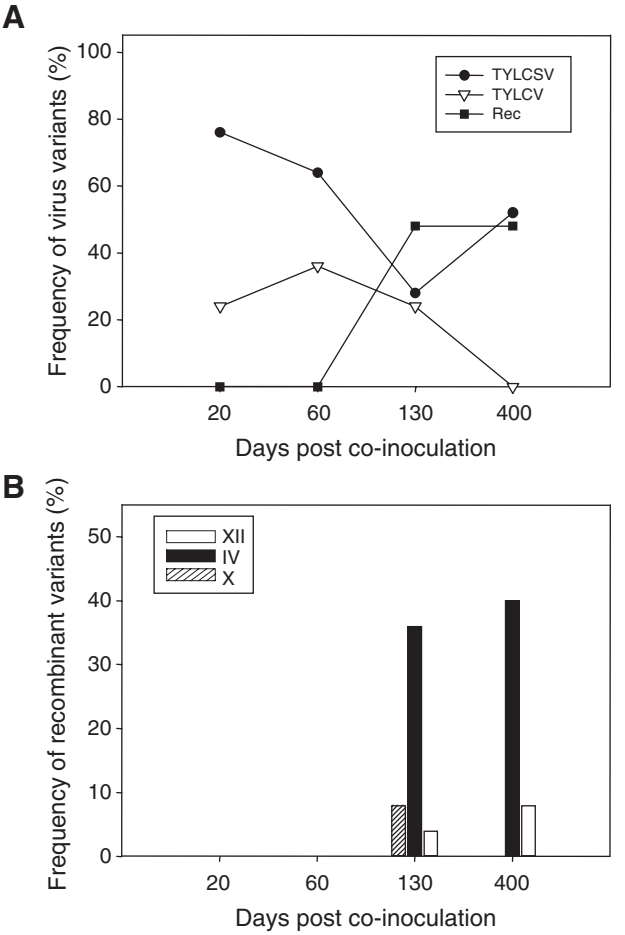

Figure 6. Appearance and frequency of recombinant genomes generated *de novo* after co-inoculation of the [ES:1:92] isolate of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES) (Navas-Castillo et al. 1999) and the [ES:72:97] isolate of *Tomato yellow leaf curl virus* (TYLCV-Mld) (Noris et al. 1994) in a single tomato cv. Moneymaker plant. Studies were based on restriction fragment length polymorphism analysis on clones containing full-length genome fragments amplified from virus population present in total nucleic acids extracted from the mixed-infected plant at several times post co-inoculation using the commercial kit TempliPhi (Amersham Biosciences, England). The amplified DNA was digested with the restriction enzyme BamHI that singly cut either TYLCSV-ES[ES:1:92] or TYLCV-Mld[ES:72:97] double-stranded DNA forms in equivalent genome positions, and the linearized genome size DNA fragments were cloned into the BamHI-cloning site of pBluescript SK+ (pBSK$^+$, Stratagene, La Jolla, CA). Twenty-five clones per time post-inoculation studied were analyzed. The evolution of the relative proportion of parental (TYLCV and TYLCSV) and recombinant-like variants (Rec) (**A**) and the frequency of the different recombinant variants found (variants X, IV, and XII) (**B**) at different times post-inoculation analyzed is shown. For restriction mapping, enzymes were selected (BglI, BglII, DraIII, EcoNI, HindIII, KpnI, PmlI, SacII, and SphI) that used in single combinations provide information about TYLCSV or TYLCV sequence identity at different positions along the cloned genome.

three types of recombinant variants could be recovered in the plant coinfected with TYLCV and TYLCSV during the 400-day infection cycle analyzed. Therefore, constraints for recombination seemed to exist in these viral genomes. Moreover, frequency of the different recombinant variants found in the population could vary with time but, at least in the experimental conditions used, one type predominated through the entire experiment (type IV, Figure 6B). Although additional studies are needed to better understand the significance of recombination in single host infection cycles in this group of viruses, these data suggested that recombination seems to be a frequent phenomenon and could contribute significantly in generating genetic diversity and novel virus variants for local adaptation.

5. IMPLICATIONS OF RECOMBINATION FOR VIRUS CONTROL THROUGH PLANT RESISTANCE

Given the importance of recombination in the molecular evolution of viruses promoting biological adaptation, understanding the frequency at which it occurs, mechanisms involved, and ecological features that control the rate of recombination, might help to predict the emergence of new viruses. This knowledge can be used to improve effectiveness and durability of current control procedures (Bonnet et al., 2005; Lewis-Rogers et al., 2004). Efficient control of plant virus diseases is difficult, however the use of virus-resistant cultivars can provide an effective mean to limit the economic damage caused. Although the use of resistance is the most desirable plant virus control strategy, it often fails because resistance-breaking virus genotypes appear and increase their frequency in the virus population (Lecoq et al., 2004; García-Arenal et al., 2001). The durability of resistance is determined by the evolutionary potential of plant viruses (García-Arenal & McDonald, 2003) and recombination is one of the major forces driving virus evolution. In this regard, recombination events have been demonstrated to be associated with major changes in fitness and pathogenic characteristics of plant viruses, including expansion of their host range and increase in their virulence (Fernández-Cuartero et al., 1994; Stenger et al., 1994; Pita et al., 2001; Zhou et al., 1997; Gibbs et al., 2001; Monci et al., 2002; García-Arenal & McDonald, 2003; Rest & Mindell, 2003; García-Andrés et al., 2006). Thus, recombination can accompany or even be at the origin of major changes during virus adaptation. In fact, recombination is known to be a potent mechanism to create more fit genotypes (Bürger, 1999; Hu et al., 2003), that can help viruses to adapt to novel environmental conditions (Dybdahl & Storfer, 2003; Lively & Dybdahl, 2000; Stavrinides & Guttman, 2004; Zhou et al., 1997). Therefore, the risk of break of a begomovirus resistance owe to the appearance and spread of better-adapted recombinant variants exists and should be considered to predict the durability of a resistance.

The abundance of recombinant variants in a virus population should also be kept in mind for the evaluation of the potential impact of recombination in the

use of transgenic plants expressing viral sequences (Harrison, 2002; Aaziz & Tepfer, 1999b; Tepfer, 2002; de Wispelaere et al., 2005). The virus-resistant transgenic plants (VRTPs) hold the promise of enormous benefit for agriculture, however, questions concerning the potential ecological impact have been raised (Tepfer, 2002). Numerous transgenic crops resistant to a wide range of viruses have been developed (Beachy, 1997), many of them based on the application of the concept of pathogen-derived resistance (Sanford & Johnston, 1985). Different virus sequences have been used for the development of virus-derived transgenic resistance, including genes encoding coat proteins, replicases, movement proteins, proteases, or helper components (Lomonossoff, 1995). However, it is important to examine VRTP carefully and take into account the risk of the deployment from the point of view of biosafety. Interactions are possible in transgenic plants between products of the viral transgene (whether DNA, RNA, or protein) and an incoming virus, which can result in potential ecological risks like synergism, heteroencapsidation, or recombination (Tepfer, 1993, 2002; Robinson, 1996; Aaziz & Tepfer, 1999a). It has been demonstrated that recombination of a challenging virus with a transgene could have important biological consequences such as changes in virulence or host range (Kiraly et al., 1998; Frischmuth & Stanley, 1998). Therefore, if as mentioned above RDR occurs during geminivirus multiplication within plants, transgenic constructs that provide information for symptom expression, host range, tissue and vector specificities should be avoided (Jeske et al., 2001). In this sense, it is a fortunate coincidence that the resistance strategy that uses defective interfering DNAs as control elements was successful for geminiviruses (Frischmuth & Stanley, 1993; Jeske et al., 2001).

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PART III

VIRUS–VECTOR–PLANT INTERACTIONS

CHAPTER 1

REPLICATION OF GEMINIVIRUSES AND THE USE OF ROLLING CIRCLE AMPLIFICATION FOR THEIR DIAGNOSIS

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1. OVERVIEW

During natural infection, geminiviruses are injected directly into phloem cells by insect vectors. It is not clear whether the whole virion or only its ssDNA has to enter the nucleus for replication. Within the nucleus, ssDNA is complemented to dsDNA, a process which is performed by host enzymes. Viral dsDNA is then packaged into nucleosomes forming chromatin as a substrate for further transcription and replication. Since geminiviruses do not encode a gene for DNA polymerase and because they multiply in differentiated cells, they need to activate the host replication machinery in order to promote their own multiplication. The plant host process of replication has to be modified, however, to ensure a rolling circle mechanism, which generates circular ssDNA to be packaged into virions for further spread.

Geminiviral replication has been studied extensively, and comprehensive reviews are available covering most of the details of this process (Gutierrez, 2000; Gutierrez et al., 2004; Hanley-Bowdoin et al., 1999). Here, we will focus on the current knowledge as far as it concerns tomato leaf curl-inducing viruses. Results from related viruses are included if there is evidence that they behave similarly.

Our general knowledge about replication mechanisms has changed considerably during the past years. Although replication was originally seen as a continuous process, evidence has accumulated indicating that it may be rather discontinuous and therefore needs additional means to solve stalled replication forks. It is now well established that recombination plays a major role in replication to repair such insufficiently processed DNA. The reader is referred to recent reviews about the triple R (replication-recombination-repair) connection (Alberts, 2003; Johnson & O'Donnell, 2005; Olavarrieta et al., 2002; Schvartzman & Stasiak,

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 141–156.

2004; Sogo et al., 2002; Stauffer & Chazin, 2004). The fact that viruses may utilize more than one mode of replication has been originally demonstrated for bacteriophage T4, but such multitasking is a more general phenomenon in prokaryotic and eukaryotic organisms (Formosa & Alberts, 1986; Mosig, 1998; Mosig et al., 2001).

The diversity of processes is reflected by an increasing number of recently detected DNA polymerases (Sutton & Walker, 2001), currently comprising 13 members in eukaryotes. It has still to be determined which one is responsible for geminivirus replication in which tissue.

The efficiency of replication is enhanced by the sliding clamp (proliferating cell nuclear antigen, PCNA in eukaryotes), first detected as a processing factor for replication but later on recognized as an extremely versatile switchboard and signal integrator in other DNA metabolic processes as well, including repair, cell cycle regulation, and recombination (Johnson & O'Donnell, 2005). The correlation of PCNA activation and geminivirus replication has been studied in some detail in section 2.

2. MULTITASKING IN REPLICATION, RECOMBINATION, AND REPAIR

Circular single-stranded DNA and the highly conserved nonanucleotide suggested themselves that geminiviruses may replicate like bacteriophages such as ϕX 174. Correspondingly, it was proposed early that geminiviruses also utilize a rolling circle mechanism (RCR) for their replication. Genetic evidence (Stenger et al., 1991), the use of two-dimensional gel electrophoresis (Jeske et al., 2001; Saunders et al., 1991) as well as direct electron-microscopic visualization of geminiviral DNA forms (Jeske et al., 2001) have supported this conclusion. However, the RCR model does not explain all intermediate DNA forms that were originally observed (Jeske et al., 2001; Saunders et al., 1991). An improved two-dimensional gel electrophoresis technique which used the combination of SDS and chloroquine-containing gel systems (Figure 1) helped to annotate most of the unknown DNA forms and led to the conclusion that geminiviruses replicate by at least three modes: complementary strand replication (CSR), RCR, and recombination-dependent replication (RDR) (Jeske et al., 2001; Preiss & Jeske, 2003). In this multitasking, geminiviruses follow the model deduced from bacteriophage T4 (Formosa & Alberts, 1986; Mosig, 1998; Mosig et al., 2001), although RDR was observed late during infection for T4, but early for geminiviruses (Jeske et al., 2001). The use of several replication modes has been shown for various begomoviruses (including TYLCV and TYLCSV) and a curtovirus (Alberter et al., 2005; Morilla et al., 2006; Preiss & Jeske, 2003), but corresponding information about the replication of mastreviruses and topocuviruses is still lacking.

Figure 1 summarizes our current knowledge in a schematic sketch. Grey circles, ovals, bands, and solid lines represent products of replication whereas stippled

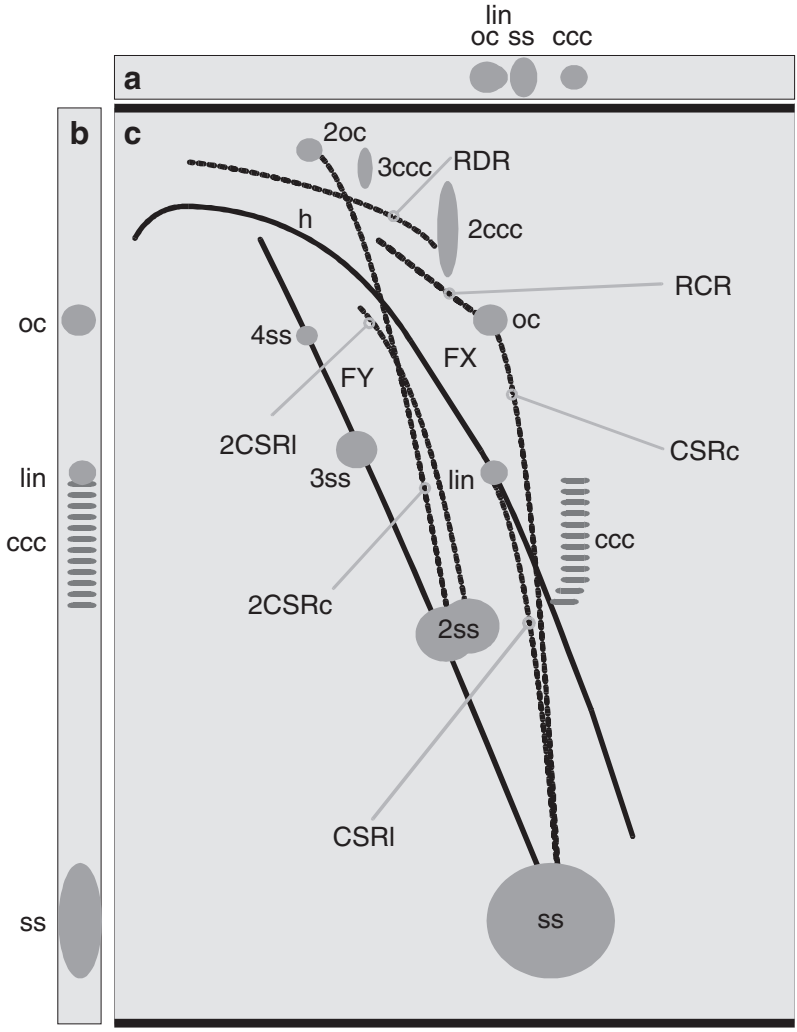

Figure 1. Separating geminiviral DNA forms. In a schematic sketch, the different geminiviral DNA forms are shown for one-dimensional gels in the presence of SDS (a) or of chloroquine as well as for a two-dimensional gel combining both gel systems. Note that the order of appearance of DNA forms has changed for (a) and (b), being open circular (oc), linear (lin), single-stranded (ss), and covalently closed circular (ccc) for the former and oc, lin, ccc, and ss for the later. Upon chloroquine intercalation the ccc DNA is resolved into its topoisomers resulting in multiple bands (b, c). Numbers 2–4 refer to dimers to tetramers of the respective conformations. Products of replication are shown as grey circles, ovals, and black solid lines. True replicative intermediates are indicated by stipled lines. Ongoing replication is marked for complementary strand replication (CSR) starting from circular (c) or linear (l) templates, for rolling circle replication (RCR), and for recombination-dependent replication (RDR). In addition, part of the heterogeneous linear dsDNA (h) may be engaged in RDR but cannot be discriminated under the chosen experimental conditions. FY and FX indicate fields of geminiviral DNA northwest of ssDNA or hDNA, respectively, which result from replicating heterogenous ssDNA (FY) or dsDNA (FX). (For a detailed description see Preiss & Jeske, 2003.)

lines refer to true replicative intermediates (Figure 1c). The true replicative intermediates are not recognized in one-dimensional gels (Figure 1a, b) because they are represented in a background smear of hybridizing material which is usually suppressed by short film exposures in hybridization analyses. Having analysed manifold samples of various geminiviruses during their courses of infection, we would like to draw attention to the discrimination of replication "products" and "intermediates" to avoid misinterpretation. For example, the presence of viral oc or ccc dsDNA may not always indicate ongoing replication. They may be engaged in transcription or represent a completely silent state, whereby oc dsDNA is in part generated from ccc dsDNA during purification and is therefore even less informative.

The underlying technical details of Figure 1 and their interpretation have been described (Preiss & Jeske, 2003). Here we will use the scheme solely for discussing the different replication processes. After infection by insect vectors, monomeric circular ssDNA is introduced into the nucleus of phloem cells where it is complemented to circular oc dsDNA (Figure 1c, ss to oc). For mastreviruses, a RNA/DNA primer was found to be attached to the short intergenic region and packaged within the virions serving as start signal for CSR (Donson et al., 1984). In contrast following infection, begomoviruses use a host RNA polymerase to generate such a primer and to start CSR within the large intergenic region (Saunders et al., 1992). The first oc dsDNA synthesized is sealed and packaged into nucleosomes, giving rise to ccc ds DNA (Figure 1c, oc to ccc) whereby each added nucleosome generates one superhelical turn (Figure 1b, c; from bottom band to top band of the topoisomers).

RCR in general starts from monomeric oc dsDNA (Figure 1c, oc) and results in a straight line (indicating the extension of ssDNA) for one round of replication. Only occasionally for specific viruses, a further elongation of RCR intermediates was observed (Jovel et al., 2007). The accumulation of heterogenous circular ssDNA (Figure 1c, straight line from ss to 4ss) may, however, indicate products of insufficient termination and/or premature termination of RCR in general, which were not detectable as RCR intermediates. These products again may be complemented by CSR (Figure 1c, 2ss to 2oc, or approaching h lin ssDNA, or represented in FY), leading to multimeric oc or heterogeneous lin dsDNA. The recycling of heterogeneous circular ssDNA to oc and subsequently ccc dsDNA, however, is underrepresented in normal geminiviral replication, whereas heterogeneous oc dsDNA was prominent for satellite DNA replication (Alberter et al., 2005). Correspondingly, ccc dsDNA multimers were always discrete, lacking heterogeneous transition states during normal geminivirus replication.

Recombination-dependent replication is useful for geminiviruses to repair every ssDNA which has been replicated only partially or has been digested by host nucleases (Jeske et al., 2001). It needs the transfer of ssDNA into a proper cccDNA, which grants a genomic full-size template. RDR intermediates are hidden in the arc of hDNA (Figure 1c, ccc to h and lin) but were visualized by

electron microscopy of nucleic acids from this area of the gel (Jeske et al., 2001). Within the 2D gel, RDR intermediates starting from dimeric ccc dsDNA (Figure 1c, 2ccc) are a better diagnostic indication of ongoing RDR. The arc starting at 2ccc represents a dsDNA elongation with no defined end. This configuration shows that just after starting RDR, the complementary strand is simultaneously produced by CSR. (Note the difference to RCR as discussed above.) The products of RDR are therefore heterogeneous linear dsDNAs found in the central most prominent diagonal arc (Figure 1c, h). hDNAs again may serve as templates in an RCR-like or RDR mode giving rise to a field of hybridizing material (Figure 1c, FX) frequently seen as background smear in the original blots.

3. VIRAL CHROMATIN STRUCTURE

To deepen the understanding of geminiviral replication, it is necessary to recall that every dsDNA in the nucleus of eukaryotic cells is readily incorporated into chromatin structure. Correspondingly, geminiviral dsDNA was predominantly found in minichromosomes (Abouzid et al., 1988; Pilartz & Jeske, 1992; Pilartz & Jeske, 2003) within purified nuclei. A typical geminiviral circular dsDNA can be wrapped around 13 nucleosomes at maximum, thereby converting the negative superhelical into a toroidal conformation of the DNA, thus relaxing topological stress. To interact with replication and transcription factors, the minichromosomes need to be opened at certain genomic positions. Nuclease hypersensitivity assays have revealed two such open positions for AbMV, each in DNA A as well as in DNA B: one within the common region and one colocalized with the promoters serving for AC2/AC3 or BC1, respectively (Pilartz & Jeske, 2003). The opening was accompanied with the loss of one or two nucleosomes of the minichromosomes as reflected in the decreased number of negative superhelical turns in one-dimensional and two-dimensional gels (Pilartz & Jeske, 2003). Interestingly, the most exposed nuclease-sensitive region in the genome was the conserved hairpin-structure of the replicator.

4. INITIATION OF REPLICATION

To start a replication, an origin has to be defined on the template DNA of all organisms and viruses analysed so far. It is now referred to as "replicator" in parallel to "promoter" for the start of transcription (Watson et al., 2004). The replicator is typically composed of three elements: a specific sequence which is bound first by an initiator protein, a region which is easily melted, and one or more preferred replication start sites. This concept holds true for the leading strand of double-stranded (ds) DNA and has to be modified for single-stranded (ss) DNAs as they are present in viruses or as they are built during lagging strand synthesis. DNA-dependent RNA polymerases are able to synthesize primers without the need of specific sequences in the latter case.

The replicator of geminiviruses is located within the 200 bp common region for bipartite members or within the large intergenic region for monopartite members. A specific duplicated sequence (called "iteron") has been identified as recognition site for the viral Rep for begomoviruses and curtoviruses, but not for mastreviruses. How far the binding of a specific Rep is necessary for replication may be different for the Old World and the New World geminiviruses (Hanley-Bowdoin et al., 1999). The region to be easily melted is represented by a hairpin structure which can adopt a cruciform structure in dsDNA upon the action of helicases or topological stress of circular dsDNA. The third general element for geminiviruses is an exactly defined sequence within the loop of the hairpin which is cleaved by Rep to start unidirectional rolling circle replication.

Being first identified for WDV (Heyraud et al., 1993; Kammann et al., 1991), the replicator nick site was analysed for TYLCV (Heyraud-Nitschke et al., 1995; Laufs et al., 1995a, b) and ACMV (Stanley, 1995). Using Rep protein which had been ectopically expressed in *Escherichia coli* and chemically synthesized primer DNAs, Laufs et al. (1995b) showed that ssDNA primers were precisely cut within the TYLCV nonamer sequence TATAATATT#AC which is conserved among all geminiviruses. Mutated loop sequences yielded a lower efficiency but did not change the specificity of this cleavage. The stem-forming sequence was not necessary at all for this *in vitro* reaction. Hybridization of primers in the viral sense and in the complementary sense prevented the cleavage reaction indicating that the hairpin structure must be melted before it can be processed. Remarkably, not only the viral sense ssDNA primer, but also the complementary sense ssDNA primer was cleaved in this assay, although with lower efficiency and specificity. A similar pattern of nicks within the viral and complementary sense sequences was later on identified in DNase-sensitivity experiments using purified nuclei infected with AbMV (Pilartz & Jeske, 2003) lending additional weight on this first observation.

Concomitantly with the nicking of the viral sense primer, TYLCV Rep binds covalently to the newly created 5′ phosphate, saving the binding energy of the phosphodiester bond and protecting the 5′ end in this way (Heyraud-Nitschke et al., 1995; Laufs et al., 1995a, b). The newly created 3′OH end would be ready now to serve as a primer for the actual DNA-dependent DNA polymerization, not tested in these assays. The fidelity of replication is granted if a second Rep nicks the newly replicated viral DNA at the same sequence and the 5′ phosphate-bound Rep re-ligates 5′ and 3′ end. Using various primers to mimic this joining reaction in the assays (Heyraud-Nitschke et al., 1995; Laufs et al., 1995b), it was shown that the authentic as well as other primers can be fused by Rep, indicating that Rep may not only serve for proper replication but also function in recombination and repair. Once attached specifically to the 5′ end of a DNA, Rep thus can join it to every prematurely terminated replication intermediate giving rise to heterogeneous circular ssDNAs as they were observed *in vivo* (Preiss & Jeske, 2003).

Besides the specific nicking site, the hairpin structure is the most conserved feature of viruses with ssDNA. The secondary structure of the stem is necessary

for replication and covariations of the primary sequences are allowed (Hanley-Bowdoin et al., 1999). Reflecting the general concept of replication initiation, it serves as an easily melting sequence either with the help of a helicase, still to be defined, or as a consequence of topological constraints generated by wrapping viral DNA around nucleosomes and interaction with binding viral and/or host proteins (see below).

The recognition of the geminiviral replicator is mediated by the interaction of Rep with a cognate sequence upstream the conserved hairpin loop (Figure 2). This DNA sequence is generally virus-specific and functional to trans-replicate the proper DNA B component preferentially (Hanley-Bowdoin et al., 1999). A more promiscuous relationship has been shown for ToLCV and DNA satellites (Alberter et al., 2005; Lin et al., 2003). Although sequence-specific Rep-binding occurs as in all other geminiviruses, it seems less important to start replication (Lin et al., 2003).

For tomato yellow leaf curl-inducing geminiviruses (TYLCV, TYLCSV), replication is only possible if the cognate pair of Rep and Rep-binding sequence is present on the same viral DNA (Jupin et al., 1995) or on transreplicated chimeric constructs (Morilla et al., 2006). The relevant DNA sequence was delimited to a region of 146 nt. upstream of the hairpin loop (Jupin et al., 1995) that harbours the canonical duplication, now called "iteron", which has been characterized for SLCV, TGMV, and BGMV in closer detail (extensively reviewed in Hanley-Bowdoin et al. (1999). Binding of Rep to iterons is not only involved in replication but also in autorepression of transcription of the *Rep* gene (Hanley-Bowdoin et al., 1999).

5. REP PROTEIN FUNCTIONS

Rep is a multifunctional protein and the only factor that is absolutely necessary for the initiation of replication. It fulfills tasks of specific nicking and joining of DNA, autorepression of its own transcription, reprogramming the cell cycle to induce DNA-dependent DNA polymerase expression in differentiated cells as well as ATP hydrolysis, the exact role of which has to be determined (reviewed in Hanley-Bowdoin et al., 1999). Based on sequence comparisons, it has been early postulated that Rep possesses helicase activity (Koonin & Ilyina, 1992), but only recently this activity has been biochemically proven (Choudhury et al., 2006; Clerot & Bernardi, 2006).

Rep is composed of two gross portions: the N-terminal one harbouring activities for specific DNA binding, nicking, and joining, whereas the C-terminal one is responsible for ATPase and helicase activity (Figure 2b). In the genome of mastreviruses, both portions are separated into two ORFs (C1, C2) which are fused by splicing (Accotto et al., 1989; Schalk et al., 1989). Rep forms oligomers which are necessary for the two-step replication mode that involves two nicking events triggered by two separate protein units. In this respect geminivirus replication differs from phage replication, like that of ϕX 174, the Rep of which possesses two active sites in one protein (for discussion see Laufs et al., 1995a).

a

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                                      ------->                      -------> ------->
TYLCV_IR  2620 CAT--------GTTGAAATGAATCGGTGTCC-CTCAAAGCTCTATGGCAATCGGTGT-ATCGGTGTCTTATTTATA
TYLCSV_IR 2615 CATTTTTGC-TGTCGTTCTGAATCGGGGGACACTCAAAG-TATCCAGCAATTGGGGGAATTGGGGGGCAATATATA
                                    --- ---->                      -------->--------> 

TYLCV_IR  CCTGGACACCTAATGGCTATTTGGTAATTTCGTAAAAGTACATTGCAATTCAAAATTCAAAATTCAAAAATCAAATCATTA
TYLCSV_IR TGATGCCCCCTAAA-------TGGCATAGATGTAATTATTCAAAGTAAT---AAATTTATTTTTTAATTTTTTT-TTGGTA

           ---------->          1 <----------
TYLCV_IR  AAGCGGCCATCCGTATAATATT|ACCGGATGGCCGCGCCTTTTCCTTTTATGTGGTCCCCACGAGGGTTCCACAGACGTCA
TYLCSV_IR AAGCGGCCATCCGTATAATATT|ACCGGATGGCCGCGC--TCCCCGATAAAGAAGT------GGGTCCTACGCAGTAGTTT


TYLCV_IR  CTGTCAACCAATCAAATTGCATACTCAAACGTTAAATAAGTGTTCATTTGTCTTTATATACTTGGTCCCCAAGTATTTTGT
TYLCSV_IR CTGTCGACCAATGAAATTGCAGCCTCAAAGCTTAAATAACTGTTCAGCTGTGTT-ATAAACTTGCTCCCTAAGTTTAAAAA


TYLCV_IR  CT-TGCAATATG.153
TYLCSV_IR ATATACAAAATG.149
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b

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                     Motif I|-- α-1 -|                47      Motif II
TYLCSV  MPRSGRFSIKAKNYFLTYPKCDLTKENALSQITNLQTPTNKLFIKICRELHENGEPHLHILIQFEGKYNCTNQRFFDLVSPTR
TYLCV-M MAQPKRFQINAKHYFLSFPKCSLSKEEALEQLLQLQTPTNKKYIKICRELHEDGQPHLHMLIQFEGKFNCKNNRFFDLVSPTR
TYLCV-A MTRPKSFRINAKNYFLTYPKCSLTKEEALSQLNNLETPTSKKYIKVCRELHENGEPHLHVLIQFEGKFQCKNQRFFDLVSPTR
TYLCV-I M--PRLFKIYAKNYFLTYPNCSLSKEEALSQLKKLETPTNKKYIKVCKELHENGEPHLHVLIQFEGKYQCKNQRFFDLVSPNR
ACMV-N  M-RTPRFRVQAKNVFLTYPNCSIPKEHLLSFIQTLSLPSNPKFIKICRELHQNGEPHLHALIQFEGKITITNNRLFDCVHPSC
ACMV-K  M-RTPRFRIQAKNVFLTYPKCSIPKEHLLSFIQTLSLQSNPKFIKICRELHQNGEPHLHALIQFEGKITITNNRLFDCVHPSC
        *  .  * : **: **::*:*.:.**. *. :  *.  :.  :**:*:***::*:**** *******   .*:*:** * *.
                    Motif III                          <=============== interaction ========
TYLCSV  SAHFHPNIQGAKSSSDVKSYIDKDGDVLEWGTFQIDGRSARGGQQTANDAYAKAINAGSKSQALDVIKELAPRDYVLHFHNIN
TYLCV-M SAHFHPNIQGAKSSSDVKSYIDKDGDVLEWGTFQIDGRSARGGQQTANDAYAKAINAGSKSEALDVIKELAPRDYILHFHNIN
TYLCV-A SAHFHPNIQGAKSSSDVKSYLEKDGDTLEWGEFQIDGRSARGGQQSANDAYAQALNTGSKSEALNVLRELAPKDYVLQFHNLN
TYLCV-I SAHFHPNIQAAKSSTDVKTYVEKDGNFIDFGVSQIDGRSARGGQQSANDAYAEALNSGSKSEALNILKEKAPKDYILQFHNLS
ACMV-N  STNFHPNIQGAKSSSDVKSYLDKDGDTVEWGQFQIDGRSARGGQQSANDAYAKALNSGSKSEALNVIRELVPKDFVLQFHNLN
ACMV-K  STSFHPNIQGAKSSSDVKSYLDKDGDTVEWGQFQIDGRSARGGQQSANDAYAKALNSGSKSEALNVIRELVPKDFVLQFHNLN
        *: ******.****:***:*::***: :::*  ************:*******:*:*:****:**::::* .*:*::*:***:.
        ============>                                    Motif IV
TYLCSV  SNLDKVFQVPPAPYVSPFLSSSFDQVPDELEHWVSENVMDAAARPWRPVSIVIEGDSRTGKTTWARSLGPHNYLCGHLDLSQK
TYLCV-M SNLDRVFQVPPAPYVSPFLSSSFDQVPDELEHWVSENVMDAAARPWRPVSIVIEGDSRTGKTMWARSLGPHNYLCGHLDLSQK
TYLCV-A SNLDRIFTPPLEVYVSPFLSSSFDRVPEELEEWVAENVKDAAARPLRPISIVIEGESRTGKTVWARSLGPHNYLCGHLDLSPK
TYLCV-I SNLDRIFSPPLEVYVSPFLSSSFNQVPDELEEWVAENVVYSAARPWRPISIVIEGDSRTGKTMWARSLGPHNYLCGHLDLSPK
ACMV-N  SNLDRIFQEPPAPYVSPFPCSSFDQVPDELEEWVADNVRDSAARPWRPNSIVIEGDSRTGKTIWARSLGPHNYLCGHLDLSPK
ACMV-K  SNLDRIFQEPPAPYVSPFPCSSFDQVPVEIEEWVADNVRDSAARPWRPNSIVIEGDSRTGKTIWARSLGPHNYLCGHLDLSPK
        ****::*  *   ***** .***::** *:*.**::**  :**** ** ******:****** ****************** *

TYLCSV  VYSNNAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPIQIKGGIPTIFLCNPGPQSSFKEYLDEEKNQALKNWATKNAI
TYLCV-M VYSNNAWYNVIDDVDPHYLKHFKEFMGSQRDWQSNTKYGKPIQIKGGIPTIFLCNPGPQSSFKEYLDEEKNQTLKNWAIKNAI
TYLCV-A VYSNDAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPVQIKGGIPTIFLCNPGPNSSYKEYLDEEKNSALKAWALKNAE
TYLCV-I VYSNDAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPIQIKGGIPTIFLCNPGPTSSYREYLDEEKNISLKNWALKNAT
ACMV-N  VFNNDAWYNVIDDVDPHYLKHFKEFMGSQRDWQSNTKYGKPVQIKGGIPTIFLCNPGPTSSYKEFLDEEKQEALKAWALKNAI
ACMV-K  VFNNAAWYNVIDDVDPHYLKHFKEFMGSQRDWQSNTKYGKPVQIKGGIPTIFLCNPGPTSSYKEFLAEEKQEALKAWALKNAI
        *:.* **********************:*************:**************** **::*:* ***: :** ** ***

TYLCSV  FVTIHQPLFADTNQNTTSHRQEEASEA--- 359
TYLCV-M FVTIHQPLFTNTNQDPTPHRQEETSEA--- 359
TYLCV-A FITLNEPLYSGTYQGPTQNSEEEVHPEEEN 362
TYLCV-I FVTLYEPLFASINQGPTQDSQEETNKA--- 357
ACMV-N  FITLTEPLYSGSNQSQSQTIQEASHPA--- 358
ACMV-K  FITLTEPLYSGSNQSHSQTSQEASHPA--- 358
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Figure 2. Comparison of selected replicator DNA (a) and Rep amino acid (b) sequences. Intergenic regions of *Tomato yellow leaf curl virus* (TYLCV-IR) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV-IR) are compared in (a). Sequences were obtained from NCBI Virus resource for TYLCV_IR (NC_004005) and for TYLCSV_IR (NC_003828). Start codons for Rep (CAT) and V2 (ATG), TATA Box, and the stem of the conserved hairpin loop are underlaid with grey. Elements relevant for replication are shown in bold, like iteron (direct repeats, arrows) and CA-box in analogy to *Tomato golden mosaic virus* (Orozco et al., 1998). Number 1 identifies the first nucleotide after Rep cleavage. Rep protein sequences (b) are compared for *Tomato leaf curl-inducing viruses* from

Although TYLCV Rep possesses two tyrosines in conserved positions, tyrosine 103 (Y103) alone is necessary and sufficient as active amino acid to perform the nucleophilic attack at the target sequence and to covalently attach Rep to the 5′ phosphate of the cleaved DNA (Laufs et al., 1995a). Mutants of Rep (Y103F) were unable to replicate in protoplasts confirming the biological significance of these *in vitro* results (Laufs et al., 1995a). The cleavage reaction needs divalent cations (Mg^{2+}, Mn^{2+}), and conserved acidic amino acids are present in the vicinity of the active Y103 (DVKX**Y**XXKD or **Y**XXKD/E/N) (Laufs et al., 1995a). However, structural analysis of TYLCSV Rep implied E49 in coordinating divalent cations (Campos-Olivas et al., 2002a, b).

When ectopically expressed in *E. coli*, the first 211 amino acids of TYLCV Rep (corresponding to 24 kDa) were sufficient to perform the specific nicking-joining reaction with chemically synthesized primers, irrespectively of whether Rep was fused N-terminally to maltose-binding protein, a tag which was used to purify the protein (Heyraud-Nitschke et al., 1995). The N-terminal portion of Rep harbours three conserved motifs (I–III) identified for various geminiviral Rep proteins (Hanley-Bowdoin et al., 1999), but lacks the fourth motif, the P-loop and nucleotide-binding site (see below). As discussed for the complete protein, truncated Rep is able to rejoin primers of different origin, underlining its possible role not only for replication but also for recombination and restoring circular DNA (Heyraud-Nitschke et al., 1995).

The capability of specific recognition of the cognate DNA sequence has been delimited for TYLCV and TYLCSV to the first 116 amino acids of Rep (Jupin et al., 1995), whereas the first 57 amino acids of ACMV Rep were sufficient for binding to autorepress the AC1 promoter (Hong & Stanley, 1995). Mutational analysis of TGMV Rep (Gladfelter et al., 1997; Orozco & Hanley-Bowdoin, 1998) narrowed down the DNA-binding domain to amino acids 25–52, a peptide sequence predicted to be composed of two α-helices, one of which is confirmed by structure determination (Figure 2 b, α-2).

Nuclear magnetic resonance (NMR) analysis of partial TYLCSV Rep comprising amino acids 4–121 and corresponding to 13,7 kDa (Campos-Olivas et al., 2002a, b) have provided the clue to elucidate the interaction of the conserved motifs in nicking and joining reaction as well as in DNA binding. According to the deduced three-dimensional model, the key amino acids of motifs I–III reach close vicinity upon folding of the N-terminal Rep domain

Figure 2. (continued) Sardinia (SwissProt P27260), Spain (Murcia, P38609), Australia (P36279), and Israel (P27259) as well as *African cassava mosaic-inducing viruses* from Nigeria (P14972) and Kenya (P14982). Conserved motifs as discussed in the text are indicated as well as α-helix 1 (α-1) and an oligomerization domain (< = = = = >) in analogy to TGMV (Orozco et al., 2000). The oligomerization domain overlaps with interaction domains for other viral proteins (REn) and host factors as shown for TGMV, TYLCV (Arguello-Astorga et al., 2004; Kong and Hanley-Bowdoin, 2002; Settlage et al., 2005), and TYLCSV (Castillo et al., 2004). It may therefore serve as a platform for mutual interactions of Rep with other proteins during replication.

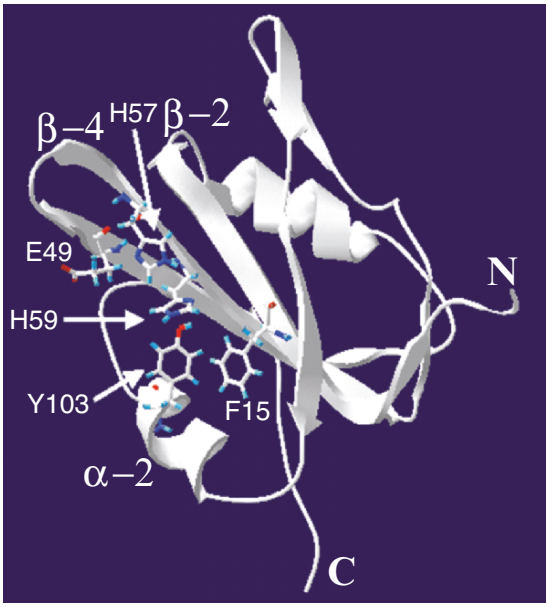

Figure 3. Three-dimensional structure of the N-terminal portion of TYLCSV Rep. NMR-resolved structure of amino acids 4–121 of TYLCSV Rep (Campos-Olivas et al., 2002a, b) redrawn from PDB ID1L2M to show the vicinity of the conserved sequence motifs in β-sheets β–2 and β-4 as well as α-helix α-2. Selected key amino acids, as discussed in the text, are shown with their chemical structure.

into four to five β-sheets and two α-helices (Figure 3). α-2 exposes Y103 of motif III just opposite to H57 and H59 in β-4 of motif II and F15 in β-2 of motif I. All together form a binding and reaction pocket which is structurally conserved in many RNA and DNA-binding proteins from all kingdoms of living organisms and viruses (Campos-Olivas et al., 2002a, b). Basic as well as hydrophobic (aromatic) amino acids may be involved in binding of ds and ssDNA and may orient ssDNA into the neighbourhood of Y103 where it can be cleaved. Divalent cations may be bound at E49 in close vicinity (Figure 3) to facilitate nucleophilic attack of Y103 to the conserved A1 nucleotide within the hairpin loop of the replicator (Campos-Olivas et al., 2002a, b).

In contrast to the N-terminal portion, the function and the structure of the C-terminal portion of Rep is less well understood. The latter harbours an NTP-binding motif (P-loop for phosphate binding fold) with the consensus of GXXXXGKT/S which is a functional site for NTP binding and ATPase activity of TYLCV Rep (Desbiez et al., 1995). The central Lys 227 was necessary for high *in vitro* ATPase activity and *in vivo* replication of TYLCV in protoplasts, but replacement of lysine by an arginine still allowed residual reactivity and replication (Desbiez et al., 1995). The C-terminal portion has been identified as

helicase domain recently (Choudhury et al., 2006; Clerot & Bernardi, 2006). ATP binding has also been discussed with reference to RecA (Desbiez et al., 1995), a recombination protein for which the release from DNA needs the conversion of ATP to ADP (Stauffer & Chazin, 2004). However, ATPase activity of TYLCV Rep was independent of ssDNA and dsDNA, otherwise a prerequisite for recombinationally active proteins.

6. RECRUITING VIRAL AND HOST PROTEINS BY REP

Geminiviral Rep has been shown to interact with several host proteins for different, more or less well-understood purposes. The most intriguing interaction occurs between the plant retinoblastoma-related protein (pRBR). Retinoblastoma protein has been detected as a tumour suppressor in animal cells, but is generally involved in cell cycle regulation (for review see Durfee et al., 2000; Gutierrez, 2000; Gutierrez et al., 2004; Hanley-Bowdoin et al., 1999). Its normal function relies on the binding of a transcription factor (EF2) thus inhibiting the expression of EF2-dependent genes which are involved in the start of DNA synthesis at the G1/S-phase transition. Viral proteins are able to recruit RB and release EF2 which can activate S-phase-specific genes. Whether plants and their DNA viruses use similar mechanisms to activate host DNA synthesis has been analysed for WDV mastrevirus (Xie et al., 1996), TGMV (Ach et al., 1997; Egelkrout et al., 2002; Kong et al., 2000; Nagar et al., 1995; Settlage et al., 2001), and TYLCV (Arguello-Astorga et al., 2004). Most RB-binding proteins harbour a highly conserved motif (LXCXE), which is present in mastrevirus Reps but not in Reps of other geminiviruses (Gutierrez et al., 2004). The role of the LXCXE in mastrevirus replication is still discussed (Collin et al., 1996; Liu et al., 1999; Xie et al., 1996). TGMV Rep lacking this sequence element, nevertheless, binds maize and arabidopsis pRBR (Ach et al., 1997; Kong et al., 2000), and essential amino acids for pRBR-binding have been mapped, with special focus on KEE146 (Kong et al., 2000) and L148 (Arguello-Astorga et al., 2004). A problem with this analysis lies in the multifunctionality of the interaction domain within which these amino acids are located (Figure 2b, KSE/Q, L). Whereas AL1–AL1 interaction is also reduced by alanine substitution at these sites, the binding of pRBR is not completely abolished. Correspondingly, these mutations reduce but do not prevent viral replication of infectious clones. They restrict TGMV to the phloem (Kong et al., 2000), where the majority of geminiviruses replicate exclusively.

In addition to pRB, TGMV Rep is able to bind a plant Ser/Thr kinase, a kinesin, and histone H3 in yeast and insect assays. The proof of these interactions *in planta* and their implications in viral replication are still pending (Kong & Hanley-Bowdoin, 2002). In yeast and insect assays, TYLCSV Rep binds to PCNA (Castillo et al., 2003) which may assist in the formation of replisomes on geminiviral DNA. Moreover in the same assays, TYLCSV Rep interacts with a SUMO-conjugating enzyme from *Nicotiana benthamiana* (Castillo et al., 2004).

Sumoylation does not destine proteins for proteasomal degradation like the related ubiqitin, but modifies proteins for specialized functions. In this context, it is interesting to note that the switch from replication to recombination and repair is accompanied by differential SUMOylation and ubiquitination of PCNA, at least in budding yeasts (Hoege et al., 2002). SUMO-modified PCNA recruits a helicase (Srs2) to prevent a stalled replication fork during S-phase from recombination (Papouli et al., 2005; Pfander et al., 2005; for review see Watts, 2006). It would be extremely interesting to know, whether the dual binding of TYLCSV Rep to PCNA and the SUMO-conjugating enzyme implicates a similar switch for plants which are less well investigated for this aspect.

Besides host factors, TGMV, TYLCV Rep (Settlage et al., 1996; Settlage et al., 2005), and TYLCSV Rep (Castillo et al., 2003) bind the viral replication enhancer protein (REn) which is not necessary for, but increases the efficiency of viral replication.

Interestingly, the self-interaction of Rep during oligomerization, as well as the interaction with other proteins is mediated by a central Rep protein domain (around aa 130–180) (Castillo et al., 2004; Kong & Hanley-Bowdoin, 2002; Orozco et al., 2000; Settlage et al., 1996; Settlage et al., 2005). This domain may serve as a general platform for mutual binding in order to differentially regulate certain functions during multitasking of replication.

7. ROLLING CIRCLE AMPLIFICATION AND DIAGNOSIS

Due to the great variety of circular replication intermediates and products, geminiviral DNA is prone to amplification by bacteriophage ϕ 29 polymerase, an enzyme that combines polymerase and strand-displacement activity (Blanco et al., 1989). CSR, RCR, and RDR intermediates were converted to high-molecular weight DNA (Jeske, 2006, unpublished data) and the products were easily identified by restriction fragment length polymorphism (RFLP) (Haible et al., 2006). This rolling circle amplification (RCA) has been used to clone geminiviral DNA (Inoue-Nagata et al., 2004), however, advanced usage of RCA will help to circumvent many bacterial cloning steps, since their products can be sequenced, modified, and inoculated directly in a cell-free system (Haible et al., 2006; Schubert et al., 2007). RCA can replace most of PCR and ELISA techniques in future, because it is easier to handle (needs no thermal cycler or specific primers) and amplifies all DNA components of a virus without the knowledge of their sequences, including defective DNAs and satellites. If an RFLP signal is obtained and contamination is avoided, RCA assays reveal virtually no false-positive results in contrast to PCR, and the fragment pattern of unknown samples can be directly compared to the expected fragment sizes deposited in a sequence database. Last but not the least, RCA works with dried leaf samples and multiple-infected plants allowing its widespread application in agriculture with lower technical equipment (Schubert et al., 2007). Since TYLCV and related viruses make up one of the major threats for tomato crops, RCA should be especially valuable for practical quarantine measures.

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CHAPTER 2

INTERACTIONS OF TOMATO YELLOW LEAF CURL VIRUS WITH ITS WHITEFLY VECTOR

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1. OVERVIEW

Whiteflies cause damages to many economically important agricultural crops because of their feeding habits and their begomovirus transmissions. The whitefly *Bemisia tabaci* is a genetically diverse group, which includes a large number of different biotypes (see Part I, Chapter 3). It is extremely prolific; a single female may lay approximately 400 eggs during her lifetime. Unfertilized eggs give rise to haploid males, whereas fertilized eggs develop into diploid females (arrhenotoky). The male/female ratio naturally changes throughout the course of the year, in fields and in insectaries (Horowitz & Gerling, 1992). *B. tabaci* develops into a flying adult from an egg, through four instars. Although *B. tabaci* nymphs are able to ingest and transmit begomoviruses, flying adults are those who spread the disease in the field (Gerling & Mayers, 1996). In this chapter we discuss the characteristics of acquisition, transmission, and retention of *Tomato yellow leaf curl virus* (TYLCV) and related begomoviruses by the whitefly vector *B. tabaci*.

2. INGESTION AND INOCULATION OF TYLCV

2.1. Acquisition and transmission by B and non-B biotypes of *B. tabaci*

Most begomoviruses are restricted to the phloem of infected plants. Hence, to acquire a begomovirus from an infected plant or to transmit a begomovirus to a host plant, the stylets of *B. tabaci* need to find their way between the epidermal and parenchymal cells before penetrating the vascular tissues and reaching the phloem that they feed on (Pollard, 1955). The parameters of acquisition and transmission of a begomovirus were first defined for the

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 157–170.

monopartite begomovirus TYLCV and were based on biological tests (Cohen & Harpaz, 1964; Cohen & Nitzany, 1966). A single insect is able to acquire TYLCV and transmit it to tomato plants. The reported minimum acquisition access period (AAP) and inoculation access period (IAP) of TYLCV isolates by *B. tabaci* biotype B varied from 15 to 60 min and from 15 to 30 min, respectively (Cohen & Harpaz, 1964; Mansour & Al-Musa, 1992; Mehta et al., 1994). Similar values were reported for other monopartite geminiviruses infecting tomato such as *Tomato yellow leaf curl Sardinia virus* (TYLCSV) from Italy (Caciagli & Bosco, 1997). Efficient AAP and IAP of bipartite begomoviruses infecting tomato such as *Tomato yellow leaf curl Bangalore virus* (ToLCBV) from India are not drastically different (Muniyappa et al., 2000).

The development of molecular tools has allowed to refine these studies. The genome of TYLCV was readily detected by Southern blot hybridization of DNA extracted from a single viruliferous whitefly (Zeidan & Czosnek, 1991). PCR allowed detecting amounts of TYLCV DNA in a single insect below the threshold of infectivity (Navot et al., 1992). The frequency of detection increased as the length of the AAP increased, from 10–20% after 30 min to 100% of the insects tested after 8 h. The intensity of the hybridization signals indicated that insects that had access to the same tissues for the same period of time could acquire variable amounts of viral DNA (Zeidan & Czosnek, 1991). A similar study conducted previously with the bipartite *Squash leaf curl virus* (SLCV) showed a similar albeit slower increase in the frequency of virus detection with time (Polston et al., 1990).

Monitoring the electronic waveforms produced during insect feeding (electrical penetration graphs (EPG), Tjallingii, 1978) allowed dissecting the virus transmission process. Following a short probing period, the minimum phloem contact period for successful inoculation of TYLCV was 1.8 min (Jiang et al., 2000). Using print-capture PCR, we have detected TYLCV DNA in 20% of the individuals tested as early as 5 min after access to the infected plant (Atzmon et al., 1998). Anatomical differences between virus source plant and target host plants (e.g., accessibility of the phloem in the leaf) may be reflected in differences in acquisition and transmission parameters associated with various begomoviruses.

The appearance of new exogenous biotypes in regions where TYLCV is endemic has allowed comparing the transmission efficiency of different *B. tabaci* biotypes from the same locality and on the same tomato cultivar. Comparison of two whitefly colonies from Murcia Spain by EPG indicated that the B biotype probed more often but ingested for less time than the Q biotype on the same tomato cultivar (Jiang et al., 1999). However, the B and Q biotypes did not significantly vary in terms of transmission efficiency of a virus isolate from Murcia, Spain (TYLCSV-ES), from infected tomato to noninfected tomato and to the weed plants *Datura stramonium*, *Solanum nigrum*. Another biotype, S, was unable to survive in tomato long enough to acquire or transmit TYLCSV-ES (Jiang et al., 2004). Acquisition and transmission efficacies of *Tomato Yellow*

Leaf Curl China Virus (TYLCCNV), a virus from the Zhejiang province in China virus closely related to TYLCV, by an invasive B and the local non-B *B. tabaci* biotype (China-ZHJ-1) were similar. A single insect was sufficient to infect tomato. Viruliferous B and ZHJ-1 adults retained TYLCCNV for their entire life when placed on healthy cotton plants (Jiu et al., 2006).

2.2. Latent period

Once ingested, begomoviruses are not immediately available for infection. They need to translocate from the digestive tract to the salivary glands from which they are excreted with the saliva during feeding. The time it takes for a begomovirus to complete this path and to infect susceptible plants is called the latent period. The latent period may not reflect the speed of virus translocation but rather the time it takes for an insect to accumulate enough virions to be able to transmit the disease to plants. For some begomoviruses this threshold may be reached much earlier than for others. For example, SLCV has been detected by PCR in the saliva 8 h after the beginning of the AAP (Rosell et al., 1999) while the minimal latent period was approximately 19 h (Cohen et al., 1983). In contrast TYLCV has been detected in the salivary glands of *B. tabaci* 7 h after the beginning of the AAP, 1 h only before the insects were able to infect tomato plants (Ghanim et al., 2001). The estimated latent period for a given virus may vary due to the experimental conditions or to changes in virus and/or vector with time. For example the latent period of TYLCV from Israel was reported to be 21 h in the early 1960s (Cohen & Nitzany, (1966), while it was found to be 8 h, 35 years later (Ghanim et al., 2001).

2.3. Transmission efficiency of begomoviruses: The effect of gender and age

It has been reported that a single insect was able to infect a tomato plant with TYLCV following a 24 h AAP; efficiency of transmission reached 100% when 5–15 insets were used (Cohen & Nitzany, 1966; Mansour & Al-Musa, 1992; Mehta et al., 1994). A similar number of insects were necessary to achieve 100% transmission of TYLCV from Italy (TYLCSV) and from China (TYLCCNV), and for the bipartite SLCV (Caciagli et al., 1995; Jiu et al., 2006; Cohen et al., 1983). However, in most cases the age and/or the gender of the insects used has been ignored. It has been previously reported that female whiteflies transmit the monopartite TYLCV (Cohen & Nitzany, 1966) and the bipartite ToLCBV (Muniyappa et al., 2000) with higher efficiency than males. We have studied the effect of the gender and of the age of synchronized populations of adult *B. tabaci* on the efficiency of transmission of TYLCV acquired during a 48 h AAP (Czosnek et al., 2001). Nearly all the 1- to 2-week-old adult females were able to infect tomato plants during a 48 h IAP. In comparison, only about 20% of the males of the same age were able to infect plants. Infection capacity decreased with age; while 60% of the 3-week-old females infected plants, the males were

totally unable to infect tomato plants. Only 20% of the 6-week-old females were able to infect tomato plants. Aging insects acquire fewer viral particles than younger individuals: 17-day-old adult females ingested less than half the virus ingested by 10-day-old insects and 24-day-old adults ingested only about 10% (Rubinstein & Czosnek, 1997). It has to be noted that female and male *B. tabaci* transmitted SLCV with the same efficiency (Polston et al., 1990). The reason for these differences is unclear.

3. THE PATH OF GEMINIVURSES IN THE WHITEFLY HOST

3.1. Organs and cells involved in circulative transmission of begomoviruses

Once ingested, begomoviruses follow a path that has been described in some details. The extensive anatomical analysis of the begomovirus nonvector whitefly *Trialeurodes vaporariorum*, performed in the 1930s, still serves as a reference for analyzing the internal anatomy of whitefly species (Weber, 1935). The description of *B. tabaci* mouthparts (Rosell et al., 1995), anterior alimentary canal (Hunter et al., 1996), digestive tract, filter chamber, and salivary glands (Harris et al., 1996; Cicero et al., 1995; Ghanim et al., 2001) has helped defining the pathway of begomoviruses in their insect vector. The organs and tissues involved in begomovirus circulative transcription are described in Part III, Chapter 3. These authors also show the immunolocalization of TYLCV in whitefly key organs.

3.2. Velocity of TYLCV translocation in *B. tabaci*

Using DNA from extracts of *B. tabaci* raised in Arizona as substrates for PCR, the bipartite SLCV DNA was detected in insect extracts after a 30 min AAP on infected pumpkin, and was found in the haemolymph after 2 h and in the saliva and honeydew after 8 h (Rosell et al., 1999). We have measured the velocity of translocation of TYLCV genomic DNA and coat protein (CP) in whiteflies from a colony raised in Israel. Stylets, head, midgut, haemolymph, and salivary glands dissected from a single insect were used as substrate for PCR and immunocapture-PCR (Ghanim et al., 2001). TYLCV was detected in the head 10 min after the beginning of the AAP and in the midgut after 40 min. The virus reached the haemolymph 90 min after the beginning of the AAP and was detected in the salivary glands approximately 5.5 h thereafter, approximately 1 h before the insects were able to infect tomato plants. TYLCV translocation timing defined by PCR and by immunocapture-PCR, overlapped, suggesting that the viral DNA is present within virions. Hence, begomoviruses transit in the body of *B. tabaci* according to an invariable sequential path: head-midgut-haemolymph-salivary glands (Ghanim et al., 2001). Moreover, it is likely that the path and the velocity of begomovirus translocation are independent of the identity of the begomovirus (as long as it is transmissible) and of the geographical origin of the *B. tabaci* vector.

3.3. Fate of nontransmittable begomoviruses

During the transit of begomoviruses in their whitefly vector, the capsid is the structure that is exposed to the whitefly tissues and interacts with insect receptors and chaperons (Morin et al., 2000). Vector specificity of geminiviruses is determined by the CP and there is no evidence for the involvement of other virus-encoded proteins in transmission. Loss of begomovirus transmission by *B. tabaci* can be caused by a small number of amino acid replacements in the CP. Natural TYLCSV mutants have been isolated. Some are ingested but not transmitted by *B. tabaci*. Loss of TYLCSV transmission was due to the replacement of two amino acids in the CP at positions 129 (P for Q) and 134 (H for Q) (Noris et al., 1998). These two amino acids are in a stretch of 12 (123–134), which are mostly external. P129 is predicted to introduce a bend in the polypeptide chain, potentially modifying the protein structure of the CP subunits. This region of the CP is also implicated in transmission of the bipartite *Watermelon chlorotic stunt virus* (Kheyr-Pour et al., 2000). *Abutilon mosaic virus* (AbMV) is another bipartite begomovirus that has lost the ability to be transmitted (Wu et al., 1996), probably, because it has been maintained and propagated by cuttings. Mutagenesis of AbMV CP showed that exchange of three amino acids at positions 124, 149, and 174 restored transmissibility by whiteflies (Höhnle et al., 2001). Replacing the CP of AbMV with that of the closely related transmissible *Sida golden mosaic virus* (SiGMV) produced a whitefly-transmissible chimeric AbMV (Höfer et al., 1997).

Although not transmittable, the pattern of association of AbMV with *B. tabaci* was similar to that of TYLCV. Following a 4-day AAP on infected abutilon plants, AbMV DNA remained associated with *B. tabaci* during the 15-day experiment, while the CP was detectable only for up to 7 days (Morin et al., 1999). AbMV was detected in the vector digestive system, but not in the haemolymph, indicating that this virus was unable to cross the gut/haemolymph barrier (Czosnek et al., 2002). We speculate that, following acquisition, AbMV binds to the putative *B. tabaci* receptors present in part of the digestive tract. However, because of a change in the conformation of the capsid due to mutations in the CP, AbMV cannot be internalized in the epithelial cells by the microvilli system and delivered to the haemolymph.

4. TYLCV AFFECTS THE FITNESS OF THE WHITEFLY HOST

4.1. Long-time association of TYLCV with the whitefly vector

Following a 1- to 2-day AAP on infected plants and transfer to non-host plants, begomoviruses may be retained in their whitefly vector for several weeks and sometimes for the entire life of the insect. TYLCV and TYLCCNV remain associated with *B. tabaci* during the entire life of the vector (Rubinstein & Czosnek, 1997; Jiu et al., 2006), while TYLCSV is undetectable after approximately 20 days

(Jiang et al., 2000). In most instances the viral DNA remained associated with the insects much longer than infectivity indicated. For example while TYLSCV DNA was detectable up to 20 days after the end of the 48 h AAP, infectivity was retained for up to 8 days only (Caciagli et al., 1995). TYLCV DNA and CP are not retained in *B. tabaci* for the same time periods. Following the end of the 48 h AAP, TYLCV DNA remained conspicuous during the 5 weeks life span of the insect, while the amount of TYLCV CP steadily decreased until it was undetectable at day 12; the disappearance of the virus CP was associated with a fast decrease in whitefly ability to infect host plants (Rubinstein & Czosnek, 1997). It is possible that most of the viral DNA dissociated from the capsid, left the circulative pathway and invaded insect tissues.

4.2. Deleterious effects of TYLCV on longevity and fertility of *B. tabaci*

In an early study, it was shown that the life span of female whiteflies (biotype not specified) fed for 24 h on bipartite SLCV-infected plants was in average 25% shorter than that of whiteflies fed on the same virus source for 4 h only (Cohen et al., 1989). To examine the effects of the direct association between the whitefly vector and TYLCV we have compared longevity and fertility of viruliferous and nonviruliferous insects reared on cotton (a virus non-host plant), following a short exposure to TYLCV-infected tomato plants (Rubinstein & Czosnek, 1997). Adult whiteflies that emerged during a 24 h time period were caged with TYLCV-infected tomato plants for a 48 h virus AAP. Control insects that emerged the same day were caged for 48 h with noninfected tomato plants. The two insect populations were then reared in a net house concurrently on eggplants, with climatic conditions close to those prevalent outdoors. At the population level, the difference at the 50% mortality point between infected and noninfected insects was between 5 and 7 days: 27 vs. 34 days in January–February, 20 vs. 26 days in April–May, 26 vs. 32 days in May–June and 29 vs. 35 days in August–September. These results showed that the life expectancy of viruliferous insect populations was significantly lower (~20%) than that of the nonviruliferous controls.

The long-term association of TYLCV with female *B. tabaci* was also correlated with a decrease in fertility (Rubinstein & Czosnek, 1997). Following a 48 h AAP on TYLCV-infected tomato plants, the mean number of eggs laid either on tomato or on eggplant during a 7 or 20 days long period was significantly lower than that laid by nonviruliferous insects of the same age. The decrease in fertility was not observed during the first 24 h following AAP (6.0 vs. 5.1 for 3-day-old insect, 9.8 vs. 10.0 for 11-day-old insects), indicating that the target was maturing eggs. The mean number of eggs laid by viruliferous insects during a 7-day period was significantly lower than that laid by nonviruliferous insects (22.7 vs. 38.1 for 1-day-old insects; 14.1 vs. 28.0 for 9-day-old insects). Similar results were obtained with 3-day-old insects during a 20-day

period (33.4 vs. 56.0). The host plant did not have a significant effect on the insect fecundity. The percentage of eggs that developed into instars was similar, whether they were laid by infected or noninfected insects. Therefore, TYLCV influenced the number of eggs laid but not the emergence of the instars.

In a similar experiment the effect of TYLCCNV on two *B. tabaci* biotypes (invasive B and local ZHJ1) was appraised (Jiu et al., 2007). Emerged whiteflies were caged with TYLCCNV-infected tobacco plants for 48 h and transferred on cotton plants. The mean longevity and fertility of viruliferous B biotype insects on cotton were significantly lower than that of nonviruliferous insects (19.8 vs. 31.0 days and 62.3 vs. 84.8 eggs per female). A similar deleterious effect of the virus on the longevity and fertility of the ZHJ1 biotype was observed (14.7 vs. 25.2 days, 55.9 vs. 83.6 eggs). In the same study, the effect of another monopartite geminivirus, the *Tobacco curly shoot virus* (TobCSV) on the two biotypes was appraised following a 48 h AAP on TobCSV-infected tobacco and transfer to cotton plants. The results were just the opposite of those obtained with TYLCCNV. Viruliferous B biotype whiteflies exhibited higher longevity and fertility than nonviruliferous whiteflies (33.8 vs. 31.0 days, 116.1 vs. 84.8 eggs per female). The effect of TobCSV on ZHJ1 insects was minor (25.7 vs. 25.2 days, 75.5 vs. 83.6 eggs per female).

In contrast to TYLCV and TYLCCNV, the bipartite begomovirus *Tomato mottle virus* (ToMoV) did not affect fertility of whitefly B biotype (McKenzie, 2002). Whiteflies infected with ToMoV deposited significantly more eggs on healthy tomato leaves than nonviruliferous whiteflies. There was no significant difference between viruliferous and nonviruliferous whiteflies for the number of adults emerged or the proportion of those adults surviving from the egg stage. There was no significant correlation between the number of eggs deposited per viruliferous and nonviruliferous females and progeny survival rates on healthy tomato. These observations indicate that some begomoviruses have deleterious effects on their insect host while others do not.

5. FATE OF TYLCV IN THE WHITEFLY HOST

5.1. Association of viral particles with insect chaperons

Begomoviral particles need to cross the gut wall into the haemolymph on their way to the salivary gland. The haemolymph consists of plasma in which haematocytes digest foreign proteins, microorganisms and tissue debris (Chapman, 1991). Hence transiting virions face a particularly hostile environment. A GroEL homologue produced by endosymbiotic bacteria of aphids has been shown to play a crucial role in the transmission of luteoviruses (van den Heuvel et al., 1994). Similarly, endosymbiotic bacteria housed in the whitefly mycetocytes have a cardinal role in protecting begomoviruses in the haemolymph (Gibbs, 1999). As demonstrated for TYLCV, the GroEL homologue seems to

bind to and protect begomoviruses from degradation in the haemolymph. Disrupting the GroEL-TYLCV association leads to the degradation of the virus and to a marked decrease in transmission efficiency (Morin et al., 2000, 1999). We have shown that in the yeast two hybrid system, *B. tabaci* GroEL interacted with the CP of TYLCV as well as with the CP of the nontransmissible AbMV (Morin et al., 2000), indicating that the amino acid residues at position 124, 149, and 174, which prevented AbMV from crossing into the insect haemolymph (Höhnle et al., 2001) did not prevent binding to GroEL. It has been suggested that viruses belonging to unrelated taxonomic groups have taken advantage of endosymbiotic bacteria proteins produced by their insect vector to avoid degradation in the haemolymph (Gibbs, 1999).

5.2. Does TYLCV replicate in its insect vector?

Begomovirus replication in its vector remains a controversial issue. It has been postulated that geminiviruses do not replicate in their insect vectors (Harrison, 1985). However, studies to determine virus titer over time in whiteflies have shown that TYLCV DNA persists in the insects longer than infectivity would suggest (Caciagli & Bosco, 1997; Rubinstein & Czosnek, 1997; Sinisterra et al., 2005). Hence, the persistence of begomoviruses in *B. tabaci* as infective entities for longer than the latent period, sometimes for the entire life of the insect, raises the question of replication of the virus in the insect.

Accumulation of viral DNA in *B. tabaci* reared on a TYLCV-non-host plant, after first feeding on plants infected with a TYLCV isolate from Egypt, has been interpreted as multiplication of TYLCV in its vector (Mehta et al., 1994). We have found that after a short AAP the amount of TYLCV DNA associated with whiteflies detectable by Southern blot hybridization steadily increased after a lag period of 8 h, reaching maximum levels approximately after 16 h and decreasing thereafter (Czosnek et al., 2001). These results could be explained by the ingestion of viral replicative complexes, which complete their replication cycle in the insect. It has to be noted that following acquisition of the closely related TYLCSV, accumulation of viral DNA was not observed in the whitefly host (Caciagli & Bosco, 1997).

5.3. Is TYLCV transcribed in the whitefly vector?

Transcriptional activity of two begomoviruses in the *B. tabaci* vector, the monopartite TYLCV and the bipartite ToMoV have been evaluated (Sinisterra et al., 2005). After feeding on virus-infected tomato plants and after subsequent transfer to the virus non-host cotton, quantitative RT-PCR was performed using specific primers for three ToMoV genes (AV1, BC1, and BV1) and three TYLCV genes (V1, V2, and C3). The ToMoV gene transcripts rapidly became undetectable in whiteflies following transfer from

tomato to cotton, probably because degradation was not accompanied by new synthesis. On the other hand, TYLCV transcripts increased after transfer of whiteflies to cotton, and were readily detected after 7 days indicating active TYLCV transcription. Interestingly, the difference observed in ToMoV and TYLCV transcripts in the vector parallel observations on the different biological effects of these viruses on whiteflies, i.e., TYLCV, but not ToMoV, reduced whitefly fitness (Rubinstein & Czosnek, 1997; McKenzie, 2002).

6. ACQUISITION OF TYLCV BY WHITEFLIES INDEPENDENTLY OF AN INFECTED PLANT SOURCE: TRANSOVARIAL INHERITANCE AND TRANSMISSION DURING MATING

6.1. Transovarial transmission

Transovarial transmission of plant viruses by their insect vector is a rare event and has been associated with replication and with deleterious effects on the insect host (Sylvester & Richardson, 1969; Sylvester, 1973). Usually, the virus was transmitted to some, but not to all progeny. Geminiviruses have not been considered to be transmitted transovarially to progeny (Harrison, 1985). Using PCR, Southern blot hybridization and transmission tests, we have found that TYLCV was transmitted to the progeny of viruliferous insects with various efficiencies. Moreover, the progeny of viruliferous insects was able to infect tomato test plants. Dissection and analysis of the reproductive system of viruliferous whiteflies showed that both the ovaries and the maturing eggs contained TYLCV DNA (Ghanim et al., 1998). The closely related TYLCSV was also found to be transmitted transovarially to the first-generation progeny (only). Similarly to TYLCV (Ghanim et al., 1998), TYLCSV was detected in eggs and nymphs as well as in adults (Bosco et al., 2004). However, in contrast to TYLCV, the adult progeny of TYLCSV viruliferous insects were unable to infect tomato plants. It is interesting to note that in the later experiments, while TYLCSV DNA was associated with eggs, instars, and adults of the first generation progeny, TYLCV was detected neither in instars nor in adult progeny of viruliferous females. These divergent results may be due to intrinsic differences in the highly inbred insect colonies raised in the laboratory and used in these experiments. Different endosymbiotic bacteria fauna might also be involved in these contradictorily processes.

The way in which TYLCV and TYLCSV enter the whitefly reproductive system is unknown. It is possible that during the maturation of eggs in the ovaries, geminiviral particles penetrate the egg together with the endosymbionts, via an aperture in the membrane (Costa et al., 1995). Invading TYLCV may affect the development of some of the eggs, causing a decrease in fertility (Rubinstein & Czosnek, 1997).

6.2. Transmission during mating: Another route of acquisition of begomoviruses

We have shown that TYLCV can be transmitted between whiteflies in a sex-dependent manner in the absence of any other source of virus (Ghanim & Czosnek, 2000). TYLCV was transmitted from viruliferous males to females and from viruliferous females to males, but not between insects of the same sex. Transmission took place when insects were caged in a feeding chamber or on TYLCV non-host cotton plants. TYLCV was detected in the haemolymph of the recipient insects about 1.5 h after caging, but was detected neither in the midgut nor in the head. Hence, the virus bypassed the pathway followed after feeding on infected plants, and probably infected the recipient insect by means of haemolymph exchange. From there TYLCV translocated in the salivary glands, but never crossed the gut membranes back into the digestive system.

The key role of the haemolymph was demonstrating by caging nonviruliferous *B. tabaci* males with females fed on AbMV-infected abutilon plants. AbMV DNA was never detected in the males. Identical results were obtained in the reciprocal mating scheme (Ghanim & Czosnek unpublished) 2007. Since AbMV remains in the digestive tract and is unable to cross the gut barrier into the haemolymph, these results confirmed that virus cannot be acquired from the feeding solution and that mating is the obligate route for sexual transmission of TYLCV, which probably occurs by exchange of haemolymph during intercourse. Transmission of the bipartite begomoviruses SLCV and WmCSV during mating was also observed by detecting viral DNA A and DNA B in the recipient insects (Ghanim & Czosnek unpublished) 2007.

7. VIRAL AND CELLULAR DETERMINANTS INVOLVED IN BEGOMOVIRUS CIRCULATIVE TRANSMISSION

Whiteflies and begomoviruses have a long history of cohabitation which has left various traces. On the one hand fossils anatomically similar to modern whiteflies have been found in ~120 million-year-old (MY) amber from Lebanon (Schlee, 1970). On the other, multiple repeats of geminiviral DNA sequences highly homologous to sequences of the modern bipartite *Tomato golden mosaic virus* (TGMV) seem to have integrated into the genome of some tobacco ancestors during *Nicotiana* speciation, about 25 MY ago (Bejarano et al., 1996). In this context it is interesting to note that the endosymbiotic bacteria that produce the GroEL homologue on which depends the survival of begomoviruses in their insect vector (Morin et al., 1999), have been associated with whiteflies for the last 200 MY (Bauman et al., 1993).

During this long-lasting virus–vector relationship begomoviruses might have optimized the conformation of their capsid to fit the receptors that mediate their circulation in the insect host and to interact with the chaperonins produced by the whitefly endosymbiotic bacteria. It is interesting to note that the adaptation of the local vector to the local begomovirus is reflected in the parameters of acquisition and transmission. Transmission of a begomovirus by an insect from

the same geographical region is more efficient than in the case where virus and insect originated from two different regions (McGrath & Harrison, 1995).

8. PERSPECTIVES: THE WHITEFLY FUNCTIONAL GENOME PROJECT

The *B. tabaci* genome has been hardly explored. It has been reported that nuclei of haploid males contain 10 chromosomes (Blackman & Cahill, 1998). Using flow cytometry we have estimated the DNA content of nuclei from haploid *B. tabaci* males as 1,020 million base pairs (Brown et al., 2005), which is approximately five times that of the fruitfly *Drosophila melanogaster*.

A functional genomics approach has been taken to understand the patterns of gene expression during whitefly development and during association of whiteflies with begomoviruses. In particular, the insect receptors which are thought to mediate translocation of begomoviruses from the gut to the haemolymph and from the haemolymph to the salivary glands are unknown. Also unknown are the genes affected by the virus, whether during circulative transmission or during long-term storage in the insect tissues. We have constructed three cDNA libraries for non-viruliferous whiteflies (eggs, immature instars, and adults) and two from adult insects that fed on tomato plants infected by two geminiviruses: the monopartite TYLCV and the bipartite ToMoV. The sequence of approximately 20,000 clones has been determined, which may represent approximately 4,000 different genes (Leshkowitz et al., 2005). Comparisons with public databases indicated that the libraries contained genes involved in cellular and developmental processes. Some sequences were specific of developmental stages while others were specific to viruliferous insects. A microarray containing 6,000 entries is available for this research. The functional analysis of these genes is in progress.

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CHAPTER 3

LOCALIZATION OF TOMATO YELLOW LEAF CURL VIRUS IN ITS WHITEFLY VECTOR *BEMISIA TABACI*

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1. OVERVIEW

Very little is known on the interactions of TYLCV and its *Bemisia tabaci* vector. Although many parameters of acquisition and transmission have been established (see Part III, Chapter 2), the localization of TYLCV particles in the whitefly has been barely studied. TYLCV is transmitted in a circulative manner. Once ingested during feeding on phloem of infected plants, the viral particles cross the midgut wall barrier, are transported through the haemolymph to the salivary system, cross the salivary gland barrier and are transmitted to the plant during feeding (Ghanim et al., 2001a). Few research articles describe the anatomy of *B. tabaci* organs and cells in relation to virus translocation, retention, and transmission. In this chapter we will describe the anatomy of the different *B. tabaci* tissues pertinent to TYLCV circulative transmission and illustrate the localization of viral particles in the body of the insect vector. Figure 1 provides the reader with the location of the major organs of *B. tabaci:* the piercing–sucking mouthparts, digestive, and salivary systems. These organs will be presented in details. Other organs will be described where needed. Localization of TYLCV in the different organs will be shown according to the temporal order by which the viral particles translocate in the insect body (Ghanim et al., 2001b; Medina et al., 2007).

2. THE STYLET AND MOUTH PARTS

The structure of the stylet and mouthparts of *B. tabaci* resemble similar structure in other piercing–sucking insect pests. These structures were extensively studied (Harris et al., 1995, 1996; Rosell et al., 1995; Hunter et al., 1996). *B. tabaci* has a stylet that penetrates the plant tissues to reach the phloem, from

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 171–183.

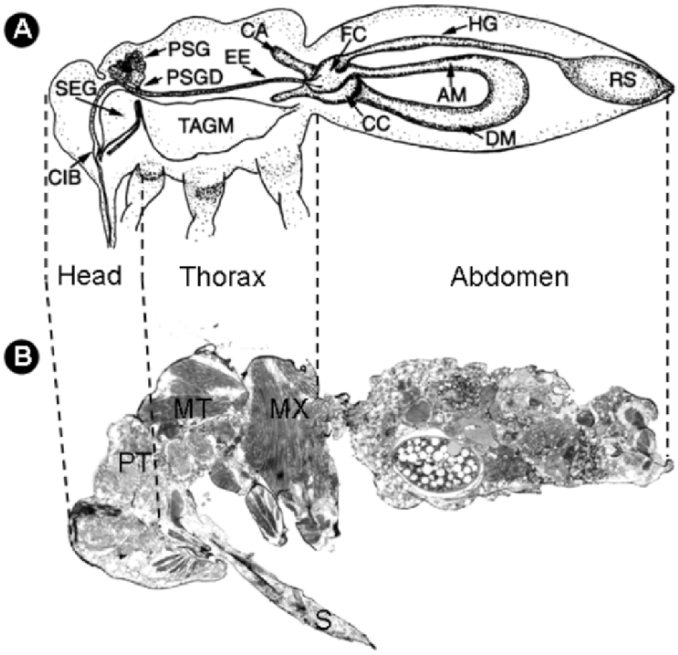

Figure 1. Drawing of a parasagittal view of an adult *Bemisia tabaci* (A), and a whole-body parasagittal section across the insect (B). AM, ascending midgut; CA, caeca; CC, connecting chamber; CIB, cibarium; DM, descending midgut; EE, external esophagus; FC, filter chamber; HG, hindgut; PSG, primary salivary gland; PSGD, primary salivary gland duct; RS, rectal sac; SEG, subesophageal ganglion; TAGM, thoracic-abdominal ganglionic mass; S, stylet; PT, prothorax; MT, mesothorax; MX, metathorax.

where sugars are transported into the insect body. This transport is thought to happen because of the pressure differences between the cavities of the plant cell and the insect body. Probing the plant tissue is a critical step in finding the right location for nutrition. The stylet is a bundle that consists of paired mandibles and paired maxillae. The paired maxilla are interlocked to form the food canal in which plant fluids are transported into the body, and the salivary canal from which saliva is excreted to smoothen the penetrating process into the plant tissues. While feeding on phloem contents; TYLCV particles are transported rapidly into the stylet food canal and then into the esophagus. Once injured, the contents of the phloem are emptied into the stylet cavity. TYLCV coat protein has been localized in the stylet cavity by immunogold labelling (IGL) on electron microscope (EM) sections (Figure 2). The localization was observed mainly in the food canal cavity, and was not associated with other parts in the stylet. This observation suggests that the passage of TYLCV virions or naked DNA is

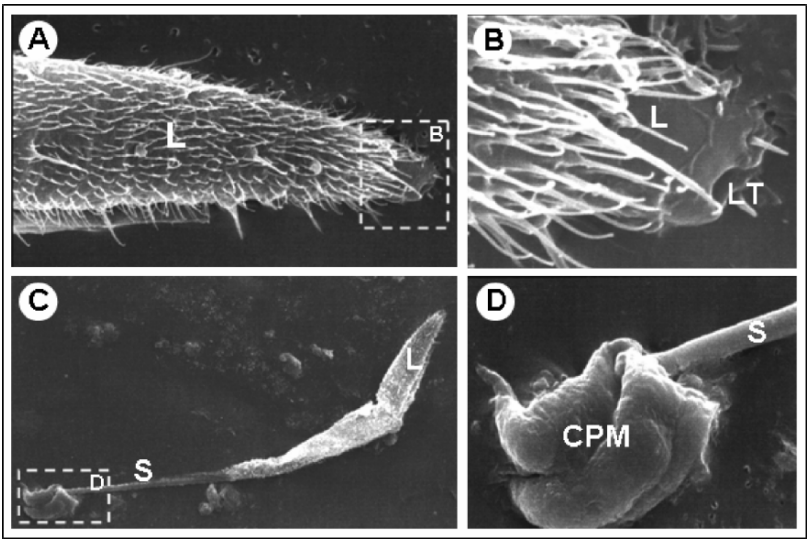

Figure 2. Scanning Electron Microscopic (SEM) images showing the external morphology of *B. tabaci* stylet (S). (A) Labium of the stylet (L); (B) a higher magnification of the inset in A showing the labial tip (LT); (C) an overview of the whole stylet; (D) a higher magnification of the inset in C showing the cibarial pump muscles (CPM).

passive and happens with the food flow towards the digestive tract. The food canal of *B. tabaci* located within the stylet harbours gustatory sensilla that vary in their location and come in close contact with passing fluids through the food canal for sensing.

Although TYLCV is transmitted in a circulative manner, an interesting phenomenon related to non-circulative virus transmission by homopteran vectors was also observed. The "ingestion–egestion" hypothesis suggests a mechanism in which the vector acquires non-circulative viruses by ingestion, carry them on the lumina lining the forgut (mainly the esophagus part before it reaches the midgut), and inoculates them to plants by egestion. This hypothesis has not been supported by experimental findings. Several researchers have suggested that an extravasation helps transmission of non-circulative viruses (McLean & Kinsy, 1984; Ammar & Nault, 1991; Harris et al., 1995). They argued that, like aphids, *B. tabaci* is also able to actively ingest and egest using the cibarial pump and postcibarial lumen manipulations. The fact that aphids and leafhoppers can ingest and egest for fairly long times with no interruptions, continuously filling the chamber with fluids, supports the idea that these vectors can sustain positive or negative pressure in the precibarial pump for prolonged time periods during which they can fill (ingest) or empty (egest) the pump (Harris & Bath, 1973; Harris et al., 1981) (Figure 3).

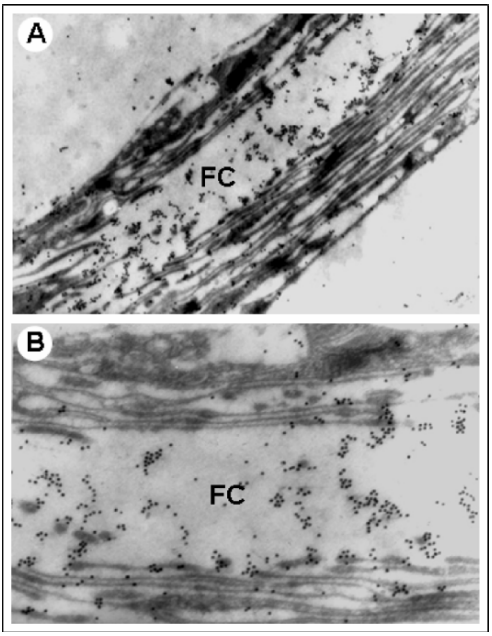

Figure 3. Immunogold localization of TYLCV coat protein in the stylet food canal (FC) of *B. tabaci*. Note that localization is mainly in the food canal and not observed in other places.

3. THE ESOPHAGUS

The esophagus of *B. tabaci* is extremely slender and is composed of external and internal esophageal tissues (Ghanim et al., 2001a). The external esophagus passes along the thorax, dorsally of the subesophageal ganglionic mass to meet the junction of the filter chamber where the descending and the ascending parts of the midgut and the hindgut meet. The external esophagus is formed by a thin layer of epithelial cells and a very well-defined cuticular intima surrounding the lumen. The external esophagus ends where it meets the connecting chamber (midgut and hindgut). Some of the tissues of the connecting chamber are esophageal, thus are called internal esophagus. The transport of food particles in the external esophagus is relatively fast if compared to other parts of the digestive system. Therefore visualization of food particles and virions inside this organ is particularly difficult. Food elements are not absorbed by esophageal tissues and are rapidly transported to the abdomen, and from there to the haemocoel.

4. THE FILTER CHAMBER AND MIDGUT

Determining the structure of the filter chamber has been challenging (Cicero et al., 1995; Harris et al., 1996; Ghanim et al., 2001a). Identification of the two arms that join the filter chamber has been controversial. Harris et al. (1996)

considered these structures as Malpighian tubules similar to those found in other insects, which have an excretory function. Cicero et al. (1995) observed large Malpighian-like cells in the area of the filter chamber. The authors suggested an excretory role for these cells because of their relationships with the hindgut tissues. Ghanim et al. (2001a) described in details the ultrastructure of the filter chamber, and demonstrated that the filter chamber is a highly specialized organ where fluids are filtered into highly concentrated sugars that are absorbed in the internal ileum of the hindgut, which constitutes an integral part of the filter chamber. Fluids absorbed into the internal ileum are directed to the rectal sac and excreted outside the body as honeydew (Figure 4A). The remaining fluids are pushed into the looped midgut and important food substances such as amino acids are absorbed into the haemocoel. Pushing food materials into the hindgut is achieved mainly by the two structures extending from the connecting chamber. Ghanim et al. (2001a) and Cicero et al. (1995) defined these paired structures as *caeca* (Figures 4B & 4C). The *caeca* have extensive musculature and a large labyrinth-like lumen (Figure 4C).

Together with food materials, TYLCV virions are expected to be found in the filter chamber locations described above. TYLCV DNA could be amplified from honeydew collected from leaves infested with viruliferous whiteflies (unpublished). This finding demonstrated the ability of TYLCV virions to cross the filter chamber barriers, including the walls of the continuous lumen, the ascending midgut and the internal ileum, and reach the hindgut and the rectum. Circulative transmission of TYLCV implies that the virus is able to cross the midgut wall barrier, to be transported into the haemolymph, to cross the salivary gland barrier and to be transmitted to plants (Ghanim et al., 2001b). Therefore extensive virus-related signal should be observed in the filter chamber and midgut tissues. A lower signal is expected in the paired *caeca* and in the hindgut because food and viral particles are rapidly transported through these tissues. The paired *caeca* push food materials into the midgut and the hindgut, from where they are rapidly excreted outside the body. Therefore we were unable to detect any TYLCV signal in the hindgut. Conversely, we observed extensive IGL of the virus in midgut and filter chamber tissues. Figure 5 shows immunofluorescent labelling of TYLCV in the filter chamber and the midgut, detected with FITC-goat anti-rabbit secondary antibody. The stain was mainly observed in the filter chamber and the descending midgut. When food material is pushed by the paired *caeca*, it is filtered in the filter chamber and the remaining continues to the descending midgut where most of the important substances are absorbed into the haemocoel. These are the two main locations where food is kept for longer time than in other locations of the digestive tract. Figure 5 reveals that the antibody stain is concentrated in the gut wall. To confirm these findings we prepared EM sections from midguts of viruliferous whiteflies that fed on infected tomato for 48 h. These sections were reacted with anti-TYLCV-CP antibody prepared in rabbits. This antibody was detected with a secondary antibody conjugated to 15 nm gold particles. The sections presented

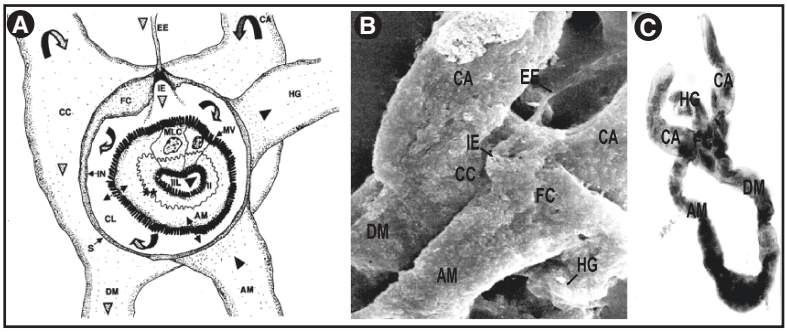

Figure 4. External and internal morphology of the filter chamber and the midgut of *B. tabaci*. (A), Drawing of a cross section through the filter chamber (FC) illustrating the components of the digestive system present in this region and the probable flow of ingested plant materials. Hatched arrowheads indicate how ingested plant materials empty from the external esophagus (EE) into the continuous lumen (CL) of the filter chamber via the internal esophagus (IE). Curved arrows indicate the movement of plant from the continuous lumen of the filter chamber into the lumen of the adjoining connecting chamber (CC). Ingested plant materials then enter the lumen of the caeca (CA) (curved arrows). The caeca are blind-ended thus the ingested plant materials circulate back into the connecting chamber lumen and then enter the descending midgut (DM) (hatched arrowhead). The solid black arrowhead indicates movement of plant materials back to the filter chamber via the ascending midgut (AM). The dotted lines with arrowheads at each end indicate that ingested fluids, before or after circulation, may also be absorbed by the ascending midgut cells. The basal membrane of these cells interdigitates the basal membrane of the internal ileum (II) cells (double asterisk). Fluids that enter these cells are presumably filtered across the internal ileum and exit the filter chamber via the internal ileum lumen (IIL) which enters the lumen of the hindgut (HG) (solid black arrowheads). The internal ileum forms a tube within the filter chamber that connects to the hindgut, where it exits the filter chamber. Hence, once fluids enter its lumen they do not reenter the continuous lumen of the filter chamber. MLC, Malphigian-like cells; MV, microvilli. (B), SEM of *B. tabaci* showing a dorsal view of the filter chamber where it joins the connecting chamber. The external esophagus is slender and expands where it becomes the internal esophagus in the filter chamber and joins the connecting chamber (not visible externally). The caeca extends into the haemocoel from the connecting chamber and the descending midgut exits the connecting chamber and joins the ascending midgut, which in turn joins the continuous lumen within the filter chamber. The arrow on the hindgut points towards the anus. (C), LM showing a ventral view of portions of the alimentary tract after dissection. The caeca and descending midgut extend from the connecting chamber. The lumen of the connecting chamber is convoluted and connects the caeca and the descending midgut. The filter chamber is not visible because it was underneath the connecting chamber in this dissection. The ascending midgut passes underneath the connecting chamber to join the filter chamber. The hindgut snapped free of the anus during dissection and was consequently folded accordion-fashion underneath the connecting chamber.

in Figure 6 show that the midgut of *B. tabaci* is rich with brush border (microvilli) extending to the lumen of the midgut. These structures are elongations of the extensive epithelial cells building the gut wall. The main role of the microvilli is to absorb and transport food materials from the lumen of the midgut to the haemocoel. The microvilli are capable of increasing the absorbing

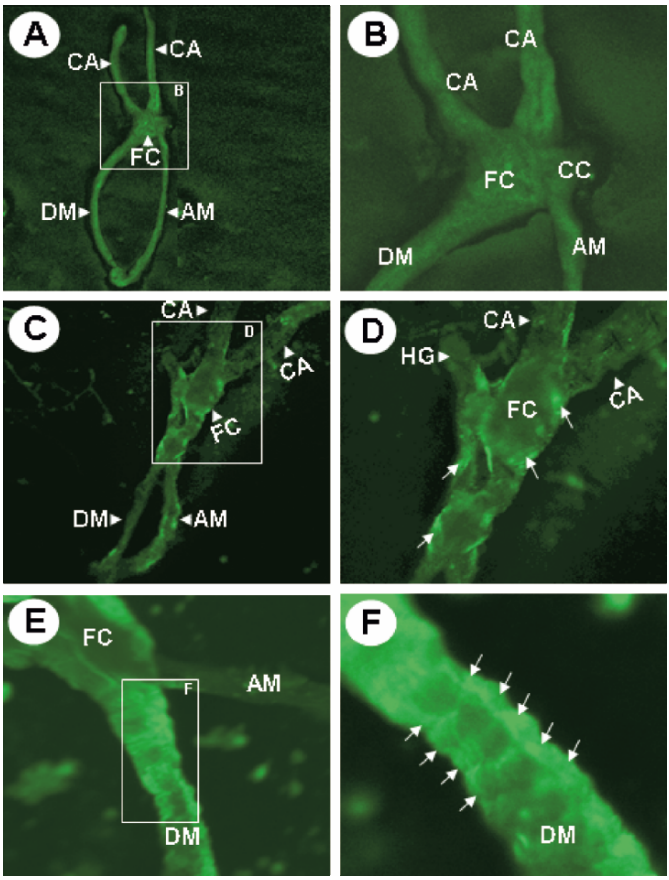

Figure 5. Immunofluorescent detection of TYLCV in dissected midgut. (A) and (B), midgut reacted with secondary antibody FITC-conjugated, and not reacted with anti-TYLCV-CP antibody. (C) and (E), detection of TYLCV-CP in filter chamber (C) and descending midgut (E). (D) and (F), higher magnifications of the insets in C and E. CA, caeca; DM, descending midgut, AM, ascending midgut; FC, filter chamber; CC, connecting chamber; HG, hindgut.

surface area in the midgut 1,000–10,000 times. Figure 6A shows the extensive microvilli originating from the gut and longitudinal and cross sections of these microvilli in different portions of the gut. Figure 7 shows IGL of TYLCV in different portions of the midgut, mainly in epithelial cells within the gut wall. In these cells, the virus-related signal is associated with chromatin, supporting the assumption that TYLCV replicates in the insect vector. These results also suggest that the microvilli may constitute one of the sites rich in begomoviral receptors and may serve as the primary site allowing internalization of viral

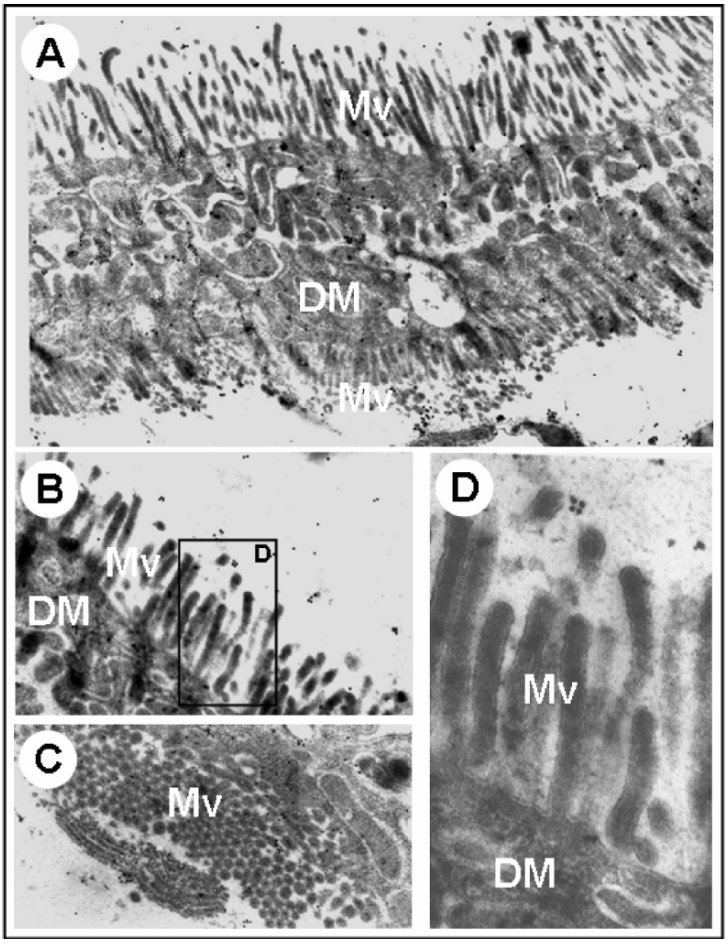

Figure 6. Overview of the descending midgut of *B. tabaci* showing its epithelium and the rich microvilli lining the boarders of the midgut. (A), longitudinal section along the descending part of the midgut showing extensive brush-boarder on its two sides. (B) and (D), higher magnifications showing the microvilli and epithelial cells. (C), cross section across the microvilli. Mv, microvilli; DM, descending midgut.

particles. Hence these cells may constitute a transit site for the virus on its way to the haemocoel, or may serve as a virus long-term storage site.

We were unable to detect TYLCV in hindgut tissues. Unlike the aphid *Rhopalosiphum padi*, which has a barrier for the *Cereal yellow dwarf virus-RPV* (CYDV-RPV) in the hindgut (Gildow, 1985; Gray & Gildow, 2003), it seems that *B. tabaci* does not have a similar barrier. Since TYLCV was detected in many epithelial cells of the midgut, it seems that the barrier sets on midgut cells such as

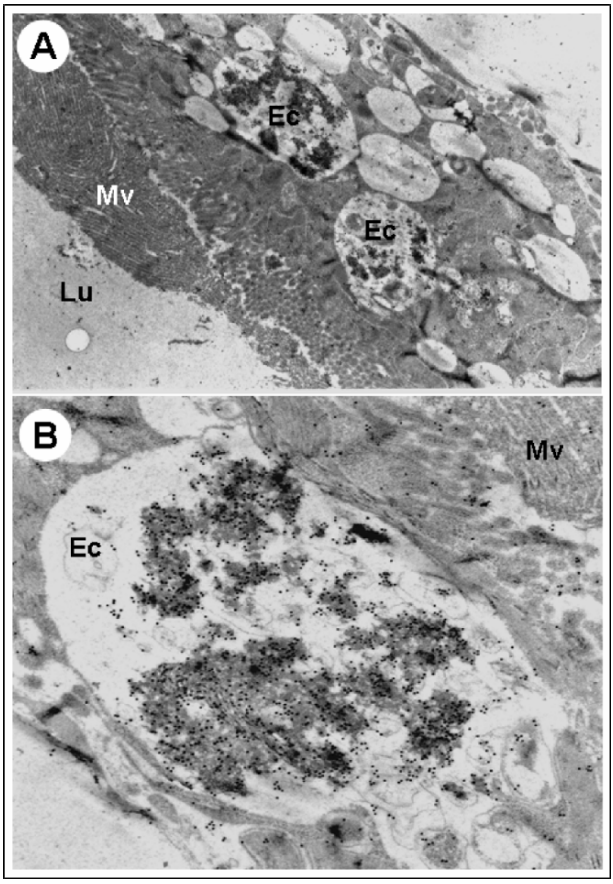

Figure 7. Longitudinal section across the descending midgut showing extensive immunogold labelling inside epithelial cells. (A), longitudinal section showing extensive labelling inside two epithelial cells; the labelling is not associated with the microvilli. (B), extensive immunogold labelling against TYLCV-CP in one epithelial cell. DM, descending midgut; Mv, microvilli; Lu, lumen; Ec, epithelial cell.

those involved in the interaction between the aphid *Myzus persicae* and *Potato leafroll virus* (*PLRV)* (Garret et al., 1993). Unlike viruses that are transported from the hindgut lumen of aphids to the haemocoel, non-transmissible viruses such as *Brome mosaic virus* and *Cowpea mosaic virus* were only observed in the lumen, and were not absorbed into the gut membrane or epithelial cells (Gildow, 1993). The specificity of virus transmission and receptor-mediated transmission was extensively studied in aphids; transmission of CYDV-RPV by its vector *R. padi* is a good example. Coated vesicles are formed in cell membranes in response to ligand binding. These vesicles are usually part of the protein transport mechanism. The virus

particles accumulate in tubular vesicles and cross the cytoplasm. They are then released into the haemocoel by budding of the tubular vesicles across the basal plasmalemma (Gildow, 1985, 1993).

5. THE SALIVARY SYSTEM

Harris et al. (1995, 1996) have provided a detailed description of the salivary ducts and the common salivary canal of *B. tabaci* at the light microscopic level. The salivary system consists of paired primary and paired accessory glands. Each primary gland has an accessory counterpart and from both a salivary duct originates to meet its counterpart. Both salivary ducts from each paired primary and accessory glands join to form the common salivary canal. In the aphid *M. persicae*, the ducts from all primary and accessory glands join immediately after exiting the gland in one point to form an afferent duct (Forbes, 1964; Ponsen, 1972, 1977).

The ultrastructure of the primary and salivary glands of *B. tabaci* has been studied by Ghanim et al. (2001a). The primary salivary glands are looped and kidney-shaped as seen in Figure 8. On each side of the prothorax, lying are one primary salivary gland (PSG) and one accessory salivary gland (ASG). The salivary ducts that originate from each PSG and ASG meet on each side (Figure 8A).

Each PSG is made from 13 nearly asymmetrical large cells that empty into one duct lined with microvilli. These cells stain differentially. Each ASG is made

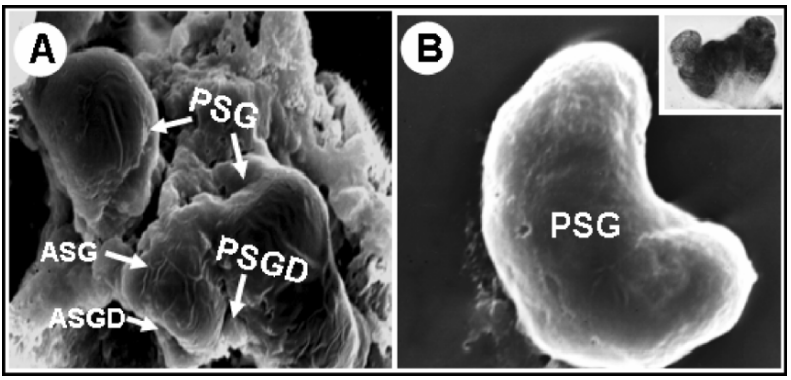

Figure 8. Scanning electron microscopic images showing external morphology of the primary and accessory salivary glands. (A), shows the close relation between primary and accessory glands, and the location they join to form the afferent salivary duct. Paired primary glands and one accessory gland are shown in this image. (B), SEM image showing one primary salivary gland and its kidney shape. In the inset, a light micrograph showing a primary salivary gland with the same shape. PSG, primary salivary gland; ASG, accessory salivary gland; PSGD, primary salivary gland duct; ASGD, accessory salivary gland duct.

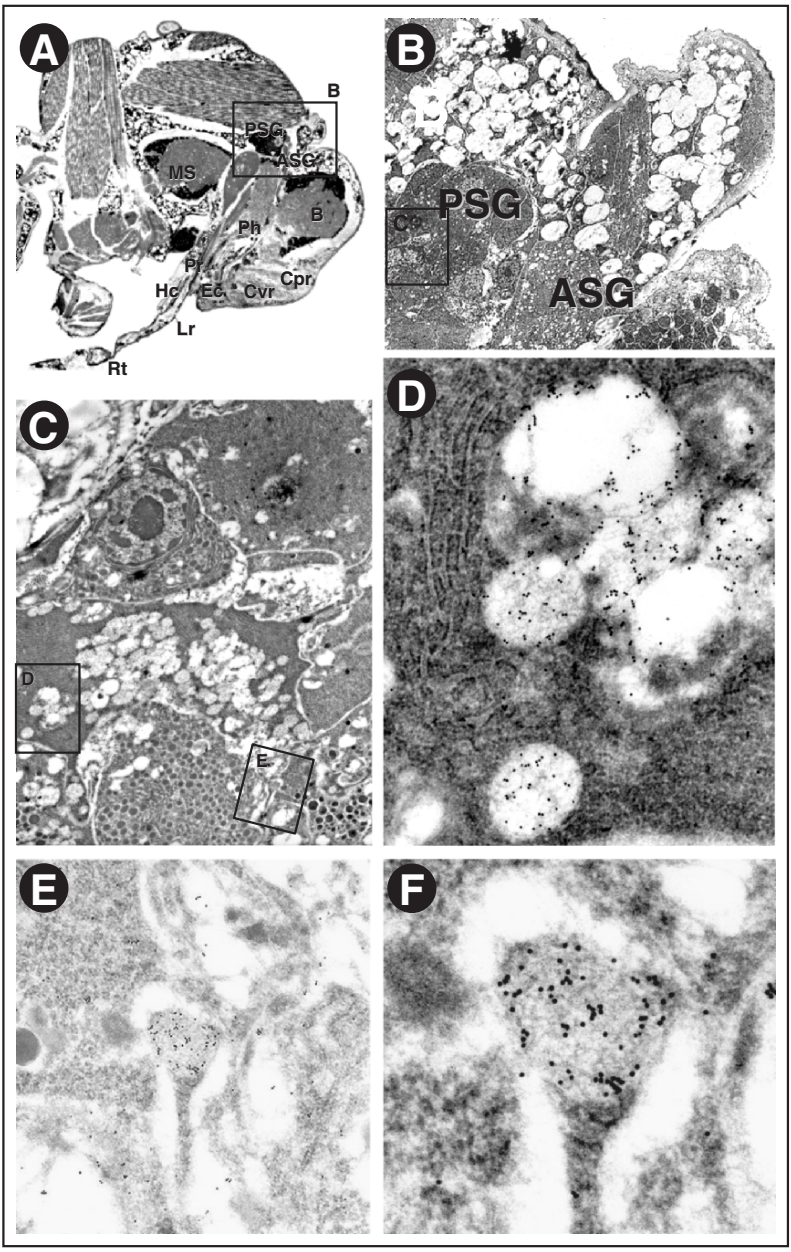

Figure 9. Localization of tomato yellow leaf curl Sardinia virus (TYLCSV) at primary salivary glands (PSG) by immunogold labelling (IGL). (A) and (B) are given for orientation. (B, brain; Cpr, cibarial pump retractor muscles; Cvr, cibarial valve retractor muscles; Ec, epicibarial gustatory organ; Hc, hypocibarial gustatory organ; Lr, labrum; Ph, pharynx; Pr, piston retractory muscle; Rt, rostrum). (C) IGL of PSG. (D) and (E) are higher magnification of the insets in (C). Strong IGL of saliva drops in saliva secretory cells is observed. (F) Higher magnification of the insert in (E) labelling of initial saliva drop.

from four large symmetric cells similar and simple in their structure. In aphids it was previously shown that transmission specificity is determined at the salivary gland barrier. Two observations led to this conclusion. First, aphids were able to transport non-transmissible viruses from the gut lumen to the haemocoel indicating that the barrier to transmission is subsequent to this event (Rochow & Pang, 1961); second, injecting non-transmissible virus into the haemocoel did not lead to transmission of the virus (Mueller & Rochow 1961; Rochow, 1969). Particles of *Barley yellow dwarf virus-MAV* (BYDV-MAV) were always associated with the anterior end of the ASG basal lamina, and not with basal laminas of other organs, suggesting receptor-mediated recognition events taking place in the ASG barrier (Gildow & Rochow, 1980). The ASGs of aphids consists four large secretory cells, with structures similar to those of *B. tabaci* (Ghanim et al., 2001a).

TYLCV has been localized in PSGs using immunofluorescent labelling (Brown & Czosnek, 2002), and by *in situ* amplification of TYLCV DNA (Ghanim et al., 2001b), no data is available regarding localizing TYLCV or other begomovirus in the salivary glands of *B. tabaci*. It is not known whether ASGs of PSGs, or both, are involved in the process of TYLCV transmission.

We were able to localize TYLCV using IGL in the PSGs. Figure 9 shows extensive gold labelling in PSG excretory cells. Although these observations do not shed light on the specificity of TYLCV at the barriers of PSG or ASG, both glands could serve as the selective barrier for TYLCV transmission. Additional studies are necessary to find out whether whitefly salivary glands are functionally similar to those of aphids.

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CHAPTER 4

MOVEMENT AND LOCALIZATION OF TOMATO YELLOW LEAF CURL VIRUSES IN THE INFECTED PLANT

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1. LOCALIZING TYLCV INSIDE HOST PLANTS: HISTORY, KEY TECHNIQUES, LANDMARKS – AN OVERVIEW

After its release into the phloem of a young leaf by feeding *Bemisia tabaci*, TYLCV systemically invades most plant organs above and below ground within 1–2 weeks (Ber et al., 1990; Czosnek et al., 1988b; Kheyr-Pour et al., 1994; Michelson et al., 1994; Picó et al., 1999, 2001; Rom et al., 1993). Different virus titers will accumulate depending on the organ type and its position at the plant. In tomato, viral replication and translocation usually precede symptom appearance by days or even weeks. Tolerant tomoto varieties were developed following epidemics of devastating tomato yellow leaf curl disease (TYLCD) in Israel and the Middle East since the first half of last century (Cohen & Antignus, 1994; Czosnek, 1999, and references herein). In tolerant breeding lines, TYLCV spread is almost or fully latent, producing only mild and delayed phenotypic alterations, if at all (Picó et al., 1996; Rom et al., 1993).

In order to understand TYLCD pathogenesis, detailed analyses on transmissibility, symptom induction, and host range of the causative agent named *Tomato yellow leaf curl virus* were performed in the 1960s (Cohen & Nitzany, 1966). In this study, eight symptomless crops and weed hosts were discovered, which were able to serve as source plants for whitefly inoculation of test plant species. These early findings substantiated the frequently "hidden nature" of the virus upon translocation inside its hosts. Hence, unraveling time course, preferential routes, and final distribution patterns of the virus in the different types of tissues and cells has been attempted in several consecutive studies, whenever more advanced techniques were available.

In the 1980s, transmission electron microscopy (TEM) was carried out on yellow leaf curl-diseased tomato and yielded first strong evidence for the presence

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 185–206.

of a phloem-associated geminivirus with reference to similar ultrastructural findings published in the 1970s for other geminivirus-infected plants (Russo et al., 1980, and references herein). TEM also verified the twinned particle morphology and the presence of a circular ssDNA genome after TYLCV was isolated (Czosnek et al., 1988a), and elucidated further details of its tissue and organelle specificity as described later.

The disadvantages of TEM analysis, such as its limitation to samples of very small dimensions, the need for extensive tissue preparation, and the lack of any proof for the identity of the observed structures with primary TYLCV components led to new strategies for localizing viral nucleic acids and proteins, as molecular biology and immunology methods were developed or improved. Hybridization of (radio-)labeled virus-derived DNA probes to nucleic acid preparations from different plant tissues became a key technique to trace viral nucleic acids inside plants and to carry out time-course analyses, in some cases accomplished by serological detection of TYLCV antigen in extracts (Ber et al., 1990; Czosnek et al., 1988b; Kheyr-Pour et al., 1994; Michelson et al., 1994; Picó et al., 1999; Picó et al., 2001; Rom et al., 1993). The studies helped to narrow down peaks of latent DNA accumulation and of plant susceptibility for whitefly inoculation to certain developmental stages, and thus yielded important data for effective quarantine and cultivation measures against TYLCD.

Tissue printing onto membranes in combination with nucleic acid or immunological detection methods allowed for a direct visualization of the viral DNA distribution in different plant organs (Czosnek & Navot, 1988; Fargette et al., 1996; Navot et al., 1989). The respective findings supported the phloem preference of TYLCV, and were able to resolve even rough numbers of infection foci in internal and external phloem squashed onto nitrocellulose membrane (Fargette et al., 1996).

Molecular localization techniques on the light-microscopic level finally yielded detailed pictures of TYLCV accumulation patterns inside extended areas of systemically invaded plant tissues, covering at minimum several millimeters in extension. Cellular or even subcellular resolution was achieved with methods specific for either viral antigens or nucleic acids. A fundamental study of Rojas et al. (2001) confirmed a strict phloem-limitation of TYLCV in different tomato organs by visualizing coat and C4 protein antigens in paraffin sections, and by *in situ* PCR detecting ORF C1. The latter also yielded first evidence for some viral DNA present in cambium tissues of stem sections, which may contribute to efficient virus release into vascular elements as discussed later. A similar phloem tropism was found for another TYLCV strain and for TYLCSV by genome-specific *in situ* hybridization (Morilla et al., 2004). By applying a dual-colour detection method, the investigation additionally demonstrated that in doubly infected plants, TYLCV and TYLCSV enter only a very limited number of nuclei, of which they share about 20% (Figure 1). On the background of inherent viral replication mechanisms exploiting the recombination machinery of plants (Jeske et al., 2001; Preiss & Jeske, 2003), this may

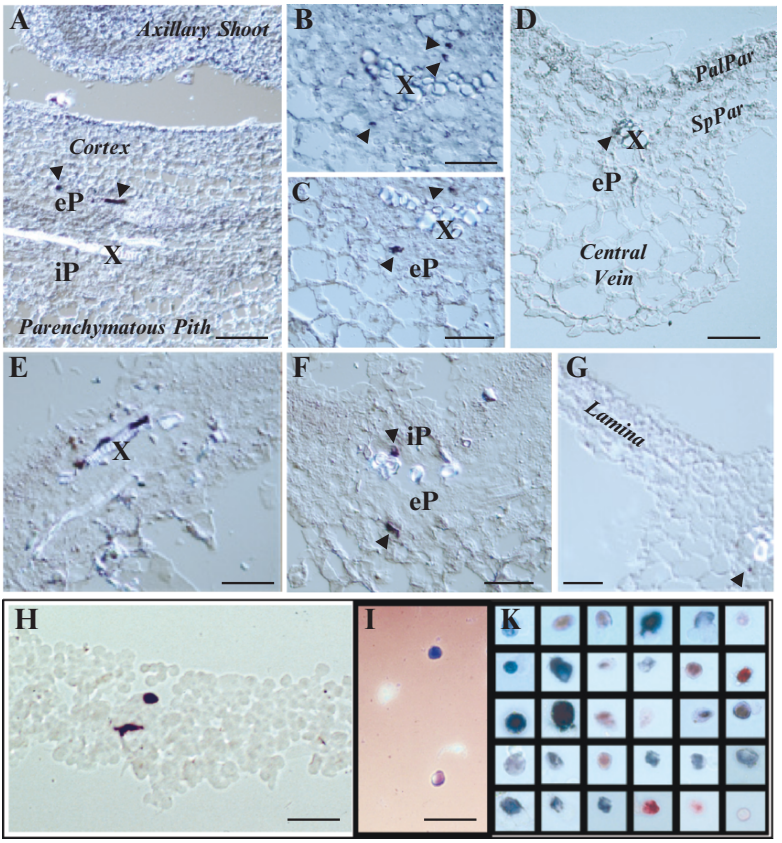

Figure 1. In situ hybridization detecting TYLCV and TYLCSV DNA in infected *Solanum lycopersicum* (A–C, E,F) and *Nicotiana benthamiana* (D,G) tissues or purified nuclei from *N. benthamiana* plants (H–K) of different infection status, sampled at 21 or 28 days post agroinfection. Arrowheads point at infected nuclei in A–G; H–K: dual-colour detection visualizes singly or doubly infected nuclei. A–C: TYLCSV-infected tomato (A: longitudinal section of stem; left hand side: apex; B, C: cross sections of central leaflet veins). Infected nuclei are located in vascular parenchyma (VP) and companion cells (CC) in internal and external phloem tissues. D: TYLCSV-infected *N. benthamiana* leaf; a virus-DNA containing nucleus is located close to a xylem element. E–G: Leaf specimens from plants doubly infected with TYLCV and TYLCSV (E,F: tomato, G: *N. benthamiana*), analysed by use of a probe visualizing both viruses in the same tissues as in single infections. H: Isolated nuclei adsorbed to slides and hybridized with TYLCSV-specific probe, or I: with mixed probe staining TYLCSV DNA red and TYLCV DNA blue. 4′,6-diamidino-2-phenylindole (DAPI) counterstain of nuclear DNA, combined brightfield/fluorescence microscopy. K: Range of colours obtained upon virus-specific DNA detection in isolated nuclei in a representative experiment. Tissue sections were examined by differential contrast (DIC) microscopy. (e/i)P: (external/internal) phloem; X: Xylem; Pal/SpPar: palisade/spongy parenchyma. Scale bars: A–G: 50 µm, H,I: 25 µm. For experimental details, refer to Morilla et al. (2004).

be a clue finding to explain the economically relevant frequency of epidemics caused by new geminiviral strains which have arisen from genome recombination events (Fondong et al., 2000; Harrison & Robinson, 1999; Moffat, 1999; Padidam et al., 1999; Pita et al., 2001; Ribeiro et al., 2003; Sanz et al., 2000; Schnippenkoetter et al., 2001; Zhou et al., 1997, 1998, 2003). Recombinants of TYLCV and TYLCSV play an important agronomic role in Spain since the beginning of this century (Monci et al., 2002) and have become prevalent only after a few years' period of coexistence of both viruses in southern parts of the country (Navas-Castillo et al., 1997, 1999; Sanchez-Campos et al., 1999). Analogous situations in which different TYLCD-causing virus strains or species with different host range and pathogenicity meet each other may be expected not only for extended regions around the Mediterranean and northern Africa, but also for tropic and subtropic regions in Asia and the USA (Moriones and Navas-Castillo, 2000; Picó et al., 1996). Hence the study of Morilla et al. (2004) indicates an enhanced need for rapid international measures to effectively reduce the worldwide incidence of TYLC(S)V, in order to prevent the establishment and spread of increasing numbers of new viral variants in the future.

Specimen preparation and *in situ* hybridization techniques have been further refined recently for begomovirus-infected plants, including TYLCSV-infected *N. benthamiana* (Rasheed et al., 2006). With buty-methyl methacrylate-embedded plant material, preservation of cytoplasmic cellular components was improved in comparison to paraffin sections, which tend to lose part of the cell contents upon paraffin removal. Hence, the authors were able to localize different viral DNA species to distinct regions of single cells. Furthermore, they present evidence for an association of TYLCSV DNA not only with phloem, but also with xylem elements, which expands observations of Rojas et al. (2001) who detected TYLCV-specific DNA in cambium nuclei of tomato. An extended model for TYLC(S)V spread was deduced, discussing a potential role of the xylem in the long-distance transport of begomoviruses (Rasheed et al., 2006).

2. EXTENDED TRACING OF TYLCV INTO THE FOURTH AND THE SMALLEST DIMENSIONS – RECENT PROGRESS TOWARDS HIGH RESOLUTION

Whereas molecular *in situ* localization studies so far have elucidated many details of TYLCV distribution patterns in two to nearly three dimensions (the latter, however, only indirectly via comparisons of cross, and longitudinal, and series of consecutive sections), they have barely been used for collecting data of the fourth dimension. Time-course experiments are either impossible with the techniques described above, since individual samples have to be processed and therefore cannot be observed at later stages, or they bear the risk of misinterpretations due to inconspicuous differences, whenever they are carried out with "comparable" tissues of other plants or of the same plant, but later on. To even out insignificant discrepancies, large numbers of specimens were

to be analysed – which is nearly impossible with modern histological *in situ* localization methods due to the immense expenditure of work they require and the limited number of samples which may be processed at a time.

Nevertheless, real-time monitoring of TYLCD in the host is an important prerequisite for tailoring cultivation measures against epidemics, and for evaluating and characterizing resistance traits of crop plants. Therefore special emphasis has been placed on developing an *in vivo* tracing strategy for the monopartite TYLCV during the last years, like it had been achieved for bipartite begomoviruses by replacement of their coat protein (CP) gene, e.g., with the marker gene for Green Fluorescent Protein (GFP; Levy & Czosnek, 2003; Sudarshana et al., 1998). Since, however, the CP is indispensable for TYLCV or TYLCSV movement inside plants (Noris et al., 1998; Rojas et al., 2001, and references herein; Wartig et al., 1997), the monopartite virus had to be visualized by help of an accompanying, trans-replicated second component expressing the tracer. First results to be noticed in this context have been reported for *Tomato yellow leaf curl China virus* (TYLCCNV), which induces symptoms only in the presence of an associated satellite DNAβ expressing ORF βC1 (Cui et al., 2004, 2005). Tao & Zhou (2004) modified TYLCCNV isolate Y10 DNAβ to develop a gene silencing system and inserted host gene sequences, which upon trans-replication by the helper virus led to bleaching of invaded tissues due to loss or blocked synthesis of chlorophyll as a consequence of target gene silencing. The system indicated in a roughly contemporary manner the viral routes inside different hosts, since the silencing signal seemed not to be transported into virus-free areas to any significant extent. These experiments, however, were designed for other purposes and therefore did not focus on highly resolved time-course observations.

Those were achieved only recently by an experimental design which intimately connects the expression of an *in vivo* tracer to the presence of replicating TYLCSV DNA in the respective cell (Morilla et al., 2006). *N. benthamiana* plants were stably transformed with a GFP-expression cassette flanked by TYLCSV origins of replication. In cells harbouring transcriptionally active and replicating TYLCSV, the transgene was released from plant genomic DNA to form freely trans-replicated GFP-expressing episomes, which under UV exposure led to strongly fluorescent areas wherever wildtype virus was multiplying. Monitoring GFP expression in individual plants over periods of several weeks confirmed that TYLCSV replication was confined to veinal tissues and revealed that it took place only very transiently in small windows of leaf development. Moreover, the experimental setup for the first time proved that the virus was able to replicate also in floral, stem, and root tissues.

All studies published so far have contributed to a quite close-meshed picture about TYLC(S)V replication and spread inside tissues of important host plants, details of which will be presented in the following paragraphs accompanying the virus on its long-distance journey through the host. Although most of the respective experiments have specifically detected either viral DNA or proteins, in no case

were they able to define the full composition of the moving viral agent. Additional analyses using different molecular biology techniques were necessary to identify viral and non-viral components involved, and to bring about details of short-distance cell-to-cell transport mechanisms and of intracellular tropisms of viral proteins and forms of nucleic acids (see also Gafni, 2003, and references herein). They include comparisons of ORF sequences and functions (Kheyr-Pour et al., 1991; Navot et al., 1991), usage of genetically modified viral clones (e.g., Jupin et al., 1994; Noris et al., 1998; Wartig et al., 1997), localization of microinjected or transiently expressed individual viral proteins and/or nucleic acids in plants (e.g., Dong et al., 2003; Rojas et al., 2001; van Wezel et al., 2001) or analyses in heterologous yeast or insect cell model systems (Kunik et al., 1998, 1999; Rhee et al., 2000). Thereby, the types of viral nucleic acid–protein complexes transported between cells, tissues, and organs could be delimited to a small number of possibilities which may occur in parallel or alternatively, depending on host plant or cell type. It is generally accepted that TYLC(S)V CP plays an important role in at least long-distance transport, and that precoat- and C4-protein are involved in viral movement (the latter, however, being of minor importance in *N. benthamiana* (Jupin et al., 1994). Chapter 1 in Part B describes the functions of individual proteins involved in viral transport in and between cells, and further information on molecular interactions of these proteins with plant factors is given in chapter 5 of Part C. Therefore, only a brief overview will be given in this chapter on the molecular processes of virus trafficking. It is, however, noteworthy to mention a few aspects of early TEM studies below, with respect to their potential relevance for supporting recent results on viral movement mechanisms as obtained by modern molecular biology methods.

Thus, the next sections will accompany TYLCVs inside a systemically infected host plant step by step, leaving out all details on individual protein or nucleic acid functions, and molecular virus–host interactions. Instead, the results from a range of completely different studies will be combined for every stage of the infection in order to yield a highly resolved "motion picture" of the viral lifecycle in the different organs and tissues.

3. TYLC(S)V ON ITS ROUTE THROUGH THE PLANT: THE START – ESTABLISHMENT WITHIN FIRST CELLS

TYLCD-causing viruses are transmitted by the whitefly *B. tabaci* in a persistant circulative manner. After virus uptake from infected plants, latent periods between 7 and 24 h occur in which the particles are translocated through different organs of the vector, finally entering its salivary glands to be injected into the phloem of newly infested plants with the saliva. Since detection of TYLCV DNA inside glands by immunocapture-PCR was dependent on the presence of anti-CP antibody, the infectious agent entering the phloem probably exists in the form of virus particles (Ghanim et al., 2001, and references herein). In order to establish a feeding site, whiteflies slowly move their stylets intercellularly between epidermal and mesophyll parenchyma cells directly into the phloem,

occasionally puncturing xylem elements (Harris et al., 1996; Janssen et al., 1989; Pollard, 1955). Virions will therefore be delivered in most cases into sieve elements (SE), in some instances into CC, or VP cells, respectively, of host plants inside the bundle sheath (BS). For starting the viral "lifecycle", however, genomic DNA needs to enter a nucleus, which is mediated by TYLCV-CP (Kunik et al., 1998, 1999; Rojas et al., 2001). It is not clear whether intact virus particles transit through the nuclear pores, or target the nuclear pore complex to release DNA into the nucleoplasm, or if completely or partially uncoated viral nucleic acids are transported into nuclei as was suggested on the basis of microinjection experiments (Noueiry et al., 1994). Similarly, it is not known if complete virions may enter nucleate cells from the enucleate sieve tubes. At the onset of a natural infection, this may occur either close to the feeding site of the viruliferous whitefly, or, after phloem transport of the virion, somewhere else in an, at that time, SE export-competent vascular region. In general, both sink and source leaves exhibit phloem and thus sieve tube unloading capacities, though they differ in several aspects (Waigmann et al., 2004). Exit from the sieve tube into an adjacent CC or, with some viruses in some plant species, alternatively a phloem parenchyma (PP) cell (Nelson & van Bel, 1998) probably happens via specialized asymmetric branched plasmodesmata (PD) at the SE/CC (or SE/PP) boundary. PD of this shape were found in apoplastic as well as symplastic phloem-loading plants and exhibit unusually large size exclusion limits which may allow for trafficking of complete virions under certain circumstances (Oparka & Santa Cruz, 2000; Silva et al., 2002). They are also called pore/plasmodesm units (PPU; van Bel & Kempers, 1997) and indeed have been shown to contain particles of a spherical luteovirus (Murant & Roberts, 1979; Waterhouse & Murant, 1982). However, studies of virus export from sieve tubes via SE-companion or SE-parenchyma cell interfaces are scarce. It might be aided by virus-induced gating of the PPUs, which could involve transient events or alterations performed by help of plant cell-wall degrading enzymes (for detailed overviews on PD and systemic plant virus spread, refer to Ding, 1998; Nelson & van Bel, 1998; Waigmann et al., 2004).

After a primary whitefly-injection of TLYC(S)V particles into a sieve tube, no movement proteins of the virus are available yet which might form DNA–protein transport complexes other than virions or increase the size exclusion limit (SEL) of PPUs. Therefore, either virions can pass PPUs from the SE lumen into CC cytoplasm by themselves, at least in some susceptible tissue regions, or the begomovirus may start a productive infection only after being introduced into a nucleate phloem cell. Any differentiation between both putative routes has not been possible so far.

Early molecular hybridization studies showed first locally multiplied TYLCV DNA inside the whitefly-inoculated organ at 8 days post inoculation (dpi) when the apex was subjected to the insects, or 2 to several days later when older leaves were used (Ber et al., 1990). With the improvement of nucleic acid detection on Southern blots, different authors consistently reported significant TYLCV

DNA accumulation in insect-inoculated tomato leaves at usually 3–4 dpi (Michelson et al., 1994; Picó et al., 1999; Rom et al., 1993), reaching a peak of maximum viral DNA content about 11–13 dpi which preceded symptom formation by another 2–4 days in susceptible plant lines. By exposing individual leaves of tomato plants at the 8-leaf stage to viruliferous whiteflies in leaf cages, Ber et al. (1990) found out that plant apices exhibited the highest TYLCV susceptibility and accumulated the largest amounts of new viral DNA in comparison to all other leaves. Both susceptibility and efficiency of TYLCV replication decreased with the age of the inoculated leaf, and were almost or completely absent when the primary leaves were treated. After *Agrobacterium*-mediated TYLC(S)V inoculation into stems of tomato plants, newly replicated viral DNA was detectable close to the site of delivery already at 2 dpi (Picó et al., 2001). This might be attributed to an increased number of cells entered by the infectious DNA construct, which is guided into nuclei via the bacterial T-DNA-transfer machinery.

In a natural infection, this step is mediated by the viral CP, which also accounts for intranuclear targeting and, later in the infection cycle, nuclear export of newly replicated virus DNA (refer also to Part B, Chapter 1; Gafni, 2003; Rhee et al., 2000; Rojas et al., 2001, and references herein; Wartig et al., 1997). Most TYLC(S)V CP functions are comparable to those of BV1 proteins of bipartite begomoviruses (for a detailed discussion on BV1 NSP functions, refer to Hehnle et al., 2004). Multiplication and transcription of geminiviruses inside nuclei involve formation of minichromosomes (to be described in detail in Part C Chapter 1; Pilartz & Jeske, 1992; Preiss & Jeske, 2003). After transcripts have been exported into the cytoplasm and non-structural TYLC(S)V proteins translated for the first time, additional movement functions can be taken over by viral proteins other than the CP, with further capacities of interacting with host components. TYLC(S)V precoat protein was shown to be one of the key players in cell-to-cell transport, probably acting in association with the ER in analogy to BC1 movement protein of bipartite geminiviruses (Navot et al., 1991; Rojas et al., 2001). An additional BC1-like role has been revealed for TYLC(S)V ORF C4, which seems to support viral cell-to-cell transport from a position at the cytoplasmatic periphery; but it is not absolutely necessary for systemic invasion of *N. benthamiana* (Jupin et al., 1994).

After movement-associated proteins have been synthesized in the primary infected cells, local and the systemic long-distance (LD) spread of the virus will start. To invade new organs via the phloem, the transported viral agent exits the initially infected cell, probably again via PPUs, into an SE. The systemically transported TYLC(S)V DNA form has not been identified yet, but CP seems to contribute to LD movement in different host plants and binds to nucleic acids (Palanichelvam et al., 1998; and Part B, Chapter 1).Evidence for a crucial role of virus-like particles comes from TYLC(S)V CP mutants which revealed a clear correlation between the abilities to form virions and to be transported systemically (Noris et al., 1998). The findings are further substantiated by

immunocapture-PCR results indicating the presence of TYLCV–DNA–CP complexes in whitefly stylets after one hour of phloem sap acquisition on infected plants, which were assumed to be virions (Ghanim et al., 2001). Additional support comes from EM studies on TYLCD-affected tomato plants, in which virus-like particles were found in mature SE (Cherif & Russo, 1983). These results, however, do not rule out that CP interactions necessary for multimerization during particle formation also contribute to a differently structured LD transport complex. In later stages of the infection, virions might also be released into sieve tubes upon degradation of virus-invaded nuclei of differentiating apical SE, as discussed below. Geminate TYLCV particles have in fact been detected in mature sieve tubes and in developing phloem cells of diseased tomato leaflets (Cherif & Russo, 1983; Russo et al., 1980).

Whereas several studies on systemically infected plants consistently have shown that the virus remained confined to vascular tissues in all subsequently invaded organs (as specified below), Michelson et al. (1997) reported for the insect-inoculated leaves of two tomato lines that viral DNA from the primary infected cells passed the BS barrier and infiltrated mesophyll tissues: On whitefly-inoculated first true leaves, TYLCV-specific DNA probes exhibited signals on parenchyma cells outside the veins, either after a collapse of those tissues, or after ageing. It may be possible that viral spread inside insect-exposed leaf laminae can differ from that in systemically invaded organs, if for example feeding damage leads to physiological disturbances (Buntin et al., 1993, and references herein). Alone or in combination with processes induced during ageing and tissue processing, it might account for abnormally enhanced SELs of PD, or efflux of virus-loaded cytoplasm into neighboured tissue domains.

4. EXPLOITING VASCULAR FLUXES: TRAVELING THROUGH THE CORMUS

After viral LD transport forms have gained access to a mature SE, they will be translocated in the phloem solute together with a large variety of different macromolecules, basically following the source-to-sink carbon transport routes inside the cormus. After a long debate on the *in vivo* permeability of the sieve plate pores interrupting the sap stream inside the tubes, it is now generally accepted that the traversing P protein filaments do not obstruct longitudinal movement of molecule complexes such large as plant virions (Knoblauch & van Bel, 1998; Oparka & Santa Cruz, 2000, and references herein). Hence, the main driving force for LD transport inside the phloem is the mass flow of macromolecules which results from the osmotic pressure gradient generated by sugars and nonsugar osmotic equivalents loaded into SE of carbohydrate-exporting source tissues (Münch, 1930; van Bel, 1993). Since plant organs at different positions of the cormus differ in their relative source strength, speed and direction of the initial TYLC(S)V spread inside sieve tubes towards sink regions depend on the viral entry site. Induced responses can locally reduce or block the passive

streaming of macromolecules in the phloem, as it was shown for mechanical injury and irradiation damage (Knoblauch & van Bel, 1998).

Several independent studies on the TYLC(S)V LD movement agree with a passive viral diffusion inside the photoassimilate stream (Ber et al., 1990; Michelson et al., 1994; Picó et al., 1999; Rom et al., 1993). Experiments comparing whitefly-inoculated tomato species or cultivars (*S. lycopersicum*) of different susceptibility indicated that certain plant genotypes did not allow for equally effective viral LD spread (Michelson et al., 1994; Picó et al., 1999; Rom et al., 1993). These studies monitored TYLCV spread throughout the cormus by means of repeated sampling and blot hybridization techniques. Whitefly-mediated infection was carried out at different developmental stages of the plants, which harboured between two and eight true leaves and were inoculated either by mass exposition of the whole plant or by cage inoculation of individual leaves, respectively. A susceptibility gradient was obvious, with the highest success of infection in the youngest apical tissues and negligible susceptibility of fully developed older leaves or cotyledons (Ber et al., 1990). The total age of the plants also played a role and seemed to be also crucial in bean hosts of TYLCV, which were shown to exhibit only narrow windows of maximum susceptibility about 2 weeks post germination (Lapidot & Friedmann, 2002, and references herein). Subsequent accumulation patterns and systemic spread routes of TYLCV were consistent in all studies: after a primary peak of virus replication in the first infected organ 3–4 dpi (Ber et al., 1990, as discussed above), a portion of TYLCV is transported through the stem phloem straight into the roots, where small amounts could be detected in fully susceptible hosts as early as 4 days after whitefly-mediated inoculation of their top leaves (Michelson et al., 1994). In parallel, in accordance with source-to-sink gradients, a much higher accumulation of viral DNA takes place in the apices and the youngest leaves from 4 dpi on (Ber et al., 1990; Michelson et al., 1994; Picó et al., 1999). Depending on the individual physiological status of the plant and on the initial inoculation dose, levels of TYLCV dsDNA and newly generated ssDNA, as well as CP further increased in young organs of susceptible hosts up to several weeks post inoculation. Concomitantly, the productive infection gradually spread downwards to older regions of the cormus, but remained strictly confined to the vascular system. Delivery of TYLCV via agroinofection resulted in a similar distribution pattern; viral DNA passed at least about one internode per day during downward movement in the stem (Picó et al., 2001). Infective viral forms most likely exit from the sieve tubes via PPUs wherever they get access to a suitable replication-competent companion or PP cell, and may also enter adjacent cells inside the BS after the next round of replication, transcription and movement protein translation. The resulting foci of viral accumulation could be visualized for the first time by high-resolution immunolocalization of TYLCV CP in stem imprints of different *S. esculentum* species (Fargette et al., 1996) and consist of either single or groups of companion and/or PP cells embedded in a majority of virus-free cells by *in situ* localization detecting non-structural viral protein C4 or viral DNA (Morilla et al., 2004; Rasheed et al., 2006; Rojas et al., 2001).

Invasion of those nucleate vascular cells may occur upon longitudinal transit inside the stems, in subsequently infiltrated leaves, or along the roots. By transforming *N. benthamiana* with a TYLCSV-derived vector cassette releasing free GFP-expressing transreplicons in cells containing actively replicating virus, Morilla et al. (2006) could directly prove that a significant portion of nucleate vascular cells inside stems and roots multiply, and not just trap, TYLCSV. Hence, replication proceeds along the vascular tissues upon viral passage through the stem, and in all leaves which are still markedly growing and therefore import the virus, including their petioles and petiolules (Fargette et al., 1996). It is not clear whether the phloem-limited begomovirus restarts the cell cycle in all of the newly invaded cells, or whether it takes advantage of not yet fully differentiated cells which may be present in veins of growing organs, descending from cambium tissues or residual meristems. Some evidence indicates that suppression of antiviral host defences contributes to successful viral gene expression and thus replication and movement inside the vascular tissue domains, probably involving the silencing suppressor function of the protein encoded by ORF C2 (for recent reviews on geminiviral silencing suppression, including references for TYLC(S)V, refer to Bisaro, 2006; Vanitharani et al., 2005). A TYLCV mutant in C2 was not able to infect tomato systemically any more and exhibited reduced pathogenicity in *N. benthamiana* (Wartig et al., 1997). Recent experiments hint at a role in silencing suppression also for the TYLCV precoat protein (Zrachya et al., 2007). ORF C4 might influence the expression of relevant host genes (Rojas et al., 2005, and references therein), in addition to its putative function in moving viral DNA to the PD (Rojas et al., 2001).

During the primary systemic infiltration period described so far, infected tomato plants usually remain symptomless. Typically about 11 to 13 dpi, maximum amounts of viral DNA and CP are reached in the youngest tissues of shoots and roots, and only 4–7 days later, symptoms will appear for the first time (Ber et al., 1990; Rom et al., 1993). At that time, TYLCV accumulation in the newly developing plant organs usually has levelled out: The long-time titres found during the ontogenesis of all subsequent leaves or roots may be substantially lower than during the first peak of systemic virus invasion (Ber et al., 1990; Morilla et al., 2006; Picó et al., 1999).

Nevertheless, as the systemic infection proceeds in the growing plant, maximum viral accumulation keeps going on in the strongest sink tissues and thus will most efficiently take place in the veins of developing leaves close to the apex, and of axillary shoots. In tomato and in three *Nicotiana* species, first indirect evidence for the time course of further systemic virus movement has arisen from studies on TYLCCNV in combination with a DNA β derivative modified in order to induce silencing of either phytoene desaturase (PDS) or a magnesium chelatase component (Tao & Zhou, 2004). The resulting chlorosis of vascular tissues was observed first in newly developing apical leaves and upper stem regions, and then in young lateral shoots. Since PCR verified the presence of TYLCCNV-DNA β in the affected tissues, the findings roughly correlated with

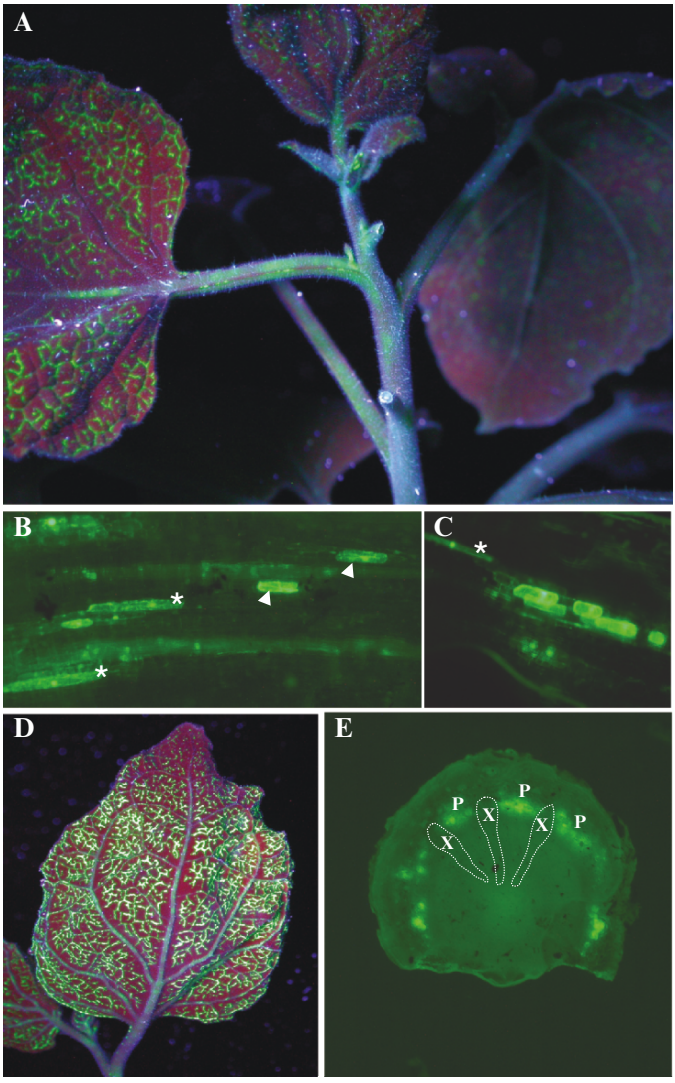

Figure 2. Tracing of TYLCSV replication in different plant organs, as revealed by GFP-accumulation in cells of transgenic *N. benthamiana* plants multiplying the virus (for experimental details, refer to Morilla et al., 2006). GFP-specific fluorescence is detected 3–4 weeks post agroinfection in leaves, petioles and stem tissues (A) and can be attributed to different cell types inside the vasculature of a petiole (B, C) such as companion cells (*) and PP cells (arrowheads in B, no label in C). B,C: Photographs were taken at 20–40 fold magnification under a fluorescence microscope, showing individual infected cells interspersed by non-fluorescing ones (B) or groups of TYLCSV-replicating parenchyma cells adjacent to each other (C). D: A leaf in which fluorescence starts to gradually disappear from the tip towards the base, strongly resembling the spatial pattern observed for sink-source transitions in *N. benthamiana* (Roberts et al., 1997). Note that viral replication goes on in veins of all classes. E: Cross section visualizing TYLCSV-multiplication inside a root. P: Phloem areas, and X: typical xylem distribution in the radial vascular bundle of a root are indicated. (All pictures in this panel: courtesy of G. Morilla and E. Bejarano.)

the movement of viral DNA in the plant. Transreplicon-induced GFP fluorescence in transgenic *N. benthamiana* plants (Morilla et al., 2006) finally unravelled precise details of systemic begomovirus accumulation in that it indicated the exact sites of viral replication during the whole lifetime of infected plants. First, TYLCSV DNA is not only passively transported into root tissues, but is amplified along the whole root in multiple spots in the phloem, as discussed above for stem and petioles (Figure 2). Second, significant TYLCSV replication is confined to the uppermost four to five expanded leaves of a plant and is an extremely transient process. GFP accumulation and thus virus replication in those leaves was observed in veins of all size classes (i.e., first to fifth order) and disappeared gradually upon ageing of the leaf. The observed patterns resemble the course of sink/source transitions as they have been visualized by carboxyfluorescein tracing and potato virus X-mediated GFP expression (Roberts et al., 1997). This indicates that TYLCSV replication is highly favoured to occur in sink tissues, which should allow for an efficient export into new organs exploiting the inherent vascular fluxes in the phloem.

5. RELEASE FROM SECONDARY INFECTIONS SITES: A ROLE ALSO FOR THE XYLEM?

When entering a developing sink leaf, or the apical or root tip sink regions via the phloem, viral movement complexes or virions may not only invade nucleate CC or VP cells upon exiting the sieve tubes. At their very ends, in the primary differentiating regions just descended from the procambium, they might be able to enter via newly formed sieve plate pores also immature nucleate SE. It has been proposed that from these nucleate cells, geminiviruses might even get access to the adjacent SE/CC progenitor cells via PD, resulting in enhanced accumulation and cell-to-cell-spread via segregation of the infected nuclei, as discussed by Latham et al. (1997) for *Beet curly top virus* and Rojas et al. (2001) for TYLCV. *In situ* PCR experiments performed by Rojas et al. indicated the presence of TYLCV DNA also in nuclei of procambial cells and in the vascular fascicular cambium, which during plant development derives from the procambial tissues (Fosket, 1994). Since the virus neither is able to invade apical meristems, nor to adapt its life cycle to callus (Pelah et al., 1994), the tissue identity of the cambial cells might be a prerequisite for begomovirus uptake. The findings of Rojas et al. (2001) are in accordance with recent results on three tomato-infecting begomoviruses, including TYLCSV, discovering viral ssDNA even in mature xylem vessels (Rasheed et al., 2006). A presence of begomoviral DNA in both non-phloem vascular domains, fascicular cambium and xylem, might be explained as a result from the movement processes supposed to function in apical regions and, similarly, major veins in young leaves. If, after exiting from the protophloem, the infection traversed into closely located xylem precursor cells, newly formed virions and/or differently organized ssDNA-containing nucleoprotein complexes could be released into primary xylem elements upon their differentiation from nucleate

cells. So far, however, no detailed data on incidence and distribution of begomoviral DNA-containing procambium or fascicular cambium cells inside the plant cormus are available.

If infected cambial cells occurred in older tissues, viral egress into newly formed phloem or xylem precursor cells would not necessarily be limited to apical regions. New conductive elements are produced during extended periods in the main veins of growing leaves in plants with a high carbohydrate content in the *Solanaceae* (Avery, 1933), and - though to a considerably lower extent in annual plants- along stems or roots upon thickening. The sites at which the TYLCSV DNA detected by Rasheed et al. (2006) in roughly 10% of the stem, and 40% of the leaf xylem elements had been released into these vessels still remain to be determined.

The authors discuss that the presence of begomoviral nucleic acid inside xylem vessels might not represent a dead end, but could allow for re-uptake of TYLC(S)V into VP or cambium cells in analogy to interpretations for unrelated viruses. A model for its export from individual xylem elements was proposed for the monocot-infecting *Rice yellow mottle sobemovirus* (RYMV), which is thought to egress from xylem vessels via destabilized pit membranes not only in young, but also in fully differentiated veins (Opalka et al., 1998). Furthermore, tracheids in the protoxylem frequently are mechanically disrupted upon organ elongation (Esau, 1969). If infective virus was concomitantly taken up by tracheid-surrounding, transiently injured parenchyma cells, this might lead to begomovirus multiplication in healed cells close to the xylem and serve as additional spread mechanism in the plant's cormus. Notwithstanding, any experimental evidence for virus egress from dead xylem vessels into living adjacent cells is lacking so far. For *Soilborne wheat mosaic sobemovirus* (SBWMV), which was shown to move long distance in the wheat xylem, any lateral transport from differentiated tracheids into the parenchyma was questioned (Verchot et al., 2001). In analogy to the processes described for apical phloem regions, however, the authors discuss that virus may exit from not yet degraded tracheid precursor cells invaded at the veins' ends into neighboured cells such as parenchyma, meristematic, or even CC of the phloem.

Another question still awaiting detailed analyses for TYLC(S)V is the utilization of internal and external phloem tissues in the bicollateral veins harboured by several agronomically important hosts, e.g., in the *Solanaceae* and the *Cucurbitaceae*. Tissue prints yielded evidence that, at least in later stages of systemic infections, the numbers of TYLCV-Sen replication foci in internal phloem tissues of tomato stems significantly exceed those in external phloem by a factor of 1.5–2, which differed from the data obtained in parallel for *Indian tomato leaf curl virus* (ITmLCV) predominantly present in external phloem (Fargette et al., 1996). Since both viruses are whitefly-transmitted and thus preferably delivered into external (abaxial) sieve tubes, the reasons underlying those differences are not obvious. In the solanaceous host *Capsicum annuum* it was determined for *Pepper mottle potyvirus* (PepMoV), spreading long distance also in the phloem,

that primary movement down the stem occurred in the external phloem which transports photoassimilates from the leaves towards the roots, whereas ascending translocation took place in the internal phloem typically supplying carbohydrates to sink leaves (Andrianifahanana et al., 1997, and references on phloem transport herein). The cotyledonary node seemed to be the main viral exchange site between both phloem domains, although phloem anastomoses connecting internal and external strands are known to exist not only in most nodes of the plants, but also in the internode regions (Andrianifahanana et al., 1997; Esau, 1969), and may be potentiated in their transport activity upon stress as shown for wounded *Dahlia* plants (Aloni & Peterson, 1990). So the TYLCV-Sen-specific partitioning between internal and external phloem may either result indirectly from its kinetics of DNA replication and release of viral transport forms, or it may reflect its capacities of traversing phloem anastomoses, perhaps in combination with functional plant responses to the presence of the virus.

6. HOW THE JOURNEY MIGHT GO ON – ENTERING FLOWERS, FRUITS, AND FURTHER HOSTS, ALONE OR IN MIXED INFECTIONS: PHLOEM LIMITATION FOR EVER? AN OUTLOOK

Though TYLC-diseases frequently cause severe harvest damage, most systemically infected plants produce flowers and fruits which are transported worldwide to a high extent: In 2002, Delatte et al. (2003) discovered 57% of TYLCV-infected tomato fruit in 13 out of 27 batches from different countries. The presence of begomoviral nucleic acid in fruit had been detected for the first time by squash blot analyses (Czosnek & Navot, 1988), who also showed that the virus can invade different flower organs like petals, anthers, and pistils (Navot et al., 1989). Since it is not transmitted by seeds, however, spread will be limited to the veins of parental tissues, as it was visualized by *in situ* hybridization in paraffin-embedded ACMV- and TGMV-infected flower buds of *N. benthamiana* (Wege et al., 2001). As indicated already by the tissue blots (Navot et al., 1989), recent experiments localized individual infection foci in sepals and petals and thereby confirmed that flowers contain considerable amounts of infected phloem cells actively replicating the begomovirus (Morilla et al., 2006; Morilla et al., 2004). Interestingly, however, the final TYLCV distribution inside a tomato berry seems to differ between individual *L. esculentum* lines. ELISA analyses on small pieces of distinct fruit regions, including the stem of the bunch and the sepals, revealed characteristic differences between two representative vine tomato cultivars, especially with respect to the relative virus titers in stems and sepals compared to those inside the ovular tissues pulp (derived from carpel regions, placentas, and integuments) and skin (exocarp of former carpels; Delatte et al., 2003). Having established itself in a tomato fruit, the journey might well go on for TYLCV, into new countries and further host plants. Delatte et al. have determined that most whiteflies survive at least 24 h on bunches of tomato, which are transported worldwide to increasing numbers since selling complete "naturally-looking"

bunches has come into vogue. After feeding on infected bunches, whiteflies were able to transmit TYLCV to healthy plants with significant percentages.

So far, this chapter has focused on singly infected plants because the large majority of data available on TYLC(S)V tissue distribution was obtained with experimentally infected hosts, using a defined inoculum, or with well-characterized test plants derived from fields infested by a single virus species. Upon crop production or in the surrounding environment, however, the begomoviruses frequently meet related as well as unrelated viruses in newly infected plants. In order to investigate if some aspects of viral systemic spread change in mixed infections with a distinct monopartite begomovirus, we studied the tissue tropism of TYLCV and TYLCSV in doubly infected tomato and *N. benthamiana* by *in situ* hybridization, detecting specifically either of both viruses by use of a dual-colour assay on tissue specimens and isolated nuclei (Morilla et al., 2004). Numerous tomato plants co-infected with these viruses have been detected in southern Spain (Sanchez-Campos et al., 1999), so our analysis addressed an agronomically relevant begomovirus combination which, furthermore, is associated with a synergism in symptoms. Despite of this, and quite unexpected, the total number of virus DNA-containing nuclei isolated from doubly infected plants was equal to that obtained with either of the singly infected ones: about 1.4% in tomato, or 6% in *N. benthamiana*. At least 20% of those nuclei contained DNA of both viruses, with variable ratios between TYLCV and TYLCSV. The "tête à tête" makes up a good prerequisite for intermolecular recombination between viral genomes, which may result in increased fitness of new virus variants under conditions of selection. Inside tissues, nuclei of different infections status frequently occurred close or adjacent to each other, interspersed between a majority of uninfected cells, and in any case strictly confined to vascular tissues, sometimes neighboured to sieve tubes containing large amounts of viral DNA (Morilla et al., 2004). These findings indicate that only a limited and host-specific portion of nuclei is attainable by TYLC(S)V, all of them located in veinal domains. The molecular mechanisms underlying the resulting tissue restriction have not been determined yet. The monopartite begomoviruses lack DNA B-encoded movement proteins, which in some cases were shown to mediate export from the phloem (Morra & Petty, 2000; Rojas et al., 2005, and references therein). So any failure to cross the vascular BS PD which directly arose from insuffient movement functions would have to be attributed to deficiencies of coat and/or precoat protein, which maybe also operate in physical interaction with the protein encoded by ORF C4 (Jupin et al., 1994). For the similarly phloem-limited bipartite begomovirus *Abutilon mosaic virus* (AbMV), however, recent experiments with transgenic plants have ruled out that a simple lack in DNA B-encoded movement functionalities is responsible for confining the virus to the vascularity (Wege & Pohl, 2007). Alternatively, or in combination with qualified movement protein functions, tissue limitations might be caused by insufficient viral capacities in influencing the plant cell cycle, which is typically mediated by replication (Rep)-associated proteins of geminiviruses (Kong et al.,

2000). For the mastrevirus *Maize streak virus* (MSV) it was shown that mutation of a retinoblastoma-related protein (RBR)-binding motif in its protein RepA confined the otherwise not strictly phloem-limited virus to this tissue (McGivern et al., 2005). Amongst further interactions with host proteins, the binding of TYLCSV Rep protein to SUMO-conjugating plant enzymes might determine an additional role of the viral protein in regulating viral tissue infiltration characteristics (Castillo et al., 2004). Furthermore, the TYLCV replication enhancer protein (REn, encoded by ORF C3), which is involved in the replication-cell cycle connection, may also contribute to viral tissue tropism (Castillo et al., 2003; Settlage et al., 2005). Finally, certain features of the TYLC(S)V mode in RNA silencing suppression, as mediated by ORFs C2 and C4, and described in Section 4 in this chapter, may play an important role in determining the final viral distribution pattern inside plants. It may be evolutionary adapted to vascular cells, resulting in a balanced pathogenicity of the virus which allows for long-time persistence inside the plant's cormus over extended growth periods. Interestingly, the phloem-limited AbMV which exhibits a comparable pathogenicity in tomato could be "mobilized" to increased numbers of cells by co-infection with *Cucumber mosaic virus* (CMV) in different hosts in the *Solanaceae* (Wege & Siegmund, 2006). The CMV 2b silencing suppressor protein significantly contributed to the enhanced tissue infiltration, although it was not sufficient to mediate phloem release under the respective conditions. It is tempting to speculate that, in analogy to these findings, also TYLC(S)V could be supported by unrelated viruses which harbour superior, or distinct, capacities in silencing suppression.

Figure 3. Systemically TYLCV-infected (right) compared to mock-inoculated *Petunia hybrida* plant (left). Is the virus also limited to vascular tissues inside this host?

Virtually no clear data are available on the tissue tropism of TYLC(S)V in plants other than *Nicotiana* sp. or tomato (Figure 3). Therefore, also host-specific differences in the systemic distribution patterns of these monopartite begomoviruses cannot be excluded at present, as they were shown for a number of different geminivirus species (refer to Wege & Siegmund, 2006 for the respective examples).

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CHAPTER 5

IDENTIFICATION OF PLANT GENES INVOLVED IN TYLCV REPLICATION

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1. OVERVIEW

Since there are still no chemicals that can be applied routinely to control plant virus diseases, TYLCV control strategies have been mainly focused on methods to prevent the occurrence of infection and on genetic resistance. Attempts to reduce the incidence of TYLCV by eliminating the sources of inoculum or controlling vector transmission are often ineffective (Picó et al., 1996). Attempts to derive TYLCV resistant tomato cultivars constituted the main effort of extended breeding programmes to introgress resistance from wild *Lycopersicon* species. Although some wild relatives of tomato are resistant, introduction of resistance traits into commercial tomatoes is however complicated by several factors. Some tolerant cultivars have been released (Lapidot et al., 1997; Friedmann et al., 1998), but no fully resistants *Lycopersicon esculentum* are still available.

The identification of plant genes involved in the viral life cycle may offer the opportunity to disrupt the interaction between the virus and the plant cell, thus preventing infection without introducing foreign genes in the plant. Despite differences in the properties of their genomes, all plant viruses face the same two fundamental challenges during the establishment of systemic infections in their plant hosts. The first necessity is to replicate in the infected cells. The second requirement is to move through adjacent plant cells to the vascular system, before spreading throughout the plant. Both processes depend on highly specific interactions with host proteins. Protein–protein interactions are the underpinnings of a vast number of these cellular processes. In recent years, the convergence of biochemistry, cellular and molecular biology has made available a number of powerful techniques for studying such interactions. These techniques

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vary in their sensitivity, efficiency and rapidity, but judicial deployment of a combination of them has proved to be effective and reliable.

The viruses of the *Tomato yellow leaf curl complex* that include *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) isolates are monopartite members of the *Begomovirus* genus. Like other begomoviruses, TYLCV virus infection is initiated by whitefly-mediated transmission of virions from an infected plant to a recipient plant. The single-stranded DNA is converted to a double-stranded form, which then serves as a transcription template for the production of the viral replication proteins Rep (also designated AL1, AC1, and C1) and REn (also named AL3, AC3, and C3). The Rep protein acts as a rolling circle initiator to catalyse a site-specific cleavage and rejoining reaction in a conserved hairpin loop in the viral replication origin (Laufs et al., 1995). The REn protein greatly enhances viral DNA accumulation and symptoms in infected plants (Hanley-Bowdoin et al., 2000; Hanley-Bowdoin et al., 2004; Settlage et al., 2005). Geminiviruses do not encode their own DNA polymerases and rely on the nuclear DNA replication machinery, like many mammalian DNA tumour viruses do. They replicate in nuclei of mature cells, which are inactive in DNA replication.

Accumulating evidence strongly supports the notion that geminivirus proteins have a significant impact on a variety of host cell pathways (reviewed in Gutierrez et al., 2004; Hanley-Bowdoin et al., 2004), including cell differentiation, cell cycle control, DNA replication, plasmodesmata function, RNA silencing, etc. Several studies have shown that the begomovirus Rep and REn proteins bind to viral and host proteins (Table 1). It has been demonstrated that besides the

Table 1. Plants proteins that interact with Rep and/or REn proteins from begomoviruses. IMYMV: Indian Mung bean yellow mosaic virus; CaLCuV: Cabbage leaf curl virus

Plant proteins	Virus	Gene	Reference
pRBR (maize)	TGMV	Rep	Ach et al., 1997
“”	TYLCV	Rep	Arguello-Astorga et al., 2004
“”	CaLCuV	Rep	Arguello-Astorga et al., 2004
“”	TGMV	REn	Settlage et al., 2001
“”	TYLCV	REn	Settlage et al., 2005
PCNA (tomato)	TYLCSV	Rep	Castillo et al.,. 2003
“””	TYLCV	REn	Settlage et al., 2005
“(tomato/ N. benthamiana)	TYLCSV	REn	Castillo et al., 2003
“(pea)	IMYMV	Rep	Bagewadi et al., 2004
SCE1(Arabidopsis/N.benthamiana)	TYLCSVT	Rep	Castillo et al., 2004
”	GMV	Rep	Castillo et al., 2004
	ACMV	Rep	Castillo et al., 2004
Histone H3 (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
Mitotic kinase (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
GRIK kinase (Arabidopsis)	TGMV	Rep	Kong and Hanley-Bowdoin, 2002
SINAC1 (tomato)	TYLCV	REn	Selth et al., 2005

interaction with themselves and with each other (Lucioli et al., 2003; Settlage et al., 2005), Rep and REn interact with the proliferating cell nuclear antigen (PCNA), an essential component of the DNA replisome (Castillo et al., 2003; Settlage et al., 2005), and with the host protein pRBR, the plant retinoblastoma homologue (reviewed in Hanley-Bowdoin et al., 2004). Rep also interacts with a novel protein kinase (GRIK), a kinesin, the histone H3 (Kong & Hanley-Bowdoin, 2002), and with the SUMO conjugating enzyme NbSCE1/Ubc9, a component of the sumoylation pathway (Castillo et al., 2004). Finally, REn from a related virus, *Tomato leaf curl virus* (TLCV) was recently shown to interact with a transcription factor in the NAC family (Selth et al., 2005).

We highlight here the use of the yeast two-hybrid assay (see Fields, 2005; Gietz, 2006, for recent reviews) to identify plant proteins that interact with geminivirus proteins. The two-hybrid method detects the interaction of two proteins by their ability to reconstitute the activity of a split transcription factor, thus allowing the use of a simple growth selection in yeast to identify new interactions. In spite of some problems (large number of false positives), two-hybrid is a powerful technique, widely used for the last 15 years and it has been recently scale-up in combination with computational analysis to a high-throughput analysis of protein interaction.

In this chapter, we summarized the results obtained using yeast two-hybrid technique, to identify plant proteins that interact with TYLCV/TYLCSV proteins involved in the replication of the virus (Rep and REn). We also described a system recently developed to easily identify if a host factor is required for geminivirus replication using Virus induced gene silencing (VIGS).

2. TWO-HYBRID SCREENINGS

To identify cellular proteins that interact with Rep and REn, several screening have been carried out on *Arabidopsis thaliana*, *Nicotiana benthamiana*, and *L. esculentum* cDNA prey libraries using Rep or REn fused to the GAL4 DNA-binding domain as bait.

To clone the viral proteins we used three different plasmids pAS2, pBD-GAL4, and pGBKT7 all containing the GAL4 DNA-binding domain. We selected the yeast strain PJ696 for the screening as it has three reporter genes, *HIS3*, *ADE2*, and *lacZ*, to detect the interaction. Positive interactions were indicated by yeast growth in the absence of histidine and adenine. Once, we confirmed that none of the viral-protein-fusions activated histidine/adenine auxotrophy by themselves yeast cells were then cotransformed with the Rep or REn bait plasmid and the plant cDNA, prey libraries. Among transformants that appeared 5–10 days after transformation, we selected those for which the 3 yeast reporter genes were activated. Next, plasmids from the cDNA library were isolated from the selected colonies and reintroduced into the yeast by transformation. Empty bait plasmids, as well as bait plasmid expressing a non-related virus sequence are used to confirm the specificity of the interaction.

Finally, we sequenced the cDNA clone, to confirm that it was cloned in frame, and identify it.

2.1. REn interacting proteins

REn screening lighted up four proteins: one from tomato, one from *N. benthamiana*, and two from *Arabidopsis* cDNA libraries. Sequence and databases analysis from the positive clones isolated from *Arabidopsis*, revealed the existence of three different partial cDNA clones of the same protein, PCNA, an essential component of the eukaryotic replication machinery. PCNA is a ring-like protein that tethers DNA and functions as a moving platform that modulates the interactions of other proteins with DNA. This sliding clamp interacts with many proteins that are involved in important cellular processes like replication and repair of DNA, DNA methylation, cell cycle control, and chromatin assembly (Warbrick, 2000). The interaction between TYLCSV REn and PCNA was confirmed using PCNA from plant species infected by this virus (*N. benthamiana and L. esculentum*) (Castillo et al., 2003).

PCNA-binding proteins can be divided into two groups: enzymatic proteins that participate in DNA metabolism and regulatory proteins that are involved in cell cycle progression, checkpoint control, and cellular differentiation. Most of the interactions are located in three loop structures protruding on the C-side that are very well conserved in all eukaryotic PCNAs (Tsurimoto, 1999). In tomato PCNA (LePCNA) those structures correspond to residues Asp41 to His44 (central loop), Leu 118 to Glu124 (long loop), and Lys254 to Glu256 (C-terminal tail). REn-binding domain of LePCNA was mapped on using a truncated LePCNA, to a region between Lys132 and Thr187, where no other interactions have been previously described (Castillo et al., 2003). This region is located at the end of the PCNA monomer with amino acids placed to the C-side and loop side (Figure 1).

By analysing the solvent accessibility of the amino acids and identifying possible receptor-binding domains, we mutagenized three amino acids residues (Arg172, Arg183, and Glu184) of the REn-binding region that could be involved in protein–protein interactions. Unexpectedly, none of the mutations reduced the interaction with REn, and moreover four of them increased protein binding, indicating that none of those residues is involved in the interaction (Collinet, unpublished data).

Although the PCNA interaction domain of TYLCSV REn was also analysed by two-hybrid assays using truncated forms of the protein the results were not clear enough to identify it (Castillo et al., unpublished data). A detailed analysis of TYLCV REn domains involved in the interaction with PCNA has been published recently (Settlage et al., 2005). In this work the authors have examined the impact of mutations in amino acids that are conserved across the REn protein family on protein interactions, and replication enhancement of REn. Analysis of the mutated proteins by two-hybrid assays has localized the PCNA interaction domain between residues 7 and 95.

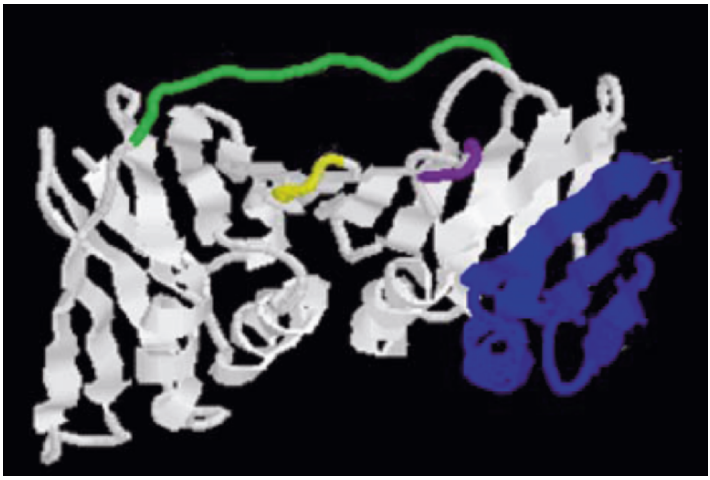

Figure 1. 3D model of the *L. esculentum* PCNA monomer. Loop structures are indicated: central loop in yellow, long loop in green, and C-terminal tale in purple. The minimal interacting domain of *L. esculentum* PCNA (amino acids 132–187) is in blue.

Notwithstanding its small size (134 amino acids), TYLCV REn also interacts with Rep, with itself and with and the retinoblastoma-related protein (pRBR). The REn-binding domain with PCNA overlaps with the REn region that binds Rep, and itself, but not with the one interacting with pRBR (Settlage et al., 2005).

The mechanism by which REn enhances viral DNA accumulation may reside in its ability to interact with Rep. The REn protein sequence shows no homology to any known enzymatic motifs. Thus, it is more likely that the structure of the REn/Rep complex is important for replication rather than a catalytic activity of REn that could affect Rep. The results obtained using mutated versions of REn protein of TYLCV, established the importance of REn–REn, REn–Rep, and REn–PCNA interactions in geminivirus replication. While REn–pRBR interaction is not required for viral replication in cycling cells, it may play a role during infection of differentiated cells in intact plants (Settlage et al., 2005).

Another recent study proposed that TLCV REn binding to a transcription factor, SlNAC1, and induction of its expression is the mechanism whereby REn enhances geminivirus replication (Selth et al., 2005). However, it is unlikely that REn–SlNAC1 interactions are sufficient for REn replication enhancement activity. REn mutant replicons are typically constructed by deleting REn sequences that do not overlap with the other geminiviral open reading frame, (Trap), to yield a truncated proteins of 83–111 amino acids. This region of REn retains the capacity to bind to SlNAC, but the REn mutant replicons support little if any detectable replication in protoplasts and are severely attenuated in infectivity assays in planta. Instead, the data suggest that the loss of replication

enhancement activity by the truncated REn proteins reflects their inability to form oligomers with themselves, Rep, and/or PCNA.

Several explanations for the effect of REn on virus replication have been proposed (Hanley-Bowdoin et al., 2000). There are some experimental observations that suggest that REn might increase the affinity of Rep for the origin of replication. Another possibility is that REn directs Rep from the Rep/DNA-binding domain to its cleavage site in the origin during the initiation of replication. REn and Rep interact with PCNA and by encircling DNA and interacting with polymerases, PCNA forms a sliding clamp that keeps the polymerases associated with the DNA template during processive DNA synthesis. By interacting with PCNA and Rep, REn could help Rep to recruit the replication machinery necessary to replicate the viral DNA. In the absence of REn, Rep can still bind PCNA although the efficiency of DNA replication decreases. Placing the DNA replication machinery in the viral origin of replication could also involve interactions of Rep and/or REn with other cellular proteins than PCNA. In fact, it has been demonstrated that Rep from the geminivirus *Wheat dwarf virus* interacts with RFC-1, the large subunit of PCNA clamp loader (Luque et al., 2002) and the existence of a multimeric REn complex is supported by gel filtration analysis of native *Tomato golden mosaic virus* (TGMV) REn, which fractionated with a complex of >100 kDa (Hanley-Bowdoin, unpublished data). It is also possible that REn or Rep binding to PCNA prevents the interaction of PCNA with cell cycle regulators such as p21, cyclin D and p57 that inhibit chromosomal replication by interacting with PCNA during cell cycle progression (Kelman, 1997).

2.2. Rep interacting proteins

Yeast two-hybrid screenings using Rep from TYLCSV and TYLCV as bait, were laborious, as this protein seems to be partially toxic in yeast. We carried out various screenings expressing full length and truncated versions of Rep. We also clone Rep in several bait plasmids that differ in the promoter strength. The best results were obtained when the full-length protein was cloned into the bait plasmid pGBKT7.

From the positive clones isolated after screening *Arabidopsis* and *N. benthamiana* cDNA libraries, we identified three *N. benthamiana* proteins able to interact with Rep from TYLCSV. The subsequent procedures to confirm the interactions were similar to those described above for REn. For further studies we selected one of the proteins present in two library clones that specifically interacts with DB-Rep. The two clones contained a single open reading frame of 160 amino acids, named NbSCE1, which had high homology to the *AtSCE1a* gene, an E2 ubiquitin-conjugating-like enzyme from *Arabidopsis*, and to UBC9 from *Saccharomyces cerevisiae*. Like UBC9/SCE1 from *S.cerevisiae*, humans and *Arabidopsis*, NbSCE1 is predicted to conjugate SUMO, not ubiquitin, to target proteins in a cellular process called sumoylation. Sumoylation is a

post-translational process that modifies function, activity, or localization of the target protein by the covalent attachment of an Ubiquitin-like polypeptide (Ubl) called SUMO (Bossis & Melchior, 2006). Contrary to ubiquitination, SUMO conjugation is mediated by a single E2 enzyme (UBC9/SCE1) that is essential for cell viability and sumoylation in yeast and animals. In yeast, there is just a single gene that codifies for SUMO (*SMT3*), but in metazoan, SUMO proteins can be divided in two families – SUMO1 and SUMO2/SUMO3. Although SUMO2 and SUMO3 share 50% sequence identity with SUMO1, they are functionally different. In plants little is known about SUMO pathway or the nature of its targets. Many of the core components for sumoylation have been identified in *Arabidopsis* (Novatchkova et al., 2004).

The NbSCE1 protein also interacts with Rep proteins from other bipartite begomoviruses TGMV (AL1) and *African cassava mosaic virus-kenya* (RepAC or AC1), both of which infect *N. benthamiana* (Castillo et al., 2004). Many proteins, including some from mammalian viruses, interact with UBC9/SCE1 in yeast two-hybrid assays. Although most of these UBC9/SCE1 interacting proteins are also sumoylated, some are not sumoylation substrates. It is not known yet if Rep/AL1 is a substrate for sumoylation. In plants, it is not easy to prove Rep sumoylation because of the difficulty detecting Rep from infected tissues on immunoblots and problems associated with its overexpression. In mammalian cells and yeast, SUMO is covalently attached to specific lysines in the target protein. The precise lysine residues modified by SUMO have been identified in more than a dozen known substrates (Muller et al., 2001). The majority of these modification sites conform to a consensus sequence defined by four amino acids "YKXE", where Y is a large hydrophobic residue and K serves as the acceptor for SUMO. However, there are sumoylated proteins that do not share this exact sequence (Yeh et al., 2000). Rep from TGMV, TYLCSV, or TYLCV does not contain any protein motifs that exactly match the consensus, but has lysine residues surrounded by similar amino acids that are potential sumoylation sites.

Sumoylation plays a role in the geminivirus replication as transgenic tobacco plants showing altered levels of SUMO disturb TGMV replication (Castillo et al., 2004) and silencing of NbSCE1 also reduces TYLCSV replication (see Section 3 of this chapter). It is difficult to provide a simple hypothesis that explains these results, as the biological effects of sumoylation are quite diverse, and the mechanisms and signal pathways involved in most of them remain unclear. SUMO conjugation has been implicated in cellular responses to environmental stress, subcellular protein translocation, nuclear body formation, centromere segregation, protection from ubiquitin-mediated proteolysis, and regulation of transcriptional activity (Hay, 2005)

SUMO may play an important role in pathogen plant defence responses. The tomato SUMO orthologue (*LeSUMO*) was isolated in a yeast two-hybrid screen by its interaction with ethylene-inducing xilanase (EIX) from the fungus *Trichoderma viridae* (Hanania et al., 1999). The expression of *LeSUMO* in tobacco transgenic plants suppressed the induction of the defence response by

EIX (a strong elicitor of the rapid defense response in tomato). A virulence factor (AvrBsT) from the plant pathogen *Xanthomonas campestris*, has SUMO protease activity that interferes with the plant defence response, probably by desumolating a key defence regulator (Gurlebeck et al., 2006). It also has been proposed that SUMO conjugation in *Arabidopsis* plays a regulatory role in the stress response, modifying the activity or localization of critical effectors. SUMO conjugation could impact a battery of nuclear regulatory proteins when plants are exposed to stress signals. Potential targets under negative regulation could include factors that promote cell division and other general physiological processes that are repressed while plants cope with adverse environments. Thus, interference with viral DNA replication observed in overexpressing *SUMO* transgenic plants or in NbSCE1-silenced plants could be related to changes in the levels and/or the profiles of SUMO conjugates. These changes may repress proteins required for viral replication or induce a stronger defence response.

However, a direct effect on Rep cannot be ruled on virus cell cycle. Viruses have evolved numerous mechanisms to overcome host defences and to utilize host biochemical pathways to their advantage. One type of viral–host interaction that it is well established and widespread is the modulation of viral protein function by post-translational modification systems such as phosphorylation, glycosylation, ubiquitination, and sumoylation. The interaction between viral proteins and the cell sumoylation system has been previously described for mammalian viruses, and the effects are target specific and very diverse (Boggio & Chiocca, 2006). As with sumoylated cellular proteins, the biological effect of sumoylation of viral proteins is target specific. Rep/AL1 interaction with SCE1 could affect geminiviral replication by preventing de novo sumoylation, or enhancing desumoylation of host proteins, or by using SUMO for viral benefit, as long as the outcome is an environment that is more favourable for viral propagation.

3. A VERSATILE TRANSREPLICATION-BASED SYSTEM TO IDENTIFY CELLULAR PROTEINS INVOLVED IN GEMINIVIRUS REPLICATION

In the genomic age, molecular biologists are looking for new alternatives to study gene function on a genome-wide scale. High-throughput techniques for gene discovery and expression analysis, such as whole genome sequencing and micro-arrays, demand efficient procedures to unravel gene functions to render them useful for both basic and applied applications. The information generated by these high-throughput technologies could be combined with post-transcriptional gene silencing (PTGS) approaches to determine gene function on a genome-wide scale.

Traditional gene knock-out techniques use transformation as a delivery system, and they usually require tissue culture procedures to regenerate silenced mutants. PTGS, and particularly virus induced gene silencing systems, can be

used as tools to speed up studies of gene function by reverse genetic analysis, since they allow us to bypass the time-consuming transformation and tissue culture procedures. The resulting phenotypes can thus be evaluated within days after the inoculation, vs. the months or even years required when using more traditional transformation methods. VIGS technology has already been used for function analysis of defence-related genes (reviewed in Lu et al., 2003; see e.g., Peart et al., 2002); however, phenotype evaluation remains one of the major constraints to the use of VIGS to identify host genes involved in viral infection. Traditionally, the level of geminivirus infection has been determined evaluating symptom development, and quantifying viral DNA accumulation by nucleic acid hybridization. Both methods present substantial inconveniences for their use in large-scale VIGS analysis. Geminivirus-induced symptoms could be partially or completely obscured by the phenotype produced as consequence of the host gene silencing, or by the symptoms induced by the viral vector used for the VIGS system. Furthermore, hybridization analysis is time-consuming and difficult to interpret when using VIGS as a gene-silencing method. Suppression of host gene expression by VIGS does not affect the whole plant. Thus, in a plant where a host gene essential for viral replication had been silenced by VIGS, a geminivirus could still replicate in areas where silencing was not implemented. Viral molecules produced in such non-silenced areas of the plant could then be transported to the silenced parts of the plant and accumulate there, thus producing misleading results when detecting viral DNA accumulation by hybridization analysis. Additionally, several samples from each VIGS-silenced plant should be analysed, since silenced leaves cannot be previously identified.

The use of recombinant viruses containing a reporter gene is a better alternative to evaluate whether gene silencing of a target gene interferes with viral infection. Many RNA viruses have already been labelled with reporter genes such as GFP. One successful example is *Potato Virus X* (PVX) (Cruz et al., 1996), an RNA virus which like many other rod-shaped viruses does not have limitations in the insert size that is able to package. However, few recombinant geminiviruses tagged with reporter genes have been reported, and GFP targeting has only been successful with *Bean dwarf mosaic virus* (Sudarshana et al., 1998; Levy & Czosnek, 2003). There are many limiting features, which hinder the tagging of geminiviruses. DNA viruses suffer a stronger DNA packaging size limitation than RNA viruses, actually the smaller viral genomes with partial deletions are selected in the movement through plasmodesmata (Hayes et al., 1989; Gilbertson et al., 2003). Furthermore, in monopartite begomoviruses such as TYLCV all six ORFs are essential for an efficient replication and long distance movement within the plant, making rather difficult the replacement strategy used with bipartite begomoviruses.

Geminiviruses, with their simple genome organization, broad host range, high copy number, and ability to generate infective clones, have many advantages as recombinant virus-based gene amplification system in infected transgenic plants (Timmermans et al., 1992). Extrachromosomal amplification

from gemimivirus-based constructs has been exploited for the production of valuable peptides and proteins (Palmer et al., 1999; Mor et al., 2002; Hefferon et al., 2004), or to analyse the function of Rep in replication (Hong et al., 2003).

We have developed a TYLCSV-based GFP amplification system to identify the plant organs or tissues where viruses replicate in real-time and without their destruction (Morilla et al., 2006). In combination with the VIGS technology, this tool could be an attractive instrument in functional genomics to easily identify host proteins required for geminivirus infection.

3.1. Amplification of mGFP transreplicons during TYLCSV infection

The TYLCSV-based GFP system is based on the construction of *N. benthamiana* transgenic plants (2IRGFP) that contain a construct with direct repeats of the intergenic region (IR) of TYLCSV flanking a GFP expression cassette (Figure 2A). These transgenic plants developed typical systemic symptoms when infected with TYLCSV or TYLCV and accumulates an episomal DNA (called mGFP replicon) when they were infected with TYLCSV but not with TYLCV.

The mGFP replicon are circular episomal transreplicons generated from the transgene during viral infection, as a consequence of the interaction between Rep and its cognate origin of replication. TYLCV is unable to mobilize the TYLCSV-derived transreplicon because TYLCV Rep does not recognize TYLCSV IR-binding motifs.

TYLCSV replication is easily detected under long-wavelength UV light in 2IRGFP plants. Three weeks after infection, GFP fluorescence is concentrated at the leaf veins (Figure 2B). An increase on GFP expression is also noticeable in other TYLCSV-infected organs such as stems, roots, or flowers. GFP signal in roots and stems was also concentrated in transport tissues. The changes observed on the GFP expression pattern of 2IRGFP plants are dependent on: (i) TYLCSV-specific infection, since they were not detected when plants were mock inoculated or infected with TYLCV; and (ii) the presence of the IR in the transgene, since GFP expression of transgenic plants containing a GFP cassette without IR is not affected by infection.

Although induction of GFP expression is noticed already 2–3 days postinfection (dpi), the characteristic GFP expression pattern in the leaves produced by the TYLCSV infection appear several days after (10–12 dpi), reach a maximum between the second and the fourth week postinfection, and slowly decay afterwards. The intensity and spread of the vein expression is stronger in younger leaves. GFP expression is associated with the generation of the episomal replicon. Increase in GFP fluorescence and amplification of mGFP replicon could also be reached expressing only TYLCSV Rep/C4, as Rep is the only viral protein required for IR recognition and virus replication.

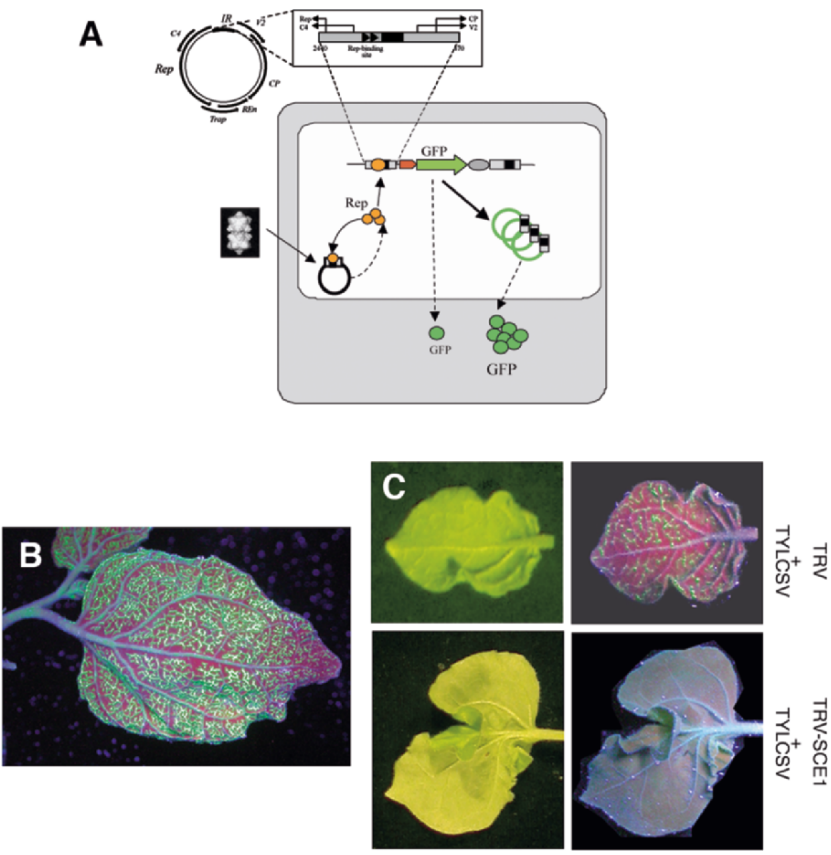

Figure 2. (A) Formation of episomal replicon in 2IRGFP transgenic plants infected with TYLCSV. Transgen contains a direct repeat of IR encompassing a GFP expression cassette. Rep expressed from the virus is able to recognize IR in the transgen and induce the formation of episomal replicons to express more GFP. (B) Leave from a 2IRGFP transgenic plants 3 weeks after infection with TYLCSV. (C) Effect of SCE1 *Tobacco rattle virus* (TRV)-induced silencing on GFP expression. Transgenic plants were infected with TYLCSV and TRV or with TYLCSV and the TRV vector containing the SCE1 fragment (TRV-SCE1). Leaves were photographed under visible (left) or UV light (right) 3 weeks after infection.

3.2. 2IRGFP plants as a tool to detect plant proteins require for TYLCSV replication

The distribution of the green fluorescence in infected 2IRGFP transgenic plants highlights the plants organs or tissues where the virus has expressed Rep, and is therefore actively replicating its DNA. So, these transgenic plants could be used to identify plant genes required for TYLCSV replication. If a cell function necessary for virus replication is silenced in TYLCSV-infected transgenic plants, we

could readily detect it by illuminating the plants with UV light, as green fluorescent vein-pattern will not be produced. Silencing of host functions could be obtained by dsRNA-mediated suppression of genes through the production of sense or antisense transcripts, or even more efficiently, using single-stranded self-complementary (hairpin) RNA containing an intron (Lee & Roth, 2003). However, both strategies rely on the generation of transgenic plants, which is a time-consuming task. Alternatively, temporal silencing of the genes could be obtained by VIGS. This technology has been widely used for analysis of gene function, particularly for genes involved in defence against pathogens (reviewed in Lu et al., 2003)

Hence, the 2IRGFP plants were assessed by VIGS-silencing two host genes: *PCNA* (Morilla et al., 2006) and *NbSCE1* (Lozano et al., unpublished results). PCNA was used to test the system, since it was already known that this protein is required for begomivirus replication (Peele et al., 2001). NbSCE1 was selected an example of functional assay to determine if a candidate gene identified by its ability to interact with Rep was required for virus replication

1. **PCNA**: *N. benthamiana* PCNA was silenced using a *Tobacco rattle virus* (TRV) vector (Ratcliff et al., 2001) designed to induce VIGS. Several time lapses between TRV and TYLCSV infections were previously assayed and finally the best results were obtained when transgenic 2IRGFP plants were agroinfected TYLCSV immediately after the infection with TRV-PCNA. Ten days after the infection a phenotype described for PCNA silencing in *N. benthamiana* was observed. Primary growth is interrupted at the apical meristem, and new leaves show progressively reduced expansion. Although transgenic plants infected with TRV-PCNA seemed to develop less symptoms of TYLCSV infection than control plants, the PCNA-silenced phenotype displayed by these plants somehow obscured the analysis of this difference. However, a difference is clearly noticed when the plants were illuminated with UV, since GFP vein-pattern fluorescence is almost undetectable on most leaves of PCNA-silenced plants located above the inoculation point, only isolated dots of green fluorescence were noticed (Morilla et al., 2006). The absence of fluorescence in these plants indicated that TYLCSV replication is impaired when the level of PCNA in the cell is reduced. This result was confirmed by Southern blot, TYLCSV DNA accumulation showed a significantly decrease but not completely suppressed. This is not an unexpected result, as silencing of an endogenous gene by VIGS is not uniform and it does not occur in whole plant.
2. **SCE1**: A 345 bp fragment of *N. benthamiana* NbSCE1 was cloned in a TRV vector to infect 2IRGFP plants 2 days before that the same plants were agroinfected with TYLCSV. As it was mentioned above, the timing of both infections was selected based on the results obtained with different timing lapses. Plants infected with TRV-NbSCE1 showed a distinctive phenotype with reduction in internodes distance and in the expansion of leaves. When TYLCSV infected leaves from SCE1-silenced plants are observed under UV

light and compare with non-silenced leaves, a clear difference in green fluorescence is noticed, lighting up that SCE1 expression is required for TYLCSV infection (Figure 2C)

The experiments achieving *PCNA* and *NbSCE1* silencing demonstrate the great potential of the system to perform a wide screening to identify plant proteins required for viral infection, as well as to determine the effect of the suppression of a certain host gene in viral replication or movement. Although the specificity of the interaction between Rep and the IR limit the use of the plant to TYLCSV, it is expected that most of the host factors identified with TYLCSV will be also needed for other TYLCV and geminivirus infections. The search with other geminivirus will require the construction of new transgenic plants carrying the construct with its own IR.

Several points should be borne in mind when interpreting a VIGS phenotype to avoid any misreading of the results. First, the absence of a phenotype does not necessarily rule out the involvement of the target gene in the trait of interest. VIGS is never complete, thus some silencing phenotypes might be missed due to some residual level of mRNA might still be supporting target-gene function in the viral vector-transformed plants. Second, it is conceivable that fortuitous sequence similarity between the cloned insert and an unknown mRNA might be responsible for the phenotype. To rule out this kind of artefact, a second non-overlapping insert from the same target gene should be used: if the target gene has been correctly identified this second insert would reproduce the original VIGS phenotype. Finally, the development of pleiotropic effects is an issue that needs to be addressed when interpreting VIGS experiments.

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CHAPTER 6

BIOTIC AND ABIOTIC STRESS RESPONSES IN TOMATO BREEDING LINES RESISTANT AND SUSCEPTIBLE TO TOMATO YELLOW LEAF CURL VIRUS

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1. OVERVIEW

In the eyes of a tomato grower, resistance to TYLCV, as opposed to susceptibility, is defined by the absence of, or mild, disease symptoms, and acceptable yield. In resistant cultivars and breeding lines, the amount of virus that can be detected with molecular tools is usually smaller than that in the susceptible plants, especially during the first 4 weeks after inoculation. Genetic studies have indicated that several genes, expressed as quantative trait loci (QTL), are involved in providing the resistance phenotype described above. Several QTLs have been localized to tomato chromosomes using polymorphic DNA markers (see Part V, Chapter 2). However, the molecular basis of resistance to TYLCV remains totally unknown. Moreover, the physiological state of susceptible vs. resistant plants, before and after inoculation, has never been compared.

To provide some clues on what makes a plant resistant to TYLCV and another susceptible, we have considered the virus as a particular case of stress, among many that a tomato plant may face, and resistance as a particular case of successful response to stress. The response of plants to biotic and abiotic stresses has been studied intensively. A stress response is initiated when plants recognize stress at the cellular level, activating signal transduction pathways that transmit information within the individual cell and throughout the plant, leading to the changes in the expressing of many gene networks. Hence plants respond to biotic and abiotic stresses by activation of R-gene mediated and "signal transduction" defense response pathways (Figure 1).

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 223–237.

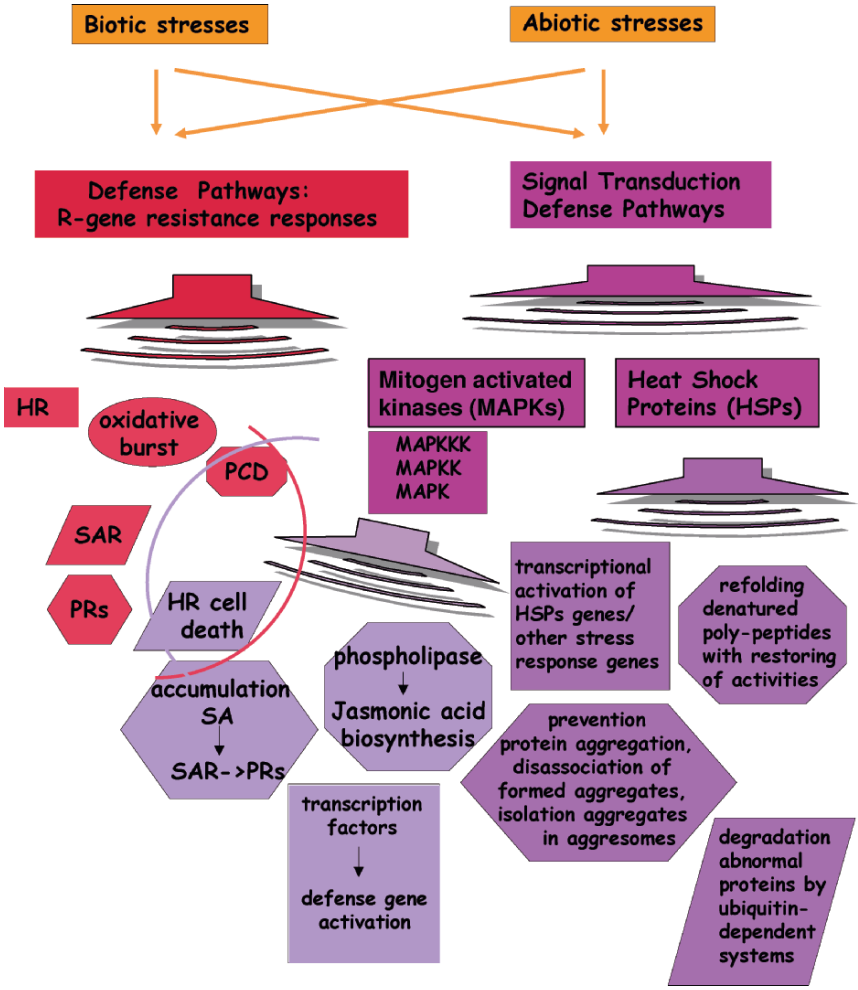

Figure 1. Schematic drawing showing the major plant defense pathways.

Wild ancestors of domesticated agricultural plants are well adapted to many environmental stresses and to a wide range of pathogens. During breeding for high yield and fruit quality, many of the gene networks conferring general resistance to stress have been lost, probably because they were linked to undesirable traits. As a result, domesticated agricultural crops are susceptible to many biotic and abiotic stresses. By crossing domesticated crops with their wild relatives, breeders have succeeded in introgressing chromosomal fragments from wild species that contain stress-resistant genes into superior lines and hybrids and reconstituting some of the gene networks that provide stress tolerance in plants. By crossing domesticated tomato (*Solanum lycopersicum*) with

their wild relatives *S. chilense*, *S. peruvianum*, and *S. habrochaites* and selecting for both horticultural quality and stress resistance, breeders have succeeded in reconstituting some of the gene networks that provide tolerance to various stresses such as viruses, drought, and salinity (Tal and Shannon, 1983).

We have used two tomato lines issued from the same breeding program, one susceptible (S) and the other resistant (R) to TYLCV, to compare the expression of genes involved in stress response. These lines are issued from a cross between *S. habrochaites* plants (accessions LA1777 and LA386) and a superior cultivar (Vidavski and Czosnek, 1998). Line 902 (R) is resistant to the virus while line 906-4 (S) is susceptible. R remains symptomless and yields upon inoculation, while S remains stunted, presents severe disease symptoms and does not yield. S behaves similarly to susceptible tomato cultivars such as Daniella (FA144). In addition to TYLCV-resistance, line R has shown adaptation to a range of deleterious condition such as heat, humidity, drought, and salinity (in Central America, Southern Africa, and the Middle East).

In this chapter, we discuss the expression of stress response genes in R and S plants upon biotic stresses (TYLCV and its vector, the whitefly *Bemisia tabaci*, and the fungus *Sclerotinia sclerotiorum*) and abiotic stresses (NaCl, H_2O_2, ethanol, heat). We discuss the implications of our findings which as a whole showed that R plants have a stronger protein homeostasis capacity to sustain stresses of many kinds than S plants. We also present evidence that some of the more potent stress response genes in R plants (especially HSP70) may originate from the wild tomato genitor *S. habrochaites* LA1777.

2. PLANT DEFENSE PATHWAYS REGULATING THE RESPONSES TO DIFFERENT ENVIRONMENTAL STRESSES IN RESISTANT (R) AND SUSCEPTIBLE (S) TOMATO LINES

2.1. R-gene-mediated resistance defense pathways

Plant stress response pathways implicate the activation of R proteins and result in hypersensitive response (HR) and plant immunity. This phenomenon is associated with the accumulation of H_2O_2 at the site of pathogen attack (oxidative burst) and rapidly induced programmed cell death (PCD) affecting the cells near the site of infection (Bolwell, 1999). The oxidative burst induces several defense responses and is expressed in most if not all plant species (Figure 1). We have compared the intensity of the oxidative burst in S and R genotypes upon whitefly-mediated inoculation of TYLCV (and mock inoculation). We have also compared the response of the S and R lines upon inoculation with a completely different pathogen, the fungus *S. sclerotiorum* (Cessna et al., 2000).

The intensity of oxidative burst or H_2O_2 accumulation can be detected by 3,3′-diaminobenzidine (DAB) histochemical staining (Alvarez et al., 1998). DAB staining was prominent in S tomato leaves at 60 days post inoculation (dpi) by viruliferous whiteflies and thereafter, when disease symptoms were

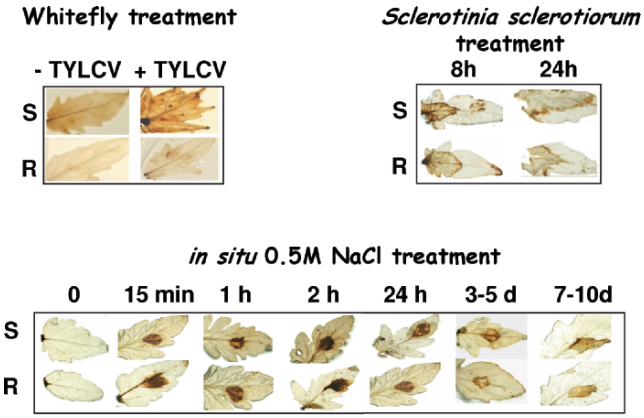

Figure 2. Oxidative burst-related damages detected with DAB. Leaves of S and R genotypes have been assayed 2 months after inoculation with viruliferous and nonviruliferous whiteflies, 8 and 24 h after *S. sclerotiorum* infection, and over time after *in situ* salt application.

conspicuous (Figure 2). Significantly less damage was detected in leaves of R plants inoculated with viruliferous whiteflies, as well as in plants mock-inoculated with nonviruliferous insects. In comparison, *S. sclerotiorum* produced DAB-detectable damages a few hours after inoculation; in this case, the intensity of the oxidative burst was similar in S and R plants (Figure 2). Many abiotic stresses induce endogenous antioxidative defense mechanisms, associated with oxidative burst and release of H_2O_2 (Anderson and Davis, 2004). We have incubated (*in vitro* treatment) or infiltrated (*in situ* treatment) tomato leaves with NaCl (0.5 M), H_2O_2 (10 mM), ethanol (8%), and H_2O as control. A short time after incubation with NaCl a strong DAB staining of *in vitro* treated leaves was observed; DAB staining was less intensive following EtOH and H_2O_2 treatments (not shown). Injections of the same reagents into leaves caused mechanical damages, though most leaves did survive and the plants continued to grow. DAB staining was observed at the site of NaCl injections and was confined to the lesions, to the surrounding cells and to clusters of cells along the veins (Figure 2). The DAB-detected damages were similar in S and R plants. Since the intensity of DAB staining correlates with the strength of the HR and PCD, we concluded that S and R tomatoes behaved similarly at the level of the first step of R gene-mediated response. The increased staining in S after prolonged TYLCV infection correlated with virus-induced disease symptoms.

The second step of R-gene mediated resistance is named systematic acquired resistance (SAR). SAR occurs in tissues that are distant from the initial stressed site and is characterized by an increase in the expression of several genes encoding pathogenesis-related (PR) proteins. The expression of PR proteins can be regulated by a variety of stresses including wounding, chemical elicitors, hormones, and UV-light (van Loon, 1999). PR proteins include at least 11 families, among them β-1,3-glucanases, chitinases, and peroxidases.

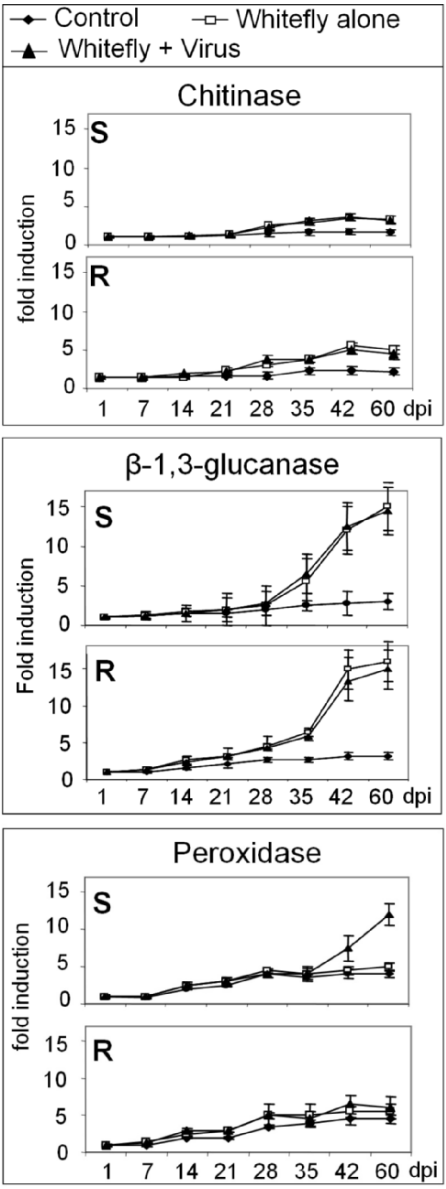

Figure 3. Chitinase, β-1,3-glucanase and peroxidase activities over time in S and R following inoculation of S and T tomatoes with viruliferous and nonviruliferous whiteflies.

We have followed the activities of chitinase, β-1,3-glucanase, and peroxidase in tomato leaves inoculated with viruliferous and nonviruliferous whiteflies, from 1 to 60 dpi. Figure 3 shows a slight increase with time in the activity of all three enzymes in leaves of noninoculated plants, which might be related to aging of tissues. Insect

and virus treatments lead to a minor increase in chitinase activity. In contrast, a pronounced increase in β-1,3-glucanase activity was correlated with whitefly feeding, starting at 28 dpi and amplifying thereafter; yet viruliferous and nonviruliferous whiteflies had similar effects. S and R plants presented similar patters of chitinase and β-1,3-glucanase activities upon treatments. The pattern of peroxidase activation was quite different: S leaves challenged with viruliferous whiteflies showed a level of peroxidase significantly higher than in uninfected or mock inoculated plants starting at 36 dpi and thereafter. Thus, TYLCV infection caused an increase of peroxidase activity in S but not in R tomato lines. The activation of the same PR proteins has been also assayed in response to some abiotic stresses (not shown). Salt applied *in situ* led to the activations of all three PRs after several days of treatment, followed by a pronounced decrease. No significant differences have been observed at the stage of activation of the PRs in S vs. R plants, but the intensity of decline of β-1,3-glucanase and peroxidase activities was somehow less pronounced in R than in S tomatoes. Chitinase, β-1,3-glucanase, and peroxidase activities in *in vitro*-treated tomato leaves showed similar patterns (data not shown).

Under our experimental conditions, the downstream events in biotic and abiotic stress response, such as activation of HR/PCD and expressions of PRs (chitinase, β-1,3-glucanase, and peroxidase), were similar in S and R tomatoes, except at the very late (more than 7–8 weeks) of TYLCV infection.

2.2. Signal transduction response pathways

2.2.1. Mitogen-activated protein kinase (MAPK)

Control of HR, PCD, and SAR, all characterized by increased expression of several genes encoding PR proteins, could be a major outcome of mitogen-activated protein kinase (MAPK) cascades (Figure 1). MAPK cascades are present in higher plants and play an important role in signal transduction in response to hormones and biological signals as well as in environmental stresses such as wounding, cold, salt, drought, oxidative stress, and ozone (Bogre et al., 1997; Ichimura et al., 2000). MAPK cascades are also involved in signal transduction leading to activation of plant defense responses against pathogen attacks (Nuhse et al., 2000; Desikan et al., 2001). Based on sequence analysis, the known plant MAPKs are most similar to ERKs, which are implied in various forms of biotic and abiotic stress responses (Bogre et al., 2000).

We have used an immunodetection approach to compare the patterns of a redundant class of ERKs in infected S and R tomato lines in response to biotic and abiotic stresses. ERK1/2 antibodies recognized 45–50 kDa polypeptides in extracts of tomato leaves (Figure 4, A and B). The ERKs patterns did not change compared with noninoculated plants at 1 dpi. At 5–7 dpi and thereafter, a decrease in ERK amounts was observed in plants caged with viruliferous as well as nonviruliferous whiteflies. This decrease was less pronounced in R than in S tomatoes (Figure 4A). The amount of ERK-like proteins increased 0.25–1 h after

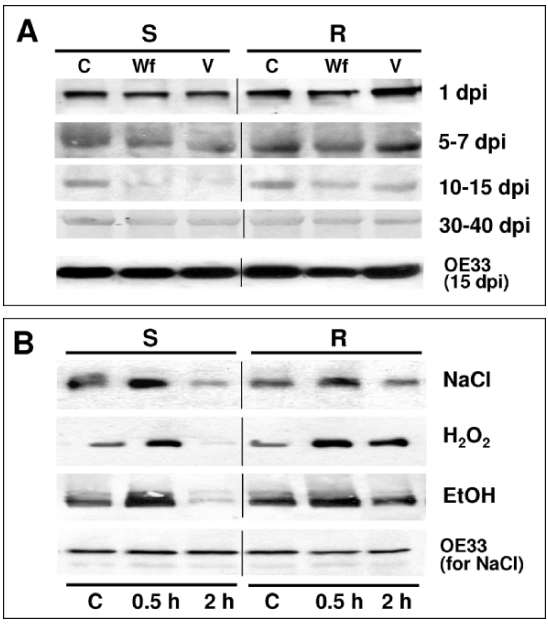

Figure 4. ERK-like proteins in S and R tomatoes upon biotic and abiotic stresses. (A) whitefly-mediated inoculation; C: untreated, Wf: mock inoculation with nonviruliferous whiteflies, V: whitefly-mediated inoculation of TYLCV. (B) exposure to NaCl, H_2O_2, and EtOH. Anti-ERK antibodies recognize tomato polypeptides with approximate molecular mass 45–50 kDa. OE33 (33 kDa subunit of oxygen evolving system of photosystem II) was used as an internal protein marker.

the application of abiotic stresses, followed by an abrupt decrease thereafter (Figure 4B). The S and R tomato lines behaved similarly during ERK activation. However, while the amount of ERKs dramatically decreased in S plants, this decrease was milder in R plants. The pattern of OE33 (subunit of oxygen evolving system of photosystem II), used as an internal protein marker, was not affected neither in S, nor in R plants under abiotic stresses (shown for NaCl; Figure 4B). Expression of two other MAPKs families, detected by antibodies raised against mammalian JNK and P38 kinases, reflected a similar pattern upon whitefly inoculation-related stresses and abiotic stresses (data not shown). The patterns of MAPK-like proteins imply that R tomato is more stable than S tomato in response to the biotic and abiotic stresses we have tested.

2.2.2. Heat shock proteins (HSPs)

A group of highly conserved proteins, termed Heat shock proteins (HSPs) (chaperones and proteases), has evolved to cope with environmental stresses. Chaperones control the folding status of proteins and, together with ATP-dependent proteases, form the cellular protein quality control system (Hartl and Hayer-Hartl, 2002). Many work in concert to facilitate correct protein folding,

assembly, and repair, while others interact with proteolytic components to degrade terminally damaged proteins that might otherwise accumulate to potentially harmful levels (Parsell and Lindquist, 1993) (Figure 1). HSPs also assist in the cell recovery from stress either by repairing damaged proteins (protein refolding) or by degrading them, thus restoring protein homeostasis and promoting cell survival (in plants, Pareek et al., 1995; Katiyar-Agarwal et al., 2003).

HSPs have been classified into six major families according to their molecular size: HSP100/CLP, HSP90, HSP70, HSP60, HSP40, and small HSPs (Jolly and Morimoto, 2000). HSPs/chaperones/proteases are expressed in plants experiencing a variety of stresses such as high and cold temperatures, drought, salinity, osmotic shock, oxidative stress, and pathogen attacks (Wang et al., 2004). Several plant proteases are induced during drought and salinity (Seki et al., 2002), desiccation, and high light (Hausuhl et al., 2001). Many stresses trigger chlorophyll decline accompanied by degradation of several photosystem-II proteins. Within the chloroplast stroma, the enzymes responsible for most protein degradation are the ATP-dependent Clp and FtsH proteases (Adam and Clarke, 2002).

The best investigated member of HSPs family is HSP70. We have analyzed the pattern of HSP70 in S and R tomatoes upon inoculation with viruliferous and nonviruliferous whiteflies. A decrease in the amount of HSP70 started at 5–7 dpi (Figure 5A); this decrease was less pronounced in R than in S plants. At 60 dpi and thereafter, HSP70 almost disappeared in S, coinciding with a massive decline of total proteins (see marker protein, OE33) which accompanies the development of the disease symptoms (Figure 5A). The other two broadly distributed members of the HSP family, HSP60 and HSP90, behaved similarly to HSP70 in response to the biotic stresses assayed (Figure 5B).

Housekeeping Clp proteases facilitate the normal turnover of many stromal enzymes and regulatory proteins, as well as removing and recycling irreversibly damaged polypeptides (Halperin and Adam, 1996). FtsH protease plays a role in proteolytic removal of oxidized components from the photo-damaged PSII reaction center after variety of environmental stresses (Adam and Clarke, 2002). Recently, in transgenic tobacco plants under- and overexpressing the *ftsh* gene, the size of necrotic lesions correlated with FtsH content (Seo et al., 2000). ClpC amounts were stable during the first weeks after mock and TYLCV inoculation of S and R plants (Figure 5C). After 60 dpi, ClpC decrease could be observed in mock inoculated and virus inoculated plants. In contrast to ClpC, the amount of FtsH started to decline after 5–7 dpi and this decrease accelerated at 10 dpi and thereafter and was more pronounced in S than in R plants (Figure 5C).

We have compared the behavior of HSP70 and of the chloroplast proteases ClpC and FtsH in detached leaves (*in vitro*) and in whole R and S plants (*in vivo*) upon heat, salt, and ethanol stresses. HSP70 accumulated in detached leaves during the first 2 h at 45°C (Figure 6A). The protein declined subsequently and almost disappeared in S tomato after 6 h; the extent of reduction was milder in R tomatoes. Application of NaCl, H_2O_2, and ethanol caused similar patterns of HSP70 expression in S and R (Figure 6B). The expression of HSP60 and HSP90

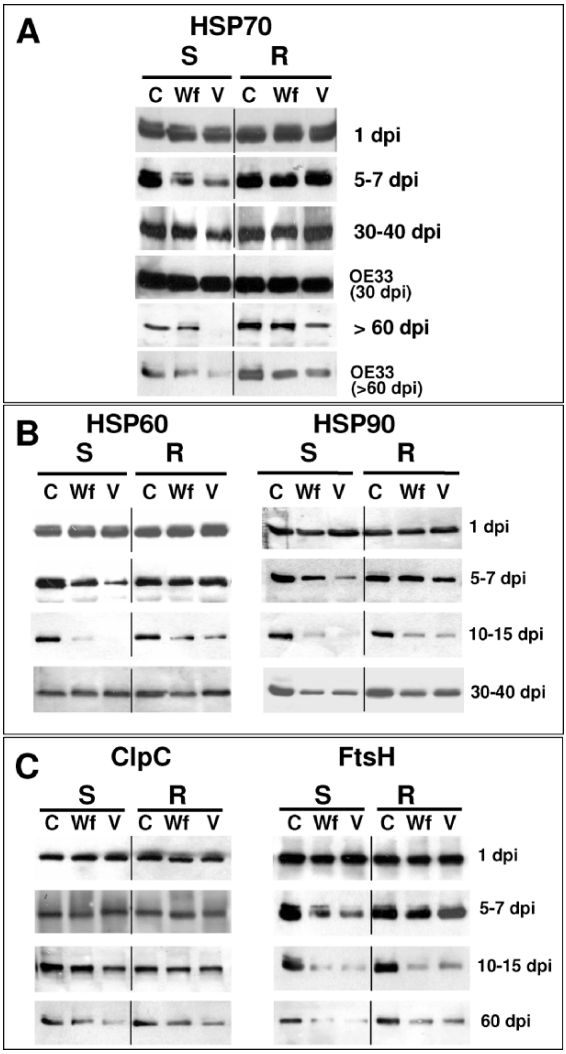

Figure 5. Heat shock proteins (HSP70, HSP60, HSP90) and proteases (ClpC and FtsH) in S and R tomato without (C) and with mock inoculation by nonviruliferous whiteflies (Wf) and whitefly-mediated inoculation by TYLCV whitefly (V). OE33 was used as an internal protein marker.

was induced in R and S plants within 1 h of salt application; the two HSPs declined soon after, more abruptly in S than in R tomato (data not shown). It has to be noted that the amounts of HSP60 and HSP90 decreased less dramatically than those of HSP70. ClpC proteins were constitutively expressed in S and R plants during NaCl treatments (Figure 6C). In contrast, in response to salt stress the levels of FtsH increased after 1 h of treatment; it sharply

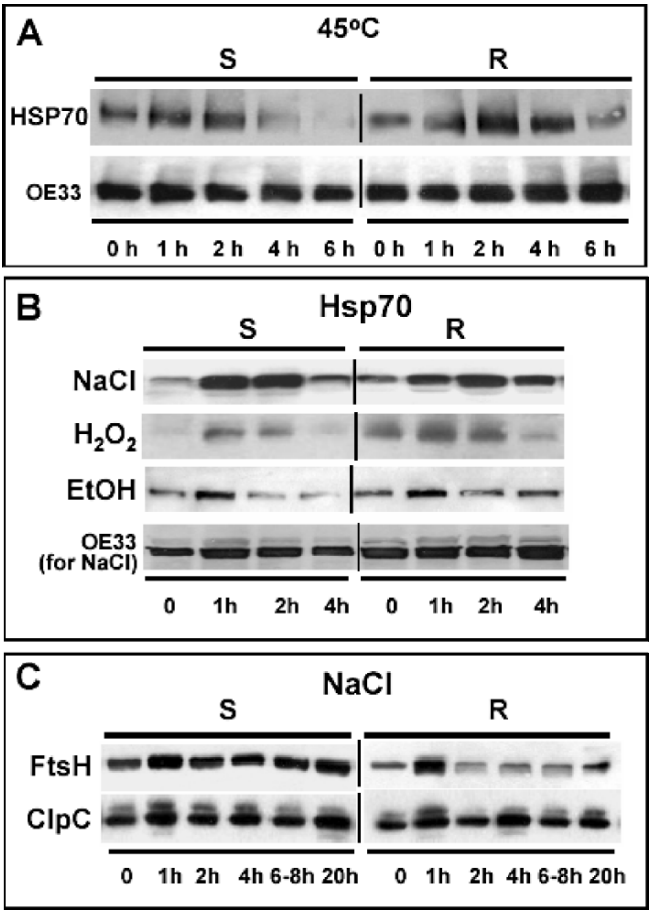

Figure 6. HSPs responses to abiotic stresses in leaves of S and R tomato. (A): HSP70 patterns after heat shock (45ºC); (B) HSP70 patterns after NaCl, H_2O_2, and EtOH treatments; (C) FtsH, ClpC patterns after NaCl treatment.

decreased thereafter in R but not in S plants. Prolonged stress (16–20 h and more) led to recovery of this protease in R tomato (Figure 6C). H_2O_2 and ethanol treatments caused similar changes in FtsH abundance (not shown). The behavior of chloroplast protease FtsH may reflect the immediate response of R plants to stress, namely a sharp decrease and shot-off of protease expression for several hours followed by recovery, which did not happen in S plants. Most relevant, when plants are exposed to different environmental stresses, chlorophyll biosynthesis and chloroplast development are inhibited (Adam and Clarke, 2002). The impaired chlorophyll biosynthesis can be part of a protective mechanism against stress during limited time periods.

Again, analysis of HSPs patterns, as the patterns of the other class of regulatory stress proteins, MAPKs, revealed the less extent of decline in R vs. S tomatoes in response to different used in this study stresses.

3. DISCUSSION

Plants have evolved strategies to perceive and to cope with deleterious conditions, whether biotic (attacks by pathogens, including fungi insects and viruses) or abiotic (including temperature, water, and salt stresses). In most cases the induced defense responses are regulated by a network of interconnecting signal transduction pathways, in which a variety of stress proteins play cross-reactive roles (Hammersmidt and Kuc, 1995). A rapid respond to these stresses determines the adaptive capacity and, therefore, the likelihood of survival of the threatened plants (Rattan, 2006).

We have compared the response of TYLCV resistant and susceptible lines to a variety of biotic and abiotic stresses. As a whole, R-gene mediated resistance at the stages of oxidative burst and PRs activation was comparable in S and R tomato. The activation of regulatory proteins was similar in the two lines, however the stress-induced decrease in the abundances of MAPK-like proteins, HSPs 60, 70, 90, and FtsH was more pronounced in S than in R plants. Hence shortly after application of stress, the plant defense mechanisms of S and R plants are activated. Thereafter, these defenses are overwhelmed in S plants and stress-induced damages appear on the treated leaves. In contrast, R plants show a milder decrease in stress response proteins expressions followed by a certain level of recovery. We propose that the higher ability of reestablishing cellular homeostasis in response to stresses makes R tomatoes tolerant to numerous environmental stresses.

After exposures to different stresses the members of HSP/chaperone/protease families seem to play a key role in the reestablishing of cellular homeostasis. We propose that the higher abundance of HSP70, HSP60, HSP90, as well as FtsH and the regulating proteins MAPKs, in R tomato lines vs. S, is one of the reasons of increased tolerance to several environmental stresses, not only in the laboratory conditions, but also in the field. As a whole, it appears that virus-resistant plants have a better buffer capacity to cope with stress than susceptible plants. Indeed, the physiological status of R plants reflected by their chlorophyll content (a commonly used marker) was higher than in S plants after exposure to the salt stress, H_2O_2, and EtOH (not shown). Moreover, our results support the idea of overlapping between signaling involved in host response against biotic and abiotic stresses. For example, leaves of tomato plants exposed to NaCl 10 days after whitefly-mediated inoculation of TYLCV did not show the decline in HSPs amounts observed without the virus preinoculation step, especially in S plants (Figure 7). Treatment with H_2O_2 and ethanol showed similar results (not shown).

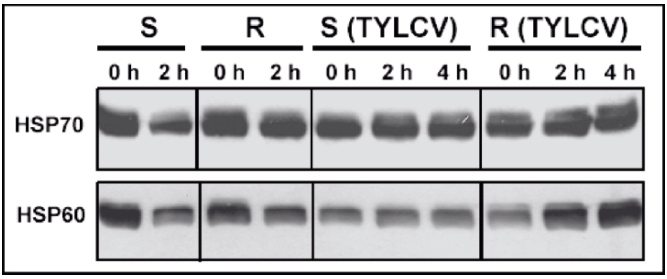

Figure 7. Protection against salt stress by a prior TYLCV inoculation. HSP70 and HSP60 in S and R tomatoes, infected (and not) by TYLCV with and without 0.3 M NaCl stress.

Whiteflies by themselves, whether viruliferous or not, induced a stress response translated by a decrease in the amount of the stress proteins MAPKs, HSPs and of the chloroplast protease FtsH (ClpC showed exceptional stability). This decrease was already observed at 5–7 dpi in S plants, much less in R plants. The specific effect of TYLCV was conspicuous at 10–15 dpi for HSP 60 and HSP90, and at 60 dpi for HSP70; this decline occurred more abruptly in S than in R tomatoes. Among the PRs studied, only the induction of peroxidase could be correlated with TYLCV, at 30–40 dpi. Activation of β-1,3-glucanase, and at a lesser extent chitinase, was similar upon mock- and TYLCV-inoculation by *B. tabaci*. These biotic stresses induce PRs expression after prolonged treatments (not earlier than 4–5 weeks), while infection by the fungus *S. sclerotiorum* led to a sharp induction already at 2–3 dpi (not shown). Infected S and R tomatoes responded similarly to biotic stresses at the level of PRs activation, except for a very late increase in the peroxidase activity more than 7–8 weeks after infection of S plants. We cannot exclude the possibility that there are other PRs underlying tolerance to TYLCV by restricting viral multiplication in the R line.

Recently, the downregulation of many expressed genes in *Arabidopsis*–phloem aphids interactions was shown (De Vos et al., 2005), moreover, large fraction of the downregulated genes were involved in plant metabolism and not directly in plant protection (as PR proteins).

The maintenance of stress protein profiles and the stimulation of proteolytic degradation of damaged proteins both contribute to improved cellular resistance to different stresses. The analysis of the signal transduction pathways to determine alterations in the phosphorylation and dephosphorylation states of ERK, JNK, and p38 MAP kinases, and consequently, functional activities of numerous MAPKs' regulated substrates, can be a measure of cellular responsiveness to mild and severe stresses. The stability of these biomarkers in response to biotic or abiotic stresses causes the increased adaptive abilities, maintenance of repair mechanisms in the living cells.

Up to now, we have discussed the response of R and S plants to biotic and abiotic stress and have shown that HSPs and MAPKs are less affected by TYLCV

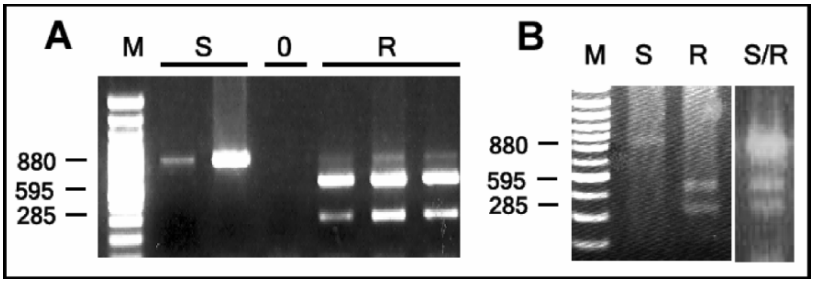

Figure 8. SNP in the intron of a HSP70 gene, related to resistance and susceptibility. (A) The 880 bp amplicon obtained by PCR with R and S DNA was treated with MunI. R DNA was digested, S was not. (B) Seed DNA collected during the breeding program for resistance; the *S. habrochaites* LA17777 genitor is heterozygote.

whitefly-mediated inoculation in R than in S germplasm. Therefore, we may assume that the alleles of R plants are more "potent" than their S counterparts. Since TYLCV-resistance is the result of introgression of (unknown) chromosomal fragments from resistant *S. habrochaites*, we have hypothesized that these alleles have been introgressed from the wild tomato species into the domesticated tomato during breeding for TYLCV resistance. To substantiate this hypothesis we have searched for polymorphism (SNPs/Indels) in the HSP70 gene family (which provided a clear difference in expression in R vs. S genotypes, see Figure 8A) that could be related to *S. habrochaites*. Since DNA sequences of tomato HSP70 were not available, we have used *Arabidopsis* HSP70 genes to find a consensus sequence flanking an intron, which served to design primers that allowed PCR amplification of a ~880 bp amplicon shared by R and S plants, including an intron. A *Mun*I site allowed discriminating between R and S by RFLP PCR: *Mun*I digested the 880 bp from R, two fragments of 595 and 285 bp, but not from S plants (Figure 8). Using seeds from plants selected at different stages of the breeding program (Vidavski and Czosnek, 1998), the R-specific *Mun*I SNPs was traced back to the *S. habrochaites* LA1777 parent, but not to the other *S. habrochaites* LA386 parent. LA1777 presented a *Mun*I heterozygous R/S profile, probably because seeds from this accession gave rise to resistant as well as susceptible and tolerant plants. Seeds from the first *S. habrochaites* × *S. lycopersicon* cross had the R/S profile. Along the R and S pedigree, there was a strict correlation between the resistant/susceptible phenotypes and the *Mun*I SNP. This polymorphism was not present in other tomato TYLCV-resistant lines that had introgressions from wild tomato species other than *S. habrochaites*.

We suggest that the maintenance of a higher stability of protein homeostasis in R compared to S tomatoes, derives from its ability to manage stress caused by the accumulation of denatured proteins. Future research should investigate the plant cellular protective machinery that copes with the buildup of

abnormal polypeptides. Compared to S plants, R tomatoes may have a greater capability (1) to refold denatured polypeptides and restore their activities, (2) to selectively degrade abnormal proteins by ubiquitin-dependent systems in proteosomes, (3) to prevent protein aggregation that can be toxic for the cells, (4) to disassociate the aggregates of denatured proteins, (5) to isolate the aggregates in special structures called aggresomes, and in the case if all the above is insufficient, (6) to prevent the accumulation of abnormal proteins, to initiate PCD (apoptosis) by using MAPKs signaling. It will be of interest to found out how TYLCV infection influences these stages.

4. ACKNOWLEDGMENTS

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PART IV

INTEGRATED PEST MANAGEMENT MEASURES AND PROTECTION OF TOMATO CULTURES

CHAPTER 1

DETECTION METHODS FOR TYLCV AND TYLCSV

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1. OVERVIEW

The tomato yellow leaf curl disease (TYLCD) has been known for many years. The cause was, premature but with commendable intuition, put down to an entity named *Tomato yellow leaf curl virus* (TYLCV) (Cohen and Nitzany, 1966) although the viral etiology was recognized only in the late 1970s, and a virus with geminate morphology detected even later. Electron microscopic (EM) observations of thin sections from TYLCV-infected tomato leaves indicated that geminate particles were located in the nuclei of phloem parenchyma cells (Russo et al., 1980; Cherif & Russo, 1983), with intranuclear occurrence of fibrillar rings and small virus-like particles like those in the new virus group named "geminiviruses" (Goodman, 1981). In those times EM was therefore the only possible way to detect TYLCV.

However, what are considered "detection methods" for this virus complex had to wait for isolation of viral particles and demonstration that they are the causal agent of TYLCD. The virus was first isolated and purified in 1988 (Czosnek et al., 1988), and its association with the disease was demonstrated by membrane feeding of the whitefly vector on purified virus preparations. Since then, several detection methods for what is now recognized as a virus complex have been developed, both for mass screening and for more specific characterization.

In this review only methods for mass screening will be discussed, omitting specific applications, such as *in situ* hybridization and immuno-enzymatic methods for light or electron microscopy.

Depending on the kind of investigation, different questions can be asked, and no single detection method can fulfil all needs. Are the tomato plants which show yellowing and curling on leaves infected by TYLCV? Or are they infected by a different begomovirus? Which virus strain or variant is present? Are the plants infected by more than one begomovirus (mixed infection)?

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 241–249.

Difficulties arise mainly because there are several begomoviruses that cause similar symptoms in tomato (Fauquet et al., 2005) and they are not sufficiently different to allow easy and reliable discrimination using techniques such as ELISA, familiar to plant virologists and agricultural extension services.

2. SEROLOGICAL TECHNIQUES

Serological methods have not had much success in detecting TYLCV and whitefly-transmitted begomoviruses more generally. This is due both to difficulty in obtaining pure virus preparations and to the low immunogenicity of virus particles. Although the first purification procedure was described in 1988 (Czosnek et al., 1988) and later improved (Luisoni et al., 1995), the polyclonal antibodies obtained, while adequate for Western blotting, were not suitable for reliable detection of TYLCV by ELISA in field samples (Al-Bitar and Luisoni, 1995). Today some commercial ELISA reagents are available, detecting both TYLCV and the related but separate species *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Dalmon et al., 2000; Crescenzi et al., 2004). Some monoclonal antibodies (MAbs) raised against particles of *African cassava mosaic virus* (ACMV) have been reported to detect three isolates of TYLCSV, but were not tested against TYLCV in TAS–ELISA (Macintosh et al., 1992).

A different TAS–ELISA format has been described in a EWSN datasheet (Winter & Louro, 2000) and in an EPPO Bulletin (EPPO, 2005). It uses a polyclonal antibody prepared against ACMV for the coating step, followed by either a MAb that can detect TYLCV and TYLCSV isolates present in Europe (DSMZ AS-0546/2) or another MAb (DSMZ AS-0546/4) which does not react with TYLCSV. To differentiate the two virus species, a sample must be analysed with both MAbs: if it is positive only to the first, it is probably infected only by TYLCSV; if it is positive to both, certainly TYLCV is present, but it is impossible to conclude on the presence of single infection by TYLCV or mixed infection by both viruses. To resolve mixed infections molecular techniques are necessary.

Immunoblotting methods have also been reported, both in the form of tissue-printing (squash immunoassay) and dot-blotting (dot immunoassay) (Hajimorad et al., 1996; Pico et al., 1999; Dalmon et al., 2000). In comparison with ELISA, these methods suffer from a relatively high background, also found in healthy controls, that masks weak signals. Furthermore, they have not been tested for their ability to distinguish among similar species.

Recently some companies have introduced lateral flow assays for TYLCV (see www.neogeneurope.com; pdiag.csl.gov.uk). However, the sensitivity and ability of these assays to detect all or some species or isolates have not yet been tested thoroughly.

A different approach has also been used for trapping virus particles: the coating step of an ELISA is performed using GroEL protein rather than antibodies to the virus. Indeed, GroEL is much more potent in binding TYLCV than commercial anti-TYLCV antibodies (Akad et al., 2004). The method

exploits the strong interaction between the GroEL protein of the whitefly *Bemisia tabaci* and the coat protein of TYLCV and other begomoviruses (Morin et al., 1999).

3. MOLECULAR HYBRIDIZATION

The use of labelled DNA probes for detecting TYLCV dates back to 1988 (Czosnek et al., 1988; Navot et al., 1989; Nakhla et al., 1993), when a cDNA clone representing part of the viral genome was radiolabelled and used in Southern blots to detect the different viral DNA forms present in infected plants. The same probe was also employed on leaf squashes obtained by tissue printing, where it showed very good specificity (no reaction with the other viruses tested and no reaction with healthy plants). These characteristics and the ease, with which nylon membranes can be prepared, even in field conditions, immediately indicated the potential of molecular hybridization assays for mass screening and diagnosis. Interestingly, TYLCV can also be efficiently detected in squashes of single whiteflies. The method was proposed for large-scale epidemiological studies and for use in breeding programmes for virus resistance (Lapidot et al., 1997, 2001). One step of the molecular hybridization, however, made it impossible for many diagnostic laboratories to run the assay: probes were radioactively labelled.

To overcome this problem non-radioactive labelling techniques were explored. The most popular, even today, is based on digoxigenin (Crespi et al., 1991; Abou Jawdah et al., 1995). Probes are labelled with digoxigenin–dUTP, and the signal is detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by incubation with an enzyme substrate. The first substrates were chromogenic, producing a pink–violet colour. Sensitivity was comparable with that of radiolabelled probes (Crespi et al., 1991; Quiñónez et al. 2004). Chemiluminescent substrates brought a further improvement. This allowed: (a) visualization of the signal on a film, therefore permitting optimal exposures and better interpretation of results, especially in cases where a weak hybridization signal is superimposed on the colour of the tissue print, and (b) more important, easy removal of the probe for a second hybridization. Reprobing membranes is particularly useful when mass screening is performed in areas where TYLCD can be caused by more than one virus species (see below). Non-radioactive hybridization was also successfully used for quantitative determination of TYLCV in dot blots of plant and whitefly extracts (Caciagli. & Bosco, 1996, 1997).

Probes, radioactively labelled or not, able to recognize the entire viral genome or selected portions of it can easily be produced, and proved very useful in organizing the taxonomy of the numerous whitefly-transmitted geminiviruses affecting tomato cultures in many tropical and subtropical regions. Hybridization tests with two DNA probes derived from a cloned isolate of TYLCV from Israel have been used to assess the affinities of viruses in naturally

infected tomato plants with yellow leaf curl or leaf curl symptoms from 25 countries (Czosnek & Laterrot, 1997). By a careful choice of probes and hybridization conditions, it was possible to conclude that samples from countries in the Middle East, Cuba, or the Dominican Republic were closely related to TYLCV from Israel, whereas samples from nine countries in the western Mediterranean area, Africa, or SouthEast Asia were more distantly related and probably represent one or more additional geminivirus species; a further group of samples contained very distantly related geminiviruses. The great number of geminivirus sequences available today have confirmed that TYLCD is caused by a number of geminiviruses whose genomes share a wide range of similarity levels (Fauquet et al., 2005).

The versatility of probes – and the need to make the best choice for everyone's purpose – is demonstrated by the cases of Spain and Italy, where TYLCSV strains were present and well established when TYLCV strains appeared in 1997 in Spain (Navas-Castillo et al., 1997) and 2002 in Italy (Accotto et al., 2003). For each species several strains have been described and their DNA sequences determined. Between the two species the nucleotide identity is about 74%, while among strains within each species it is above 90%. Sequence similarities are not uniformly distributed along the genome, so probes can be designed that will cross-react to different degrees with heterologous sequences. For example, probes based on the intergenic region (IR), the less conserved portion of genome, are the most specific. For example, an IR-specific probe made on the Sardinia isolate of TYLCSV does not recognize TYLCV and gives little or no reaction with other TYLCSV strains, so is not suited for most screening purposes. On the other hand, a probe designed on a more conserved region, such as the CP gene, will reliably detect all strains of the viral species from which it was designed, and can therefore be considered a good choice for mass screening (Accotto et al., 2000a). It should be noted, however, that samples giving weak signals may contain a low concentration of the homologous virus or a high concentration of the heterologous. Hybridization of the same membrane with a second probe, specific for the CP region of the other virus species, will generally help in final interpretation, but a definitive answer can only be given by a PCR-based method, such as PCR/RFLP described below. One remedy for the excessive specificity of IR-specific probes is to use an artificial mixture of them (Accotto et al., 2000b), and to use less stringent conditions during post-hybridization washing.

Both in Spain and Italy epidemiological studies on TYLCSV and TYLCV were conducted using tissue printing on membranes that were consecutively hybridized with two species-specific probes (Sanchez-Campos et al., 1999; Davino et al., 2006). Molecular hybridization employing non-radioactive probes, in spite of some limitations, definitely remains the best choice for simultaneous analysis of hundreds of samples: it does not require expensive equipment or a laboratory authorized for radioisotopes, and tissue-blotted membranes can be prepared in the field, with no extraction steps, and then stored for long

periods or sent to diagnostic laboratories. Furthermore, digoxigenin-labelled non-radioactive probes are stable for years, and can be reused at least five times without loss of sensitivity (Accotto et al., unpublished).

4. PCR-BASED METHODS

Numerous PCR-based methods have been reported for detecting whitefly-transmitted geminiviruses, using specific or degenerate primers, or combining PCR with tissue printing, immunoblotting, restriction enzyme digestion, molecular hybridization, etc. Navot et al. (1992) developed primers for detecting TYLCV in plants and whiteflies; for one of the primer combination tested, they estimated that a single infected whitefly could be detected in a bulk sample of 1,000.

Several publications describe degenerate primers for detecting whitefly-transmitted begomoviruses. One of the most cited uses primers PAL1v1978 and PAR1c713 to amplify a 1.4 kbp fragment (Rojas et al., 1993). Further analysis of this DNA provides information on the begomovirus under investigation. This approach to a suspected geminivirus infection in tomato can be found in many "first reports" in the literature. Other protocols have been proposed for detecting all begomoviruses (Deng et al., 1994; Wyatt & Brown, 1996), but not all of them have been tested on TYLCV and TYLCSV isolates, and might be unsuitable in some cases.

As mentioned above, a more challenging situation happens when there is need to rapidly and reliably detect and differentiate two related begomoviruses, that infect the same host (tomato) producing the same symptoms. For identification and differentiation of the two species infecting tomato in Europe, TYLCV and TYLCSV, the EPPO standard (EPPO, 2005) describes a PCR/RFLP protocol (Accotto et al., 2000a, c), that utilizes two degenerate primers – TY1(+) and TY2(–) – to amplify a 580 bp fragment from both species, followed by digestion with AvaII, which produces one pattern for TYLCV isolates and a clearly different one for those of TYLCSV. In another study (Martinez-Culebras et al., 2001), following extensive sequence alignments of several begomoviruses, two primer pairs were designed and successfully tested, one for detection of both TYLCV and TYLCSV, the other for TYLCSV only. A duplex PCR was also reported, that can detect either TYLCV or TYLCSV in a single step: However this protocol cannot distinguish the two viruses when mixed infections are present.

Print-capture PCR protocols have been successfully employed for several purposes. For example, squashes of plant or whitefly tissue on small pieces of nylon membranes were directly used in amplification reactions (Atzmon et al., 1998) to study TYLCV acquisition and transmission. This approach was modified by Navas-Castillo and coworkers (1998), who used pieces of Whatman 3 MM paper instead of nylon, with similar results. An important advantage consists in omitting the DNA extraction step needed before standard PCR.

Immunocapture-PCR, when applied to transmission studies, showed that the capsid protein of TYLCV was present in the insect organs at the same

time as DNA, suggesting that at least part of the virus circulates as virions within the insect (Ghanim et al., 2001). However, this technique is not widespread in diagnostics.

Finally, it is worth mentioning a new technique, named loop-mediated isothermal amplification (LAMP), that has been applied to TYLCV detection (Fukuta et al., 2003). It amplifies DNA with high efficiency under isothermal conditions without being significantly influenced by co-presence of non-target DNA, and its detection limit is a few copies, being comparable to that of PCR. Although LAMP applications are today mostly in clinical microbiology, its future development in plant virology cannot be excluded.

5. COMPARISONS AMONG METHODS

Not much effort has been devoted to compare the different detection methods. It is assumed that PCR is more sensitive than hybridization, which is more sensitive than serological methods. But sensitivity does not always go together with reliability. In the most comprehensive study, Pico et al. (1999) compared TAS–ELISA, squash immunoassay, dot immunoassay, squash and dot blot hybridization, and PCR for their sensitivity, reliability, and possibility of quantification, in order to measure resistance levels in tomato breeding lines. The squash and dot immunoassay were neither sensitive nor reliable. TAS–ELISA can be used in large-scale field screening, but hybridization methods are generally more appropriate, and, in the dot blot version, are quantitative. PCR, in principle the most powerful technique, sometimes fails to detect TYLCV reliably. A good protocol could be a squash blot followed by the more sensitive PCR applied to inconclusive samples. Dalmon et al. (2000) confirmed the superiority of squash and dot hybridization methods, because of their reliability and low cost. PCR gave some false negatives, probably due to the presence of inhibitors in the plant extract.

6. CONCLUDING REMARKS

Detection of begomoviruses causing TYLCD is best achieved by integrating two or more methods, as shown by the two following examples. When a new disease is suspected to be caused by a begomovirus, PCR with degenerate primers (better more than one combination) is the first step, followed by sequencing the amplified DNA. Comparing the new sequence with the DNA databases will give a strong indication on the nature of the begomovirus. In cases where mass screening is required, such as epidemiological studies, high-throughput is necessary; the best choice is squash blot hybridization, which allows analysis of hundreds of plants or insects on every membrane, without extraction steps, and with the option of reprobing the same membrane with different probes. For samples where results are not clear, PCR can then be applied.

However, some situations cannot be resolved with a simple strategy; virus populations are dynamic, and recombinant viruses have been shown to evolve

and spread in the parental population (Monci et al., 2002). If the new recombinant virus is present together with one or both parents, results of hybridization can easily be misinterpreted. In these cases, unfortunately, PCR using carefully selected primers is probably the only way to study the population dynamics. Rolling circle amplification (RCA), a method which utilizes a DNA polymerase from a bacteriophage, has recently been proposed for diagnosis of geminiviruses (Haible et al., 2006). Although it is probably the best way to face the challenge of characterizing populations of viruses with single-stranded DNA genomes in situations where mixed infections, recombinants, or new viruses are present or expected, RCA will hardly substitute the other techniques, at least in the near future, in diagnostic laboratories.

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CHAPTER 2

MANAGEMENT OF TOMATO YELLOW LEAF CURL VIRUS: US AND ISRAEL PERSPECTIVES

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1. OVERVIEW

Over the last 15 years, TYLCV has been a serious problem for tomato production in many parts of the world. The virus has been known in Israel for over 40 years and in Florida since 1997 (Cohen & Nitzany, 1966; Polston et al., 1997). In Israel, tomato crops are severely affected by epidemics of TYLCV and despite almost daily spraying with insecticides, 100% yield losses have often been recorded in cases where the whitefly populations were high (Cohen & Antignus, 1994). In Florida, there have been numerous crop failures due to TYLCV and costs of production have risen. TYLCV is considered the most important pathogen of tomato in Israel and in Florida (Lapidot & Friedmann, 2002). The management of TYLCV in tomato is difficult and expensive both in protected and open field production. Often management techniques are not sufficient and economic losses are incurred. Many approaches have been used to try to decrease losses due to TYLCV although only a few are frequently effective and some cannot be used in all climates and locations. In general, no single approach is effective to manage TYLCV. Combinations of chemical and cultural techniques are employed to (1) reduce the number and movement of the whitefly vector, and (2) minimize or eliminate inoculum sources of TYLCV.

Management of TYLCV is often expensive and difficult but not always successful. In both Florida and Israel, multiple techniques are employed simultaneously to reduce incidences of TYLCV-infected plants. In Israel, TYLCV is managed primarily through the use of resistant cultivars, pesticides, cultural practices, and exclusion through the use of 50 mesh screens, and regular or UV absorbing plastics in the case of protected production. In Florida, where the

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 251–262.

majority of tomatoes are produced in open fields, the virus is managed through cultural practices and a heavy reliance on insecticides (Cohen & Antignus, 1994; Lapidot & Friedmann, 2002). The development of pesticide resistance and the loss of natural predators and parasites after repeated insecticide applications contribute to control problems and environmental concerns. The most practical control of TYLCV is the use of resistant cultivars. These unfortunately are limited and not available for all production conditions, climates and market preferences. Growers are forced to rely on other approaches to minimize yield losses.

2. ECOLOGY OF TYLCV

Management techniques are dependent upon the ecology of the virus. TYLCV is transmitted in a persistent manner by adult whiteflies of the *Bemisia tabaci* species complex. TYLCV has one of the largest described host ranges for begomovirus and is capable of infecting more than 30 species in over 12 plant families. Known hosts can be cultivated vegetables ornamentals, or wild or weed plant species. The TYLCV reservoirs often vary among tomato production regions and must be identified on a case-by-case basis. Although TYLCV can produce severe symptoms in tomato, it is also able to establish symptomless infections in both wild and nontomato crop species. TYLCV reservoirs may not be obvious. For instance, in many tomato-growing areas pepper is grown in close proximity. Since pepper was known as a nonhost of TYLCV, whitefly management was not practiced rigorously in pepper plots. There have been conflicting reports regarding the susceptibility of peppers to TYLCV (Mansour & Al-Musa, 1992; Reina et al., 1999). Only recently was it clearly demonstrated that some but not all cultivars of pepper are in fact symptomless hosts of TYLCV (Morilla et al., 2005; Polston et al., 2006). This is similar to the situation with beans (*Phaseolus vulgaris*) in which only 57% of the genotypes tested were susceptible to TYLCV, unlike tomato where all genotypes tested were susceptible (Lapidot, 2002). Some pepper genotypes were infected at much higher rates than others while other genotypes only became infected using higher densities of whiteflies (Polston et al., 2006). This lack of complete susceptibility and differences in ease of establishment of TYLCV infection among genotypes may explain many of the discrepancies in the literature regarding the status of pepper as a host and reservoir of TYLCV. Despite the lack of TYLCV-induced disease symptoms whiteflies were able to acquire TYLCV from infected pepper plants and transmit it to tomato (Polston et al., 2006). The symptomless but infected pepper plants can serve as virus reservoir for the acquisition and transmission of TYLCV. Other asymptomatic hosts of TYLCV may act as reservoirs in a similar manner.

The whitefly vector has a very large number of hosts upon which it can feed and reproduce. Reservoirs of the vector may also vary among production regions. TYLCV is transmitted persistently by the *Bemisia tabaci* species complex. Approximately 15–30 min of feeding by an adult whitefly are required for

acquisition of TYLCV, which is followed by a latent period of approximately 6 h. Transmission can then occur following a single 15–30 min feeding period (Cohen & Nitzany, 1966). Adult whiteflies can retain the virus for several weeks, spreading the virus as far as they range to feed. TYLCV has been shown to be acquired by immature whiteflies developing on infected plants. The adults however are responsible for the spread of virus to healthy susceptible host plants. Whiteflies are believed to be able to move relatively long distances (up to 7 km) over land and water using wind-assisted flight (Byrne & Bellows, 1991; Cohen et al., 1988).

Often, the most important source of TYLCV and whiteflies is old tomato fields. Whiteflies move readily from old plants to younger plants. The virus can spread very rapidly in older or abandoned fields where whitefly management has ceased. The earlier the infection occurs the greater the yield loss. Grape tomatoes, which have a long production cycle in Florida fields, can act as reservoirs of TYLCV and have been shown to cause an increase in incidence of TYLCV in the regions where they are produced. Although resistant cultivars produce an acceptable yield after TYLCV infection, they still support viral replication and can act as reservoirs of TYLCV for susceptible crops (Lapidot et al., 2001).

3. CROPPING PRACTICES

Management techniques are dependent upon the ecology of the virus as well as on the cropping production practices employed. Tomatoes are produced using different approaches in Florida and Israel due to differences in climate, economies of production, and the type of tomato produced.

Florida tomatoes are produced in five major production regions; two produce year-round, three have a 2–3 month tomato-free period. The climate is generally hot and wet in the summer, and cooler and drier in the winter. Tomato plants are initially sown in open-sided plant houses which are then transplanted to the fields after 4–6 weeks. Tomato transplants are planted into plastic covered raised beds of fumigated soil and later staked. A crop is in the field about 4–5 months before removal. The majority of the tomatoes produced are large globe types although there has been a significant increase in the production of grape tomatoes. Grape tomatoes are produced in a similar manner to the larger types, but are harvested for a minimum of 6–8 months. Throughout the extended harvest period few applications of insecticides for whitefly management are allowed.

In Israel, the majority of tomato production consists of indeterminate cultivars grown year-round in greenhouses or 50-mesh net-houses. In the past, tomatoes were grown in open fields during the spring and summer. Heavy infestations of whiteflies and high incidences of TYLCV were the major reasons for the transition from open field production to protected (greenhouse or net-house) production. Nearly all tomatoes produced in Israel are sown in commercial nurseries. Almost 4–5 weeks later they are transplanted to the field or greenhouse.

The major types of tomatoes produced are globe or flattened globe but the production share of cherry and cluster tomato is growing rapidly. Most open field tomatoes are staked. Processing tomatoes are grown without any support.

4. MANAGEMENT APPROACHES USED IN FLORIDA AND ISRAEL

4.1. Avoidance

4.1.1. Avoidance in time

Planting dates or locations that avoid high whitefly populations will often have a significant impact on incidences of TYLCV-infected plants. In addition, planting during these times can significantly increase the impact of the management tactics and may reduce the costs associated with them. In Israel, processing tomatoes are transplanted to the field in early spring, usually the end of March or the beginning of April, and are harvested 3 months later. In Israel, TYLCV spreads mainly during the late summer and autumn due to whitefly populations which peak from September to November. Processing tomatoes are harvested prior to the build up of large whitefly populations when incidences of TYLCV-infected plants are lower. When TYLCV appears late in the plants' development, impact on yields are minimal.

4.1.2. Avoidance in space

New plantings should not be located near old plantings. New tomato plantings should not be placed near any crops known to be hosts of TYLCV nor should they be located next to older fields of tomato, older fields of known susceptible crop species or any crop species where whiteflies are not managed. This is especially true of resistant tomato cultivars which may not show symptoms but may still act as sources of TYLCV for susceptible cultivars (Lapidot et al., 2001).

4.2. Plastic mulches

In open field production in Florida, reflective plastic mulches are used successfully to reduce incidences of TYLCV-infected tomatoes. The most effective reflective mulches are entirely or partially aluminized and reflect a lot of daylight. These are believed to reflect both visible and UV light which disorients whiteflies and decreases the landing of whiteflies on plants in the field. Like other mulches, the effectiveness decreases as the tomato canopy increases and covers the mulch. Reflective mulches are effective even when whitefly populations are expected to be high. This approach has the added benefit of interfering with other virus vectors (aphids and thrips) and is associated with lower incidences of several other tomato viruses.

The use of yellow plastic mulch to protect open-field tomato plants from the whitefly-borne TYLCV is a common practice in Israeli agriculture (Zaks, 1997; see also Part IV, Chapter 3). In 1962, Mound demonstrated that the color yellow

attracts whiteflies (Mound, 1962). It was suggested that yellow radiation may be a component of the whitefly's host-selection mechanism (Mound, 1962). It was found that the protection effect of the yellow mulch lasted about 20–30 days after transplanting, probably due to change with time in the ratio of canopy to mulch (Cohen and Melamed-Madjar, 1978). Nonetheless, 38 days after transplanting only 10% of the plants protected by yellow mulch showed TYLCV symptoms, compared to nearly 100% of the nonmulched control tomato plants (Cohen & Melamed-Madjar, 1978). The controlling effect of yellow mulch is due to a combination of the whitefly's attraction to the yellow color of the mulch and its consequent death due to dehydration induced by the high temperature of the mulch (Cohen, 1982; see also Part IV, Chapter 3). It should be noted that the typical Israeli climate is semiarid – high temperature and low humidity. In the tomato-growing regions, soil temperatures exceeding 30°C are quite common. It was demonstrated that at temperatures above 30°C, in low-humidity conditions, whiteflies not feeding on a plant dehydrate within an hour (Cohen, 1982).

Interestingly, yellow plastic mulches were not found to be effective in Florida (Csizinszky et al., 1996, 1999). The reason for this may be due to the very high level of humidity in Florida. Whiteflies which are attracted to the yellow mulch probably are not dehydrated as quickly in Florida as they were in Israel, where relative humidity is much lower. Whiteflies attracted to the yellow mulch in Florida were still able to fly to a plant and feed on it. In a climate with high relative humidity the yellow mulch may actually attract whiteflies to the crop rather than protect it from whiteflies. Although the yellow plastic mulches were ineffective in Florida, reflective or aluminized plastic mulches have been used very successfully to reduce incidences of TYLCV-infected plants (Csizinszky et al., 1996, 1999). In addition to reducing incidences of whitefly-transmitted viruses such as TYLCV, reflective mulches can also reduce incidences of aphid- and thrips-transmitted viruses.

4.3. Physical barriers

4.3.1. Whitefly-proof screens

Physical barriers such as fine-mesh screens have been used in the Mediterranean Basin since 1990 to protect crops from TYLCV (Berlinger & Lebiush Mordechi, 1996; Berlinger et al., 2002; Cohen & Antignus, 1994). Net houses covered by 50-mesh screens became a necessity due to the spread of TYLCV and its whitefly vector. The 50-mesh whitefly-proof screens decreased dramatically the number of invading whiteflies into covered net or greenhouses. Combined with a few insecticide sprays, the incidence of TYLCV in the covered structures 18 weeks after planting was only 1%. This was well below the level required for significant economic damage. In the control unscreened greenhouse TYLCV incidence reached 100% despite daily insecticide sprays (Berlinger & Lebiush Mordechi, 1996). Moreover, thanks to the reduced insecticide usage, it was possible to use bumblebees for pollination. It was shown that bee pollination for tomato is

cheaper and more efficient than hand pollination and increases yields by increasing fruit number per plant (Pressman et al., 1999). Adoption of physical barriers does add to production costs and screens can create problems of shading, overheating, and poor ventilation. The combination of 50-mesh screens and a positive pressure ventilation system may reduce whitely penetration as well as reducing the ventilation problems and over heating. It should be noted that although 50-mesh screens are indeed highly efficient in excluding whiteflies, these screens alone may not sufficiently protect against TYLCV since some whiteflies are still able to enter houses through gaps in entrances and on personnel. Some insecticide application should be combined when using screens.

4.3.2. UV absorbing plastics and screens

Good results have been reported in protected production in Israel when ultraviolet-absorbing plastic films were used as greenhouse covers or insect-proof nets (Antignus et al., 2001, 1998). UV-absorbing plastics reduce levels of UV light, blinding the whiteflies which use the light UV wavelengths to navigate. These UV-absorbing films have been shown to inhibit penetration of whiteflies into greenhouses and to reduce movement of whiteflies within greenhouses. TYLCV incidence in tomato grown under the UV-absorbing sheets was only 1% compared with approximately 80% in control conditions, without any application of insecticides (Antignus et al., 1996). Furthermore, filtration of UV light was shown to hinder the dispersal activity of whiteflies, and consequently reduce TYLCV spread (Antignus et al., 2001; see also Part IV, Chapter 3). It is important to note that besides the higher production cost, the use of these screens, may also result in increased temperature and humidity inside greenhouses.

4.4. Use of resistant cultivars

The use of TYLCV-resistant tomato cultivars, when available, is the best approach to reduce losses due to infection by TYLCV. Resistant commercial cultivars are available in a limited number of genotypes. A 30-year breeding effort by multiple programs has resulted in numerous cultivars with variable levels of resistance using genes derived from wild tomato species (Lapidot & Friedmann, 2002). Progress in introgression of TYLCV resistance has been slow. This is due to linkage with poor fruit quality, complex inheritance patterns, and the difficulty of transferring the resistance to commercial cultivars due to the presence of interspecific barriers between the wild and domesticated tomato species. Some of these resistances collapse under early or severe infection pressure and require additional cultural and chemical control measures that reduce whitefly populations to protect the plants from infection. The challenge today is a cultivar that combines high levels of resistance with high fruit quality. High-quality TYLCV-resistant tomato cultivars suitable for production in the Mediterranean region have become available only recently. Many of these

resistant cultivars cannot be grown in Florida due to the lack of bacterial and fungal resistances. In Florida, growers are still forced to rely on the use of cultural and chemical approaches.

4.5. Production and use of virus-free transplants

The production season should begin with the use of virus-free transplants. This can be accomplished by purchasing or producing tomato transplants in isolated areas away from production fields or houses. The greater the distance between production areas and nurseries, the lower the incidence of infected transplants will be. An antifeeding insecticide (such as pymetrozine) can be applied to transplants during their production, reducing transmission rates by whiteflies. Each application will give approximately 1 week of protection. Transplant production houses which are enclosed by 50 mesh or smaller screens can effectively exclude whiteflies and reduce the frequency of TYLCV-infected transplants. These houses must be well sealed and entrances constructed in such a way as to prevent whitefly intrusion. A positive pressure ventilation system used with 50 mesh screening can be even more effective. In the absence of transplant nurseries, floating row covers that exclude whiteflies have been used to produce transplants with lower rates of virus-infected plants (Cohen & Berlinger, 1986).

The use of a protective dose of a neonicotinoid in the transplant house a week before plants are set in the field will protect plants for the first 2 weeks (open field production). Young tomato plants are very attractive to whiteflies and are highly susceptible to TYLCV. Neonicotinoids have been shown to have a negative impact on pollinators; therefore, they are not always used in protected production. Application of a neonicotinoid insecticide in the setting water at the rate recommended could provide 8 weeks of protection (open field production).

4.6. Other cultural controls

4.6.1. Crop-free periods

A mandatory vegetable crop-free period of 2 months was found to be very instrumental in eliminating TYLCV or other vegetable-virus epidemics in the Arava region in Israel (Ucko et al., 1998; see also Part IV, Chapter 4). However, it should be noted that the Arava region is an arid area, where cultivated fields were the major sources of whiteflies in the region. A mandatory 3-month whitefly host-free period issued in the Dominican Republic was one component of a new TYLCV management effort that helped to drastically reduce losses due to TYLCV in the first 5 months of the tomato growing season (Polston & Anderson, 1997; Salati et al., 2002). However once whitefly populations reached their peaks in February and March, high incidences of TYLCV-infected plants could be seen in the fields, and losses could be significant in those later plantings (Polston & Anderson, 1997; Salati et al., 2002).

4.6.2. Sanitation

Old tomato plants are one of the best known reservoirs of TYLCV. Old tomato plant can support whitefly reproduction and TYLCV replication. Since old plants are not ideal hosts for whiteflies, the whiteflies produced on these plants are likely to be migratory morphs which are more likely to leave the field after emergence in search of better quality hosts. The removal of tomato plants immediately after harvest reduces whitefly populations in the area and helps reduce the movement of TYLCV into nearby plantings. Volunteer tomato plants in and around fields and production houses should be removed as they can also serve as reservoirs.

4.6.3. Weed management

Weeds in the fields should be kept at a minimum as they interfere with the application of insecticides and can harbor whitefly populations as well as virus. In Florida growers are recommended to eliminate or reduce abandoned crops, volunteer tomato plants and whitefly weed host reservoirs located on field edges and ditch banks. However, it has been found in Florida that wild plants outside the field that are not sprayed with insecticides can act as reservoirs of natural predators and entomophagous fungi that can be very effective in reducing whitefly populations. So it is recommended that growers not follow a "scorched earth" policy in pastures and wild areas near their fields. These areas should not be treated with herbicides and the natural whitefly predators and pathogens be allowed to function.

4.6.4. Roguing

In Florida growers have learned to identify early symptoms and rogue infected plants in the field early in the season by pulling and placing in a plastic bag, sealing tightly and discarding the bag. No studies have been conducted on this approach, but observational data suggests that this approach can be effective in some circumstances. Roguing young infected plants appears to reduce the amount of secondary spread within a field when incidences are low in the beginning of the season, such as might occur when TYLCV-infected transplants are used. This approach is not successful when higher rates of infection (greater than 10%) are present early in the season. Also this method becomes impractical when the plants have been in the field more than 6 weeks.

4.6.5. Use of integrated pest management scouting program

Particularly in open field production, scouts can be used very effectively to minimize incidence of TYLCV-infected plants. Although action thresholds for whitefly control have been developed, these are useful for limiting direct damage caused by whiteflies but are insufficient for the purposes of reducing incidences of plants infected with whitefly-vectored viruses. In Florida, many scouts take a zero tolerance approach to whiteflies especially early in the tomato production cycle. Scouts monitor whitefly populations weekly or twice

weekly for numbers of adults and for whitefly development on the tomato plants. They can recommend the optimal time to begin applying insecticides as well as alternative chemical controls.

4.7. Chemical approaches

Chemical approaches can be effective in reducing economic losses in tomato to TYLCV. A number of different classes of chemicals have been used to reduce whitefly populations including chlorinated hydrocarbons, organophosphates, neonicotinoids, pyridine-azomethines, and pyrethroids. Many of these chemicals are used primarily in field production. For protected production, fewer chemicals in fewer applications are required and desired due to the nontarget effects of these chemicals on pollinators. In many locations whiteflies have developed resistance to many of these chemicals and efficacies have decreased over time (Ahmed et al., 2001; Faria et al., 2000; Mason et al., 2000; Palumbo et al., 2001; Polston & Anderson, 1997; Villas Boas et al., 1997). In addition, the repetitive and frequent use of these insecticides in whitefly control has been responsible for outbreaks of secondary pests such as leafminers (Rafie et al., 1999). In addition to these insecticides, oils, insecticidal soaps, and insect growth regulators have been used. The most effective and widely used class of insecticides to reduce whitefly populations is the neonicotinoids of which at least three (thiomethoxam, imidacloprid, and dinotefuron) have been used to reduce incidence of TYLCV-infected tomato plants (Ahmed et al., 2001; Cahill et al., 1996; Polston & Anderson, 1997).

In Florida and in Israel neonicotinoids applied as drenches and less often as sprays, is the main line of TYLCV management. Neonicotinoids are used at a reduced rate in the plant house on tomato transplants for protection for the first 2 weeks in the field, and then are applied at higher rates in the setting water at the time of transplant. The setting water application is applied at a rate that gives approximately 8 weeks of whitefly control. Once whiteflies begin to develop on the tomato plants then a rotation of non-neonicotinoid insecticides such as insect growth regulators, oils and soaps, and several contact insecticides can be employed through final harvest. Resistance to neonicotinoids has been shown in several locations around the world (Cahill et al., 1996; Elbert & Nauen, 2000; Schuster & Gilreath, 2003). Guidelines for the management of resistance to nicotinoids have been compiled by the Insecticide Resistance Action Committee (IRAC) at www.irac-online.org for Florida tomato growers.

In Israel the majority of tomato is produced under nets or in greenhouses, and bumblebees are used routinely for pollination. It was demonstrated that bumblebee is the most efficient pollinator in greenhouse tomato (Zaks, 1997). However, the use of bumblebees in a greenhouse requires a different pest protocol approach, preferably a nonchemical approach. Insecticides application should to be monitored carefully so as not to adversely affect the activity of the bumblebees. Zeidan (2005) recommends the use of insecticides be stopped prior

to introduction of bees for crop pollination. However, many insecticide suppliers provide specific information regarding their effect on pollinators and on the timing of applications to minimize the effects on pollination.

5. CONCLUDING REMARKS

Tomatoes are grown in many climates and in many forms by both large and small growers. Unfortunately for tomato production, TYLCV has become widely distributed and is now found in many different tomato production areas. The management of TYLCV in these susceptible tomato cultivars is very difficult. For field production, a combination of a rotation of insecticides to control the whitefly vector in addition to cultural practices to reduce virus reservoirs and whitefly populations has been the most effective approach. In protected production, exclusion, resistant cultivars, roguing, and the limited use of insecticides is used. For either type of production when whitefly populations are high and a source of TYLCV is nearby these approaches are usually inadequate to save the crop. Resistant cultivars have been very effective in reducing losses to TYLCV. Unfortunately for many growers, resistant cultivars are not available in the type of tomato they produce nor are there resistant cultivars adapted for all production climates. Management will become much easier as more resistant cultivars are made available.

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CHAPTER 3

THE MANAGEMENT OF TOMATO YELLOW LEAF CURL VIRUS IN GREENHOUSES AND THE OPEN FIELD, A STRATEGY OF MANIPULATION

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1. INTRODUCTION

Tomato yellow leaf curl virus (TYLCV) (Cohen & Antignus, 1994) and some other viruses of the genus *Begomovirus* has become a limiting factor in tomato production worldwide. The wide global distribution of tomato crops and the dramatic outbreaks of the populations of the exclusive vector of TYLCV, *Bemisia tabaci*, led to a pandemic of this devastating disease. In the USA, the estimated losses caused by whitefly-transmitted geminiviruses reach about 20% of tomato production, but in the Dominican Republic, Cuba, Mexico, Guatemala, Honduras, Nicaragua, Costa Rica, Venezuela, and Brazil the damage is much greater, ranging between 30% and 100% of the yield. The losses in the Dominican Republic during 1989–1995 were estimated at $50 million (Polston & Anderson, 1997). In African countries, a total of 11,694,000 t of tomatoes were produced on an area of 602,744 ha with an average yield of 19.4 t/ha. As most of the production is in open fields, plants are exposed to infection by TYLCV, which results in severe epidemics of the disease (personal observations). In 1988, losses from this disease in Egypt were estimated at 32,000 ha (Nakhla et al., 1994).

The introduction of genetic resistance to commercial tomato cultivars has improved significantly the ability to reduce losses of the disease. However the horticultural quality of many of these varieties is still unsatisfactory and therefore there is a need for an integrated pest management (IPM) strategy. Viral plant diseases are not curable, and therefore prevention is the strategy of choice for their control. This chapter will outline the IPM elements that were developed during the last three decades to reduce the spread and damage of viruses belonging to the TYLCV complex.

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2. CHEMICAL CONTROL

Chemical control was reported successful against persistent and semipersistent viruses. Presumably in these cases there was sufficient time to expose the insect to a lethal dosage of pesticide, or enough pesticide to alter the behavior, which interrupted transmission (Perring et al., 1999). As in the case of other persistent viruses, the spread of TYLCV can be partially controlled by killing the vector through insecticide sprays (Sharaf, 1986; Cohen et al., 1974).

Control of *B. tabaci* to a level that will result in a significant reduction in virus infection has been difficult (Sharaf, 1986). Whiteflies feed on the under surface of leaves, and the waxy covering of immature insect stages provides protection from insecticides. However the introduction of the neonicotinoids group of insecticides (Horowitz & Isshaaya, 2004) into the market helped to improve control of the increasing whiteflies populations. This group of insecticides was found efficient in controlling sucking insects such as whiteflies, aphids, and leafhoppers. They have been used widely during the last decade for the control TYLCV and other members of the *Geminiviridae* family of viruses. This group of insecticides has a mild effect on beneficials, their efficacy for controlling insect pests and their versatile use render them important components in IPM programs. The frequent applications of neonicotinoids as well as insecticides of other groups led to development of resistance in the whitefly population, illustrating a selection process that diminishes significantly their efficacy (Dittrich & Ernst, 1990; Byrne et al., 2003). In Israel at the end of summer and during autumn, when the whiteflies population is at a peak it is impossible to prevent the spread of geminiviruses even if insecticide applications are frequent (e.g., three applications per week). The use of chemical control by itself against the spread of TYLCV and other begomoviruses proved inefficient and normally it is used to block the establishment of the vector *B. tabaci* within the crop to protect plants from direct damage.

3. CULTURAL MEANS

3.1. Eradication of source plants

Studies on the epidemiology of TYLCV in Israel have confirmed a positive correlation between population size of *B. tabaci* and the levels of virus spread. It was shown that *Cynanchum acutum* which is a common weed growing on the banks of the Jordan river, is the only perennial host in the region and serve as a source for TYLCV. Migrating viruliferous whiteflies are responsible for the spread of the disease in tomato production areas 7 km away from virus reservoirs. It was suggested that interference in the epidemiological cycle of TYLCV can be achieved by eradication of C. *acutum* in June–July before the beginning of the *B. tabaci* migration, and thereby reduce the primary spread of TYLCV in the region (Cohen et al., 1988).

In Cyprus in a relatively isolated region, the spread of TYLCV was successfully controlled by eradicating in the spring the primary inoculum sources in overwintered tomato, before emergence of adult whiteflies. In 3 consecutive years when this measure was undertaken, primary virus spread to spring plantings was almost completely prevented while further secondary spread to summer plantings was below 5%, compared with 40–50% in previous years (Ioannou, 1987).

The interference with the epidemiological cycle of viruses can be best implemented in desert ecosystems where cultivated crops are surrounded by sparse vegetation that does not support the creation of significant virus reservoirs. The success of this approach is demonstrated in Israel in the Arava valley which stretches along 180 km from the Dead Sea to the Red sea. An area wide sanitation program named "Pest-free Arava" is attempting to resolve the viral infestation problem. The program addresses the utilization of sanitation techniques for the alleviation of the damage caused by different viruses including TYLCV. The sanitation practices consist of synchronized sowing of different vegetable crops area-wise and the timely removal of crop residues from the fields. To this end, a crop free period of at least 4 weeks is imposed by law in mid-summer. The enforcement of this policy since 1986 diminished severe virus epidemics in this region of Israel. The occurrence of TYLCV in this area is significantly lower compared to other parts of the country (Ucko et al., 1998).

3.2. Bait crops

It was shown that by planting alternate rows of tomatoes and cucumbers (planted 30 days before tomato transplantation) the spread of TYLCV in the tomatoes was significantly delayed during the first 2 months (Al-Musa, 1982). The controlling mechanism of the cucumber bait plants is probably due to the combined effects of (1) the huge difference between the canopy volumes of cucumbers and tomatoes, and (2) cucumber is a much better host for whiteflies and once they land on this host they do not leave it as long as the plants are fresh. Tests in a flight chamber showed no preferences of adult whiteflies in landing on tomato or cucumber leaves, but once they land, cucumbers are preferred over tomatoes (Cohen, 1988).

The same approach was used later by Schuster (2004), who protected tomato from TYLCV by using squash as a bait plant. A greater cumulative proportion of TYLCV infected plants were observed in plots that were surrounded by tomato than on tomato plants surrounded by squash.

3.3. Physical barriers

The contribution of physical means to the IPM system is substantial. In general, any materials that are fine enough to physically deny insects' infiltration, but not too fine to provide the plants with light and adequate ventilation can be used for

this purpose. Among the materials that are being used are perforated polyethylene and polypropylene sheets (Agryl) (Cohen & Berlinger, 1986). The use of physical barriers was motivated by the heavy outbreaks of TYLCV epidemics in Israel that led to the development of insecticide-resistant *B. tabaci* populations. Fifty mesh screens were used to protect greenhouses walls and were proved highly efficient in blocking insect invasion (Berlinger et al., 1991). This type of protection is compulsory in many tropical and subtropical countries. However, the reduced ventilation in the screened structures may create heat stress that may affect crops like tomatoes during mid-summer when temperatures reach maxima. The lack of proper ventilation may result in increased humidity and enhances the spread of foliage diseases. It is recommended that during these critical periods, ventilation and/or cooling systems should be operated in the protected greenhouses to avoid overheating of the plants (Weintraub & Berlinger, 2004).

3.4. Optical barriers

3.4.1. The visual mechanisms of the insect eye

The use of optical barriers is associated with interference with the insect vision. Evolutionary contacts between insects and plants have created mechanisms that enable insects to detect and select their favorite plant hosts for feeding and oviposition. Vision (colors, shape, size) and olfaction (host odor) are the primary cues used by insects to orient to their plant hosts; sometimes the two cues work in concert (Prokopy et al., 1983; Dobson, 1994). Most adult insects have compound eyes, which are equipped to distinguish colors. Among compound-eye insects, though, the majority are bichromatic. This means that these insects have just two types of color pigment receptors and, as a result, they are not so efficient at distinguishing pure colors from mixtures of colors. Trichromatic insects, such as honeybees, have three types of pigment receptors, enabling to distinguish a wider spectrum of colors than bichromatic insects.

3.4.2. The role of UV in insect behavior

Unlike human beings insect can perceive in the UV range of the spectrum due to specific photoreceptors within their compound eye (Mellor et al., 1997). The UV component plays an important role in aspects of insect behavior, including orientation, navigation, feeding, and interaction between the sexes (Mazokhin-Porshnykov, 1969; Stark & Tan, 1982; Seliger et al., 1994). The involvement of UV rays in the flight behavior of some economically important insect pests has been studied by several researchers (Moericke, 1955; Mound, 1962; Kring 1972; Vaishampayan et al., 1975, Matteson et al., 1992; Issacs et al., 1999). The UV range (360–400 nm) forms a strong stimulus for whiteflies to fly. For example, the greenhouse whiteflyy *Trialeurodes vaporariorum* took off more readily and walked faster when exposed to light of wavelengths under 400 nm than

when exposed to that between 400 and 500 nm (Coombe, 1982). Mound (1962) correlated the reaction to UV of *B. tabaci* to the induction of migratory behavior, and showed that yellow wavelengths induced vegetative behavior, which may be part of a natural host selection mechanism.

Insect visual behavior is linked to a chain of events, which begins with their orientation to the plant from a distance and ends with their establishment on plants for feeding and oviposition (Coombe, 1982). By interfering with different links along this pathway, we may prevent a contact between the vector and the plant, thereby preventing plant infestation and/or viral infection.

3.4.3. Protection of open field crops by yellow mulches

The use of yellow mulches for the control of whitefly-borne viruses including TYLCV (Figure 1) has been reported (Cohen & Berlinger, 1986; Cohen & Melamed Madjar, 1974, 1978; Nitzany et al., 1964), The protection effect of yellow mulches was explained by the attraction of the whiteflies to the yellow color of the mulches and their subsequent killing by the reflected heat

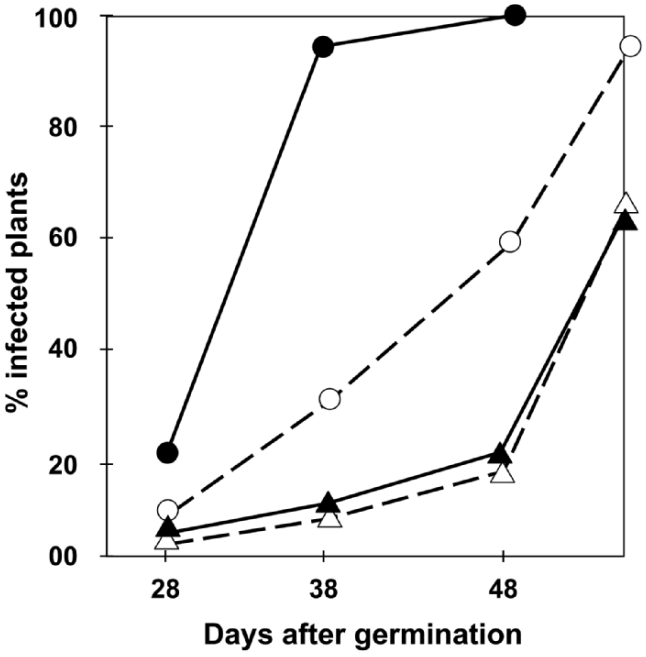

Figure 1. Effect of soil mulches on the spread of TYLCV in tomatoes grown in the open field. Open circles: straw mulch plus sprays of azinphos-methyl starting 10 days after germination; open triangles: mulching with yellow polyethylene sheets plus sprays of azinphos-methyl, starting 10 days after germination; filled triangles: mulching with yellow polyethylene sheets plus sprays of azinphos-methyl starting 20 days after germination; filled circles: unmulched control. (Copied from Cohen S. 1982).

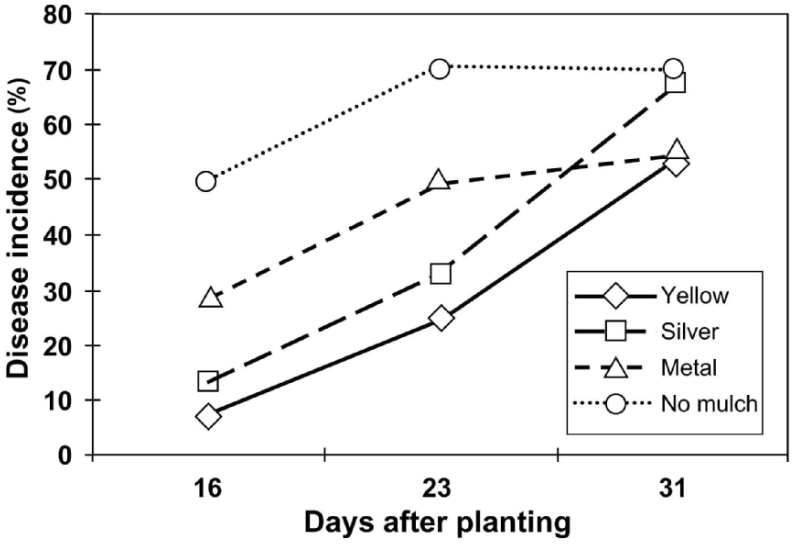

Figure 2. The effect of colored polyethylene mulches on the delay of squash leaf curl begomovirus (SLCV) infection in zucchini plants grown in the open field.

(Cohen & Melamed-Madjar, 1974). The control effect of mulches lasts for a relatively short period of 20–30 days. Changes in the ratio of canopy to mulch were suggested as a reason for the time-limited protection effect of the mulches (Cohen, 1982). In later studies carried out in Florida, Csizinsky et al. (1985), found that tomatoes were better protected against TYLCV by aluminum and orange plastic mulches compared to yellow mulches.

Field experiments carried out recently, demonstrated the protection effect of yellow and silver polyethylene mulches. Ten times fewer whiteflies were found on zucchini plants that grew over yellow or silver polyethylene mulches compared to plants that grew over bare soil (Antignus et al., 2005). In these treatments the delay in infection by *Squash leaf curl begomovirus* (SLCV) was close to 20 days (Figure 2). This delay resulted in a significant increase of yield in the mulched treatments compared to the unmulched control.

To study the mechanism of protection by soil mulches, light reflections from plants, soil, and the plastic mulches were analyzed to characterize the optical cues of the mulched plots. Spectral analysis of these reflections indicated various intensities of reflection along the entire UV and visible light spectrum (Figure 3).

Zucchini leaves form a peak of reflection at around 550 nm while light reflections of the yellow and silver mulches at this wave length are more intensive. Based on these results it is suggested that the protection effect of the yellow and silver soil mulches is associated with their relative high reflections

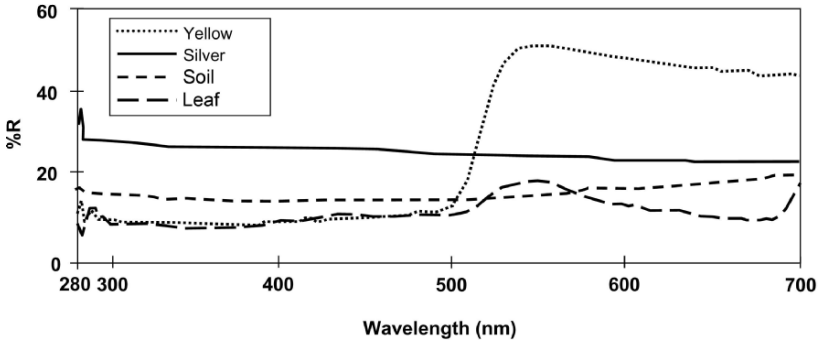

Figure 3. Analysis of light reflection by colored polyethylene mulches, soil, and leaves in the UV and the visible range of the spectrum.

at 560 nm. It is hypothesized that the plant image is more visible to the whitefly eye when contrast between the plant foliage and soil background is high. In other words plants that grow over the yellow or silver polyethylene are camouflaged from the insect eye by the relative high reflections of the plastic that form their background, resulting in a lower number of landing insects. The use of yellow or silver plastic mulches is highly recommended when tomatoes or other susceptible crops are grown in the open field in regions that are inflicted by high whitefly populations. The combination of soil mulches, chemical control, and the use of tolerant varieties is a reasonable combination of control means that enable efficient protection against TYLCV and other begomoviruses in crops grown in the open field.

3.4.4. Protection of greenhouse crops by UV-blocking cladding materials

Inhibition of insect infiltration: Polyethylene films are used as greenhouse cladding materials to provide efficient protection against wind, rain, and hail; they also act as radiation filters that enable growers to control light quality and intensity within the greenhouse. Originally UV-blocking polyethylene films were commercially produced to protect of greenhouse grown roses from petal blackening. However UV-blocking cladding materials have been found useful not only for horticultural purposes, but also as a tool to combat fungal diseases (Elad, 1997; Reuveni, 1997; Raviv & Antignus, 2004). Further studies have demonstrated the dramatic inhibitory effect of UV blocking, on the invasion of greenhouses by insects (Nakagaki et al., 1982; Antignus et al., 1996a, b, 2001a). UV-blocking polyethylene films are produced by the introduction of a UV-absorbing additive into the polyethylene raw material. The resulting modified polyethylene blocks over 95% of light transmission in the range of 280–380 nm, while allowing 80% transmission of radiation in the range, 380–700 nm (Figure 4). UV-blocking films allow the transmission of 5% of the total UV irradiation of

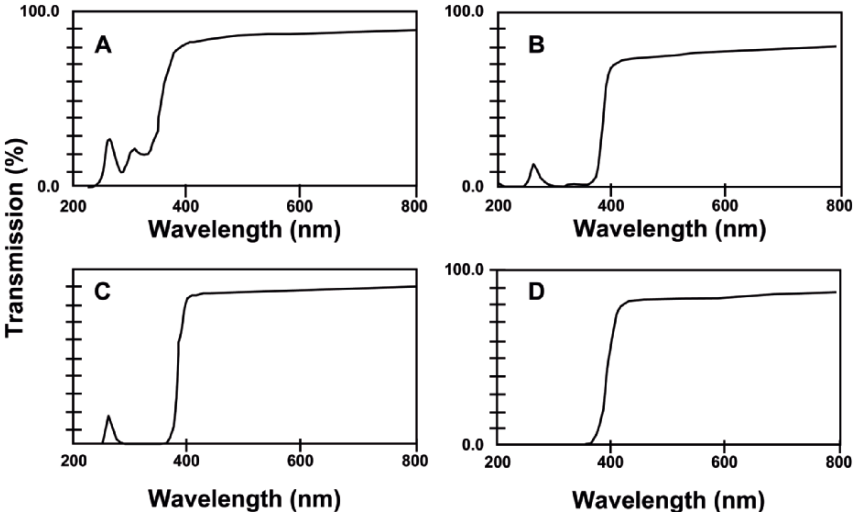

Figure 4. Light transmission spectra of standard and UV-blocking plastic films. (A) A standard polyethylene (Ginegar Plastic Products, Israel). (B) Solarig, a UV-blocking polyethylene (Palrig, Neot Mordechai, Israel). (C) IR-Veradim, a UV-blocking polyethylene (Ginegar Plastic Products, Israel). (D) Rav-Hozek, a UV-blocking PVC (Erez, Thermoplastic Products, Israel).

the sunlight compared to 13–20% that flow through films of the ordinary type. It has been shown that UV-blocking greenhouse cladding materials can reduce the entry of insects and the spread of insect-transmitted viral diseases of plants (Antignus et al., 1996b).

In experimental "walk-in" tunnels (6 × 6 m) the number of whiteflies (*B. tabaci*) trapped on yellow sticky traps under a UV-blocking film was 4–10 times lower than the number trapped under a standard film (Figure 5). In a similar experiment, the number of aphids (*A. gossypii*) recorded under UV-blocking films was ~100 times lower than that recorded under standard films (Figure 6; Antignus et al., 1996b) and these films reduced the invasion of thrips (*Frankliniella occidentalis*) by a factor of 10 (Figure 7; Antignus et al., 1996b).

In a similar set of experiments, UV-blocking films dramatically reduced the infestation of mint with nocturnal moths (*Spodoptera littoralis*, *Laphygma* spp.) and of leafminer flies (*Liriomyza trifolli*) that infested the same crop heavily under standard films (Antignus et al., 1997; Messika et al., 1999). The reduction in pest populations under the UV-blocking films enabled growers to reduce the number of pesticide applications by 50–80% from the usual level (Antignus et al., 1997).

Inhibition of the spread of viral diseases in greenhouses by UV-blocking films: The protective effect of UV blocking has a highly significant effect in reducing the spread of insect-borne viral diseases of plants. Tomatoes grown in "walk-in"

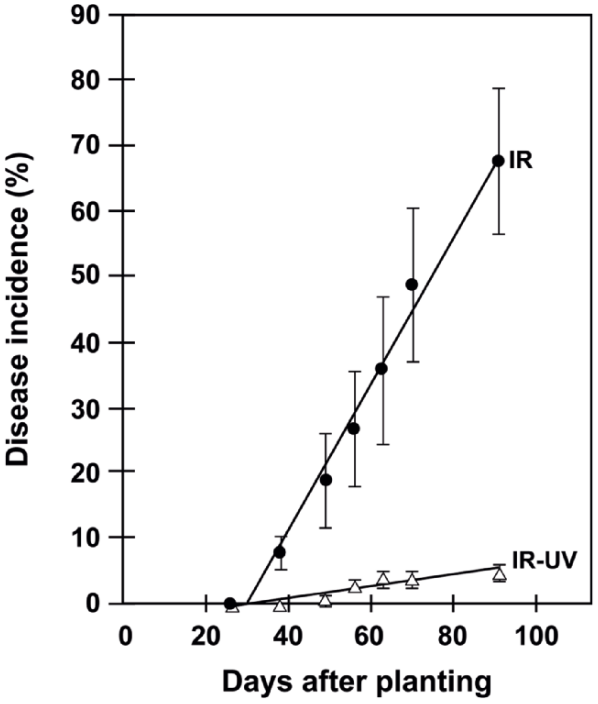

Figure 5. Catches of whiteflies on yellow sticky traps in "walk-in" tunnels covered with either standard (IR) or UV-blocking (IR-UV) polyethylene films. Means (+/–SEM) differ significantly at $P < 0.05$ when analyzed by one-way ANOVA.

tunnels covered with a UV-blocking film (without insecticide application), had a 1% infection rate with (TYLCV), compared with 80% infection for tomatoes grown under standard films (Figure 8; Antignus et al., 1996a).

In all experiments that tested the protection of tomato from whiteflies and TYLCV, the reduction in the rate of viral infection was always greater than the reduction in whitefly population density, which may indicate that beside the reduced invasion into the protected structures, the viral transmission efficiency is lowered under UV-blocking films.

Parameters affecting the efficiency of protection by UV-blocking films: The degree of the UV blocking by a plastic film determines its protective efficiency (Doukas, 2001). PVC films, which are highly efficient UV blockers, gave significantly better protection against insect pests than standard polyethylene films (Antignus et al., 1996a, b).

To achieve an adequate level of protection against large populations of insects, in addition to the UV-blocking roof, the greenhouse side walls should be covered with 50-mesh screens; a combination of a UV-blocking roof and

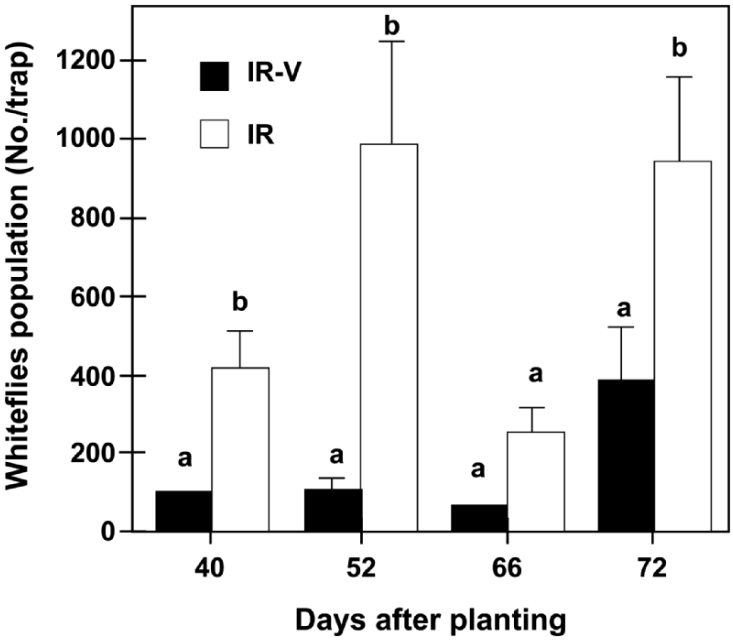

Figure 6. Catches of aphids on yellow sticky traps in "walk-in" tunnels covered with either standard (IR) or UV-blocking (IR-V) polyethylene films. Means (+/–SEM) followed by different letters differ significantly at $P < 0.05$ when analyzed by one-way ANOVA.

30-mesh screens failed to provide protection against whiteflies and the spread of TYLCV (Antignus et al., 1999). This is consistent with a report from California that UV-blocking roofs did not reduce the numbers of whiteflies in open-sided greenhouses, although reductions in the numbers of aphids and thrips were achieved in those greenhouses (Costa et al., 2002).

The effects of UV blocking on plants: No significant differences were found in growth, yield, maturing time, fresh or dry weights of tomatoes grown in greenhouses under standard and UV-blocking films. Physiological disorders were reduced by 38% under the UV-blocking plastic compared with their incidence under the standard material (Amagai et al., 1984). Similarly the yield and quality of peppers and cucumbers were not affected by UV blocking (Onuma & Nakagaki, 1982). No differences were found in pigment intensity and TSS of tomato and pepper fruits grown under regular and UV-blocking films, and the percentage of viable pollen grains in these plants were not affected by the type of polyethylene film covering (Pressman et al., 1996). The firmness and shelf life of tomato fruits were also unaffected by the type of polyethylene cladding (Antignus et al., 1999) and UV blocking had no effect on the yield and marketing quality of tomato and pepper (Antignus et al., 2001c). However, UV-irradiation is

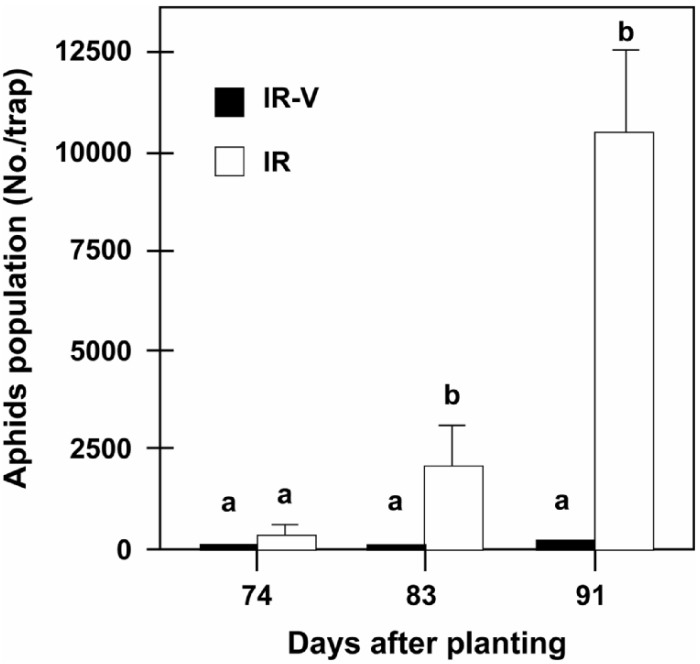

Figure 7. Catches of thrips on blue sticky traps in "walk-in" tunnels covered with either standard (IR) or UV-blocking (IR-V) polyethylene films. Means (+/–SEM) with different letters differ significantly at $P < 0.05$ when analyzed by one-way ANOVA.

required for the synthesis and accumulation of anthocyanins and flavonoids (Marcel et al., 1998), therefore, UV-blocking films may not be used to protect crops in which anthocyanin pigmentation is a determinant of their quality.

The effects of UV blocking on beneficial insects: The effect of UV filtration on the parasitizing activity of three commercially available parasitoids (*Aphidius colemani* Viereck, *Diglyphus isae* Walker and *Eretmocerus mundus* Mercet) was studied. The prasitoids preference between natural light and UV-filtered light was tested in the laboratory using Y-shaped pipe system. The parasitoids exhibited a significant preference for non-UV-filtered light. The ability of the parasitoids to locate a host infested plant from a distance (approximately 10 m) was tested in field trials. Host location by *A. colemani* and *D. isae*, expressed by parasitization rates was not affected by greenhouse covering plastic type (regular vs. UV-absorbing plastic). Conversely *E. mundus* was unable to locate the host infested plant when the latter was placed in the center of UV-absorbing plastic covered greenhouse. When the host-infested plants were located in the corner of the greenhouses and the wasps were released at the center, prasitization rates were lower under the UV plastic than under the regular plastic covered greenhouses.

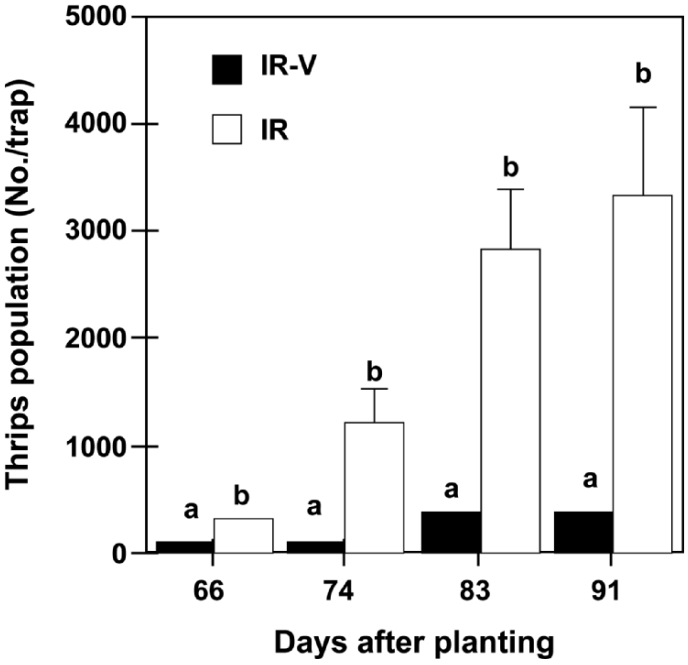

Figure 8. Tomato yellow leaf curl virus disease progression rate in tomato plants grown under standard (IR) or UV-blocking (IRV) polyethylene films. The infestation rate is described by linear regression. The estimated slope for IR-V was $b = 0.09$, whereas for IR $b = 1.06$.

From these experiments, it was deduced that UV-absorbing films can be used concurrently with *A. colemani* and *D. isae,* without harming their host location ability. *E. mundus* should probably be introduced in multiple release points, or close as possible to the *B. tabaci* infested foci in order to facilitate an efficient parasitization process (Chiel et al., 2006). The ability of parasitoids to perform in a UV-deficient environment was studied also by others who report similar results (Kajita, 1986; Doukas, 2001).

The effects of UV blocking on the activity of bumblebees: Bumblebees pollination is the standard technology for pollination of greenhouse grown tomatoes. No significant differences in bumblebee activity or in the numbers of flowers visited, under standard or UV-blocking films (Antignus et al., 2001c). The biomass and size of hives were not significantly affected by whether the greenhouses in which they were held were covered with standard or UV-blocking films (Antignus et al., 1999; Hefez et al., 1999; Seker, 1999).

Plastic model flowers of spectral properties similar to those of tomato flowers were used to evaluate bee search efficiency. The results show that the

bumblebees foraging behavior is not affected by removal of UV (Dyer & Chittka, 2004).

Putative mechanisms for the protective effects of UV blocking by greenhouse cladding materials: The protection model is supported by the following observations:

1. Significantly lower numbers of whiteflies are trapped over external greenhouse walls covered with UV-absorbing films or UV-absorbing nets than over those covered with standard materials (Antignus et al., 2001b).
2. When the arches that form the greenhouse roof are covered alternately with UV-absorbing and non-absorbing ordinary polyethylene films, a "two compartment effect" is created. Under these circumstances insects are attracted to areas located under the non-absorbing films where a relatively UV rich environment exist. Insects tend to aggregate in these sections of the greenhouse, while those under the UV-absorbing films remain insect-free.

A twofold mechanism is suggested for the protection effect of UV-absorbing films:

1. The flight orientation of whiteflies as well as many other insects is directed by the UV part of the spectrum. It is suggested that the reduced UV reflection from a greenhouse covered by a UV-absorbing roof is producing a UV-deficient compartment within the greenhouse, while the external environment that surround the greenhouse form a UV-rich zone. A disengagement of contact between the eye of a flying insect and the UV-irradiation source is diverting its flight from the UV-deficient zone toward the UV-rich compartment formed by the UV reflecting zones, away from the walls of the protected greenhouse.
2. Lack of UV radiation within the greenhouse alters the behavior of the invading insects, which exhibit reduced flight activity. Under these conditions the efficiency of virus transmission is reduced significantly.

4. CONCLUDING REMARKS

A significant progress was made in our understanding of the interactions of TYLCV with its host plants and the whitefly vector *B. tabaci*. However the impact of these outstanding findings on the ability to control this devastating viral disease is unfortunately negligible. Israel is confronting TYLCV disease since 1939 (Cohen & Antignus, 1994) and was therefore a leading country in the long struggle against the disease. The IPM system presented herein forms a successful model that can be adopted for TYLCV as well as for the control of other begomovirus-induced diseases in greenhouse and open field crops. The described elements of IPM pay attention to the sustainability of the production systems and the concerns over potential health and environmental hazards inflicted by the overuse of pesticides.

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CHAPTER 4

INTRODUCTION OF TOMATO YELLOW LEAF CURL VIRUS INTO THE DOMINICAN REPUBLIC: THE DEVELOPMENT OF A SUCCESSFUL INTEGRATED PEST MANAGEMENT STRATEGY

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1. OUTBREAKS OF A NEW WHITEFLY AND A MYSTERIOUS VIRUS-LIKE DISEASE DEVASTATE A FLOURISHING PROCESSING TOMATO INDUSTRY IN THE DOMINICAN REPUBLIC

The island country of the Dominican Republic (DO, Republica Dominicana) is located in the Caribbean Ocean, where it shares the island of Hispaniola with Haiti. The island lies to the West of Puerto Rico and to the East of Cuba and Jamaica. The DO is perhaps best known for producing some of the world's greatest baseball players, and agricultural products such as sugar and tobacco. The DO also had established a flourishing processing tomato industry, which was able to provide tomato paste to satisfy the needs of the ~7–8 million inhabitants of the country. This production was concentrated in two areas, Northern production area (around Santiago) and Southern production area (centered on the Azua Valley). The total area under production was ~8000 ha.

However, in the late 1980s, the DO began to experience large outbreaks of the sweet potato whitefly (*Bemisia tabaci*), which resulted in economic losses to common bean, cucumber, melon, and tomato production. The losses in common bean were due to damage by the whitefly and the whitefly-transmitted *Bean golden yellow mosaic virus* (BGYMV; genus *Begomovirus*, family *Geminiviridae*), whereas the losses in processing tomato were primarily due to direct damage from insect feeding, i.e., irregular ripening and sooty mold. Although the biotype of the *B. tabaci* in the DO was not determined at that point, the development of

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 279–303.

silverleaf symptoms on sentinel squash plants, planted around tomato fields in various locations, indicated that the B biotype had reached the island. By the early 1990s, mottling and mosaic symptoms in tomatoes were associated with high whitefly populations, and attributed to begomovirus infection. By 1991, high whitefly populations and this virus-like disease had caused significant losses to tomato production in the Azua Valley (Southern production area). Although these symptoms were reported to be caused by a Western Hemisphere bipartite begomovirus, it was not extensively characterized on the molecular level.

In 1992, a series of events occurred that were to change the way tomatoes were produced in the DO. Tomato production in the DO begins with the production of seedlings in nursery plots in the field. From these nursery plots, transplants are planted into production fields. In the fall of 1992, heavy rains and unfavorable environmental conditions led to the loss of seed nurseries and tomato transplants in the North. Farmers were forced to obtain transplants from other sources. Then, during the 1992–1993 growing season, unusual virus-like symptoms began to appear on tomatoes in the North, and these were associated with high populations of whiteflies. Affected plants showed stunted and upright growth, and leaves were small, curled, and crumpled and showed a striking chlorosis or yellowing (Figure 1A). Plants infected at early stages of growth were so severely stunted and bushy that they were referred to as "bonsai" or "broccoli" plants; they produced little or no fruit. Plants infected later developed symptoms on new growth, flowers aborted and there also was little or no fruit production.

This "new" virus rapidly devastated tomato production in the North (Figure 1B), and farmers that kept their fields obtained very low yields. Most of the 1993–1994 tomato production shifted to the Southern production area, but the "new" virus appeared, infecting many plants during flowering, and causing nearly 100% losses in some fields. By this point, overall tomato production in the DO had decreased by 75%, and losses were estimated at more than US$10 million. Moreover, to compensate for these tremendous losses, local canneries had to import tomato paste; for example, in 1993–1994, it was estimated that 16,000 t of tomato paste were imported. The collapse of local tomato production had a severe negative impact on the local economies in the North and South, which depended on the tomato crop as a source of employment.

2. IDENTIFICATION OF THE CAUSE OF THE "NEW" VIRAL DISEASE: INTRODUCTION OF THE EXOTIC *TOMATO YELLOW LEAF CURL VIRUS* (TYLCV) IN THE DO

By this time, experts in the areas of whiteflies and whitefly-transmitted viruses had visited the DO and observed the devastating disease. It was agreed that the disease was probably transmitted by whiteflies, and that the symptoms were different than those typically associated with Western Hemisphere whitefly-transmitted begomoviruses. Analyses of leaf samples with these symptoms, by squash blot

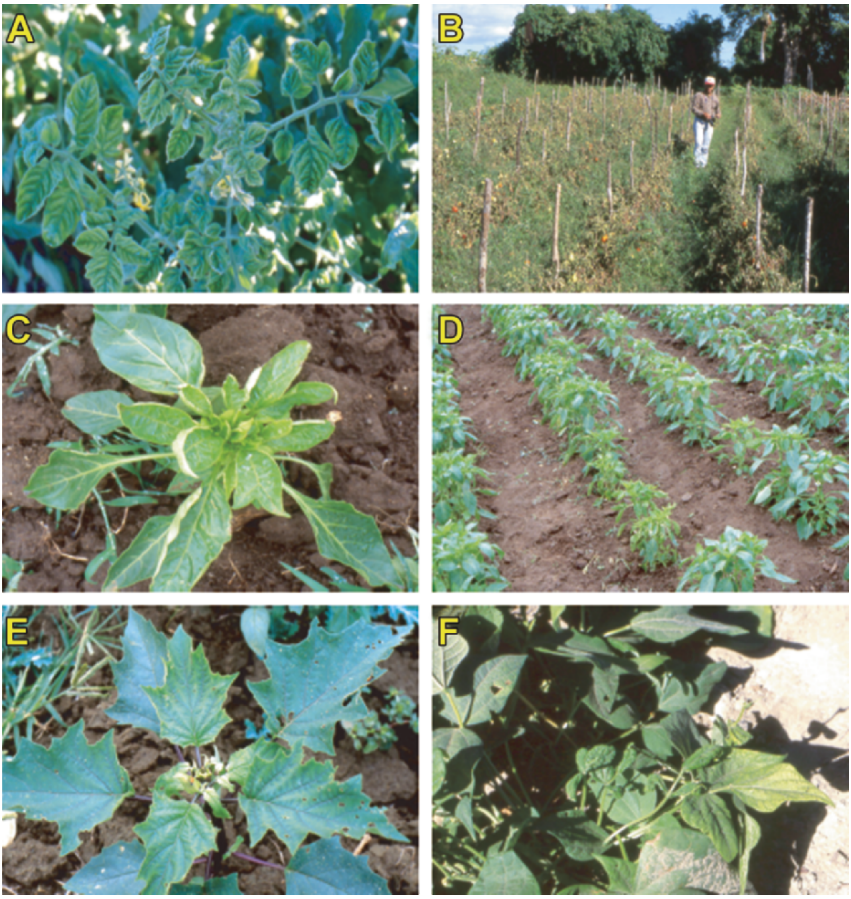

Figure 1. Disease symptoms caused by *Tomato yellow leaf curl virus* in the Dominican Republic. Typical tomato yellow leaf curl (TYLC) symptoms in (A) a tomato plant infected with TYLCV and (B) a tomato field. TYLC-like symptoms in (C) a banana pepper plant infected with TYLCV and in (D) a banana pepper field. TYLC-like symptoms in (E) *Datura stromonium* and (F) common bean (*Phaseolus vulgaris* cv. Topcrop) infected with TYLCV.

hybridization with a general probe for whitefly-transmitted begomoviruses, suggested that a geminivirus was associated with the disease (Nahkla et al., 1994). A breakthrough occurred when Dr. Douglas Maxwell of the University of Wisconsin noted the similarity between the symptoms of this "new" geminivirus disease and the tomato yellow leaf curl (TYLC) disease that he had observed in Egypt. However, the causal agent of this disease, *Tomato yellow leaf curl virus* (TYLCV; genus *Begomovirus*) was known to exist only in Old World locations.

Thus, to test the hypothesis that this mysterious disease was, in fact, caused by TYLCV, an overlapping primer pair (Patel et al., 1993) was designed that spanned a conserved *Nco* I site in the TYLCV C1 ORF (Rep protein gene), and would direct the amplification of a DNA fragment corresponding to the TYLCV genome. When these primers were used in the PCR with a DNA extract from tomato leaves showing typical symptoms of the "new" disease, a ~2.8 kbp DNA fragment (the expected size for the TYLCV genome) was amplified and cloned (Nakhla et al., 1994). The restriction map of the cloned fragment was identical to that of TYLCV (Israeli isolate; Navot et al., 1991), and the nucleotide sequence was 97% identical to that of TYLCV (Nakhla et al., 1994; Salati et al., 2002). These results provided unequivocal evidence that TYLCV had been introduced into the DO, and it was probably being spread by the indigenous *B. tabaci*. Similar findings also were being reported by Jane Polston and associates from the University of Florida (Polston et al., 1994; Polston & Anderson, 1997). The cloned TYLCV genome was then used as a DNA probe in squash blot hybridization analyses. All samples of the "new" virus disease from the North and South production areas hybridized strongly with the TYLCV probe. Thus, TYLCV had spread rapidly throughout the major processing tomato production areas of the DO.

TYLCV had become well established in the DO, but how was this devastating Old World virus able to get introduced into the New World? It could not have been in association with seed because geminiviruses, including TYLCV, are not seed-transmitted due to an inability to infect the embryo (Mink, 1993; Sudarshana et al., 1998). It also seemed unlikely that viruliferous whiteflies could have flown across the Atlantic Ocean. Thus, it seems that TYLCV had to have been introduced in association with plant materials. Indeed, it is generally believed that TYLCV was brought into the DO in transplants imported from an area with TYLCV (Polston & Anderson, 1997). Alternatively, the virus could have been introduced in another host plant, such as a perennial ornamental, or possibly in whiteflies carried on plants imported from an area having the virus. What is perhaps more important than the actual source of the virus is the larger lesson of the dangers of importing exotic damaging pests when moving living plants long distances. This highlights the need for careful regulation of the long-distance movement of live plants, particularly across borders and/or oceans (see Part 1, Chapter 4).

3. ESTABLISHMENT OF THE HOST RANGE OF TYLCV IN THE DO

An important question, in terms of developing a TYLCV disease management strategy, was to determine the distribution of the virus, in terms of geographic location and host plants (host range). To answer these questions, sampling surveys of the Northern and Southern production areas were conducted to (1) determine the spread of TYLCV and (2) identify potential TYLCV hosts/reservoirs, such as over seasoned and/or volunteer tomatoes, other crop plants

and weeds. Samples were tested for TYLCV infection by squash blot hybridization with a TYLCV probe. Tomatoes showing TYLC-like symptoms from the North, South, and Southwest (Enriquillo) production areas all were strongly positive for TYLCV infection, confirming that the virus had quickly spread throughout the island. These surveys also revealed that few over seasoned or volunteer tomatoes survived the hot dry conditions of the host-free period months (June–August) in the DO. However, the few over seasoned tomatoes that were found were strongly positive for TYLCV infection.

In terms of crop plants, about 10% of the pepper (*Capsicum anuum*) samples, showing a diversity of virus-like symptoms, were TYLCV-positive. Bean and tobacco samples were negative. One weed showed TYLCV-like disease symptoms, *Datura stromonium* (Figure 1E), and it was strongly positive for TYLCV infection in squash blot hybridization analyses. However, this weed is not very common and only a small proportion of plants actually showed TYLC symptoms. TYLCV infections also were detected in a small number of symptomless *Cleome viscosa* plants (~3%), a very common weed in the DO; and in a few other symptomless weeds (Salati et al., 2002). Weeds (*Jatropha* spp., *Sida* spp., *Malva* spp., and *Euphorbia* spp.) or common bean plants with golden mosaic symptoms all were negative for TYLCV infection. Thus, TYLCV appeared to have a fairly narrow host range, with tomatoes being the primary host, and pepper and a number of weeds being much less frequently infected.

4. DEVELOPMENT OF A TYLCV MANAGEMENT STRATEGY FOR THE DO

4.1. Implementation of a whitefly host-free period

The finding that TYLCV-DO was primarily a virus of tomatoes triggered a disease management strategy based on the idea that a tomato-free period could significantly reduce the initial inoculum pressure. This sort of approach had been previously used for TYLCV management in Cyprus and Israel, with some success (Ioannou, 1987; Ucko et al., 1998). Other important steps that were considered included: (1) evaluation of new tomato cultivars in order to identify those with potential resistance/tolerance and adaptation to tropical growing conditions, (2) production of disease-free transplants, (3) management of the whitefly vector, and (4) extensive sanitation efforts (i.e., prompt destruction of old plantings by plowing or some other method).

It turns out that, following the outbreaks of whiteflies in the Azua Valley in the mid- to late 1980s, a series of resolutions were issued through officials in the Ministry of Agriculture (Secretaria de Estado de Agricultura (SEA)) and the National Program of Integrated Management of Pests (Programa Nacional Manejo Integrado de Plagas (MIP)) aimed at instituting a host-free period (veda) in areas heavily impacted by the whitefly and, subsequently, TYLCV. Unfortunately, the extent and implementation of these host-free periods were

not sufficient to provide significant reduction in populations of whiteflies and/or incidence of TYLCV. In order to develop a more effective host-free period, a joint government–private industry committee was put together to oversee the development and implementation of a mandatory 3-month whitefly host-free period, which was imposed in the North and South processing tomato production areas (Figure 2). This period included the months of June, July, and August (the off-season for tomato production) in both production areas; and included whitefly hosts such as common bean, cucurbits, eggplant, melon, okra, pepper, and tomato. The new resolution differed from previous attempts because it had a greater regional scope; it was a cooperative effort between academia, government, and private industry; and the government agreed to actively enforce the resolution (i.e., searching for and destroying fields of violators). The enforcement of this "new and improved" resolution started in the 1995–1996 growing season and the few tomato crops that were planted, shortly after the host-free period, gave decent yields. This suggested that the host-free period might have a positive effect in reducing the TYLCV inoculum pressure.

To assess the compliance and the effect of the host-free period, field surveys were conducted in the North and South production areas early in the fall (i.e., September–October) of 1997–2003. The findings of these surveys can be summarized as follows: (1) the overall compliance with the host-free period was very good and relatively few violators were found, (2) very few volunteers and over seasoned tomato plants were found, (3) *Datura stromonium* plants with TYLC symptoms were found in these areas but at low incidences, and (4) no additional weeds with TYLC-like symptoms were observed (recall that weeds with the common yellow mosaic symptoms previously were negative for TYLCV infection).

Finally, these surveys indicated that the host-free period was gradually becoming an accepted practice for growers and crop consultants in the DO. This was because it was being recognized that the host-free period was successful in reducing whitefly populations and the incidence of TYLCV, thereby allowing for the recovery of the processing tomato industry. Furthermore, there are also a number of crops that can still be grown during the host-free period, including annuals such as onions, corn and sorghum, and perennials such as banana (plantain) and cassava.

4.2. Identification of improved varieties

From 1996 to the present, concerted efforts have been made by industry and government to screen tomato varieties for tolerance/resistance to TYLCV and adaptation to DO growing conditions. The traditional open-pollinated varieties grown in the DO (e.g., Napoli and UC82) were highly susceptible to TYLCV and yielded little or no fruit, even if infected later in development. Because relatively few TYLCV-resistant cultivars were available, the initial emphasis was on

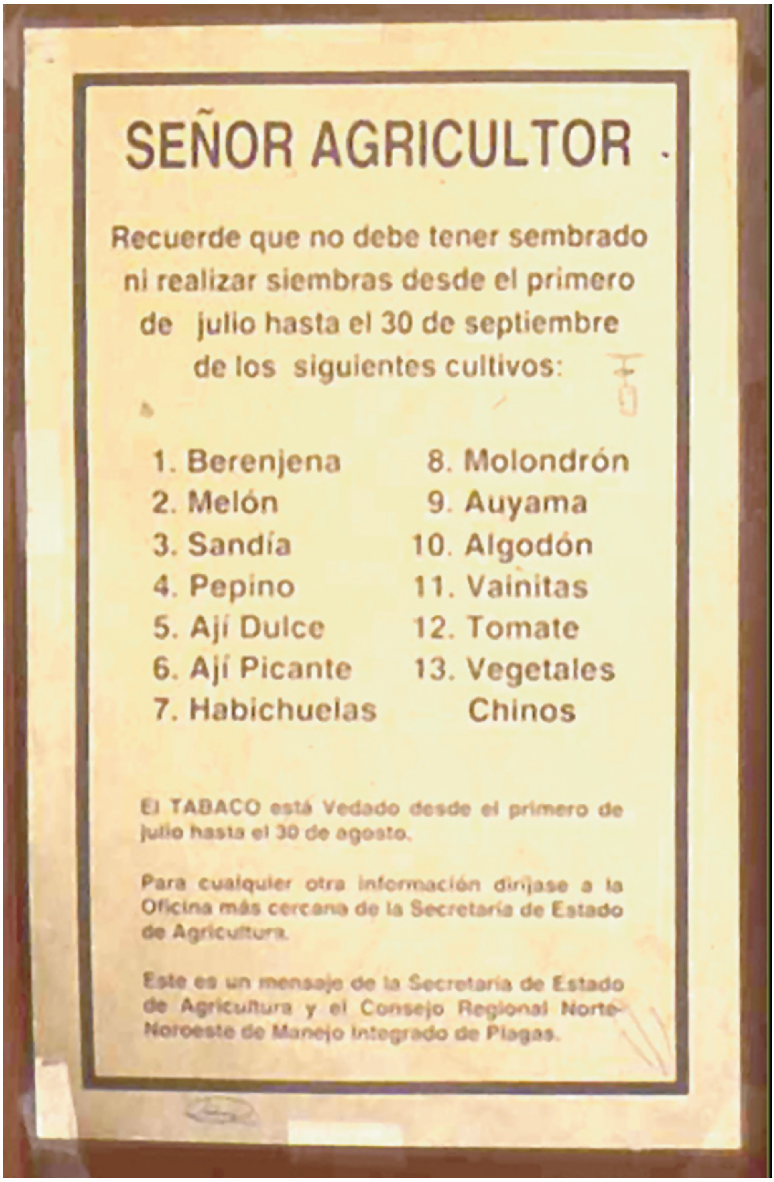

Figure 2. The resolution establishing the mandatory whitefly host-free period in major processing tomato areas of the Dominican Republic.

identifying early maturing hybrid varieties (i.e., ready for harvest within 114–116 days or earlier) that were adapted to the tropical conditions of the DO. The idea was that these varieties would flower and begin to set fruit during the window of low virus pressure provided by the host-free period. Through the efforts of the DO processing tomato industry, seed companies (e.g., Heinzseed, Campbell Seeds, Seminis, and Harris Moran) and tomato production experts (e.g., Mr. Donald May of the University of California Cooperative Extension) identified a number of well-adapted early maturing varieties. More importantly, it was also becoming clear that the host-free period was delaying the onset of TYLCV in tomato by as much as 6–8 weeks, which was sufficient time to allow these early maturing hybrids to flower and initiate fruit set.

In addition, a few *bona fide* TYLCV-resistant varieties were becoming available and one in particular, cv. Gempride (Seminis Seed Co.) was found to be relatively well-adapted and to show high levels of TYLCV resistance in the DO. Unfortunately, the fruit of this variety is not well-suited for processing (low solids or Brix), making it inferior on a horticultural level. However, by planting the susceptible early maturing varieties early in the growing season, when TYLCV pressure was low due to the host-free period, and the TYLCV resistant varieties later in the season when the TYLCV pressure was high, adequate tomato production could be achieved in the North and South production areas.

4.3. Emergence of neonicotinoid insecticides provides a valuable tool for whitefly management

Another important tool that helped in the management of TYLCV in the DO (and many other areas) was the development and emergence of the neonicotinoid class of insecticides. These insecticides have a high toxicity against whitefly adults and nymphs (and even eggs in some cases), but a relatively low mammalian toxicity. Moreover, these materials move systemically within the plant, allowing for uptake by piercing–sucking insects such as whiteflies and aphids, and can provide protection for 30–60 days. Initially, these materials had to be applied as a soil drench or via drip irrigation but recently formulations have been developed that can be applied to foliage. The first of these materials was imidacloprid, followed by others such as thiamethoxam and acetamiprid.

These materials have been extensively used worldwide for whitefly management, with great success, and effective whitefly control can also sometimes reduce the incidence and spread of begomoviruses. In the DO, the use of these materials reduced whitefly populations and, to a lesser extent, virus incidence. However, once the host-free period was implemented, field tests showed that multiple applications of these insecticides did not provide a significant benefit in terms of reduced TYLCV incidence or increased yield, thereby allowing for reduced pesticide application and production costs.

4.4. Sanitation

In order for the host-free period to be effective, it is imperative that extensive regional sanitation is practiced after the tomato crop. Thus, in the DO, extensive efforts have been made to educate farmers of the importance of destroying old crops, preferably by some form of cultivation (e.g., deep plowing or disking). Together with the hot dry temperatures that occur during the host-free period, these sanitation efforts result in little or no survival of old tomato plants into the next season in both the North and South production areas. Similarly, these same factors minimize the survival of tomato volunteers coming from seeds of fruits that have fallen during harvest.

5. THE APPLICATION OF BIOTECHNOLOGY TO SHOW THE BENEFICIAL EFFECT OF THE HOST-FREE PERIOD

The implementation of a mandatory host-free period over large areas of the DO was a draconian measure, but one that was clearly needed given the magnitude of the losses experienced by the industry. Nonetheless, this approach was controversial and there was a need for some way to assess whether the host-free period was having the intended effect: reducing the amount of TYLCV inoculum in the tomato production areas.

The approach used to address this question was to use the incidence of TYLCV in whiteflies as an indirect measure of the relative amount of virus in the agricultural ecosystem. This approach involves collecting adult whiteflies from different locations in the North and South production areas, and determining the presence of the virus by PCR with a TYLCV-specific primer pair (Mehta et al., 1994). Whiteflies (~30 adults/location) are collected at monthly intervals from tomatoes (during the growing season) or from weeds or other crop plants (during the host-free period) from each of 6 locations in the North and 9–13 locations in the South. The insects are placed into ethanol in screw-capped tubes and then shipped to UC Davis. DNA extracts are prepared from the whiteflies, and PCR analyses conducted with a TYLCV-specific primer pair that directs the amplification of a ~330 base pair DNA fragment (Salati et al., 2002). Southern blot hybridization analysis with a TYLCV-specific probe is used to confirm the identity of the PCR-amplified fragments. As a control, whiteflies are also collected from tomato growing areas that are not included in the host-free period (e.g., Ocoa and Paya) and tested for TYLCV in order to confirm that any reduction in the incidence of virus was due to the host-free period. This monitoring was initiated for the 1996–1997 growing season and is still being conducted.

As can be seen in Figure 3, the whitefly host-free period results in a marked reduction in the percentage of whiteflies carrying TYLCV in both the North and South production areas, thereby allowing for a window of low virus pressure (September, October, and part of November in some years). The results

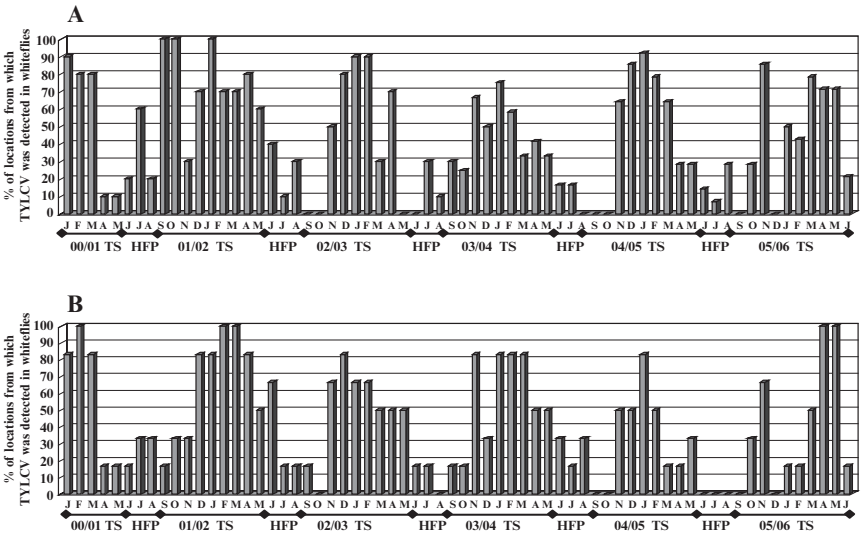

Figure 3. Percentage of locations (fields) from processing tomato production areas in the (A) north and (B) south of the Dominican Republic from which *Tomato yellow leaf curl virus* (TYLCV) was detected in samples (~30) of adult whiteflies. Adults were collected at monthly intervals during the tomato growing season (TS; September–May) and the host-free period (HFP; June–August). TYLCV was detected by PCR with TYLCV primers, and Southern blot hybridization analysis was used to confirm the identify of PCR-amplified TYLCV fragments.

have been striking; high incidences of TYLCV in whiteflies are consistently detected during the peak months of the tomato growing season (December–February), whereas relatively low incidences are detected from whiteflies collected from weeds and other hosts during the host-free period (Figure 3; Gilbertson et al., 1998; Salati et al., 2002). Importantly, the level of virus in whiteflies remains low in the beginning of the tomato growing season (September–October). Virus incidence in whiteflies increases in November, peaks in December–March, and then declines as the season ends (April–May) and the host-free period starts (June). Evidence that it is the host-free period that causes the reduction in TYLCV in the North and the South production areas was provided by the finding that whiteflies collected from Ocoa and Paya, areas without a host-free period, had high incidences of TYLCV throughout the year. Finally, it is important to note that the host-free period also results in significant reductions in whitefly populations in the North and South production areas of the DO. Further reduction in whitefly populations are also mediated by yearly rains that occur in August and September.

The detection of TYLCV in whiteflies was highly correlated with the incidence of TYLC in tomatoes. During September and October, when the incidence of TYLCV in whiteflies was low, the incidence of TYLC in tomato was generally low. By November and December, as the incidence of TYLCV in

whiteflies increases, the incidence of TYLC symptoms in tomato plants in the field also increases dramatically, often reaching 100% in some fields by the end of December. By January and February, when TYLCV is detected in most whitefly samples, the incidence of TYLCV in many fields in the North and South can be as high as 100%. Taken together, these results established that: (1) the host-free period resulted in a reduction in TYLCV incidence in whiteflies and the agricultural system as a whole, (2) the level of TYLCV in whiteflies is a good indicator of TYLC incidence in the field, and (3) tomatoes play an essential role in the build-up of TYLCV in whiteflies after the host-free period.

In summary, the "cleaning out" of the virus, mediated by the host-free period, occurs in both production areas, and has continued over a 10-year period. This suggests that there have been no major changes in the biology of the virus (i.e., adaptation to a new host; see Section 9 below). A biotechnological tool (PCR) was used to directly demonstrate the benefit provided by the host-free period. Finally, the host-free period has now been widely accepted as a part of normal agricultural practices in these regions.

6. RESERVOIR HOSTS FOR TYLCV IN THE DO: A LESSON SHOWING THE PARADIGM THAT VIRAL HOST RANGE IS A CONTINUUM OF RESPONSES RATHER THAN A SIMPLE "YES OR NO" PHENOMENON

6.1. Viral host range represents the complexity of the host–pathogen interaction

Before discussing the host range of TYLCV in the DO, it is important to define some concepts. It has become clear for geminiviruses, and other viruses as well, that host range is a continuum ranging from hosts in which severe or typical symptoms are observed to hosts where few or no symptoms are observed (Dawson & Hilf, 1992; Wang et al., 1999). Hosts in which typical symptoms are observed tend to sustain high viral titers and are hosts to which the virus is well-adapted; whereas hosts in which few or no symptoms are observed tend to have low viral titers and are hosts to which the virus is poorly adapted (i.e., the virus does not replicate or spread well) (Hou et al., 1998). There also can be a continuum of responses within a crop species, e.g., differences between resistant and susceptible varieties, or in responses of germplasm that has been derived from different origins of domestication (e.g., the differential reaction of bean germplasm from the Andean or Middle American gene pool to *Bean dwarf mosaic virus*) (Seo et al., 2004). This continuum of virus–host interactions can impact the capacity of an insect to acquire the virus. For example, insects tend to acquire the virus more efficiently from hosts to which the virus is well-adapted and/or is present in high titers.

In terms of TYLCV in the DO, the virus is clearly best adapted to tomato, as susceptible varieties have typical symptoms and high viral titers. Of course, this

situation may be altered, to the advantage of the host, in a resistant variety such as Gempride, where few symptoms are observed and the viral titer may be lower (see below). The other host to which TYLCV appears to be well adapted is *D. stromonium*. However, the case was not so clear for pepper and common bean.

6.2. TYLCV and pepper in the DO: To be or not to be a TYLCV host

There has been considerable interest in understanding whether pepper (primarily banana pepper, *C. anuum*) is a TYLCV host in the DO, particularly in terms of whether it should be included in the host-free period. One confounding factor in understanding the role of pepper as a TYLCV host in the DO is the fact the pepper is commonly infected with aphid-transmitted RNA viruses such as *Cucumber mosaic virus*, *Tobacco etch virus*, and *Potato virus Y*. Thus, the symptoms induced by these viruses can be confused with or possibly mask those caused by TYLCV. However, the results of over 10 years of field observations have clearly shown that, compared with tomato, pepper is not a good host of TYLCV. For example, in pepper fields next to tomato fields with high incidences of TYLCV, it is common to see no obvious disease symptoms or only the leaf mosaic/mottle/distortion symptoms characteristic of RNA viruses. Leaves from such plants are usually negative for TYLCV infection by squash blot hybridization tests.

The failure to observe TYLC symptoms in peppers could be due to (1) pepper not being a preferred whitefly host and/or (2) pepper not being a host to which TYLCV is well adapted. Our surveys suggested that pepper was not a preferred whitefly host, as whitefly populations are often low or even absent on pepper, even when high populations are present on beans, cucurbits, and tomatoes. However, a small number (3/30, 10%) of pepper samples, collected in a squash blot survey conducted from 1996 to 1999, were positive for TYLCV infection. Furthermore, the symptoms in the TYLCV-positive plants were stunted growth and upcurled leaves, suggestive of TYLCV infection. When the more sensitive PCR assay was used with peppers showing a range of symptoms, much higher rates of TYLCV infection were detected (23/26; 89%) and many of these were symptomless infections. This led to the hypothesis that pepper was a host of TYLCV in the DO, but perhaps one to which TYLCV is not particularly well adapted.

Our field surveys, conducted in the DO over the past ~10 years and at different times of the year, led to a finding about the TYLCV–pepper interaction that provides an interesting practical example of the complexity of the virus–host interaction and the influence of environment. As mentioned above, peppers generally do not show TYLC-like symptoms, even when growing next to tomatoes with high TYLC incidences. However, surveys conducted at the end of the tomato season in Santa Maria in the North (end of January 2000) and in the Azua Valley in the South (end of February 2003), revealed a situation where new pepper fields (plants in the early vegetative stage of growth)

were established nearby old tomato fields in which plants had high incidences of TYLCV and were senescing (dying) quickly. Examination of young pepper plants in these fields revealed populations of whiteflies (~5–10 insects/plant), and some plants that showed TYLC-like symptoms including stunted growth, shortened internodes, and upcurled and chlorotic leaves (Figure 1C and D). These symptoms were clearly different than those typically associated with RNA virus infection.

Analyses of leaves of these symptomatic pepper plants by squash blot hybridization with a TYLCV probe revealed TYLCV infection in some plants, but the hybridization signals were weak, suggesting low viral titers. Definitive evidence that some of these pepper plants were infected by TYLCV was provided by PCR and Southern blot hybridization analyses. To further confirm TYLCV infection in these pepper plants, a 1.6 kb fragment was amplified from one of the PCR-positive pepper plants with the degenerate primers PAC1v1978 and PAR1c715 (Rojas et al., 1993) and cloned and sequenced. The sequence of this fragment, which included portions of the C1, C4, V1, and CP genes, was 99% identical to that of TYLCV-DO. Furthermore, Southern blot hybridization analyses of total genomic DNA extracted from two of the PCR-positive symptomatic pepper plants revealed strong hybridization signals corresponding to begomovirus ss- and ds-DNA forms, whereas no hybridization signals were observed from DNA extracted from symptomless PCR-negative pepper plants. Taken together, these results demonstrated that TYLCV had the capability to infect peppers in the DO, but that many infections were symptomless. However, it appears that TYLCV may induce TLCV-like disease symptoms in peppers in situations where new pepper fields are established near old tomato fields with high populations of whiteflies and high rates of TYLCV infection. Under these conditions, highly viruliferous whiteflies will feed (to some extent) on peppers, thereby delivering sufficient virus at a critical stage of development (i.e., young rapidly growing transplants) to result in a symptomatic infection. In cases where tomatoes and other crops are available, whiteflies will preferentially feed on these hosts, thereby resulting in less virus pressure on peppers, and lower rates of infection and/or symptomless infections.

Our findings of TYLCV infection of pepper in the DO are consistent with reports showing an association between TYLCV infection and symptoms of dwarfing, leaf curling, and yellowing in *C. anuum* and *C. chilense* plants in Jamaica (Roye et al., 1999), and a report of TYLCV infecting peppers in Spain (Reina et al., 1999). Subsequently, TYLCV was reported to infect peppers in Cuba (Quinones et al., 2002). Although all of these reports strongly suggested that peppers were infected by TYLCV, the definitive whitefly transmission experiments had not been conducted. Recently, whitefly transmission of TYLCV to pepper has been reported and, in both cases, the resulting infections were symptomless. However, in the study of Morilla et al. (2005), whiteflies were unable to acquire and transmit TYLCV from pepper, leading to the conclusion that it was a "dead-end" host. However, in the study of Polston

et al. (2006) whiteflies were able to acquire TYLCV from pepper and transmit it to tomato. These studies confirmed that pepper is a host of TYLCV, and suggested that this host could play a role in the epidemiology of TYLC in tomato. In terms of the DO, there is a legitimate concern that inefficient whitefly transmission from pepper to tomato, shortly following the host-free period, could result in an earlier onset of the disease in tomato, thereby reducing the TYLCV-free "window." Taken together, these results strongly suggest that peppers are a TYLCV host in the DO and, thus, should be included in the host-free period.

6.3. TYLCV and common bean in the DO: disease phenotype depends on the pedigree of the cultivar

Common bean is another host that is included in the host-free period, but does not show obvious symptoms of TYLCV infection, even when fields of beans are grown adjacent to tomato fields with 100% TYLC symptoms. However, TYLCV infection in beans was detected in plants (five of six) showing mottle/mosaic symptoms from a field near a tomato field with a high incidence of TYLCV. This suggested that TYLCV might be infecting beans in the DO, but not causing TYLC-like symptoms.

To further investigate this possibility, a small plot of beans of the cultivar (cv.) Topcrop, a large-seeded genotype of the Andean gene pool highly susceptible to begomovirus infection, was planted in early January 2001, next to a tomato field with TYLC established in early October 2000 at Projecto 2C. This plot of common beans was surveyed February 2, 2001, and many plants showed symptoms of strong stunting and leaf epinasy, crumpling and chlorosis (Figure 1F). Leaves from these plants tested positive for TYLCV infection by PCR with TYLCV-specific primers. In Southern blot hybridization assays, TYLCV ss- and ds-DNA forms were detected in total genomic DNA extracted from the three PCR-positive plants. In 2001, this experiment was repeated, and a plot of cv. Othello, a medium-seeded pinto bean of the Middle American gene pool, was planted along with the cv. Topcrop in early January 2002, adjacent to a tomato field with a high incidence of TYLCV. These plots were surveyed in late January 2002. Unlike the previous growing season, there was a high incidence of bean golden mosaic disease, making it difficult to differentiate TYLC symptoms. However, PCR analyses revealed high rates of TYLCV infection in symptomatic cv. Topcrop plants (8/10), as well as infections in symptomatic (2/2) and symptomless (2/3) cv. Othello plants. Some plants had a mixed infection of TYLCV and BGYMV. Together, these results indicate that TYLCV can induce both symptomatic and symptomless infection in common bean in the DO, and that symptom development may relate to the gene pool from which the cultivar was derived (i.e., more severe symptoms in large-seeded Andean cvs.).

These findings are in agreement with those of Navas-Castillo et al. (1999) indicating that TYLCV can cause a leaf crumple disease of common bean in

Spain. Moreover, the beans that developed these symptoms in Spain were staked fresh market beans, likely of the Andean gene pool. Subsequently, Lapidot (2002) used whitefly inoculation of a collection of bean cultivars to show that (1) some bean cultivars were highly susceptible to TYLCV whereas other were not, and (2) some cultivars developed a symptomless infection. The relative susceptibility depended on the common bean genotype, but the materials were not classified according to gene pool.

Thus, common bean is clearly a host for TYLCV in the DO. The failure to observe TYLCV symptoms (i.e., the bean leaf crumple disease; Navas-Castillo et al., 1999) in beans in the field is due to the fact that many of the genotypes grown in the DO, such as pinto-type beans, develop mild or symptomless infections. However, it is clear that it is necessary to include common bean in the host-free period.

6.4. TYLCV resistant cultivars: a possible Typhoid Mary

Some commercial tomato cultivars, such as Gempride, are highly resistant to TYLCV in the DO and do not show obvious disease symptoms. However, it was not clear whether these symptomless plants are infected with TYLCV (a resistance situation), or contain no virus (immunity). To determine whether cv. Gempride could be infected by TYLCV, leaves were collected from 15 Gempride plants (5 from each of 3 replicates) that were part of a variety trial conducted in Km 13 in the South (Azua). The positive control consisted of leaves collected from a plant with typical TYLC disease symptoms. This plot had a very high incidence of TYLCV (most entries showed 100% TYLC by 35 days after transplanting), and there were moderate to high whitefly populations on all materials, including Gempride. PCR analyses revealed TYLCV infection in leaves from all 15 Gempride plants, as well as in the leaves of the control plant; moreover, the intensities of the DNA bands amplified from the resistant vs. susceptible materials were not noticeably different. Thus, cv. Gempride is clearly resistant rather than immune to TYLCV, and it should not be grown during the host-free period as it is capable of carrying a fairly heavy viral load and serving as a reservoir for TYLCV.

Further evidence for TYLCV infection in cv. Gempride came for the apparent development of TYLC symptoms in a late-planted field in February 2003. This field was still early in development (late green to early ripening fruit stage) this late in the season. Plants had very high whitefly populations (hundreds per plant), large amounts of honeydew on the leaves and fruits were showing symptoms of irregular ripening. One plant in this field had developed clear TYLC-like disease symptoms in the new growth (Figure 4). TYLCV infection in this plant was confirmed by squash blot hybridization analysis with a TYLCV-specific probe and PCR analysis with a TYLCV-specific primer pair. These results suggests that the high whitefly and virus pressure (whiteflies collected at this time were carrying heavy

Figure 4. Tomato yellow leaf curl symptoms developing in a plant of the resistant variety Gempride in a late planted field subjected to high populations of viruliferous whiteflies.

viral loads) may be able to overcome levels of resistance in cv. Gempride, as has been reported in southern Europe.

7. SYMPTOMLESS WEED HOSTS: RESERVOIRS FOR TYLCV DURING THE HOST-FREE PERIOD

Although the host-free period greatly reduced the level of TYLCV inoculum, the virus reappears every year during the tomato growing season and this occurs in the North and South production areas. This indicated that there was a reservoir host(s) harboring TYLCV, and that this host(s) was relatively widely distributed. As previously mentioned, surveys for reservoir hosts revealed *D. stramonium* as the only symptomatic weed host of TYLCV in the DO (Salati et al., 2002; Figure 1E). However, it was not widely distributed in the North or the South, nor is the incidence of TYLCV symptoms usually very high. Thus, *D. stramonium* is probably not the major reservoir host for the virus in the DO. These surveys also revealed that very few old tomatoes survived the host-free period (often none were found in a given year), nor were there many volunteer tomato plants. However, two observations led to the consideration of symptomless weeds as reservoirs of TYLCV. First, our field surveys often revealed that TYLC symptoms first appeared on the edges of fields (i.e., an edge-effect) (Figure 5). Second, as previously indicated, TYLCV infection of a small number of symptomless weeds was detected by squash blot hybridization. These weeds

Figure 5. The "edge effect" showing the initial appearance of tomato yellow leaf curl symptoms in plants at the outer edges of the field. This was consistent with the virus moving in from surrounding reservoir hosts.

included *Cleome viscosa* L. (six plants), *Polygonum* spp. (one plant), *Malva* spp. (one plant) and *Euphorbia* spp. (one plant).

The infrequent detection of TYLCV in these potential weed hosts by squash blot hybridization suggested that perhaps the viral titer was below the threshold of detection. Thus, a modification of the standard PCR method for detection of TYLCV in tomato leaf tissues was employed in which a fivefold excess of DNA extract was used in the PCR with TYLCV-specific primers, and Southern blot hybridization analysis was used to detect amplification products that were below visual detection in agarose gels (Salati et al., 2002). Indeed, when this method was used for samples of symptomless weeds collected from 1998 to 2000, TYLCV

Table 1. Detection of *Tomato yellow leaf curl virus* (TYLCV) in weed plants sampled in the Dominican Republic from 2000–2004

Plant species	2000	2001	2002	2003	2004
Caparidaceae					
Cleome viscosa	30(44)	17(34)	4(18)	43(68)	24(39)
Euphorbiaceae					
Croton lobatus	5(8)	1(3)	–	33(46)	12(16)
Euphorbia spp.	–	0(4)	–	1(4)	–
Jatropha	–	–	–	6(6)	1(1)
Leguminosae-Papilionoideae					
Macroptilium spp.	–	2(3)	0(3)	10(12)	0(1)
Malvaceae					
Malva spp.	4(5)	4(8)	–	2(6)	–
Sida spp.	–	–	–	3(4)	–
Bastardia spp.	–	–	1(2)	–	–
Abelmoschus esculentus	–	–	–	–	–
Polygonaceae					
Smart weed	–	–	–	1(2)	8(11)
Solanaceae					
Datura stramonium	2(2)	–	–	–	–
Solanum nigrum	–	1(1)	1(3)	5(12)	10(15)
S. melongena	1(1)	–	–	–	–
Unidentified weed	3(3)	0(1)	1(1)	3(4)	0(2)
Total	45(63)	25(54)	7(27)	107(164)	55(85)
Controls					
TYLCV-infected tomato	6(6)	10(10)	5(5)	17(17)	12(12)
Healthy tomato	0(5)	0(12)	0(8)	0(18)	0(12)

In some cases, the PCR-amplified TYLCV fragment was only detected by Southern blot hybridization analysis with a TYLCV probe. Results are presented as number of samples testing positive for TYLCV of total number of samples assayed. A sample was determined positive when a 334 bp amplified TYLCV DNA fragment was detected from a PCR and/or Southern blot hybridization analysis.

infection was detected in a much greater number of symptomless weeds, including *C. viscosa*, *Croton lobatus*, *Malva* spp., *Physalis* spp., and *Solanum nigrum* (Salati et al., 2002). To further confirm this hypothesis, additional testing of these potential reservoir hosts was performed from 2000 to 2003. As can be seen in Table 1, the results were fully consistent with those for 1998–2000: numerous symptomless TYLCV infections were detected in *C. viscosa*, *C. lobatus*, *S. nigrum* and *Malva* spp., and *Macroptilium* spp.; whereas less frequent infection was detected in *Bastardia* spp., *Euphorbia* spp., and *Polygonum* spp.). Together, these results indicated that symptomless infections of certain weeds by TYLCV were relatively common in the DO, and support the hypothesis that these may be important reservoir hosts for TYLCV during the host-free period.

The symptomless infections and low viral titer in these weeds indicates that TYLCV is not well adapted to these hosts. Surveys revealed that most of these weeds are colonized by whiteflies to some extent, consistent with infection via viruliferous whiteflies. However, what is less clear is whether whiteflies have the capacity to acquire TYLCV from these low-titer hosts and transmit the virus to tomato. We propose, given the delay in TYLCV appearance in tomato provided by the host-free period that whitefly acquisition of TYLCV from these symptomless weed hosts is inefficient. Furthermore, the probability of acquisition is likely also influenced by the whitefly population; thus, the overall reduction in whiteflies also would contribute to the delay in TYLCV appearance. However, definitive evidence will need to come from whitefly transmission experiments such as those conducted by Polston et al. (2006) with pepper.

8. SUCCESSFUL IPM OF TYLCV HAS ALLOWED FOR THE RECOVERY OF THE PROCESSING TOMATO INDUSTRY IN THE DO

The IPM program developed for TYLCV in the DO was based upon the precise identification of the virus involved and an understanding of the biology of the virus, which was mediated by the application of the tools of biotechnology. This program, summarized below, has allowed for the recovery of the processing tomato industry in the DO. Indeed, production levels now exceed pre-TYLCV levels, due in great part to the implementation of the host-free period and the widespread planting of high yielding early maturing hybrid tomato varieties.

Key components of the TYLCV IPM program in the DO:

1. Implementation of a mandatory 3 month whitefly host-free period.
2. Production of transplants in isolated locations, with drenches of neonicotinoid insecticides to provide early protection against whiteflies.
3. Early in the season (e.g., within 30 days of the host-free period) plant high-yielding early maturing (generally TYLCV susceptible or tolerant) varieties.
4. Later in the season (e.g., >30 days after host-free period) plant TYLCV-resistant varieties (e.g., cv. Gempride).
5. Monitoring of whitefly populations during the tomato season and application of contact insecticides when adult populations exceed thresholds (5–10 adults/leaf).
6. Extensive and thorough sanitation following the tomato growing season (i.e., immediate cultivation of tomato plants following harvest).

9. GENETIC STABILITY OF TYLCV IN THE DO

One of the key components of the successful IPM program for TYLCV in the DO is the capacity of the host-free period to provide a cleansing of the system of TYLCV prior to the tomato season. Thus, there is a concern that this may put a selection pressure on the virus to evolve forms that may more efficiently

infect weeds or other hosts, thereby resulting in higher levels of virus in these hosts and a corresponding increase in the efficiency by which whiteflies can acquire the virus from these hosts. On a practical level, this could reduce the period of low TYLCV pressure following the host-free period. Furthermore, recombination is an important mechanism of variability in begomoviruses (Rojas et al., 2005), and many TYLCV species are recombinant viruses (Salati et al. 2002, Monci et al., 2002). There also are numerous indigenous bipartite begomoviruses (infecting crops or weeds) in the DO that could be sources of genetic material for recombination. On the other hand, there is evidence from Spain showing that *Tomato yellow leaf curl Sardinia virus* has been genetically stable over a period of 8 years (Sanchez-Campos et al., 2002).

We conducted an analysis of the variability of TYLCV isolates from various locations in the DO. During a survey of tomato fields conducted in February 2006, tomato leaves showing typical TYLC symptoms were collected from various locations in the North and South production areas, and squashed onto nylon membranes (Table 2). These membranes were returned to UC Davis and analyzed by squash blot hybridization and PCR analysis with TYLCV-specific primers. In squash blot hybridization analyses with a TYLCV-specific probe, all samples were strongly positive, consistent with infection with TYLCV. Next, squash blot/PCR analysis was performed with the overlapping primer pair that directs the amplification of the full-length TYLCV genome (Nahkla et al., 1994; Salati et al., 2002). The expected size fragment (~2.9 kb) was amplified from all the samples and these fragments were cloned. Restriction enzyme analyses of 5–10 clones from each location revealed no polymorphisms; thus, the sequence of the 5′ end of the AC1, the complete C4 open reading frame and the IR (166 nt up to the stem-loop sequence) was determined from a representative clone from each location. As shown in

Table 2. Genetic variability of isolates of *Tomato yellow leaf curl virus* collected from various locations in the Dominican Republic in 2006

	Percent nucleotide sequence similarity with TYLCV-DO					
	Total nucleotide sequence	C1		C4		5′IR
Location	nt[a]	nt	aa	nt	aa	nt
Juan Gomez (N)[b]	99.1	99.8	99.5	100	100	96.4
Guyabin 1 (N)	99.7	99.7	99.5	100	100	100
Cerro Gordo (N)	99.1	99.7	99.5	100	100	97.0
Finca 4 (S)	99.6	99.7	99.0	99.7	99.0	99.4
Km-15 (S)	99.2	99.7	99.0	99.7	99.0	97.6
2CA (S)	100	100	100	100	100	100
Enriquillo (S)	100	100	100	100	100	100
Ocoa-2 (S)	99.6	99.8	99.5	100	100	98.8

[a] 746 nt sequence obtained from a representative full-length clone.
[b] N = Northern tomato production area, S = Southern production area.

Table 2, all of the sequences were nearly identical to TYLCV-DO, with C1 and C4 nucleotide and amino acid sequences >99% identical and IR nucleotide sequences >96% identical. Thus, TYLCV in the DO has been genetically stable since it was introduced in the early 1990s, and there is no evidence for selection of a recombinant that is better adapted to weed hosts. The continued effectiveness of the host-free period also argues against the emergence of such a recombinant.

10. DÉJÀ VU ALL OVER AGAIN: THE INTRODUCTION OF TYLCV INTO SINALOA, MEXICO

The state of Sinaloa, Mexico is a major producer of fresh market and, to a lesser extent, processing tomatoes. Tomatoes produced in this area are well known to be infected by a number of indigenous whitefly-transmitted begomoviruses, including *Pepper hausteco yellow vein virus*, *Pepper golden mosaic virus*, *Chino del tomate virus* (=*Tomato leaf crumple virus*), and *Sinaloa tomato leaf curl virus*. However, during the 2005–2006 growing season, TYLC-like symptoms began to appear in tomatoes produced in this area. Leaf squash/PCR analysis of leaves with these symptoms revealed TYLCV infection, and sequence analysis of PCR-amplified fragments revealed near identity to the sequence of TYLCV-DO.

To further confirm this result, PCR analysis with the TYLCV overlapping primers was used. The expected size ~2.9 kb fragment was amplified, and sequence and phylogenetic analyses revealed that the isolate of TYLCV from Sinaloa, Mexico is nearly identical to TYLCV from the DO, and clusters with a large group of TYLCV isolates from the Caribbean (Figure 6).

Thus, TYLCV has now been introduced into the major tomato-producing region of Mexico. Alone, this poses a major threat to production in this area; however, this area has a diversity of indigenous whitefly-transmitted begomoviruses as well as other tomato-infecting viruses, including *Tomato spotted wilt virus*. Therefore, it will be of considerable interest to see if TYLCV acts synergistically with these other begomoviruses to cause more severe disease symptoms and/or displaces these viruses, as has been the case in Florida with *Tomato mottle virus* (Polston et al., 1999). Finally, the lessons learned in terms of TYLCV management in the DO should be useful in the management of this newly introduced virus.

11. THE RESULTS IN THE DO ALLOW FOR THE DEVELOPMENT OF A GENERAL APPROACH FOR IPM OF TYLCV, OTHER BEGOMOVIRUSES AND INSECT-TRANSMITTED VIRUSES IN GENERAL

We will end this chapter by using the experience in the DO and other areas to present a general IPM program for whitefly-transmitted begomoviruses. In fact, with minor modifications, this approach could be used with almost any insect-transmitted virus.

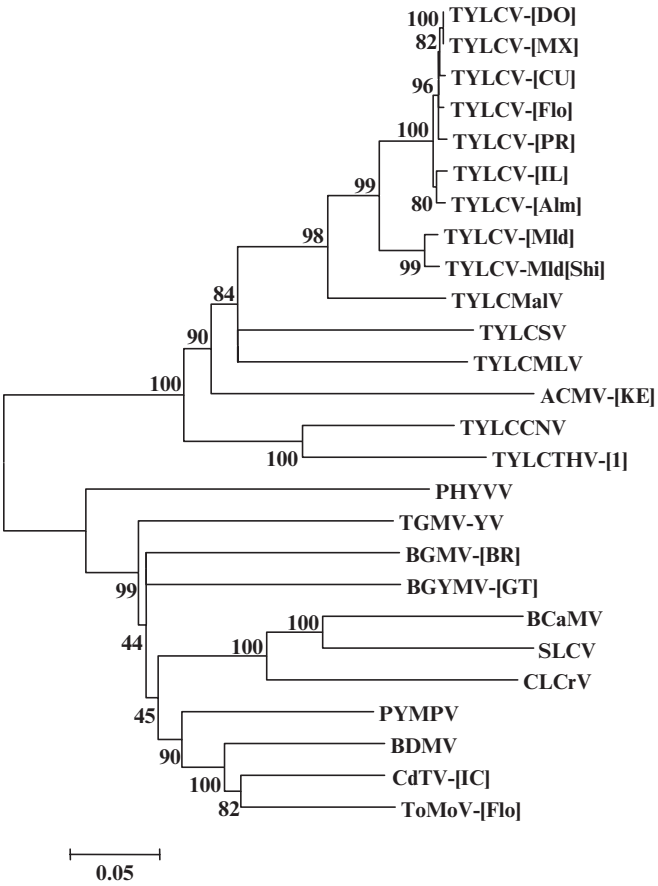

Figure 6. Phylogenetic consensus tree showing the relationship of an isolate of *Tomato yellow leaf curl virus* (TYLCV) from Sinaloa, Mexico with isolates from the Dominican Republic and other locations.

A general IPM approach for whitefly-transmitted begomoviruses.

A. Background (i.e., ideally done prior to or during the development of an IPM program)

– Identify viruses involved and develop detection tools.
– Screen varieties for tolerance/resistance/yield and utilize the most up-to-date agronomic practices for the crop (e.g., drip irrigation, fertilization etc.).
– Understand viral ecology/epidemiology and vector biology (whitefly species/biotype, and insecticide resistances).

B. Before planting

– Selection of best varieties (high yielding, disease resistant).
– Use pathogen-free, high-quality seed.

– Produce pathogen-free transplants, preferably in greenhouses or in protected nurseries (never establish nurseries adjacent to established fields).
– Apply neonicotinoid insecticide for protection against whiteflies and other insects (e.g., imidacloprid [Admire/Provado], thiamethoxam [Platinum/Actara], and acetamiprid [Assail]).

C. At planting and during the growing season
– Plant immediately after host-free period.
– Do not plant near old established fields.
– Monitor populations of whiteflies and other key insect pests and establish economic thresholds.
– Implement chemical control for whiteflies when a threshold is exceeded (e.g., neonicotinoids; insect growth regulators [IGRs] such as buprofezin [Courier/Applaud], pyriproxyfen [Knack], and spiromesifen [Oberon]; and contact insecticides [endosulfan/pyrethroids/bifenthrin]).
– Use row covers (e.g., Agribon), especially for the early vegetative stages of growth.
– Rogue plants with obvious symptoms on a weekly basis.

D. Following the growing season
– Sanitation: remove and destroy old crops/volunteers by plowing/physical removal and on a regional basis.
– Weed management (around fields).
– Implement a regional host-free period of 1–3 months, voluntary or enforced, with the crops to be included depend on the knowledge of the biology of the virus(es) and agroecosystem.

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CHAPTER 5

RESISTANCE TO INSECTICIDES IN THE TYLCV VECTOR, *BEMISIA TABACI*

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1. OVERVIEW

The whitefly *Bemisia tabaci* (Gennadius) is a serious pest of many agricultural crops (Byrne & Bellows, 1991). It is relatively new as an economic pest and has raised to increasingly higher levels of importance over the last 20–30 years in many semiarid and arid production areas. This coincided with the appearance and dispersion of the B biotype of *B. tabaci* showing distinct biochemical and host range characteristics (Costa & Brown, 1991). The B biotype was proposed as a distinct species, *B. argentifolii* (Perring et al., 1993; Perring, 2001) but the definition of *B. tabaci* as a complex of biotypes or races is more generally accepted (Brown et al., 1995; De Barro et al., 2005). The two most widespread and damaging biotypes that attack tomatoes are the "B" and "Q" biotypes. The B biotype has a broad geographical distribution and is considered to be a recent invader over much of its range. The Q biotype was originally considered to be restricted to the Iberian Peninsula, but has recently been detected in southern Europe and Middle East as well in the Far East and in the USA (e.g., Horowitz et al., 2003a; Zhang et al., 2005; Zanic et al., 2005; Dennehy et al., 2005). Although some natural biological control has been achieved, the use of insecticides remains the primary means of control for many crops. In tomatoes, insecticides are applied against *B. tabaci* especially to prevent transmission of tomato yellow leaf curl virus (TYLCV).

In many cropping systems, repeated insecticide applications used to control *B. tabaci* often result in resistance development (Dittrich et al., 1990; Denholm et al., 1996; Palumbo et al., 2001). The most deleterious practice was adopted by some tomato growers in their effort to control *B. tabaci* and TYLCV (Berlinger

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 305–325.

et al., 1993; Horowitz et al., 1994). Since whiteflies carrying the virus are able to infect a tomato plant with TYLCV within 4 h of inoculative feeding, insecticides with a quick killing effect on adults are needed to prevent virus spread. In the past, two applications per week with conventional insecticides were sufficient to control whiteflies and prevent virus damage. However, after a couple of years, even one to two applications per day were ineffective due to resistance.

Use of diverse insecticide chemistries can delay or prevent resistance (e.g., Horowitz & Ishaaya, 1996). Newly developed insecticides such as the neonicotinoids, pymetrozine, diafenthiuron, and spiromesifen are effective against whiteflies (Denholm et al., 1998b; Ishaaya & Horowitz, 1998; Liu, 2004) and can use as components of insecticide resistance management (IRM) programs.

This chapter reviews the status and the mechanisms of insecticide resistance in *B. tabaci* in tomatoes and other crops along with IRM tactics against the pest.

2. OVERVIEW OF RESISTANCE MECHANISMS

Insecticide resistance is an evolutionary genetic phenomenon caused by a variety of mechanisms based on enhanced detoxification of insecticides, or structural modifications of their target sites in arthropods. Such mechanisms are described in detail in several books and conference proceedings (e.g., Otto & Weber, 1990; Roush & Tabashnik, 1990; Mullin & Scott, 1992; Brown, 1996; McKenzie, 1996; Denholm et al., 1999; Ishaaya, 2001).

The most extensively used insecticide classes – organochlorines, organophosphates (OPs), carbamates, and pyrethroids – have generally been the most seriously threatened by resistance, and hence the major targets of research to resolve the causal mechanisms. Resistance to cyclodienes (such as dieldrin and endosulfan) usually results from a modification of the target site, the GABA (γ-aminobutyric acid) – gated chloride channel of post-synaptic nerve membranes. Resistance to OPs and carbamates can arise through enhanced detoxification by cytochrome P-450 monooxygenases, esterases or glutathione-S-transferases (GSTs), or from structural modifications of their target enzyme, acetylcholinesterase (AChE). Pyrethroid resistance can arise through enhanced esteratic or oxidative detoxification, as well from target-site insensitivity at the voltage-gated sodium channel in nerve membranes (knockdown or *kdr* resistance). Neonicotinoid resistance has appeared more recently and been shown to involve cytochrome P-450 monooxygenase (Rauch & Nauen, 2003) activity or a single point mutation in nicotinic acetylcholine receptor subunits (Liu et al., 2005).

Mechanisms of resistance to insecticides acting outside the nervous system (e.g., insect growth regulators [IGRs]), or to more novel neurotoxins are less clearly understood, but are also likely to prove attributable to enhanced detoxification and/or target site modification.

Perhaps the most significant recent progress with understanding resistance mechanisms has resulted from the application of molecular biology to resistance

research. Depending on the mechanism involved, resistance has been shown to arise from structural alterations of genes encoding detoxifying enzymes (Newcomb et al., 1997), or target-site proteins (ffrench-Constant et al., 1998), or through processes (e.g., amplification or altered transcription) affecting gene expression (Hemingway et al., 1998). Despite the complexity of receptors or enzymes responsible, mutations leading to resistance frequently recur in different species (Thompson et al., 1993; Martinez-Torres et al., 1997). This is especially the case for mechanisms based on decreased sensitivity of insecticide target sites. Molecular studies of insecticide resistance have identified the point mutations associated with target site insensitivity in genes encoding the three major insecticide targets: the GABA receptor (cyclodiene resistance), the voltage-gated sodium channel (pyrethroids), and AChE (OPs and carbamates) (ffrench-Constant et al., 1993; Mutero et al., 1994; Williamson et al., 1996). These provide important insights into the homology of resistance mutations between species and the frequency with which they arise (ffrench-Constant et al., 1996, 1998).

3. BIOASSAYS FOR RESISTANCE MONITORING

Accurate and regular monitoring of changes in susceptibility is essential for anticipating resistance problems and for assessing the effectiveness of resistance management tactics. It is highly advantageous to evaluate, define, and standardize test methods for insecticides, especially novel insecticides, prior to their introduction in the field. Monitoring tests should be as rapid and simple as possible, yield repeatable results, and be sufficiently sensitive to detect any differences in tolerance under field conditions (Denholm et al., 1998b).

Monitoring programs to detect resistance genotypes and/or phenotypes, as early as possible, and to document their distribution, should be a key component of any resistance management strategy. Whole-organism bioassays, involving topical application or exposure to pesticide residues on surfaces or in food, have long been the basis of such programs, but are limited in their application and precision (Roush & Miller, 1986). Comparisons of LD_{50} or LD_{90} values of samples from populations – the most widely adopted approach – may be useful for detecting a high frequency of resistant insects but are far too insensitive for detection of incipient resistance. Use of a "discriminating" dose (or concentration) corresponding to the LD_{99} or higher of baseline susceptible populations, is a better alternative, but is still subject to important statistical constraints. Firstly, the estimation of these doses is challenging because the fitting of probit models is usually inaccurate at the extreme ends of dose–response relationships. Secondly, unless doses are perfectly diagnostic (i.e., killing 100% of susceptible individuals but no resistant ones, which is rarely the case), sample sizes required for the reliable detection of even 1% resistance may be very large (Roush & Miller, 1986; Sawicki et al., 1989).

4. MONITORING FOR RESISTANCE IN THE WHITEFLY *BEMISIA TABACI*

There are several bioassay methods available for whiteflies (e.g., Ishaaya et al., 1988; Cahill & Hackett, 1992; Prabhaker et al., 1996, 1997; Horowitz et al., 1998; Cahill et al., 1996c; Castle et al., 1999). For adults, the most widely used bioassay is the leaf-dip test with numerous variations; the common principle is to expose adults (female or both sexes) either to a cotton leaf disk or a seedling that has been dipped in formulated insecticide solution. In the case of the leaf-disk method, the leaf may be excised and placed on a layer of agar in a Petri dish (e.g., Horowitz et al., 1988; Cahill et al., 1995). With the seedling method, the adults are confined to the treated leaf using a clip cage (Ishaaya et al., 1988; Horowitz et al., 1994). Two other approaches of more limited utility involve confining adults inside glass scintillation vials coated with an insecticide deposit (Cahill & Hackett, 1992; Prabhaker et al., 1996; Sivasupramaniam et al., 1997a), and trapping adults on yellow sticky cards impregnated with insecticide (Prabhaker et al., 1996). Lacking any source of food, both are suitable only for testing contact insecticides for short periods of time (e.g., 3 h).

Methods for testing insecticides with novel modes of action (e.g., buprofezin and pyriproxyfen) which act primarily on developing stages rather than adults, are based on dipping foliage infested with eggs or nymphs (e.g., Cahill et al., 1996d). Another variation is to confine adults to treated leaves and determine the accumulated mortality until pupation (Ishaaya et al., 1988; Ishaaya & Horowitz, 1992). The systemic effects of imidacloprid (a neonicotinoid) have led to the development of an alternative method, in which adults or nymphs are exposed to foliage treated with the insecticide either through plant roots or the petiole of an excised leaf (Cahill et al., 1996c; Horowitz et al., 1998). For testing the systemic effects of imidacloprid on whitefly adults, a hydroponic procedure has also been suggested (Williams et al., 1996; Prabhaker et al., 1997). A one-day hydroponic uptake procedure using cotton seedlings and reliable mortality criterion was devised by Williams et al. (1996). A modification of these methods using a cut leaf petiole in vials containing solutions of various concentrations of imidacloprid has also reported (Schuster et al., 2003). Similar bioassay methods have been developed for other neonicotinoid insecticides (Horowitz et al., 1998).

In assay of two novel insecticides, diafenthiuron and pymetrozine, some problems arose. The toxicity of diafenthiuron against insects depends on desulfuration in the presence of light to a carbodiimide derivative (CGA 140408) that inhibits ATP-ase activity in mitochondria (Ruder et al., 1991). The poor repeatability encountered when testing this insecticide against *B. tabaci* may be due to variable and inefficient photo conversion that occurred under laboratory lighting (Denholm et al., 1995). Repeatability was increased by conducting bioassays after exposing treated plants to sunlight (Ishaaya et al., 1993).

Pymetrozine is thought to act primarily by suppressing stylet penetration of target pests. The insecticide limits feeding by whiteflies and other homopteran pests, leading to their starvation (Kayser et al., 1994). Testing of pymetrozine against *B. tabaci* and the cotton aphid (*Aphis gossypii*) showed a holding period of at least 96 h (and preferably 120 h) to be essential for obtaining reliable dose–response data for whitefly adults or aphid nymphs (Denholm et al., 1995).

5. CURRENT STATUS OF RESISTANCE AND IDENTIFIED MECHANISMS OF RESISTANCE IN *BEMISIA TABACI*

Over the last 10 years a number of symposia, reviews and book chapters have provided comprehensive details of the documentation, monitoring, and management of resistance in *B. tabaci* and other whiteflies to conventional and novel insecticides (Dittrich et al., 1990; Horowitz & Ishaaya 1996; Denholm et al., 1996, 1998a; Cahill et al., 1996a; Horowitz et al., 1999a; Cahill & Denholm, 1999; Palumbo et al., 2001; Nauen & Denholm, 2005). Therefore, our review briefly summarizes the recent reports of insecticide resistance in *B. tabaci*.

5.1. Resistance to conventional insecticides

Dittrich et al. (1990) reviewed worldwide data on resistance in *B. tabaci* to DDT, OPs, carbamates, and pyrethroids applied singly or as mixtures. The levels of resistance, with resistance ratios (RR) ranging sometimes from hundreds to thousands, were correlated with frequency and years of insecticide usage. Resistance of this pest to conventional insecticides was observed in all countries in which monitoring of resistance was conducted (Horowitz & Ishaaya, 1996). On the other hand, reduction in use of conventional insecticides against whiteflies and switching to newer compounds resulted in some cases in a decrease in resistance to the formers (Ahmad et al., 2001).

5.1.1. Organophosphates (OPs) and carbamates

Resistance to OPs is well established in *B. tabaci*. Dittrich et al., (1990) reported high resistance in *B. tabaci* to monocrotophos, dimethoate and methamidophos, and lower resistance to profenofos in Turkey and Sudan. Other studies in the USA reported resistance to chlorpyriphos and monocrotophos to be lower than that to methyl-parathion and sulprofos (Prabhaker et al., 1985). More recently, OP resistance was shown to be geographically widespread in *B. tabaci* (Cahill et al., 1995), and attributable in part to modified AChE, the target sites of these insecticides (Byrne et al., 1994; Byrne & Devonshire, 1997). Metabolic mechanisms such as elevated monooxygenases and carboxylesterases may also contribute to OP resistance in some populations (Denholm et al., 1996).

5.1.2. Pyrethroids

Pyrethroid resistance in whiteflies is also widespread although the magnitude and pattern of resistance and cross-resistance varies considerably among countries and cropping systems (Cahill et al., 1995, 1996a; Denholm et al., 1996). Intensive use of pyrethroids in Sudanese cotton against *B. tabaci* caused resistance to cypermethrin and deltamethrin to increase from 3-fold to about 170- and 350-fold, respectively in the mid 1980s, although resistance to bifenthrin remained low. Subsequent reductions in pyrethroid use led to a corresponding decline in resistance levels (Dittrich et al., 1990). However, studies have shown high levels of resistance in *B. tabaci* from Pakistan encompassing both bifenthrin and the older pyrethroids (Cahill et al., 1995). Pyrethroid resistance in *B. tabaci* has also been observed in glasshouse or greenhouse populations from the UK, the Netherlands, and Spain, and from field crops in Israel, Turkey, and Cyprus (Cahill et al., 1996a).

Resistance to bifenthrin in a strain of *B. tabaci* from the USA was inherited as an incompletely dominant trait and probably controlled by a few genes or a single gene (Riley & Tan, 2003). Although little detailed biochemical information is available, synergism studies (Ishaaya et al., 1987; Horowitz et al., 1988; Prabhaker et al., 1988; Dittrich et al., 1990) have implicated both elevated esterases and monooxygenases in pyrethroid resistance. Using naphthyl esters as substrates, Byrne and Devonshire (1993) identified six naphthyl esterases in B biotype strains of *B. tabaci*. Of these esterases, only one (termed $E_{0.14}$) was directly associated with pyrethroid resistance. A strain lacking naphthyl esterase $E_{0.14}$, had lower resistance to both permethrin and cypermethrin, which suggested that substantial component of pyrethroid resistance in this strain was attributable specifically to the activity of $E_{0.14}$ (Byrne et al., 2000).

Mixtures of pyrethroids and OPs have been used against *B. tabaci* in many countries, and became widely adopted to manage outbreaks of *B. tabaci* that occurred in the southwestern USA in the early 1990s (Ellsworth & Jones, 2001; Palumbo et al., 2001). Consequently, extensive efforts were initiated to monitor *B. tabaci* susceptibility to pyrethroids in laboratory bioassays, as well as to explore potentially synergistic combinations of pyrethroids and OPs (Prabhaker et al., 1996; Simmons & Dennehy, 1996; Dennehy et al., 1997; Sivasupramaniam et al., 1997b; Sivasupramaniam & Watson, 2000; Castle et al., 2001). Despite very high densities of *B. tabaci* and heavy insecticide use from 1991 to 1995, bioassay data from the Imperial Valley of California indicated that field-collected *B. tabaci* populations remained susceptible to the most commonly applied pyrethroids and synergistic combinations (Castle et al., 1996a, b). Although no field control failures with synergized pyrethroids have been reported in the Imperial Valley to date, a significant shift in reduced susceptibility to fenpropathrin and acephate was detected in laboratory bioassays in 1997, and again in 1999 (Castle et al., 2001).

In Arizona, cotton and vegetable growers were experiencing similar *B. tabaci* outbreaks during the early 1990s. From 1993 to 1995, synergized pyrethroids, particularly the combination of fenpropathrin (pyrethroid) plus acephate (OP), were essential in providing control of *B. tabaci* in cotton, especially from the middle to the end of the cotton growing season. In 1994, evidence of reduced susceptibility to synergized pyrethroids in *B. tabaci* populations collected from cotton fields in central Arizona was documented (Dennehy et al., 1995). Monitoring of field-collected *B. tabaci* populations during 1995 confirmed significant reductions in susceptibility to these combinations in major cotton growing regions (Dennehy et al., 1997; Dennehy & Williams, 1997). Combining pyrethroids with OPs to control *B. tabaci* was based on the premise that the OP component can inhibit detoxification systems, thereby overpowering any pyrethroid resistance attributable to such enzymes (Ishaaya et al., 1987; Byrne et al., 1994). However, Byrne et al. (1994) proposed that mixtures should be carefully used since they can select for an alternative resistance mechanism. By the end of the 1995 growing season, growers in some areas of Arizona experienced unacceptable yield losses and sticky lint contamination following repeated use of synergized pyrethroids. It was therefore suspected that an alternative resistance mechanism, probably based on target site modification, had evolved. This suspicion was vindicated by the discovery in contemporary B-type strains, of two independent mutations in the *para*-type voltage-gated sodium channel, the target site of pyrethroids: methionine to valine at position 918 (M918V) and leucine to isoleucine at position 925 (L925I) (Morin et al., 2002). Although each mutation was isolated independently from strains showing >100-fold resistance to synergized pyrethroids, only L925I was associated with resistance in strains derived from the field in 2000 and 2001. The L925I mutation occurred in all individuals from nine different field collections that survived exposure to a discriminating concentration of fenpropathrin plus acephate. Linkage analysis of hemizygous male progeny of unmated heterozygous F_1 females (L925I X wild-type) showed that the resistance phenotype was tightly linked to the voltage-gated sodium channel locus. Recently, two independent mutations, both associated with resistance to pyrethroids, were identified in *B. tabaci* Q-biotype strains (Alon et al., 2006; Roditakis et al., 2006). One was the L925I mutation previously reported from the B biotype (Morin et al., 2002), and the other a novel threonine to valine substitution at position 929 (T929V). Through nucleotide sequence analysis of a wide range of globally collected resistant and susceptible *para*-type voltage-gated sodium channel alleles, it was shown that the patterns of variation observed in the different alleles are consistent with independent resistance mutations in the B and Q biotypes (Alon et al., 2006).

As a result of the high level of resistance to synergized pyrethroids, an emergency approval (US-EPA Section 18) for the IGRs buprofezin and pyriproxyfen was granted for US cotton in 1996 (Dennehy & Williams, 1997; Dennehy & Denholm, 1998). Restricted use of these compounds in a conservative resistance management program has, over several years, resulted in area-wide suppression

of *B. tabaci* and contributed to substantial reductions in overall insecticide use (Ellsworth et al., 1996; Ellsworth, 1998; Agnew & Baker, 2001; Ellsworth & Jones, 2001). Presently, *B. tabaci* has recovered susceptibility to synergized pyrethroids (Li et al., 2001), but results from continued monitoring with fenpropathrin and acephate suggest that a return to intensive use of pyrethroid/OP mixtures could result in the rapid selection of resistant *B. tabaci* populations and control failures (Castle et al., 2001).

Recent research has shown that applying the synergist piperonyl butoxide several hours prior to a pyrethroid gives improved control of *B. tabaci* compared with a tank mix of both products (Moores et al., 2005). In this case, the synergist fully inhibits the metabolic enzymes ("temporal synergism") before the active ingredient is applied.

5.1.3. Cyclodienes

The only organochlorine still used widely against whiteflies is endosulfan. Resistance levels in *B. tabaci* to endosulfan have ranged from 20- to 360-fold in strains from many countries (Denholm et al., 1996). The resistance factors recorded, although generally lower than for OPs and pyrethroids, did reduce the performance of endosulfan under simulated field conditions (Cahill et al., 1996b). The principal mechanism of endosulfan resistance in several insects, including *B. tabaci*, involves a single point mutation within the gene *resistance to dieldrin* or Rdl. This gene encodes a subunit of the GABA receptor, the target site of cyclodiene insecticides (ffrench-Constant et al., 1993). Sequencing of the Rdl homologue from *B. tabaci* revealed the presence of an alanine to serine replacement at position 302 (A302S), equivalent to the mutation first described in *Drosophila melanogaster* (Anthony et al., 1995). The A302S replacement was found not to be confined to the B biotype, but also distributed among a range of non-B strains (Anthony et al., 1995).

5.2. Resistance to novel insecticides

The need for a greater diversity of compounds effective against whiteflies is being met by the introduction of several insecticides with new modes of action, which are less affected or unaffected by existing resistance mechanisms (Denholm et al., 1998b; Ishaaya & Horowitz, 1998; Nauen & Denholm, 2005). Neonicotinoid insecticides – imidacloprid, acetamiprid, nitenpyram, and thiamethoxam – are generally systemic in plants, and target nicotinic acetylcholine receptors in the insect central and peripheral nervous system. IGRs include inhibitors of chitin synthesis – buprofezin and benzoylphenyl ureas such as novaluron, and the juvenile hormone mimic pyriproxyfen. Other new insecticides active against whiteflies inhibit mitochondrial ATP synthesis (diafenthiuron) or affect feeding behavior in certain sucking pests (pymetrozine). Various fermentation products of *Streptomyces avermitilis* – such as abamectin (mixed with mineral oils), emamectin, and milbemectin – have been reported

as effective against *B. tabaci* in laboratory and field trials. These insecticides and other biorational products are generally considered to be relatively safe to natural enemies, and are gradually being incorporated into whitefly control programs around the world. They offer excellent prospects for regaining control of insects resistant to conventional insecticides. However, none should be assumed to be immune to resistance, and some cases of resistance to these novel agents have already been reported.

5.2.1. Buprofezin

Buprofezin inhibits chitin synthesis in several hemipteran pests including whiteflies (Ishaaya et al., 1988). Its mode of action is not fully understood, although the principal effect is to interfere with chitin deposition during molting, resulting in nymphal mortality during ecdysis. In addition, the fecundity and egg hatch of females exposed to treated leaves is reduced (Ishaaya et al., 1988). Buprofezin is considered a major compound for controlling whiteflies in both greenhouses and outdoors, especially in locations where resistance to conventional insecticides has evolved (Horowitz et al., 1994; Dennehy & Williams, 1997). A decrease in buprofezin susceptibility occurred 3 years after its introduction on Israeli cotton in 1989 (Horowitz & Ishaaya, 1992; Horowitz et al., 1994). Most recently, significant decreases in susceptibility to buprofezin were detected in *B. tabaci* populations collected from cotton fields in the Ayalon Valley of Israel from 1992 to 1995 (Horowitz et al., 1999a). Buprofezin still provides satisfactory *B. tabaci* control in most growing areas in Israel, but its use in cotton fields in Israel is quite low.

The risk of resistance development is higher in protected crops in confined spaces, and in these habitats buprofezin resistance is now becoming widespread. Resistance levels of 10- to 50-fold have been reported from greenhouses or glasshouses in the UK, the Netherlands, Spain, and Israel (Cahill et al., 1996d; Horowitz et al., 1994). Recent bioassays of *B. tabaci* collected from greenhouses in Almeria, Spain, showed that resistance to buprofezin has apparently increased since 1994 (Elbert & Nauen, 2000).

Bioassays of *B. tabaci* populations collected from cotton indicated a trend of reduced susceptibility from 1996 to 1998 (Dennehy et al., 1999). Susceptibility to buprofezin increased significantly in 1999, but returned to lower levels in 2000, where a tenfold reduction was reported in several populations (Li et al., 2001). Similarly, *B. tabaci* populations collected from several regions in California and Arizona in 1998 and 1999 showed an increase in susceptibility to buprofezin (Toscano et al., 2001).

5.2.2. Pyriproxyfen

The use of pyriproxyfen during the last decade against *B. tabaci* in Israel provides a striking example of how genetic and ecological factors can combine to promote resistance, despite concerted efforts to prevent its occurring. This compound inhibits hatching of whitefly eggs, directly or transovarially, and also

affects nymphs by suppressing adult emergence, resulting in pupal mortality (Ishaaya & Horowitz, 1992, 1995). Since 1991, it has been one of the main agents for controlling *B. tabaci* in cotton fields in Israel (Horowitz et al., 1999b), and from 1996 in the southwestern USA (Dennehy & Williams, 1997).

The dynamics of pyriproxyfen resistance in *B. tabaci* have been studied intensively in cotton fields and greenhouses in Israel (Horowitz et al., 1999b, 2002). Seasonal trends of susceptibility to pyriproxyfen in field populations have been monitored annually from June (prior to treatment) through late summer at different locations in Israel. Initially, only a slight decrease in susceptibility was observed during the cotton season. Due to a restriction in its use on cotton, and a consequent reduction in selection pressure, pyriproxyfen could be reapplied in the following season when susceptibility has been restored. However, in a greenhouse roses, after three successive applications, higher than 500-fold resistance to pyriproxyfen was recorded (Horowitz & Ishaaya, 1994). After 7 years of pyriproxyfen use on cotton within a resistance management strategy that limits its use to a single application per season, susceptibility has been maintained in some areas. In other locations, such as the Ayalon Valley in central Israel, where populations of *B. tabaci* are relatively isolated geographically, moderate to high levels of resistance have been observed (Horowitz et al., 1999b).

The findings from Israeli cotton have potentially important implications for managing resistance to pyriproxyfen in *B. tabaci* elsewhere. In general, a restriction to one application per season appears essential for sustaining the effectiveness of pyriproxyfen. Regions with climates, cropping systems or histories of whitefly resistance such as those of Ayalon Valley of Israel may need to implement pyriproxyfen-free years in order to contain resistant genotypes effectively.

Recent findings may implicate the occurrence of different biotypes of *B. tabaci* as determinants of resistance development in southern Europe and the Middle East. To date, all confirmed cases of strong resistance to pyriproxyfen in Israel have been associated with the Q rather than the B biotype (Horowitz et al., 2002, 2003a, 2005). It is therefore possible that the present distribution of genes for pyriproxyfen resistance reflects the current gene flow associated with Q-type populations.

The mechanisms of resistance to pyriproxyfen in *B. tabaci*, as well the mode of action of the compound itself are still unknown. Inheritance of resistance to pyriproxyfen in *B. tabaci* was studied by bioassaying F_1 heterozygous females from reciprocal crosses between a susceptible Q strain (ALM-1) originating from Spain and a pyriproxyfen-resistant Q strain (Pyri-R) from Israel. Resistance was found to be partially dominant (Horowitz et al., 2003b). This level of dominance is sufficient for heterozygous females to survive field exposure to pyriproxyfen. Under these conditions, the speed at which pyriproxyfen resistance was selected in some regions of Israel (Horowitz et al., 1999b, 2002), despite a restriction to one application of this insecticide per season, becomes more explicable. Mortality curves for F_2 males produced by virgin F_1 heterozygous females displayed a broad plateau at 50% mortality, indicating that

resistance to pyriproxyfen in the Q biotype of *B. tabaci* is conferred primarily by a mutant allele at a single locus (Horowitz et al., 2003b).

In recent seasons (1998–2001), there has been a decline in levels of pyriproxyfen resistance in cotton fields in Israel, mostly in the western Negev (southwestern Israel) but also in the Ayalon Valley (Horowitz et al., 1999b, 2002). The decline corresponds with the cessation of pyriproxyfen use in the Ayalon Valley since 1997, and increased use of neonicotinoid insecticides, especially acetamiprid (G. Forer, personal communication). The introduction of the neonicotinoids has resulted in reduced use of pyriproxyfen, even in locations with less severe resistance to this insecticide, such as the western Negev, where susceptibility to pyriproxyfen is almost restored.

In the USA, pyriproxyfen and buprofezin were first used as rotational alternatives in cotton resistance management programs beginning in Arizona in 1996 and in California in 1997. Initial monitoring of *B. tabaci* collected from cotton in Arizona from 1996 to 1998 showed no reductions in susceptibility to pyriproxyfen (Dennehy et al., 1999). However, a significant decrease in susceptibility was observed in populations collected from some Arizona cotton growing regions in 1999 and 2000 (Li et al., 2001). Monitoring of *B. tabaci* populations in southern California and southwestern Arizona revealed that regional differences in pyriproxyfen toxicity were minimal, and similarly to buprofezin, susceptibility to pyriproxyfen was maintained after 3 years of use (Toscano et al., 2001). To date, both buprofezin and pyriproxyfen remain highly effective and continue to provide economic control of *B. tabaci* in California and Arizona cotton (Ellsworth & Jones, 2001; Palumbo et al., 2001).

5.2.3. Neonicotinoids

The use of neonicotinoid insecticides (formerly termed chloronicotinyl insecticides) against whiteflies and other sucking pests is increasing rapidly. On many greenhouse tomatoes, pest management has become particularly reliant on neonicotinoids.

The first commercial compound was imidacloprid. Among others being introduced are acetamiprid, nitenpyram, thiamethoxam, and thiacloprid. A combination of neonicotinoid overuse, coupled with a strong risk of cross-resistance between these chemicals, threatens the effectiveness of the group as a whole (Cahill & Denholm, 1999; Li et al., 2001; Horowitz et al., 2004). Although only a few cases of resistance to neonicotinoids have been reported (reviewed by Nauen & Denholm, 2005), it is of utmost importance to develop recommended resistance management strategies for this important group (Elbert et al., 1996).

Resistance to imidacloprid was first reported in *B. tabaci* from greenhouses in southern Spain (Cahill et al., 1996c; Elbert & Nauen, 2000). From the late 1990s, resistance to neonicotinoids increased and field strains exhibited more than 100-fold resistance to this group (Nauen & Denholm, 2005). In many cases the resistance to neonicotinoids was associated with the Q biotype (Nauen et al., 2002; Rauch & Nauen 2003; Horowitz et al., 2004; Dennehy et al., 2005),

although a few cases of neonicotinoid resistance have been described also in B-type strains (Byrne et al., 2003).

Rauch & Nauen (2003) studied the involvement of target site modification and metabolic enzymes in neonicotinoid resistance. Radioligand competition assays revealed no significant differences in imidacloprid binding affinity to nicotinic acetylcholine receptors of susceptible and resistant strains. Biochemical analyses of metabolizing enzymes such as esterases, GSTs, and cytochrome P450-dependent monooxygenases showed that only the monooxygenase activity was correlated with imidacloprid, thiamethoxam, and acetamiprid resistance. The involvement of P450-dependent monooxygenases activity in resistance to neonicotinoids was also supported by molecular data. In preliminary experiments, one P450 gene from the CYP6 class was shown to be overexpressed only in resistant Q- and B-type strains (Morin et al., 2004). Metabolism studies of [^{14}C] imidacloprid revealed that the main metabolite in resistant strains is 5-hydroxy-imidacloprid. Compared with imidacloprid, the 5-hydroxy metabolite showed a much lower binding affinity (13-fold) to the nicotinic acetylcholine receptors (Rauch & Nauen, 2003). In contrast, Byrne et al. (2003) were unable to detect oxidative metabolism of imidacloprid in the presence of NADPH when incubating microsomes prepared from an imidacloprid-resistant B-type strain from Guatemala. This implies, at least for the selected strain from Guatemala, that elevated levels of monooxygenases may not be the only resistance mechanism of neonicotinoid resistance in *B. tabaci* (Nauen & Denholm, 2005).

Three years of acetamiprid use in Israeli greenhouses resulted in five- to tenfold tolerance of *B. tabaci* to this compound; however, acetamiprid remained highly effective in cotton fields (Horowitz et al., 1999a). On the other hand, high cross-resistance to thiamethoxam was reported in cotton fields, even though this neonicotinoid has not been used in cotton in Israel (Horowitz et al., 2004).

In the Imperial Valley of California, bioassays with imidacloprid of field-collected *B. tabaci* showed no evidence of resistance in 1996 (Prabhaker et al., 1997). In Arizona, where imidacloprid has been used since 1993, a slight decline in *B. tabaci* susceptibility to this compound was observed in laboratory bioassays (Dennehy et al., 1999). Subsequently, field monitoring showed that whitefly populations maintained their susceptibility to imidacloprid in 1999 and 2000, at levels similar to those reported in 1997 (Williams et al., 1998; Li et al., 2001). The inherent toxicity of systemic-applied imidacloprid and its metabolites, and whitefly bionomics and diverse agroecosystems may explain why efficacy of imidacloprid formulations remains relatively high against whiteflies in the desert cropping systems in southwestern USA (Palumbo et al., 2001).

In conclusion, the whitefly *B. tabaci* has the ability to develop resistance to both conventional and non-conventional insecticides. Management of this pest should be based on a rational use of insecticides, restriction of treatments and alternation with compounds of different modes of action, and the use of alternative control tactics in order to reduce selection pressure for resistance.

6. TACTICS FOR DELAYING/REDUCING RESISTANCE

Since the 1970s, various countermeasures, based largely on computer models, have been proposed for combating resistance. Most are based on manipulating operational factors defining the rate, timing, nature, and frequency of insecticide applications and on exploiting knowledge of pest biology in order to anticipate the selection pressure imposed by insecticides. As noted by several authors (e.g., Sawicki, 1981; Roush, 1989; Denholm & Rowland, 1992; Georghiou, 1994; Castle et al., 1999), there is no single prescription for combating resistance under all situations. Tactics must be tailored as carefully as possible to individual pests or pest complexes in light of ecological and genetic factors, the diversity of chemicals available and practical constraints on the precision with which they can be implemented.

Approaches to combating resistance can be viewed from different perspectives (e.g., Georghiou, 1983; Roush, 1989; Denholm & Rowland, 1992; McKenzie, 1996). The classification proposed by Georghiou (1983) is briefly summarized below:

Management by moderation – Aims to reduce selection for resistance by preserving susceptible insects in the population through use of low application doses, less frequent applications, short-lived residues, or the creation of untreated refuges. This approach is often the easiest to implement and involves the least risk. However, the value of lowering application rates to manage resistance remains debatable, since unless overall efficacy is compromised to a substantial extent and there is a threat of increasing the number of resistance genes that could be selected (Roush, 1989; Denholm & Rowland, 1992; McKenzie, 1996).

Management by saturation – Aims to overpower any resistant individuals present by using doses sufficiently high to kill resistant insects (especially resistant heterozygotes), suppressing detoxification enzymes through the use of synergists, or identifying "resistance-defeating" toxins less affected or unaffected by known resistance mechanisms.

Management by multiple-attack – Involves using two or more unrelated pesticides in ways that reduce the selection or impact of resistance to any one chemical. The compounds could be applied simultaneously as mixtures, alternately in rotation, or in more complex spatial patterns known as mosaics. Although mixtures offer greater theoretical benefits than alternations, they require a far greater number of assumptions to be met regarding the efficacy, persistence, and complementarily of partner chemicals (Tabashnik, 1989; Roush, 1989; Denholm et al., 1998b). All tactics in this category rely on the absence of cross-resistance between component insecticides.

Strategies implemented to challenge resistance in practice have tended to adopt combinations of the above three approaches. As an example, measures introduced in the early 1980s to combat pyrethroid resistance in the bollworm, *H. armigera*, on cotton (Forrester et al., 1993) involved restricting the "window"

duration when pyrethroids could be used (management by moderation), and recommending the use of non-pyrethroid alternatives outside this period (management by multiple attack). Other recommendations – to target pyrethroids against neonate larvae (thus enabling even pyrethroid-resistant phenotypes to be killed); and to use the synergist PBO with pyrethroids to suppress detoxification systems, – introduced components of a management-by-saturation approach. Unfortunately, even these measures failed to prevent a gradual increase in the frequency of pyrethroid resistance in *H. armigera*. The Australian strategy nonetheless pioneered a number of principles relating to the design, implementation, and support of large-scale resistance management, and has rightly achieved a great deal of international acclaim (Horowitz & Denholm, 2001).

A resistance management strategy introduced in Israel in the late 1980s against *B. tabaci* and coexisting cotton pests also relies heavily on restricting the use of key compounds (in this case to a single application per season), and on rotating insecticides in a sequence intended to protect beneficial organisms and to exploit non-chemical tactics as much as possible (Horowitz et al., 1994, 1995). Again, this has not completely prevented resistance, but it has resulted in a dramatic reduction in the number of insecticide sprays on cotton. Similar results have been obtained by extending components of the Israeli strategy to the cotton/vegetable cropping systems of the southwestern USA (Dennehy et al., 1996; Dennehy & Williams, 1997; Dennehy & Denholm, 1998; Palumbo et al., 2001).

One notable feature of these and many other resistance management strategies is that they were initially formulated with little or no knowledge of the resistance mechanisms already present or likely to arise. Their primary objective was and continues to be prevention of resistant phenotypes from reaching economically damaging frequencies. In principle, this objective could be further supported by biochemical or genetic input which can serve as a base for resistance management (Horowitz & Denholm, 2001).

7. ALTERNATIVE MANAGEMENT TACTICS FOR *BEMISIA TABACI* IN TOMATOES

Although the use of insecticides to control the whitefly adults prior to virus transmission is still common in tomatoes, the use of other control tactics is preferable because of the resistance and environmental concerns. Other control countermeasures such as cultivars resistant to virus and/or vectors, and physical barriers to immigration by insect pests would be most effective ways of reducing damage by virus disease (Cohen & Berlinger, 1984; Lapidot & Friedmann, 2002). The introduction of tomato TYLCV-tolerant cultivars with reasonable fruit quality, along with insect-proof netting, are a good opportunity to use integrated-biological control in protected tomatoes (Stansly et al., 2004; Chyzik et al., 2005). Hence, insecticide applications against the vector should be used as a last option in combating the virus; in addition, the use of other control tactics would certainly moderate resistance evolution to insecticides.

8. CONCLUSION

Insecticide resistance in *B. tabaci* is widespread elsewhere. The new groups of insecticides attracting most concern are the IGRs buprofezin and pyriproxyfen, which have already been proved to be prone to resistance in *B. tabaci*, and the neonicotinoids are now in widespread use against whiteflies and other pests. Although neonicotinoid insecticides offer outstanding operational versatility, their vulnerability to cross-resistance requires that resistance management tactics be directed at the group as a whole rather than at single compounds (Elbert et al., 1996, 2005; Cahill & Denholm, 1999; Nauen & Denholm, 2005).

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PART V

NATURAL AND ENGINEERED RESISTANCE

CHAPTER 1

SCREENING FOR TYLCV-RESISTANT PLANTS USING WHITEFLY-MEDIATED INOCULATION

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1. INTRODUCTION

Today, *tomato yellow leaf curl disease* has become the limiting factor for tomato production in many tropical and subtropical regions of the world. This disease is induced by a number of begomoviruses, the type member being *Tomato yellow leaf curl virus* (TYLCV), transmitted by the whitefly *Bemisia tabaci* (Gennadius), whose severe population outbreaks are usually associated with high incidence of the disease. Control measures in infected areas usually rely on seclusion of the whitefly vector, mainly through multiple applications of insecticides or physical barriers (Antignus & Cohen, 1994; Hilje et al., 2001; Palumbo et al., 2001; Polston & Anderson, 1997). Due to the large populations of whiteflies, and their ability to develop pesticide resistance, vector seclusion is not an ideal way of fighting the spread and damage induced by TYLCV. Hence, development of genetic resistance in the tomato host is the best solution for any virus problem, and especially for whitefly-transmitted viruses such as TYLCV, since it requires no chemical input and/or plant seclusion and may be stable and long-lasting. Thus, the best way to reduce TYLCV spread is by breeding tomatoes resistant or tolerant to the virus (Lapidot & Friedmann, 2002; Morales, 2001; Pico et al., 1996).

Wild tomato species have been screened for their response to the virus and a number of TYLCV-resistant accessions identified, because no resistance has been found in the domesticated tomato (*Solanum lycopersicum*) (Lapidot & Friedmann, 2002; Nakhla & Maxwell, 1998; Pico et al., 1996). Thus, breeding programs have been based on the transfer of resistance genes from accessions of wild origin into the cultivated tomato. Progress in the breeding for TYLCV resistance has been slow, due in part to the complex genetics of the resistance and the presence of interspecific barriers between the wild and domesticated

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 329–342.

tomato species. The lack of an accurate and reliable mass inoculation and selection system has also slowed breeding programs. Since TYLCV is not transmitted mechanically, it is essential that inoculation protocols be developed using whiteflies, which can ensure 100% infection rate, and a standardized (as much as possible) inoculum pressure.

To succeed in a program aimed at developing resistance to TYLCV, a number of issues must be addressed: development of inoculation protocols, screening for resistant genotypes, development of a symptom-severity scale, inheritance of resistance, and determination of the effect a resistant host may have on virus epidemiology. This chapter focuses on aspects of screening for TYLCV resistance using whitefly-mediated transmission.

2. TYLCV INOCULATION

2.1. Controlled greenhouse inoculation vs. spontaneous field inoculation

To identify resistant plants, in either wild-type species, as segregating populations following introgression of resistance to domesticated plants, full control of the pathogen is absolutely essential. Hence, the development of a protocol for a controlled inoculation method is essential. Such an inoculation method must be highly efficient and reproducible. The need to develop an inoculation protocol may seem odd, since TYLCV is the limiting factor in tomato production in many areas worldwide. Hence, why not rely on spontaneous field inoculation? Field populations of whiteflies are usually high. Surely one can grow plants in the field and all of them will be inoculated with TYLCV. Although many breeders have indeed made this assumption, it turns out to be invalid. In fact, spontaneous field-exposure infection has been shown to be largely inefficient, as many plants escape infection, even under heavy inoculation pressure (Vidavsky et al., 1998). Following planting of susceptible tomato plants in an area stricken with whiteflies and TYLCV, only 50% of the susceptible tomato plants were infected during the first month after planting. Despite high whitefly populations and available viral inoculum, 90 days after transplanting, 10% of the susceptible plants had still escaped infection (Vidavsky et al., 1998). Moreover, in another study (Cohen et al., 1988), the percentage of viruliferous whiteflies in the general whitefly population in the field was found to be rather low. Depending on the TYLCV-susceptible host from which the whiteflies were collected, only 3–6% of the whiteflies collected in the field were actually able to transmit the virus (Cohen et al., 1988). It should be noted that a susceptible plant that escapes inoculation and is thus screened (erroneously) as resistant will probably be used as a resistant parent for further crosses. Quite soon the breeding program will get clogged with a large number of susceptible plants that are supposed to be resistant. Thus, selection of tomato plants based solely on the absence of symptoms in an infested field could be misleading (Vidavsky et al., 1998).

Spontaneous field inoculation has other disadvantages besides promoting inoculation escapees. Inoculation may lead to milder disease symptoms compared to controlled inoculation, probably due to late and unsynchronized infection (Pico et al., 1998). A plant inoculated at an older age may exhibit milder symptoms than one infected at a young age. Thus, milder symptoms might be wrongly considered a manifestation of genetic resistance, rather than just late infection. Indeed, some cultivars with partial resistance to TYLCV reacted as susceptible under controlled greenhouse inoculation, whereas in field inoculation they showed a "resistance" level comparable to other more highly resistant cultivars (Pico et al., 1998). It was concluded that the response of a resistant source to TYLCV may vary with the inoculation technique used, and that controlled greenhouse inoculation corresponds to high-inoculum levels, while spontaneous field inoculation corresponds to low ones. It was also concluded that despite the low and delayed disease incidence following spontaneous field inoculation, it is possible to discard the most susceptible genotypes with field testing (Pico et al., 1998).

Another problem with spontaneous field inoculation is that TYLCV-resistant plants may be infected by an unrelated virus, or any other pathogen, and erroneously considered susceptible. In field inoculation, the whitefly pressure, intensity of inoculation, level of viral inoculum, and plant age at time of inoculation are all unknown (Table 1). The elapsed time between whitefly acquisition and transmission of the virus is also unknown. TYLCV, like all begomoviruses, is transmitted by its whitefly vector in a persistent, circulative manner. As shown for TYLCV, although transmission may continue for the life span of the vector, transmission efficiency declines with time (Cohen & Harpaz, 1964). Thus, the efficiency of field inoculation is unknown and it is not reproducible.

Table 1. Main differences between spontaneous field inoculation and controlled inoculation in a greenhouse

Parameter/aspect	Controlled inoculation	Field inoculation
Whitefly		
Gender	Known/controlled	Unknown
Age	Known/controlled	Unknown
Number per plant	Measurable and controlled	Unknown/varies
Acquisition feeding time	Synchronized	Unsynchronized
Time between acquisition and transmission	Known/controlled	Varies
Transmission feeding time	Synchronized	Unsynchronized
Virus source plant		
Plant age	Known	Varies
Virus titer	High	Varies
Inoculated plant		
Age at time of inoculation	Controlled and synchronized	Unsynchronized
Transmission efficiency	Very high, 99–100%	Unpredictable
Avoiding other pathogens	Controlled	Limited

Contrary to spontaneous field inoculation, the different parameters dictating inoculation efficiency are known and can be manipulated under controlled greenhouse inoculation. Table 1 summarizes the main differences between spontaneous field inoculation and controlled greenhouse inoculation. For instance, under controlled greenhouse inoculation, whitefly age, acquisition feeding time, transmission feeding time, number of whiteflies per infected plant and more, can all be manipulated by the researcher conducting the inoculation.

When using whiteflies as viral vectors, it is important to remember that apart from virus transmission, they may cause extensive direct damage to the plant. Upon feeding on a plant, whiteflies damage it through excessive sap removal, excretion of honeydew that promotes growth of sooty mold fungi, and induction of systemic disorders (Byrne & Bellows, 1991; Schuster et al., 1990). Hence, inoculation feeding of whiteflies on the target plant should be long enough to ensure efficient inoculation, but short enough to minimize the direct damage induced by whiteflies.

In essence, a good controlled inoculation consists of allowing whiteflies to feed for 48 h on TYLCV-infected source plants (acquisition access feeding), followed by exposing young tomato seedlings to large numbers of viruliferous whiteflies (approximately 30–50 whiteflies per plant) (Figure 1) which, upon feeding on the plants, transfer the virus with close to 100% efficiency (all susceptible controls become infected with TYLCV) (Lapidot et al., 1997).

2.2. Inoculation in cages

Even in controlled greenhouse inoculation, one may encounter the problem of non-preference. When inoculating tomato plants from different cultivars in the same location, whiteflies may not efficiently inoculate a certain type or cultivar and may prefer to feed on another. In tomato, most examples of non-preference

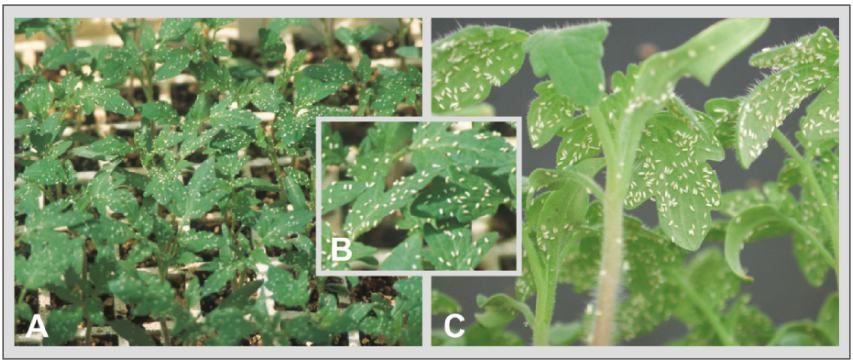

Figure 1. Mass inoculation of young tomato seedlings. (A) Whiteflies immediately after landing on the tomato plants, prior to moving to the abaxial side of the leaf. (B) Close-up on plants from A. (C) Inoculation access feeding of whiteflies on tomato seedlings.

by whiteflies are due to physical barriers, such as waxy or thick cuticles or the presence of specialized trichomes that inhibit whiteflies from settling and feeding on such a leaf (Bellotti & Arias, 2001). This problem is most prominent during inoculation of wild species of tomato; in a search for new sources of resistance, accessions of some wild species tend to escape infection as a consequence of non-preference by whiteflies (Pico et al., 1998). The problem of non-preference can be overcome by carrying out individual inoculation of different wild tomato species in cages. In this case, a single plant is introduced into a cage containing viruliferous whiteflies, forcing them to feed on the target plant (and transmit the virus in doing so) since it is the only plant the insect can feed on.

Another issue that arises following mass inoculation using whiteflies is precision work, or the lack of it. When mass-inoculating large numbers of plants with whiteflies, it is nearly impossible to determine the exact number of whiteflies per plant, and which leaf serves as the inoculation target. Clip cages (or leaf cages) enable precision work with whiteflies (Figure 2).

The advantages of clip cage inoculation are numerous; it allows controlling the number of whiteflies used per plant, their age and gender, the exact length of the acquisition access period (AAP) and of the inoculation access period (IAP), and the site of TYLCV inoculation. Clip cages also enable comparisons between different plants in terms of their response to TYLCV infection, for instance when comparing plants with different levels of viral resistance, since clip cages facilitate the same whitefly-mediated inoculation conditions for all of the different test plants. The clip cage is basically a small transparent plastic cylinder that is truncated on both sides. One side is equipped with a fine mesh cover that can be opened so that whiteflies can be put in the cage by aspiration. The other side has a clip that facilitates its attachment to the underside of the desired leaf (Figure 2). Thus, following the AAP, a known number of whiteflies

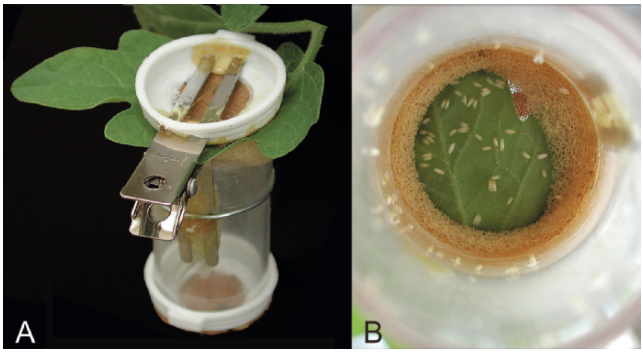

Figure 2. Inoculation of a tomato plant using a clip cage. Viruliferous whiteflies were aspirated into a clip cage, and the cage was attached to a tomato leaf. (A) Side view of the clip cage attached to a tomato leaf. (B) Bottom view of the clip cage, showing the caged whiteflies feeding from the tomato leaf abaxial side.

are aspirated into the cage which is then placed on a leaf, creating an inoculation site (Figure 2). Clip cages were recently used to follow TYLCV multiplication at the inoculation site of a resistant plant, in comparison to viral multiplication in a susceptible plant (Lapidot & Polston, 2006; Segev et al., 2004).

2.3. Non-whitefly-mediated inoculation

Due to the need to breed whiteflies in order to develop a controlled inoculation protocol for TYLCV, other, non-whitefly-mediated inoculation procedures were required. Attempts were made to transmit TYLCV mechanically, using different combinations of source and test plants (Makkouk et al., 1979). The highest TYLCV transmission achieved via mechanical means was less than 17%, and then only when TYLCV-infected datura (*Datura stramonium)* plants were used as the source plants. When datura plants were used as the test plants, the success rate was only 12%. When tomato plants were used as source and test plants, no transmission was achieved with mechanical inoculation (Makkouk et al., 1979). Hence, although it is possible to transmit TYLCV mechanically, the successes rate is far too low to justify the development of an efficient inoculation protocol for this technique.

Graft inoculation has also been used to inoculate TYLCV. With this method, either the test plant is grafted on a TYLCV-infected scion, or a leaf or apex from an infected plant is side-grafted on the test plant. Graft inoculation has been used to screen for TYLCV-resistant plants with high-transmission efficiency (Abou Jawdah et al., 1995; Fargette et al., 1996; Kasrawi et al., 1988). An advantage of graft inoculation is that it allows the continuous exposure of a test plant to high levels of viral inoculum (Friedmann et al., 1998). Grafting might be used to screen for resistance. Resistant plants on which symptomatic TYLCV-infected leaves were grafted remained symptomless (Friedmann et al., 1998). In any event, graft inoculation is labor-intensive and time-consuming and therefore not appropriate as a mass-inoculation technique.

Another inoculation assay applied for TYLCV is agroinoculation (Czosnek et al., 1993; Kheyr-Pour et al., 1994). Agroinoculation uses *Agrobacterium tumefaciens* to deliver cloned viral DNA into host cells (Grimsley et al., 1986, 1987). In the case of TYLCV and other geminiviruses, a tandem repeat (or a 1.5–1.8 mer) of the viral genome is cloned into the T-DNA of an *A. tumefaciens* Ti plasmid, which is then usually delivered by injection into the plant. As a result, genome-sized viral DNA is formed, replicates (Stenger et al., 1991), spreads systemically through the plant and induces disease symptoms. Today, agroinoculation is used routinely to inoculate plants or leaf disks with geminiviruses. Agroinoculation has been used successfully to introduce TYLCV into leaf disks and whole plants, and thus was proposed as an assay system to inoculate and screen for resistant plants with TYLCV (Czosnek et al., 1993; Kheyr-Pour et al., 1994). However, it was shown that the delivery of cloned TYLCV DNA by agroinoculation may overcome the

natural resistance of wild tomato species to this virus. It was concluded that the usefulness of agroinoculation in screening for TYLCV resistance is questionable (Kheyr-Pour et al., 1994).

In a recent study, the effectiveness of agroinoculation as a tool to test for TYLCV resistance in different wild and cultivated tomato genotypes was tested (Pico et al., 2001). Rub agroinoculation (rubbing a leaf dusted with carborundum with a *A. tumefaciens* suspension) resulted in an erratic and mild infection and did not discriminate between genotypes with different resistance levels. Stem agroinoculation (injecting the stem with a *A. tumefaciens* suspension) was more effective; although the inoculation rate of the susceptible control reached 100%, the efficiency of inoculation of the resistant genotypes was lower. It was concluded that agroinoculation can be used in a breeding program but only to complement whitefly-mediated inoculation (Pico et al., 2001).

3. REARING WHITEFLIES

3.1. The whitefly

About 1,300 whitefly species (family Aleyrodidae) in over 120 genera have been described, but relatively few transmit plant viruses (Byrne & Bellows, 1991). Presently, only three whitefly species, *Bemisia tabaci, Trialeurodes vaporariorum*, and *T. abutilonia*, are known as vectors of plant viruses. Of the three virus-transmitting whiteflies, *B. tabaci* is the most important, demonstrated to be the vector of over 100 different viral diseases in the tropics and subtropics, belonging mostly to the *Begomovirus* genus (Jones, 2003).

There are a number of biotypes of *B. tabaci* (see Part I, Chapter 3). The two known biotypes which are prevalent in the Mediterranean region and transmit TYLCV with high efficiency are the B (also known as the silverleaf whitefly) and Q biotypes. The B biotype, which is the most studied, has also been considered as a different species – *B. argentifolii*, but this separation is not universally accepted (Brown et al., 1995; Perring, 2001; Perring et al., 1993). Unless stated otherwise, this chapter discusses only the B biotype of *B. tabaci*.

The whitefly life-cycle progression, from egg to adult emergence, is governed mainly by temperature. In warm weather, such as the Mediterranean summer, the life cycle takes approximately 3 weeks, but it may take up to 2 months under cool conditions. Adult emergence does not occur when the temperature drops below 17°C. The vast majority of adult whiteflies emerge during daylight with only a few emerging in the dark. The rate of whitefly reproduction varies with the host plant, but the average is 160 eggs per female (with an approximate range of 50–400), with female crawlers emerging from about two-thirds of the eggs (Byrne & Bellows, 1991).

3.2. Host plant

The *B. tabaci* species is highly polyphagous. Although the genus *Bemisia* has a wide range of host plants (more than 500 species from 74 plant families), not all of them support large populations of whiteflies. Plants that do support large numbers of B biotype whiteflies include cotton, cabbage, cucumber, squash, melon, watermelon, tomato, eggplant, sesame, soybean, okra, bean, peanut, and many ornamentals, including poinsettia, hibiscus, lantana, verbena, garden mum and Gerber daisies, to name a few.

When considering which plant to use as a host for rearing whiteflies, a number of issues have to be considered: (1) the plant should be able to support large populations of whiteflies; (2) the plant has to be strong enough to survive these large populations of whiteflies without collapsing; (3) the plant should be a non-host for the viral diseases under investigation – in our case, TYLCV; and (4) the plant should be relatively easy to grow and maintain.

We use cotton plants routinely as a host to rear whiteflies. Cotton plants are non-hosts for TYLCV and the other whitefly-transmitted viruses native to the Mediterranean region, relatively easy to maintain and can support large numbers of whiteflies without collapsing. However, cotton plants grow relatively slowly, and develop poorly below 15°C. Therefore, cotton plants are suitable for whitefly rearing mainly in warm climates, and even then they require efficient heating in the winter.

3.3. Whitefly cages

When growing whiteflies, the insect has to be contained in a cage, due to its flying ability. Cages come in all shapes and sizes. The simplest cage is built from basic structural materials (plastic, metal, or wood) with an insect net (such as 50-mesh) attached. For routine whitefly growth we use greenhouse benches covered by stainless-steel plates: these are light, strong, and unaffected by water (unfortunately, they are expensive). Thin plastic rods in the shape of an arch are attached to the plate. The "arches" are covered by 50-mesh nets. This structure is cheap to build and maintain, easy to replace when broken, and of course, not affected by water.

Regardless of its shape or the material used to build it, the cage must be well ventilated. Whiteflies secrete honeydew on the plants, and together with high humidity, sooty mold fungi are inevitable. A high rate of fungi together with a whitefly colony usually causes plant-host collapse.

Another constant problem is the invasion of other insects in the whitefly cages. The whiteflies are provided with the best growing conditions, which attract other insects as well. Moreover, spraying with insecticides is practically impossible once the whiteflies are in the cage. The 50-mesh nets will usually prevent penetration of aphids, but not of thrips or mites. The best way to deal with insects other than whiteflies is to make sure that the plants used as whitefly hosts

are as devoid as possible of other insects prior to whitefly colonization. Ants and spiders are also attracted to the whitefly cages; ants are attracted to the secreted honeydew, while spiders find the caged whiteflies easy preys. In this case as well, the best way to deal with the problem is to keep the growth area as clean as possible, and to eliminate ants and spiders from the whitefly facility.

4. SCREENING FOR RESISTANCE LEVEL

4.1. Symptom-severity rating

The immediate manifestation of a pathogen infecting a plant is the expression of disease symptoms. Hence, many resistance-breeding programs rely mainly (and sometimes solely) on symptom expression. However, host resistance to TYLCV may be manifested by a reduction in disease symptoms rather than no symptoms at all. TYLCV-induced symptoms usually appear within 2–3 weeks after inoculation. Hence, it has been suggested that plants be scored 30 days after inoculation, when full-scale symptoms are expressed. To analyze segregating populations for TYLCV resistance, a symptom-severity rating system was developed (Friedmann et al., 1998; Lapidot & Friedmann, 2002), ranging from 0 to 4: (0) no visible symptoms, inoculated plants show same growth and development as non-inoculated ones; (1) very slight yellowing of leaflet margins on apical leaf; (2) some yellowing and minor curling of leaflet ends; (3) a wide range of leaf yellowing, curling, and cupping, yet plants continue to develop; (4) very severe plant stunting and yellowing, pronounced cupping and curling, plants stop growing (see Part V, Chapter 3).

Viral DNA content was determined for plants from each symptom level (Friedmann et al., 1998). According to the viral DNA level, three different groupings were found, which were statistically significant. Plants showing levels 0 and 1 had the same very low amounts of viral DNA, plants with level 2 and 3 symptoms had intermediate, albeit significantly higher amounts of viral DNA than at levels 0 and 1, while plants of level 4 had very high amounts of viral DNA. It should be noted that level 3 and 4 plants expressed a high level of variation in the amount of viral DNA detected (Friedmann et al., 1998).

4.2. Yield reduction

The most relevant evaluation of resistance level lies in the effect of TYLCV infection on total yield and yield components, relative to noninfected controls (Lapidot et al., 1997). Usually, tests comparing different varieties are carried out under field inoculation, and comparison is not made with the full yield potential of uninfected plants. Moreover, often there is confusion between the general performance of a given variety and the level of TYLCV resistance it displays. Hence, it is recommended to perform a yield-reduction test as follows. Seeds of the tested lines or cultivars are sown in an insect-proof greenhouse.

The seedlings are inoculated at an early stage, preferably at the first true leaf. Non-inoculated plants of the same cultivar or line exposed to nonviruliferous whiteflies serve as non-inoculated controls. After a short recovery period, the plants are transplanted to the field. The inoculated plants of each cultivar or line are compared with their respective control non-inoculated counterparts, in terms of total yield, average fruit weight and number. In this way, variation due to differences in the genetic backgrounds of the tested varieties can be overcome, and only the effects of the virus on yield and yield components are recorded. It has to be noted that such expensive and time-consuming tests can only be carried out on the most promising resistant varieties, not on segregating populations.

4.3. TYLCV-resistance scale

A major obstacle in the development of TYLCV resistance has been the lack of a standard for its assessment. The assessment of resistance levels displayed by plants being screened is even more confusing, since to date, TYLCV resistance has been multigenic and quantitative. Thus, due to the lack of a standard for resistance assessment, resistance level is usually based on the severity of disease symptoms induced by the virus. However, plant age at the time of infection, inoculation pressure and growth conditions can have major effects on the severity of the induced disease symptoms (Lapidot et al., 2000; Pico et al., 1998). Thus, variability in assay conditions leads to contradictory results, where different resistance levels have been attributed to the same genetic sources (Lapidot et al., 2000; Pico et al., 1998; Vidavsky et al., 1998). In addition, the performance of at least some of these resistance genes appears to be influenced by the environment. Significant differences in resistance performance were found when a number of TYLCV-resistant cultivars were compared under different environmental conditions; for example yield reduction was double when the resistant plants were evaluated in the field during a hot season than when evaluated in a greenhouse during a cooler season (Lapidot et al., 2000). Hence, the different performances of the same cultivars when tested under different conditions can be misleading and can frustrate efforts to select for the best TYLCV resistance from a segregating population.

To overcome these difficulties, a scale of differential TYLCV-resistant hosts was developed, which enables the determination and comparison of TYLCV resistance levels expressed by resistant tomato lines or by individual plants in a segregating population (Lapidot et al., 2006). The scale is made up of seven different homozygous tomato genotypes that exhibit different levels of TYLCV resistance, ranging from fully susceptible to highly resistant. The differential hosts composing the scale were inoculated with TYLCV under greenhouse conditions. Four weeks after inoculation, the plants were evaluated for disease-symptom severity, and virus DNA titer was determined. The different genotypes were arranged along the scale according to symptom-severity score. The different

genotypes were then tested under different environmental conditions, inoculated at different ages, and tested in a field experiment assaying TYLCV-induced yield reduction (by comparing the yield of TYLCV-infected plants to the yield of control non-inoculated plants of the same variety). While the symptom-severity score of each individual resistant genotype changed under different environmental conditions, their relative position on the scale did not, except for one genotype. Moreover, the yield reduction induced by the virus was in direct correlation with the symptom-severity score, except for one genotype (Lapidot et al., 2006). Using this scale, TYLCV-resistance level in a segregating population and/or of stable resistant lines can be determined as quickly as 4 weeks post-inoculation. Moreover, the scale can be used either in a controlled environment such as a greenhouse or under field conditions. Thus, to evaluate disease resistance of a given tomato genotype, it should be inoculated alongside the differential hosts composing the scale, and within 4 weeks one can determine the relative level of resistance of the tested genotype.

5. EFFECT OF TYLCV RESISTANCE ON VIRUS EPIDEMIOLOGY

Although disease resistance reduces the deleterious effects of the virus, the potential of resistant varieties to serve as virus reservoirs should be considered. We have used four tomato genotypes with different levels of resistance to TYLCV, ranging from fully susceptible to highly resistant, as virus source plants. We have examined the survival rates and the TYLCV acquisition and transmission parameters of whiteflies that fed on the different infected tomato genotypes. The survival rates of whiteflies that fed on the different source plants at 21 days post-inoculation (dpi), shortly after the appearance of TYLCV symptoms, were similar regardless of the source plant. Significant differences were found after whiteflies fed on infected source plants at 35 dpi; the whitefly survival rate was positively correlated with the level of resistance of the source plant. This may be due to the deleterious effects of TYLCV in susceptible plants. At 35 dpi, the susceptible and moderately resistant genotypes exhibited pronounced symptoms, presumably making the plant less suitable for whitefly feeding. In contrast, the highly resistant genotypes were symptomless, favoring whitefly survival (Lapidot et al., 2001).

TYLCV amounts in whiteflies following feeding was found to be in direct correlation with the virus amounts in the source plant: the higher the level in the source plant, the higher the TYLCV level in the whitefly. This correlation was maintained, regardless of the time of feeding – 21 or 35 dpi – and regardless of the state of the source plants. The severity of disease symptoms exhibited by the source plants did not seem to affect TYLCV acquisition by the whiteflies.

Transmission rate by whiteflies that fed on infected source plants at 21 dpi was negatively correlated with the level of resistance displayed by the source plant; therefore, the higher the resistance, the lower the transmission rate. However, at 35 dpi, transmission rates from the susceptible plants were lowest,

presumably due to their poor condition. Transmission rates from source plants displaying a medium level of resistance were highest, with rates declining following feeding on source plants displaying higher levels of TYLCV resistance (Lapidot et al., 2001).

Based on these results, we postulated that a TYLCV-infected field of susceptible tomato plants may serve as a high-risk virus reservoir early after infection (Lapidot et al., 2001). However, as the plants deteriorate due to the expression of TYLCV disease symptoms, the potential of these plants to serve as a source of virus declines. In contrast, a field of moderately resistant plants will serve as an effective virus reservoir throughout the season, as plants do not deteriorate as badly as do the highly susceptible ones. In contrast, following infection in the field, tomato plants expressing a high level of resistance to TYLCV pose the lowest risk to the surrounding plants in terms of outbreaks of viral epidemic.

6. CONCLUDING REMARKS

In this chapter, the major issues concerning whitefly-mediated inoculation and screening of TYLCV-resistant tomato plants were summarized. These include: the need to develop a controlled mass-inoculation system; the unreliability of spontaneous field inoculation as a screening tool during breeding for resistance; how to screen for TYLCV-resistant genotypes, and finally, the effect resistant plants may have on viral epidemiology. These issues are also valid for other whitefly-transmitted viruses, and to some extent for viruses transmitted by other insects as well. Hence, this chapter may serve as a basic guideline for researchers aiming at the development of resistance to TYLCV in tomato or in other hosts, as well as at resistance to other viruses.

7. ACKNOWLEDGMENTS

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CHAPTER 2

SOURCES OF RESISTANCE, INHERITANCE, AND LOCATION OF GENETIC LOCI CONFERRING RESISTANCE TO MEMBERS OF THE TOMATO-INFECTING BEGOMOVIRUSES

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1. OVERVIEW

Several management strategies have been implemented in different regions of the world for control of the tomato-infecting begomoviruses. These have included planting virus-free tomato seedlings, applying insecticides, using insect-proof netting, and implementing a whitefly-host-free period. Recently, tomato cultivars with various levels of resistance have become available for some parts of the world. The use of resistant hybrids offers many advantages; and when used with integrated pest-management methods, these resistant hybrids can ensure that the growers will have a tomato crop.

Different begomoviruses infect tomatoes in different regions of the world. Currently, there are over 35 described tomato-infecting begomoviruses (Fauquet et al., 2003; Jones, 2003; Morales & Anderson, 2001. See also Part II, Chapter 2). Begomoviruses are divided into two groups based on genome organization: those with a monopartite genome and those with a bipartite genome. *Tomato yellow leaf curl virus* (TYLCV) was the first monopartite begomovirus to be molecularly characterized (Navot et al., 1991), and this was quickly followed by *Tomato yellow leaf curl Sardinia virus* (TYLCSV, Kheyr-Pour et al., 1991). Fauquet et al. (2005) described TYLCV as a complex of different begomovirus species. In addition to the characterization of TYLCV and TYLCSV as monopartite begomoviruses, many other tomato-infecting

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 343–362.

monopartite begomoviruses have been described, such as *Tomato leaf curl virus* (Banerjee & Kalloo, 1987). Besides these monopartite begomoviruses, many tomato-infecting bipartite begomoviruses are known, e.g., *Tomato mottle virus* (ToMoV), *Pepper golden mosaic virus* (PepGMV), *Pepper Huasteco yellow vein virus* (PHYVV), *Tomato leaf crumple (chino) virus* (ToLCrV) (see Fauquet et al., 2003). Commonly, bipartite begomoviruses exist as mixed infections in tomato plants (Kon et al., 2006; Nakhla et al., 2005). For instance, seven begomoviruses were identified in a single experimental field in Guatemala (Nakhla et al., 2005). Recombination among begomoviruses has contributed to the creation of this diversity (Fauquet et al., 2005; Kon et al., 2006; Padidam et al., 1999) and will continue to be a factor in the evolution of these viruses.

Plant breeders face tremendous challenges, because of the diversity of tomato-infecting begomoviruses. A further concern for plant breeders is the introduction of exotic tomato-infecting begomovirus into a country. This occurred in the Caribbean islands with the introduction of TYLCV from the eastern Mediterranean and resulted in serious crop losses (Salati et al., 2002). Plant breeding programs can employ molecular analysis to track resistance genes in a marker-assisted selection (MAS) breeding scheme. These tools will be important in breeding the next generation of begomovirus-resistant hybrids, which are likely to have multiple-resistance genes from different wild tomato species. It is expected that these hybrids will have higher levels of resistance and be resistant to different begomovirus species (Moustafa & Nakhla, 1990; Vidavsky & Czosnek, 2006).

2. MAJOR SOURCES AND INHERITANCE OF RESISTANCE GENES

All of the domesticated tomato source germplasm (*Solanum lycopersicum*, syn. *Lycopersicon esculentum*) originally tested for resistance to TYLCV were susceptible (Pilowsky & Cohen, 1974) and no source of resistance to begomoviruses have been identified in *S. lycopersicum* since then, with the exception of a few commercial hybrids, which are discussed below. Thus, it was necessary to screen the related wild species of tomato for potential sources of resistance to TYLCV and other begomoviruses. Many accessions of the wild species are heterogeneous populations, so it is expected that different plants will have different interaction phenotypes. This has been demonstrated by interaction phenotype studies with wild species (Hassan et al., 1982) and by molecular analysis at the REX-1 locus for individual plants from a single accession (LA1969, *Solanum chilense*) (Salus & Maxwell, 2006).

An exception to the use of wild species as sources of resistance genes may be the commercial hybrids marketed by Royal Sluis who derived their begomovirus resistance from a *S. lycopersicum* landrace from Cape Verde Island resembling the French heirloom variety "Marmande" (J. Webster and J. Hoogstraten, 2005, personal communication).

Sources of resistance or tolerance to TYLCV and bipartite begomoviruses and their application in tomato breeding programs were summarized previously (Picó et al., 1996, Scott 2007).

2.1. *Solanum pimpinellifolium*

Pilowsky & Cohen (1974) were among the first to study TYLCV-resistance in wild species; they found resistant plants in several accessions of *S. pimpinellifolium* and reported that resistance was controlled by a single, incompletely dominant gene in LA121. Similar inheritance of resistance was proposed for accession A1921 (Banerjee & Kalloo, 1987). LA121 and LA373 were studied by Hassan et al. (1984) and resistance was quantitatively controlled and partially recessive. A single dominant gene was associated with resistance in the accessions hirsute-INRA and LA1478 (Kasrawi, 1989), LA1478 and LA1582 (Geneif, 1984), and PI407543 and PI407544 (Hassan & Abdel-Ati, 1999). Partial dominance was proposed for the resistance from PI407555 (Hassan & Abdel-Ati, 1999). The PIMPERTYLC population was created by crossing *S. pimpinellifolium* plants from accessions hirsute-INRA and LA1478 (Laterrot, 1992), which had been selected for resistance in different countries. Even though resistance has been detected in various accessions of this wild species, it has not become a major source of resistance genes in current breeding programs.

2.2. *Solanum peruvianum*

Because breeding lines derived from *S. pimpinellifolium* LA121 had low vigor and yield, Pilowsky & Cohen (1990) evaluated another wild species, *S. peruvianum*, and found that several recessive genes were associated with plants from TYLCV-tolerant accession PI26935. This effort resulted in the release of TY20 as a moderately resistance cultivar. Subsequently, highly resistant breeding lines (e.g.,TY172, TY197) have been developed at the Volcani Center in Israel from *S. peruvianum* (PI126926, PI126930, PI390681, and LA441 (Friedmann et al., 1998; Lapidot et al., 1997). The resistance in these lines was considered to be partially dominant; and genetic analysis of an F_2 population indicated that at least three genes were involved in resistance (Lapidot et al., 2000). In Egypt, Hassan et al. (1982) found that accessions LA372, LA452, LA462, LA1274, LA1333, LA1373, and CMV sel INRA (PI126926 × PI128648–6, Laterrot, 1984), as well as *S. peruvianum* var. *humifusum* LA385 were highly resistant to TYLCV. This group developed a resistant breeding line from an interspecific cross of *S. lycopersicum* Mortelglan × CMV sel INRA (Hassan et al., 1987). Genes from *S. peruvianum* are presently deployed in commercially grown hybrids that have provided good resistance to TYLCV (M. Lapidot, 2006, personal communication).

2.3. *Solanum chilense*

Resistance genes carried in introgressions from *S. chilense* are important in several breeding programs around the world (Mejía et al., 2005; Pinón et al., 2005; Scott, 2001; Scott et al., 1995; Zakay et al., 1991). Zakay et al. (1991) reported high levels of resistance to TYLCV in individual plants from LA1969; and plants from this accession have also been found to be resistant to TYLCV in Cuba (Pinón et al., 2005) and to ToMoV and TYLCV in Florida, USA (Scott & Schuster, 1991; Scott et al., 1995). Introgression of TYLCV resistance from LA1969 was also carried out in breeding programs of worldwide research teams (Laterrot & Moretti, 1994; Chiang et al., 1994; Zamir et al., 1994). A gene, *Ty-1*, conferring partially dominant resistance has been mapped to chromosome 6 and two modifier genes mapped to chromosomes 3 and 7 (Zamir et al., 1994). Resistance has also been introgressed into the cultivated tomato from LA1969 by some private seed companies and the resistance is located in a chromosome 6 region that includes *Ty-1* and possibly another linked resistance locus (Hoogstraten and Mercier, 2003, personal communication). This introgression appears to provide resistance to a broader array of begomoviruses than that of *Ty-1* alone. *S. chilense* accessions LA1932, LA1938, LA1959, LA1960, LA1961, LA1963, LA1968, LA1969, LA2747, LA2774, and LA2779 were found to be resistant to the bipartite begomovirus ToMoV in Florida and used to initiate a program of interspecific crosses (Scott et al., 1995). Later, LA1932, LA2779, and LA1938 have proven to be useful sources of resistance for the tomato breeding program in Florida (Scott et al., 2001). Inheritance studies using LA1932 indicated two loci with primarily additive gene action accounted for the resistance to ToMoV (Griffiths & Scott, 2001).

The CHILTYLC 92 is a BC_1F_2 population derived from self-pollination of the cross ((Momor verte × LA1969) × Tropiva 3). Selection and subsequent backcrosses to Tyking and Fiona led to the development of CHILTYLC 93 and CHILTYLC 94, respectively (Laterrot & Moretti, 1994). The latter two populations segregated for resistance to ToMoV that appears to be derived from Tyking, in part at least (Scott, 1998, unpublished data).

Currently, several commercial breeding programs are using resistance genes from *S. chilense* and horticulturally acceptable cultivars are being marketed. Among these cultivars are Anastasia, Boludo, and Carmencita in Spain, Titrit in Morocco, Llanero in Guatemala, and Tygress in Florida, USA.

2.4. *Solanum habrochaites*

Accessions of *S. habrochaites* LA0386, LA1252, LA1295, LA1352, LA1393, LA1624, and LA1691 were highly resistant to TYLCV (Hassan et al., 1982). Phenotypic evaluation of the F_1 *S. lycopersicum* cv. VF145-B7879 × LA0386 indicated that resistance was dominant but a low number of F_2 plants prevented an analysis of inheritance (Hassan et al., 1984). A high level of resistance was

reported for LA1777 (Ioannou, 1985; Fargette, 1991; Moustafa, 1991). Vidavsky & Czosnek (1998) selected TYLCV-resistant plants from LA0386 and LA1777, and these plants were crossed to produce a highly resistant F_1 population, which was used in crosses with *S. lycopersicum*. The resulting tolerant interspecific F_1 plants were backcrossed to the cultivated tomato. Through a series of self-pollinations and phenotypic selection for resistance to TYLCV, plants with immunity and tolerance were generated. They suggested that resistance was controlled by a major dominant gene and several minor genes. A breeding line Ih902 was used to create hybrids, including FAVI 9, which has been an important source of resistance for breeding programs in Guatemala (Mejía et al., 2005) and other Middle East countries (Maruthi et al., 2003). Picó et al. (2000) also found high levels of resistance in *S. habrochaites*. In India, *S. habrochaites* f. *glabratum* B6013 was shown to have two epistatic genes controlling resistance to *Tomato leaf curl virus* (Banerjee & Kalloo, 1987). Subsequently, these researchers developed breeding line H24 from this accession (Kalloo & Banerjee, 1990). "H24" has been shown to carry the resistance gene *Ty-2* (Hanson et al., 2000, 2006). "H24" confers specific tolerance to some, but not all strains of TYLCV/ToLCV (e.g., tolerance to TYLCV/ToLCV strains in Taiwan, northern Vietnam, South India, and Israel but susceptible to TYLCV strains from northern India, Thailand, and the Philippines). *Ty-2* resistance was the initial source of resistance used in tomato breeding program at the Asian Vegetable Research and Development Center (AVRDC) and has been extensively exploited by some seed companies in Asia and elsewhere.

2.5. *Solanum cheesmaniae*

A recessive and/or polygenic resistance has been associated with accessions of *S. cheesmaniae* (Hassan et al., 1984; Kasrawi, 1991). In Egypt, a moderately resistant breeding line (line 44) was derived from introgression of resistance genes from *S. cheesmaniae* with the commercial cultivar Pakmor (Moustafa & Nakhla, 1990). This species has not been a significant source of resistance in current commercial cultivars.

3. DEPLOYMENT OF RESISTANCE SOURCES IN DIFFERENT REGIONS

One of the major issues in breeding begomovirus-resistant hybrids is the utility of different resistance alleles in regions where different begomoviruses are present. This has been investigated for some lines by multiple-site testing. For example, TY52, which has resistance derived from *S. chilense* LA1969, was developed in Israel and has moderate resistance to TYLCV. When evaluated in Guatemala, where the begomoviruses are bipartite and the virus pressure was very high, this line was susceptible (Mejía et al., 2005). When evaluated in eight locations in Southeast Asia and in Florida, USA, it was susceptible in seven locations, moderately resistant in one and

resistant in another (Green and Shanmugasundaram, this book). For another line, H24, with resistance to ToLCV from *S. habrochaites* f. *glabratum* (Kalloo & Banerjee, 1990), it was resistant in five locations and susceptible or slight resistance in four other locations (Green and Shanmugasundaram, this book). Of nine germplasm sources tested at AVRDC, only *S. chilense* LA1932 was resistant at all eight sites in Southeast Asia and Florida, USA. The *S. habrochaites* LA1777 was resistant at all sites except the Sri Lanka site (see Part VI, Chapter 2). However, LA1777 is resistant to the whitefly (Momotaz et al., 2005) and this can confound determination of virus resistance. Pietersen & Smith (2002) evaluated tomato germplasm that had been selected for resistance to TYLCV by inoculation with *Tomato curly stunt virus* (ToCSV), and they found TYLCV-resistant lines with resistance derived from either *S. chilense* or *S. habrochaites* were resistant to ToCSV. Similarly, Maruthi et al. (2003) evaluated germplasm in Bangalore, India, and Rehovot, Israel. The begomoviruses at these two sites are monopartite ToLCV and TYLCV, respectively. The breeding line Ih902 with resistance derived from *S. habrochaites* was resistant at both locations. In contrast, *S. pimpinellifolium* LA121 was moderately resistant in India but susceptible in Israel. They concluded that *S. habrochaites* LA1777 and IP390659 were the best source of resistance to these two viruses. Breeding lines derived from Ih902 and Fla595 (*S. chilense* LA2779 resistance) have also been resistant in Guatemala (Mejía et al., 2005), where at least seven bipartite begomoviruses exit. The highly TYLCV-resistant breeding lines from Volcani Center, Israel, such as TY172 and TY197 (Friedmann et al., 1998; Lapidot et al., 1997), which have a resistance genes derived from *S. peruvianum*, were also highly resistant in Guatemala (Mejía et al., 2005).

Besides testing at multiple locations, another approach would be to evaluate germplasm using different begomoviruses. de Castro et al. (2005) used this approach with two monopartite begomoviruses, TYLCV and TYLCSV. Twelve breeding lines with resistance derived from *S. chilense* were selected as resistant to TYLCSV, and when these lines were inoculated with TYLCV, six were resistant. In Florida, USA, the tomato breeding program has used separate inoculations with a bipartite begomovirus (ToMoV) and a monopartite begomovirus (TYLCV) to select breeding lines with resistance derived from *S. chilense*. Lines with a high level of resistance to one virus have also been highly resistant to the other virus (Scott et al., 1995). Some of these highly resistant lines have been tested for various begomoviruses around the world and they usually have some level of resistance. However, some lines have better resistance in certain locations whereas different lines have better resistance in other locations. Nevertheless, it is generally believed that these lines have a broad spectrum of resistance provided by relatively simple (digenic) inheritance. Modifier genes may interact with environmental conditions to preclude a genotype from being universally resistant to all begomoviruses.

From multi-location testing, where different tomato-infecting begomoviruses exist, or from inoculation of the same material with different begomoviruses, it is evident that it is not always possible to predict the resistance reaction against

a certain begomovirus. Thus, a genotype by environment interaction is evident, which often results in different interaction phenotype. However, breeding lines with resistance genes from *S. habrochaites*, *S. chilense*, and *S. peruvianum* have all exhibited resistance against both monopartite and bipartite begomoviruses at locations with very different climates.

4. TAGGING LOCI FOR RESISTANCE TO BEGOMOVIRUSES

Development of tomato hybrids relies on traditional protocols of plant genetics and breeding as well as marker-assisted selection when available. There has been significant progress in the development of markers for important resistance genes (Foolad & Sharma, 2005), and this is expected to continue as the tomato genome is sequenced. Currently over 285 morphological, physiological, and disease resistance markers, 36 isozymes and over 1,000 restriction fragment length polymorphism (RFLP) markers have been mapped to the 12 individual chromosomes (Foolad & Sharma, 2005; Sol Genomic Network, www.sgn.cornell.edu). From these linkage maps, it is possible to predict the ease with which two traits might be combined. For example, the *Mi-1* gene for root knot nematode resistance in the short arm of chromosome 6, and the nearby gene for TYLCV resistance, *Ty-1,* are linked in repulsion in ca. 6 cM region (Mueller et al., 2005; Zamir et al., 1994), and thus it would be difficult to bring these two genes together in cis. However, Garcia and Maxwell (2007, personal communication) recently detected a breeding line, Gh2, that was homozygous for the *Mi-1* and *Ty-1* loci.

Marker-assisted selection is currently an important part of many commercial breeding programs. This methodology makes it possible to follow a genetic trait without a biological assay and thus allows for more rapid development of tomato hybrids. The mapping and development of markers for genes resistant to begomoviruses would facilitate breeding of hybrids by pyramiding resistance genes from various sources. For example, in the future, resistance genes from the different wild species may be combined to provide higher levels of resistance and to provide resistance to a wider range of begomoviruses.

In the following sections, information on markers was obtained from the Solanaceae Genomics Network (www.sgn.cornell.edu). The Tomato-EXPEN 2000 map, which was constructed from an F_2 *S. lycopersicum* × *S. pennellii* population, is used as a reference map in the discussion.

4.1. *Solanum chilense*

Zamir et al. (1994) were the first to report the mapping of a begomovirus resistance gene using lines derived from this species. The *S. lycopersicum* susceptible line, M82–1–8, was used as the female parent in a cross with TYLCV-resistant *S. chilense* accession LA1969. Only one interspecific hybrid plant was produced from 300 pollinated flowers. Analysis of RFLP markers and resistance in the subsequent backcross and selfed (BC_2S_1 and BC_2S_2) progenies revealed that a major

incompletely dominant gene (*Ty-1*) on chromosome 6 accounted mostly for the resistance. This gene was mapped near RFLP markers TG297 (4.0 cM) and TG97 (8.6 cM), the latter being the closest marker. A second locus, which contributed a lesser degree to the resistant interaction phenotype, was located near TG61 (9.0 cM) on chromosome 7. The RFLP markers (TG66 and TG33) on chromosome 3 had only minor association with the resistant interaction phenotype. The contribution of various loci to the resistant interaction phenotype was further investigated using a BC_3S_3 (backcross-3 and then self-3) population. Plants homozygous for the *S. chilense* allele at TG61 on chromosome 7 were as susceptible as the susceptible controls. However, plants homozygous for *S. chilense* alleles at TG297 and TG97 on chromosome 6 were symptomless, while susceptible controls had severe symptoms. PCR-based markers for the TG97 locus can be licensed from Hebrew University of Jerusalem, Israel, and are being used in MAS by research institutes and commercial companies. An alternative marker that can be used to tag the *Ty-1* gene is the CAPS (Cleaved Amplified Polymorphic Sequences) marker REX-1 (ca. 5.5 cM), which is also associated with the *Mi-1* gene for resistance to root-knot nematode (Milo, 2001; Williamson et al., 1994). REX-1 PCR fragments for *S. lycopersicum*, *S. chilense*, and *S. peruvianum* have zero, two and one *TaqI* restriction sites, respectively, which can easily be differentiated by standard agarose gel electrophoresis. These three species as well as *S. habrochaites* can also be distinguished by a number of unique SNPs for the REX-1 locus (Salus & Maxwell, 2006).

As mentioned above, LA1932, LA1938, and LA2779 also showed high levels of resistance to begomoviruses (Scott & Schuster, 1991; Scott et al., 1995; Scott, 2001). A low percentage of backcross progenies (3.4%) from the *S. chilense* introgressions resistant to ToMoV suggested a polygenetic nature of ToMoV resistance (Scott et al., 1995), which was confirmed from inheritance studies of this resistance in various populations (Griffiths, 1998). Supporting evidence also came from QTL analysis of a LA1932-derived F_2 population with RAPD markers, which indicated that at least two additive regions controlled ToMoV resistance (Griffiths & Scott 2001). Twelve RAPD markers, which were linked to the morphological markers self-pruning (*sp*) and potato leaf (*c*) on chromosome 6, were associated with ToMoV resistance and segregated into two linked regions flanking either side of the *sp* and *c* loci. A third region encompassing the *Ty-1* resistance gene was also associated with ToMoV resistance in LA2779- or LA1938-derived lines (Griffiths, 1998). Recently, Agrama & Scott (2006) confirmed at least three regions on chromosome 6 contributed to begomovirus resistance in these accessions, and each region was associated with a number of RAPD markers. Efforts were taken to search for RAPD markers tightly linked to the resistance loci, using advanced breeding lines derived from these accessions (Ji & Scott, 2005a). The tightly linked RAPD markers were converted to sequence characterized amplified region (SCAR) markers (Ji & Scott, 2004, 2005b).

Recently, Ji & Scott (2006a) reported that advanced breeding lines derived from LA2779, which are resistant to both TYLCV and ToMoV, have a large intro-

gressed segment spanning markers from C2_At2g39690 (5.3 cM) to T0834 (32 cM) on chromosome 6. A partially dominant gene, that we are now designating *Ty-3* (Ji & Scott, 2006b), was mapped to the marker interval between cLEG-31-P16 (20 cM) and T1079 (27 cM) on the long arm of chromosome 6 (Figure 1) using an F_2 population of susceptible *S. lycopersicum* × resistant advanced breeding line having this introgression (Ji & Scott, 2006a). This gene has a dominance-to-additive effect ratio of 0.47 (an additive effect of –1.1 and a dominance effect of –0.52), suggesting a nearly equal contribution to the variance of TYLCV resistance from additive and dominance effects (Purcell et al., 2003). Additionally, this gene accounts for most (~65%) of the variance in TYLCV resistance in the F_2 population, indicating this gene is a major locus responsible for the resistance.

Besides TYLCV resistance, *Ty-3* likely contributes resistance to ToMoV, but to a lesser degree, although the possibility of a different genetic locus in the same region that is responsible for ToMoV resistance cannot be ruled out. QTL analysis of an F_2 population from the same cross, but inoculated with ToMoV, revealed that ~41% of the variance in ToMoV resistance in the progeny could be explained by this gene locus, which had a dominance-to-additive ratio of 0.35 (an additive effect of –1.17 and dominance effect of –0.41). No recombinants were found between TG590 (22 cM) and T1079, which prevented a fine-scale mapping of the *Ty-3* gene using this LA2779-derived F_2 population. To address this limitation, the same authors employed another F_2 population from a cross of susceptible *S. lycopersicum* × resistant advanced breeding line derived from LA1932. The latter has a short *L. chilense* introgression from TG590 to C2_At5g41480 (26 cM). Similarly, recombination is greatly suppressed in the introgressed segment, and only two recombinants were found among 247 progeny (Figure 1). The responsible resistance gene was mapped to the interval from TG590 to C2_At5g41480, the same region as in the LA2779-derived breeding lines carrying the *Ty-3* gene. It is most likely that a resistance gene in LA1932-derived lines is at the *Ty-3* locus. Previous studies (Griffiths, 1998; Griffiths & Scott, 2001; Agrama & Scott, 2006) using RAPD markers and less advanced breeding lines did not reveal resistance from LA1932 close to the *Ty-1* locus, but located two resistance regions flanking either side of the *sp* and *c* loci as discussed above. Supporting evidence came from marker analysis of the resistant parents (960719) used in the previous studies, which revealed no *S. chilense* introgression around the *Ty-1* region from markers T1928 (0 cM) to T0774 (18 cM) (Ji et al., 2007). A SCAR marker, P169C, converted from a RAPD marker UBC169 linked to the resistance locus flanking the *sp* locus (81.5 cM) (Griffiths & Scott, 2001), mapped to the *Ty-3* region (Figure 1). However, the *sp* locus is ~75 cM from the *Ty-1* region, while there is only ~20 cM between *Ty-1* and *Ty-3* regions. This may be due to the location of the RAPD markers and a large introgression in the early breeding lines including line 960719. Noncontinuous introgressions in line 960719 spanned markers from cLEG-31-P16 (20 cM) to the *sp* locus (Ji et al., 2007), which must cover the *Ty-3* region as well as the RAPD

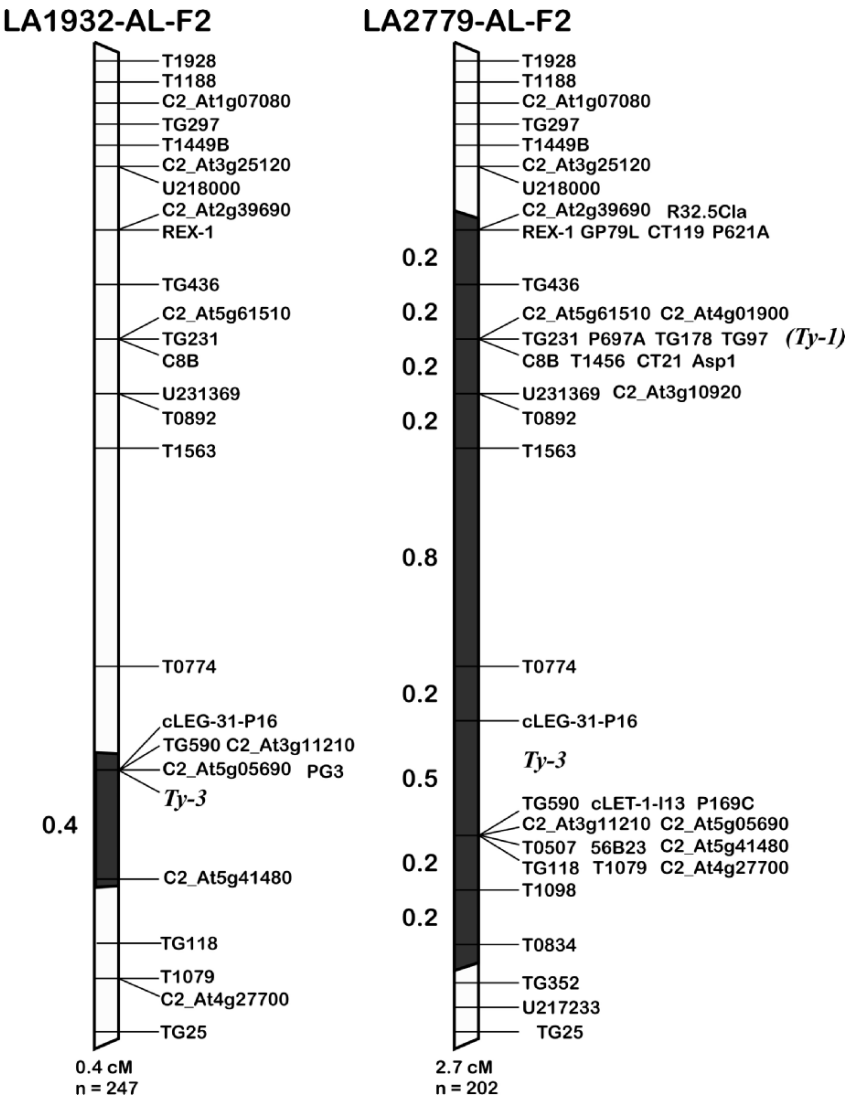

Figure 1. Map of the *Ty-3* gene on chromosome 6 of tomato. Linkage maps were constructed from F_2 populations of susceptible *S. lycopersicum* × resistant LA1932-derived advanced breeding line (on the left, labeled as LA1932-AL-F2), or susceptible *S. lycopersicum* × resistant LA2779-derived advanced breeding line (on the right, labeled as LA2779-AL-F2). All the markers are PCR-based, including SCAR markers (P621A, P697A, and P169C) converted from RAPD markers, and CAPS markers taken from either the public domain or designed from the public sequences except T1098 and T0834 (Maxwell et al. 2006). Shaded regions represented introgressions from *S. chilense*. The markers in non-introgression regions are not drawn to scale. This figure is adapted from Ji et al., 2007.

markers flanking the *sp* side. A crossover in more advanced lines removed the RAPD markers further down flanking the *sp* locus but retained the resistance from *Ty-3*. There may be another resistance gene flanking the *c* locus on chromosome 6. But if so, it must be in a small introgression that no longer has the RAPD markers used in earlier studies (Griffiths & Scott, 2001; Agrama & Scott, 2006) and has not been detected with markers that have been tested so far.

The introgression in the *Ty-3* region in the advanced breeding lines derived from LA2779 and LA1932 was further confirmed by sequencing these lines and related wild accessions. After a report by Ji & Scott (2006a), Maxwell et al. (2006) sequenced the PCR product of various breeding lines from the program in Guatemala (Mejía et al., 2005) using the COS II marker, C2_At4g27700 (27 cM) on chromosome 6, which was associated with the *Ty-3* gene for begomovirus resistance (Ji & Scott, 2006a). For line Gc9, which was derived from LA2779 and was highly resistant to the multiple bipartite geminiviruses in Guatemala, there was an introgression at this locus. Sequence alignment between Gc9 and Heinz 1706 indicated a large indel (AAGGTGGCTTCGCCC) in Gc9 and several other indels and SNPs between these two lines. Additional results with sequences of PCR fragments derived for markers from 6 cM (REX-1 locus) to 32 cM (T0834) for either Gc9 or Gc271–1 and Heinz 1706 indicated that this region contained an introgression. This included the additional markers: COS II At3g10920 (13 cM), T1563 (16 cM), T0744 (18 cM), TG590 (22 cM), T0507 (25 cM), FER locus (25 cM, included in BAC clone 56B23), COS II At4g27700 (27 cM), T1098 (30 cM), and T0834 (32 cM). No introgression was detected for the TG352 (33.5 cM) marker. At the REX-1 locus the sequence of Gc9 was identical to that of line TY52 which is homozygous for the *Ty-1* gene (Maxwell et al., 2006). These sequence data may indicate the presence of alleles at both *Ty-1* and *Ty-3* loci in the LA2779-derived breeding lines in Guatemala.

4.2. *Solanum habrochaites*

The two major sources of resistance from *S. habrochaites* include line H24 developed by Kalloo & Banerjee (1990) and line Ih902 by Vidavsky & Czonsek (1998). The introgression in "H24", which is responsible for moderate resistance to ToLCV, has been localized to the long arm of chromosome 11 delimited by RFLP markers TG393 and TG36 (Hanson et al., 2000). PCR primers were synthesized for 12 markers in the putative introgressed region. Two markers (cLET24J2 and TG393) were polymorphic in H24 but not some resistant AVRDC breeding lines developed from H24. Evaluation of a set of AVRDC lines carrying the full H24 introgression versus other lines with a shortened introgression missing the segment between TG26 and TG393 indicated that both groups were resistant to the Taiwan geminivirus (Hanson et al., 2006). This evidence suggested that the H24 resistance factor, named *Ty-2* (Hanson et al., 2006), was located in the vicinity of TG36. In order to design PCR-based markers linked to *Ty-2*, three markers (cLET24J2, TG393, and TG105A) were converted into CAPS markers, which are polymorphic for the *S. habrochaites* introgressions (Figure 2).

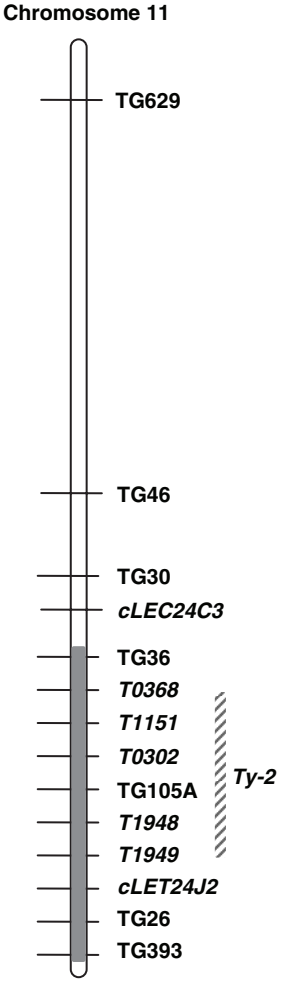

Figure 2. Location of the *Ty-2* gene on chromosome 11 of tomato. Markers are not drawn to scale, and markers in italics indicate map positions inferred from public information (www.sgn.cornell.edu). Shaded region represents *S. habrochaites* introgression identified from previous study (Hanson et al., 2000). *Ty-2* maps within 5 cM of T0302.

The locations of those markers were confirmed to the terminal region of the long arm of chromosome 11 using an independent mapping population of *S. habrochaites* LA1777 introgression lines (Monforte & Tanskley, 2000). The PCR-based marker T0302 (T0302F, 5' TGGCTCATCCTGAAGCTGATAGCGC 3', and T0302R, 5' AGTGTACATCCTTGCCATTGACT 3', at an annealing temperature of 55ºC) showed a robust amplification polymorphism between *S. habrochaites* (950bp expected size) and *S. lycopersicum* (850bp expected size) (http://www.sgn.cornell.edu). This polymorphic marker was chosen to screen the segregating populations that were

concurrently screened at AVRDC with a biological assay for resistance to TYLCV. Segregation ratios of T0302 and the *Ty-2* gene in the mapping populations were tested using the χ^2 goodness-of-fit test for conformity with the expected segregation ratios of 1:2:1 for the markers and 3:1 for *Ty-2* gene (i.e., where the heterozygous genotype could not be distinguished from the homozygous resistant genotype). T0302 and *Ty-2* segregation ratios were not significantly different from the expected values and were appropriate for use in linkage analysis. Mapmaker version 3.0 (Lander et al., 1987) was used to construct the linkage map with a threshold LOD of 3.0 and recombination fraction (RF) of 0.40 to determine linkage, and all map distances were then calculated with the Kosambi mapping function (Kosambi, 1944). The results of the linkage analysis are shown in Figure 2. The mapping analysis showed that TG105A and T0302 are tightly linked to each other and *Ty-2* is approximately 5 cM from these markers. Without flanking marker information it is not possible to determine whether *Ty-2* lies on the telomere or interstitial side of the PCR markers. Future research will either develop a marker more tightly linked or find flanking markers that will provide an efficient screen for the *Ty-2* gene. It will also be important to determine the distance between *Ty-2* and *I-2*, the gene conditioning resistance to race 2 of the Fusarium wilt pathogen (*Fusarium oxysporum* f. sp. *lycopersici*). *Ty-2* is tightly linked to Fusarium wilt race 2 susceptibility and efforts at AVRDC to identify recombinants carrying both *Ty-2* and *I-2* have not succeeded so far. The possibility that these two genes are allelic cannot be ruled out (E. Graham and P. Hanson, 2005, unpublished data).

Vidavsky & Czosnek (1998) initiated a program to develop breeding lines with immunity to TYLCV using *S. habrochaites* LA1777 and LA0386. A highly resistant line, Ih902, has been used extensively in the breeding program in Guatemala (Mejía et al., 2005). Several resistant lines, which could be traced back to the resistant lines developed by Vidavsky & Czosnek (1998), were found to have the identical sequences as Gc9/Gc2711 (discussed above) at numerous marker loci from 13 cM (Cos II At3g10920) to 32 cM (T0834) (Maxwell et al., 2006). A SCAR marker was developed from sequence obtained with primers provided by Y. Ji and J. W. Scott (2006, personal communication) designed from the left end of the tomato BAC clone 56B23 encompassing the FER locus (25 cM). The primer pair (FLUW-25F, 5′ CAAGTGTGCATATACTTCA TA(t/g)TCACC, and FLUW-25R, 5′ CCATATATAACCTCTGTTTCTATTTCGAC, at an annealing temperature of 53°C) gave a 480-bp fragment with the susceptible germplasm, Heinz 1706, two landraces from Guatemala, and Purple Russian (heritage tomato, Seed Savers Exchange) and a 650-bp fragment with the begomovirus-resistant lines (M. Salus and D. Maxwell, 2006, unpublished data). For a heterozygous plant, two fragments of the expected sizes, 480 and 650 bp, were obtained. Each fragment was sequenced, and the smaller fragment corresponded to the *S. lycopersicum* sequence and the larger to the sequence of the introgression (S. Melgar, M. Havey, and D. Maxwell, 2006, unpublished data). This PCR protocol was used to follow the

segregation of this marker in an F_2 population of Gh13 (introgression, resistant) × M82 (no introgression, susceptible) and there was a accepted fit to a 1:2:1 ratio (M. Salus, L. Mejía, M. Havey, and D. Maxwell, 2006, unpublished data). To date, there is only association data between the presence of the introgression and resistance to begomoviruses in Guatemala. Future plans will involve the evaluation of appropriate populations for resistance in the field in Guatemala. Much effort has been taken to locate resistance genes in other regions using this germplasm and to date no other introgressions have been identified. In Florida this work included testing 198 primers polymorphic between tomato and LA1777, which covered the genome at ~10 cM intervals (A. Momotaz and J. W. Scott, 2006, unpublished data).

4.3. *Solanum peruvianum*

Highly resistant breeding lines have been developed by scientists at Volcani Center, Israel, which are derived from S. peruvianum. One of these lines, TY197, (Lapidot et al., 1997), was found not to have introgressions corresponding to the Ty-1 locus (marker TG97) and the Ty-3 locus (marker from PCR primers FLUW-25F/FLUW-25R) on chromosome 6, nor an introgression on chromosome 11 at the Ty-2 locus (K. Jensen and D. P. Maxwell, unpublished data). Thus, the locations for the resistance genes associated with this germplasm are unknown.

Another source of TYLCV resistance is that from Tyking, a commercial hybrid marketed by Roral Sluis, Holland. It was speculated that the resistance might originally have come from *S. peruvianum* (J. W. Scott, 2001, personal communication). PCR-based marker analysis indicated Tyking has different sequences from *S. lycopersicum* at numerous marker loci from C2_At1g07080 (3.5 cM) to T0892 (14 cM), encompassing the *Ty-1* region, suggesting an introgression from some wild species of tomato in this region (Y. Ji and J. W. Scott, 2006, unpublished data). Five resistant and five susceptible lines generated in the early breeding program (in 1998) for TYLCV resistance derived from LA1938/Tyking were screened with a CAPS marker TG231 (in the *Ty-1* region). All the five susceptible lines were homozygous for the *S. lycopersicum* allele, whereas three resistant lines were homozygous for the resistant allele and the other two were heterozygous (Y. Ji and J. W. Scott, 2006, unpublished data). A genetic study of an F_6 line derived from Tyking revealed that resistance to a Brazilian bipartite begomovirus, *Tomato chlorotic mottle virus*, was conditioned by a single recessive gene tentatively named *tcm-1* (Giordano et al., 2005). The location of this gene is not known.

4.4. *Solanum pimpinellifolium* and *solanum cheesmaniae*

There is only one report on mapping of resistance loci for TYLCV associated with *S. pimpinellifolium* (Chagué et al., 1997). In this research, a cross was made between the resistant line (Ritz Azur), which has TYLCV resistance derived

from *S. pimpinellifolium* hirsute INRA, and the susceptible line (S Harmony), an *S. lycopersicum* cultivar bred by Clause Semences, France. Bulk segregant analysis was carried out on F_4 lines, with the five most resistant lines in one pool, and the six most susceptible lines in the other pool. Both pools and parents were screened using 600 single 10-mer oligonucleotides (Operon Technologies), resulting in the identification of 254 primers, which gave 330 polymorphic DNA fragments between the two parents. Of these, four primers were only associated with the resistant plants. These four RAPD markers were mapped to the same linkage group within a distance of 17.3 cM. QTL mapping analysis revealed that the four RAPD markers linked to a QTL located in this region, which contributed ~27.7% of the resistance. One of these RAPD markers was further mapped between TG153 (33.0 cM) and CT83 (34.0 cM) on chromosome 6 (Tomato-EXPEN 2000 map, www.sgn.cornell.edu). It is interesting to note that this is close to the *Ty-3* gene mapped by Ji and Scott (2006b) near 25 cM on chromosome 6. The RAPD marker Rc is most tightly linked to this resistance locus from *S. pimpinellifolium* and could be used in a breeding program to track this resistance locus. Zamir et al. (1994) reported as unpublished data the occurrence of a major locus for resistance from *S. cheesmaniae* on chromosome 6 as well as the *Ty-1* gene near TG97 (8.6 cM).

5. RESEARCH IN PROGRESS

One of the important goals with marker-assisted selection is to pyramid resistance genes from different sources. The diallel experiments by Vidavski et al. (2006) with sources of begomovirus resistance from *S. chilense*, *S. peruvianum*, *S. pimpinellifolium*, and *S. habrochaites* provided evidence that pyramiding of genes will contribute to hybrids with high levels of resistance (see Part V, Chapter 3). Vidavski et al. (2006) reported that the highest level of resistance was obtained from an F_1 between parents with *S. habrochaites* (Ih902) and *S. peruvianum* resistance loci. Unfortunately, there are no confirmed markers for the resistance loci associated with these two sources, but preliminary data provided in the previous sections indicate that the *Ty-3* gene is likely to be one gene associated with these lines. With the availability of PCR-based markers for the three mapped TYLCV resistance genes including *Ty-1*, *Ty-2*, and *Ty-3*, it is promising and relatively facile to bring these genes together in a single genotype to reach the maximum level of resistance. However, since *Ty-1* and *Ty-3* loci are linked a crossover between them will be required to obtain the resistant alleles in cis. Hybrid breeding may be one avenue to join the resistant alleles in heterozygous condition. A diallel analysis of different resistance sources did show improved resistance, when different loci were combined heterozygously (Vidavski et al., 2006). Current research is focused on evaluation of the *Ty-3* locus introgression in additional segregating populations of different sources, fine mapping and potential cloning of the *Ty-3* gene, detection of additional resistance genes, and development of breeder-friendly markers for these genes.

6. CONCLUSIONS

The existence of over 35 begomoviruses species with worldwide distribution have caused major losses in many regions of the world and pose a continuous threat to tomato production. Fortunately, resistance genes exist that provide a broad spectrum of protection to many of these viruses. If the resistance genes were very specific to particular begomoviruses, breeding for resistance would be ineffective in many regions of the world where multiple begomoviruses exist. Still, the complexity of resistance to the begomoviruses, especially TYLCV, renders considerable challenges to plant breeders. With the development of molecular markers (especially breeder-friendly PCR-based markers) tightly linked to the resistance genes, plant breeders can efficiently incorporate these resistance genes into elite tomato genotypes, thus accelerating the breeding of resistant cultivars. When molecular markers linked to new resistance genes are discovered, tomato breeders will have more options in providing tomato growers with durable resistance to the begomoviruses in their production region.

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CHAPTER 3

EXPLOITATION OF RESISTANCE GENES FOUND IN WILD TOMATO SPECIES TO PRODUCE RESISTANT CULTIVARS; PILE UP OF RESISTANT GENES

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1. BREEDING FOR RESISTANCE TO TOMATO BEGOMOVIRUS

Begomoviruses, whitefly-transmitted geminiviruses, are a major constraint for tomato production in many parts of the world. This is a highly profitable but costly crop, due to the labour and amount of chemical inputs usually required to protect tomato from the various pests and diseases that attack this crop. The well-documented pesticide abuse associated with tomato production has greatly contributed to the development of pesticide-resistant *Bemisia tabaci* populations (Horowitz et al., 2005). This whitefly specie is capable of vectoring over 20 different begomoviruses that attack tomato in tropical and subtropical regions of the world (Polston & Anderson, 1997; Zeidan et al., 1999).

Although most of the begomoviruses that attack tomato are found in the New World, very little breeding work has been done to minimize the severe damage that these viruses cause to tomato plantings in this region. Moreover, despite its tropical American origin, most of the tomato breeding work has been conducted in temperate countries. Hence, tomato growers in tropical and subtropical America have relied almost exclusively on pesticides to control *B. tabaci* and the geminiviruses this vector transmits. The situation in the Old World is similar, due to the severe damage caused by a group of geminiviruses transmitted by *B. tabaci* in tomato fields throughout the Mediterranean region, the Middle East, North Africa, central Africa and Southeast Asia (Czosnek & Laterrot, 1997). These related although distinct geminivirus species, are collectively referred to as *Tomato yellow leaf curl virus* (TYLCV). This geminivirus was accidentally introduced in the last decade into the Americas in the early 1990s (Nakhla et al., 1994), where it has already caused millions of dollars worth losses of industrial and fresh tomato. Early efforts to identify sources of resistance to TYLCV within the domesticated tomato *Solanum lycopersicum*

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 363–372.

(previously name *Lycopersicon esculentum*) only revealed the existence of some moderately resistant or tolerant genotypes (Cohen & Harpaz, 1964; Nitzany, 1975; Abu-Gharbieh et al., 1978). However, Cohen & Nitzany (1966) observed that some wild relatives of tomato, namely *S. pimpinellifolium and S. peruvianum,* possessed a higher level of resistance to TYLCV, although they were not immune. Crosses between *S. lycopersicum and S. pimpinellifolium* (current tomato/accession LA 121) and genetic analyses of F1–3 and backcross generations indicated the existence of incomplete dominance of resistance over susceptibility, suggesting a monogenic control of resistance (Pilowski & Cohen, 1974). A dominant gene (coined *Tylc*) was later proposed for the resistance gene in *S. pimpinellifolium* (Kasrawi, 1989). The progenies derived from this cross showed only moderate symptoms, but their yield was markedly reduced. Nevertheless, among the Lycopersicon species, *S. pimpinellifolium* is one of the most compatible for crossing with *S. lycopersicum* (Picó et al., 1996). In contrast, the inheritance of tolerance to TYLCV in *S. peruvianum* (PI 126935) is controlled by five recessive factors, according to Pilowski & Cohen (1990). This breeding programme initiated in 1977, resulted in the release of the commercial hybrid TY-20, in 1988. This hybrid delays symptom expression and viral DNA accumulation in infected plants, resulting in acceptable yields (Pilowski & Cohen, 1990). Other tolerant/resistant TY-lines generated by this breeding programme are: TY172, TY197, TY198, and TY536 (Lapidot et al., 1997; Friedmann et al., 1998). In 1991, other wild tomato species: *S. chilense* and *S. habrochaites* (formerly *L. hirsutum*) besides *S. peruvianum and S. pimpinellifolium*, were examined for the presence of viral DNA and symptom expression following their inoculation with whiteflies removed from TYLCV-infected tomato plants. Approximately 85 days after inoculation, all species have been infected and contained detectable levels of viral DNA; however *S. chilense* and *S. habrochaites* were the most resistant species, with the majority of the inoculated plants remaining symptomless, and with only few containing detectable amounts of viral DNA (Zakay et al., 1991). A TYLCV-resistance gene in *S. chilense* was identified and named *Ty-1* (Michelson et al., 1994; Zamir et al., 1994). The resistance to this virus in *S. habrochaites*, on the other hand, seems to be dominant (Vidavski & Czosnek, 1998). *S. habrochaites* has been crossed with *S. lycopersicum*, yielding tolerant and resistant lines. One of the resistant lines was crossed with *S. lycopersicum*, to produce the hybrid FAVI-9. Another promising species evaluated for TYLCV resistance, *S. cheesmanii*, possesses recessive resistance to TYLCV. Breeding projects in the Mediterranean region have also used *S. cheesmani*, *S. peruvianum*, and *S. pimpinellifolium* to control TYLCV in this region (Laterrot, 1990, 1992, Laterrot & Moretti, 1996). Some of the TYLCV-resistant lines obtained from this project are: Pimpertylc-J-13 and Chepertylc-92. Interespecific hybrids obtained from crosses between *S. pimpinellifolium*, *S. peruvianum*, and *S. habrochaites,* show different patterns of segregation upon TYLCV inoculation, suggesting the existence of different, complementary genes (Kasrawi & Mansour, 1994). In 1990 Jay W. Scott and

Dave Schuster (1991) started a breeding programme in Florida for geminivirus resistance using three accessions of *S. chilense*, LA 1932, LA 1938, and LA 2779. From this work they discovered four resistance genes where any two of them are required for resistance to *Tomato mottle begomovirus* (ToMoV) in a given breeding line or variety. The genes are additive, which means a hybrid between a resistant and a susceptible parent (heterozygous resistance) has intermediate resistance (Griffiths & Scott 2001). In 1991, Muniyapa and co-workers reported that *S. habrochaites* and *S peruvianum* were resistant to another tomato geminivirus: *Tomato leaf curl virus* (ToLCV). The resistance mechanism in these wild species was subsequently associated with the presence of exudates from trichome glands on the leaf surface, in which whiteflies became entrapped (Channarayappa & Shivashankar, 1992). This is one of the few cases where genetic resistance to a viral disease has been achieved indirectly by incorporating genetic traits against *B. tabaci*.

2. ACCUMULATION OF RESISTANT GENES IN THE WILD SPECIES

The first step towards the development of TYLCV-resistant tomato lines is searching for resistance sources among the wild tomato species. From practical purposes, each wild tomato species has been attributed an accession number (see Tomato Genetics Resource Center at URL http://tgrc.ucdavis.edu/).

These wild species in most cases are carrying genes for self incompatibility. This mechanism is the most basic and efficient way of Mother Nature to preserve the species from extinction by maintaining a wide genetic variation among the species. Screening of accessions of wild tomato species reported to be resistant to TYLCV has shown that susceptible, tolerant, and resistant individuals can be found within each accession (Table 1). This heterogeneity is probably due to the fact that most wild species in their propagation routine are subject

Table 1. Response of selected accessions of wild tomato species, 4 months after inoculation to TYLCV

			Responsible to inoculation		
Wild tomato species	Accession number	Number of plants	Susceptible	Tolerant	Resistant
S. pimpinellifolium	LA 121	10	7	3	0
	LA 1582	16	9	4	3
S. peruvianum	LA372	8	0	2	6
	LA462	9	0	5	4
S. habrochaites	LA 1777	16	0	1	15
	LA386	10	0	0	10
S. chilense	LA 1969	9	0	3	6

Susceptible: symptomatic, large amounts of viral DNA; tolerant: symptomless, detectable viral DNA; resistant: symptomless, non-detectable viral DNA.

Table 2. Response of F1 hybrids between susceptible *S. lycopersocum* and resistant *S. habrochaites* plants, and of F1 hybrids between resistant plants of two *S. habrochaites* accessions, 4 months after inoculation

Parents			Response of F1 to inoculation		
Male	Female	Number of plants	Susceptible	Tolerant	Resistant
LA 1777	*S. lycopersicum*	10	8	2	0
LA 386	*S. lycopersicum*	6	6	0	0
LA 386	LA 1777	9	0	0	9

to open pollination especially if they have self-incompatibility genes like in *S. habrochaites, S. chilense*, *and S. peruvianum*.

The recessive nature of resistance and the fact that it is a polygenic trait, combination of major and minor genes makes the introgressing of this trait into the domesticated tomato very difficult. Furthermore in the process of introgressing polygenic trait by backcrossing most likely that we will lose part or some of the gene involve in the resistant. Therefore, it is highly recommended to start piling up genes for resistant in the very first stage, meaning selecting the most resistant individual plant in each accession and crossing them together either between accession from the same wild species or between individuals plant in a specific accession.

Table 2 shows that a hybrid between two accessions of the wild tomato species *S. habrochaites* is superior to the two parents in term of resistance, suggesting a complementary mechanism. The strategy, of combining resistant gene from the two *S. habrochaites* accessions LA 386 and LA 1777 into one F1 hybrid and using this hybrid as the source of resistance provided one of the best source of resistance available (Vidavski & Czosnek, 1998).

3. ACCUMULATION OF RESISTANT GENES IN THE DOMESTICATED TOMATO

Breeding programmes aimed at producing tomato cultivars resistant to TYLCV and to other begomoviruses infecting tomato started in the 1960s and have expanded since. Most of the cultivars and breeding lines available today present variable degrees of tolerance to TYLCV, they are either symptomless or present mild symptoms, and have relatively good yields and fairly good fruit quality.

TYLCV-resistant genes were introduced from different sources by different breeders at different locations: *S. chilense* (Israel, Florida), *S. peruvianum* (Israel), *S. pimpinellifolium* (France), *S. habrochaites* (Israel, Taiwan,). Depending on the plant source, resistance was reported to be controlled by 1–5 genes, either recessive or partly dominant.

To increase the level of resistance available, we decided to combine tomato lines with different sources of resistance. Following evaluation in infested fields in

different countries and during different seasons, we choose the best breeding lines available both for tolerance and agronomic traits. The lines that showed excellent resistance to TYLCV were used to pile up resistance genes from several different origins. These tomatoes lines served as a common baseline of resistance. From there we designed a non-reciprocal dialellic set of crossings between the selected lines. Twenty-day-old seedlings were inoculated in the greenhouse by caging viruliferous whiteflies for 5 days, 10–20 insects per plant. Twenty days thereafter, the plants were transplanted in open field in ten replicates (Coastal Plain, Israel). We evaluated the level of resistance by scoring symptoms 42 days after inoculation. The plants where evaluated for yield 114 days after inoculation. The potential combining ability of the different source of resistant was evaluated.

3.1. Disease severity index (DSI)

In order to evaluate resistance we use a disease severity index (DSI) scale of 0–4:
0: Symptomless
1: Very slight yellowing of leaf edges
2: Yellowing and curling of leaves
3: Marked yellowing, curling, and cupping of leaves; plant continues to grow
4: Severe stunting, curling, and cupping, plant stops growing

Scoring was performed by two very well-trained experts (see Part V, Chapter 1). The evaluation made without knowing the source of the F1-hybrids or the parental lines.

3.2. Source of resistance

Based on the above we selected four resistant lines, each represents a different source of resistance. These lines were bred by different breeders in different locations around the globe, and are the outcome of a breeding programme aimed at

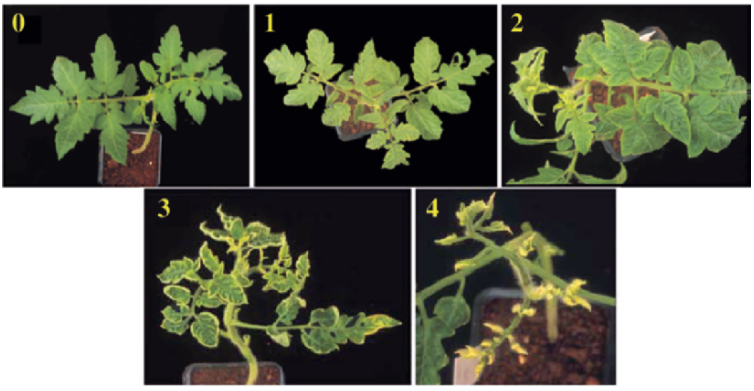

Figure 1. TYLCV disease severity index. (Lapidot, M. & Friedmann, M. (2002). Breeding for resistance to whitefly-transmitted geminiviruses. *Ann. Appl. Biol.* **140**, 109–127).

Table 3. Parental lines participating in the non-reciprocal dialelle set of crossing

Source	Name	Label	Breeders
S. peruvianum	172	PER	M. Pilowski, M. Friedmann, Volcanic Center, Israel
S. habrochaites	H-902	HIR	F. Vidavski, H. Czosneck, Hebrew University of Jerusalem, Israel
S. chilense	Fla-595	CHIL	J. W. Scott, University of Florida, USA
S. pimpinellifolium	Pim-Hir	PIMP	H. Laterrot, INRA Avignon, France
S. lycopersicum	Susceptible	SUS	

introgressing resistant to begomoviruses from the wild species into the domesticated tomato *S. lycopersicum*. One susceptible tomato line was chosen for its good agronomic qualities. Table 3 lists the lines that participated in the diallele experiments. These line representing the predominant source of resistance used by most breeders for breeding elite lines and commercial hybrids.

3.3. Pile up of TYLCV resistance genes

3.3.1. Parents line

The first step was to evaluate the DSI of the parent lines under controlled uniform inoculation procedures. Lines 172 (*S. peruvianum*) and H-902 (*S. habrochaites*) showed the highest level of resistance (DSI of 1.3). Fla-595 (*S. chilense*) showed mediocre resistance (DSI of 2.5) and the Pim-Hir (*S. pimpinellifolium*) showed a level of resistance (DSI of 3.6) close to that of the susceptible line (Figure 2).

3.3.2. F1 hybrids between susceptible and resistant line

The potential of producing hybrids with only one parent resistant to TYLCV has a huge importance from the breeding point of view. Each breeding programme has a core germplasm with a general combining ability and with excellent agronomical traits (e.g., yield, fruit size, and colour). By combining lines from the core germplasm and a resistant line one may shorten the time needed to breed tomato with a commercial value.

After crossing the resistant lines with the susceptible cultivar (Figure 2), it appeared that in the hybrids, lines H-902 (*S. habrochaites*) and Fla-595 (*S. chilense*) showed dominant resistance with DSIs of 2.2 and 2.6, compared to 3.8 for line 172 (*S. peruvianum*) and 4 for line Pim-Hir (*S. pimpinellifolium*). However, the level of resistance achieved by these hybrids obtained by crossing the resistant lines with a susceptible cultivar may not be suitable for heavily inoculated area. Hence, combining sources of resistance and following crop-management practices that reduce virus incidence may provide farmers with an acceptable solution until cultivars with higher levels of resistance are available.

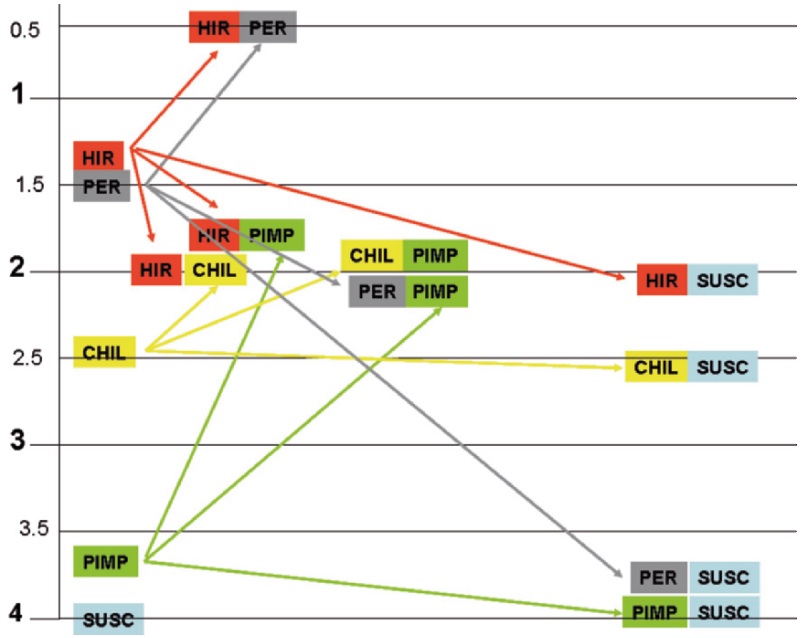

Figure 2. Disease incidence (measured as DSI) of hybrids obtained by piling up various sources of resistance (see Table 3). Sources of resistance: PER – *S. peruvianum*, HIR – *S. habrochaites*, CHIL – *S. chilense*, PIMP – *S. pimpinellifolium*; SUS – susceptible S. *lycopersicum*.

3.3.3. F1 hybrids between resistant lines

In the diallele tests, the most resistant hybrids were those that combined more then one source of resistance. Nevertheless all sources of TYLCV resistance appeared to be complementary to each other and achieved better DSI scores than each of the separate resistance source. The highest level of resistant was achieved by combining the resistant source derived from 172 (*S. peruvianum*) and H-902 (*S. habrochaites*): the hybrid had a DSI of 0.9 compared with the DSIs of 1.3 for each of the two resistant sources. Surprisingly, although the Pim-Hir line (*S. pimpinellifolium*) showed a very poor level of resistance (DSI of 3.6), when combined with Fla-595 (*S. chilense*) with DSI of 2.5, the Pim-hir x Fla-595 hybrid had a level of resistance (DSI of 1.6) higher than each of the two parents (Figure 2).

4. CONCLUSION

Breeding for TYLCV resistant is one of the most difficult task breeder are facing today. New viruses and strain of the Begomovirus group keep on emerging. This is mainly a result of changing in cultivation habits, the continuing worldwide spread of different biotypes of whiteflies and the relatively high frequency of

recombination among geminiviruses (Padidam et al., 1999).The diversity of the begomoviruses and of its vector the whitefly *B. tabaci*, forces us to keep improve our understanding and knowledge on the relation between the plant the virus and the whitefly.

Pyramiding the chromosomal regions associated with resistance in the lines from different origins will improve the degree of resistance to TYLCV and will broaden the resistance against a wider range of begomoviruses. The strategy followed to incorporate high levels of begomovirus resistance in common bean, strictly through intraspecific recombination and pyramiding of different resistance traits found in diverse gene pools of *Phaseolus vulgaris*, confirms the feasibility of this approach (Blair et al., 1993). However, there are both direct and circumstantial evidence indicating the existence of adequate genetic variability in the primary and secondary gene pools of most cultivated species. This genetic variability can be exploited within and between cultivated species and their relatives. Interspecific hybridization in tomato can be practiced not only in search of resistance to begomoviruses, but to other pathogens and pests as well (Nichols, 1947; Debouck, 1991). In the case of tomato, it is evident that the cultivars with some degree of TYLCV resistance, also exhibit resistance to distinct bipartite begomoviruses infecting tomato in the Americas and in Asia (Muniyapa et al., 1991; Piven et al., 1995, Mejia, et al., 2001).

The combination of classical breeding together with molecular markers linked to the different sources of resistance will be required in order to facilitate the pyramiding of the resistance genes. It will help the breeder to distinguish between the different sources of resistant and to combine all TYLCV-resistance genes available from the four main resistance sources in use, *S. chilense*, *S. peruvianum*, *S. pimpinellifolium*, and *S. habrochaites*.

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CHAPTER 4

TRANSGENIC APPROACHES FOR THE CONTROL OF TOMATO YELLOW LEAF CURL VIRUS

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1. OVERVIEW

Management of TYLCV and other begomoviruses is very difficult and expensive. The least expensive and most practical control of TYLCV and many other begomoviruses is the use of resistant cultivars. Begomovirus-resistant tomato cultivars would greatly reduce the use of insecticides for whitefly control, which would reduce production costs and chemical contamination of the environment. Conventional breeding of TYLCV-resistant tomato lines has been conducted for several decades and the results are discussed in Part I, Chapter 1, Part V, Chapters 2 and 3.

Resistance derived through genetic engineering offers several advantages over resistance genes obtained from plant genomes. Transgenes are usually inherited as single dominant genes. This has great advantages over conventional resistances which have multigenic with complex inheritances. Single dominant genes are much easier and less expensive to transfer to elite commercial cultivars. While very high levels of resistance (equivalent to non-host resistance) have been reported using genetically engineered resistance (Fuentes et al., 2006; Yang et al., 2004), current conventionally derived resistances collapses under early or severe infection pressure (Lapidot & Friedmann, 2002; Lapidot et al., 1997). Although some genetically engineered resistances prevent TYLCV replication, all conventionally derived resistances support TYLCV replication, and as such can therefore act as sources of TYLCV for susceptible crops (Lapidot et al., 2001). To date, pathogen-derived resistance has shown itself to be an attractive method to improve the resistance of tomatoes against TYLCV as well as other begomoviruses. In fact, some of the resistances generated by this approach suggest that engineered resistance may become the cornerstone for begomovirus management programs throughout the world.

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 373–390.

2. SOURCES OF RESISTANCE

There are a number of reports to develop genetically engineered resistance to geminiviruses through the introduction of a variety of partial and full-length coding as well as non-coding sequences. Most of these studies are on other viruses, both monopartite and bipartite, in the *Begomovirus* genus, and a few from the *Curtovirus* genus. In addition, there are many variations and contradictory results involving transgene expression and engineered resistance. For the sake of brevity, this chapter will focus primarily on those studies which report studies with TYLCV and will include only a few studies using other geminiviruses which may offer some useful information for the development of resistance to TYLCV.

2.1. Pathogen-derived resistance

Pathogen-derived resistance has been the most common approach used to obtain resistance to TYLCV as well as other begomoviruses. A number of different approaches have been used including viral sequences that generate antisense RNA, as well as the expression of full-length and truncated viral genes. Viral sequences in various arrangements have been evaluated for their ability to generate plants resistant to TYLCV, with results that vary from non-host resistance to complete susceptibility. While TYLCV has six open reading frames, most of the attention on the development of resistance has been focused on the TYLCV *Replication-associated protein* (*Rep*) and *Coat protein* (*CP*) genes.

2.1.1. Replication-associated protein

The multifunctional *Rep* encoded by the *C1* open reading frame is the only protein absolutely required for replication (Elmer et al., 1988). As such this gene has been successfully targeted by researchers seeking to produce transgenic resistance. A wide variety of *Rep* constructs have been used to produce virus resistance with an array of results. A number of reports indicate that full-length *Rep* constructs result in few or no transformants or produce transgenic plants with altered phenotypes (due to phytotoxic effects) and with no apparent virus resistance. Transgenic plants with the antisense *Rep* gene have a normal phenotype with varying degrees of TYLCV resistance. To overcome the phytotoxic effects of expressed Rep protein in transgenic plants researchers have used various truncated or mutated *Rep* constructs. Multiple labs have shown that transgenic expression of a truncated form of the *Rep* gene can confer high levels of resistance to TYCLV.

Day et al. (1991) were the first to report the production of transgenic resistant tobacco expressing an antisense sequence of the bipartite begomovirus *Tomato golden mosaic virus* (TGMV) *Rep* gene in the R_1 generation; however, the studies did not continue on further generations. Bendahmane et al. (1997) demonstrated that the use of the full-length antisense *Rep* including the 63 nt

(nucleotide) leader sequence conferred moderate resistance to the monopartite begomovirus, *Tomato yellow leaf curl Sardinia virus* (TYLCSV), in *Nicotiana benthamiana*, and that this resistance was inheritable to at least until the R_2 generation. Transgenic lines challenged by agroinoculation showed a spectrum of symptoms ranging from severe to very mild. They correlated elevated transcript level with a higher degree of viral resistance, based on reduced disease symptoms and reduced levels of viral DNA replication. Both studies showed that the degree of sequence homology between the antisense transgene and challenging virus sequence was correlated with the degree of resistance expressed. They were unable to generate plants that expressed a sense copy of *Rep*, indicating that expression of the Rep protein has phytotoxic effect on plant cells.

Noris et al. (1996) were the first to demonstrate that a truncated TYLCSV *Rep* gene could confer high levels of virus resistance in transgenic *N. benthamiana* plants. Their construct designated as Rep-210 contained 42 nt of upstream of the *Rep* and 630 nt of the 5′end of the TYLCSV *Rep*. The 630 nt construct contained the sequence for the small ORF C4. Eleven transformants with the construct in the sense and eight in the antisense orientation were evaluated by agroinoculation. Seven sense and three antisense lines were free of disease symptoms and viral DNA was not detected. All sense lines that scored as resistant were demonstrated to express transgene mRNA and truncated *Rep* protein. In a further study with this construct Brunetti et al. (1997) demonstrated that transgenic tomato plants expressing high levels of this truncated TYLCSV Rep protein were resistant to TYLCSV infection, whereas those where the protein was not expressed (lines containing the antisense *Rep* or both sense and antisense *Rep* gene) were susceptible to the homologous virus. However, resistance was associated with an undesired, altered phenotype and could only be observed in the first backcross generation plants, due to the toxicity of the construct in the homozygous state. The fact that the same antisense construct produced resistance in *N. benthamiana* but no resistance in tomato is in conflict with their conclusion that expression of transgene protein was necessary for induced viral resistance. In a study to investigate the possible molecular mechanisms of the Rep-210-induced resistance they tested a series of C-terminal deletion mutants (Lucioli et al., 2003). Constructs Rep-156 and Rep-130 lacked the oligomerization domain but were as effective as Rep-210 in repressing *Rep* transcription. They concluded that the repression of TYLCVSV *Rep* gene transcription was the mechanism of resistance in Rep-210 transgenic plants. However, the Rep-210 mediated resistance to a Portugal isolate of TYLCV (TYLCV-[PT]), a heterologous virus, required the oligomerization domain. They proposed that homologous virus resistance was due to the Rep-210 inhibition of the Rep transcription, acting as a transdominant–negative mutant that represses the viral Rep promoter. For the heterologous virus resistance they propose that the Rep-210 forms dysfunctional Rep-210/Rep (from the heterologous virus) complexes. They conclude that the virus resistance, in both cases, is due to transgenic *Rep* repressing viral transcription for homologous challenge and the assembly of

dysfunctional oligomers for the heterologous challenge. Although they detect small *Rep* specific 21–25 nt RNAs indicating that the *Rep* mRNA was a target of post-transcriptional gene silencing (PTGS) during viral infection, they minimize this as a factor in their transgenic resistance. The question whether their proposed models based on *Rep* protein-mediated resistance is correct could be resolved by grafting experiments to see if the resistance is due to protein or diffusible signals known as small interfering RNAs (siRNAs), the latter a hall mark of (PTGS).

In further studies with the Rep-210 transgene Noris et al. (2004) tested a sense x antisense hybrid and two multicopy lines for resistance to TYLCSV when challenged by agroinoculation or whitefly inoculation. In both challenges high amounts of Rep-210 siRNAs were detected, however all lines were susceptible to TYLCSV regardless of the type of inoculation with a slight delay in infection after whitefly inoculation. They concluded that TYLCSV could overcome the transgene-mediated siRNAs.

Antignus et al. (2004) constructed transgenic tomatoes using as a transgene a truncated *Rep* sequence (nt 2790 to 2404) of the mild strain of TYLCV (TYLCV-Mld). This transgene was 386 nt long, and coded for 129 n-terminal amino acids, and had a 78% sequence identity at the nucleotide level and a 77% amino acid identity with the severe strain of TYLCV. Three transgenic lines each with three copies of the transgene were tested for resistance to TYLCV. The transgenic tomatoes were completely resistant to whitefly inoculations with the TYLCV-Mld while agroinoculation with the same virus resulted in variable responses with 21% of the plants being immune, 46% with a range of intermediate resistances, and 33% fully susceptible. However, no resistance was detected when these plants were challenged with the severe strain of TYLCV. The transgenic lines with the truncated *Rep* sequences did not show phytotoxicity as seen with larger TYLCSV *Rep* sequence transgenes as reported by Noris et al. (1996) and Brunetti et al. (2001).

Yang et al. (2004) tested eight different *Rep* constructs of an isolate of TYLCV from Florida (TYLCV-[FL] for their ability to confer resistance to TYLCV in tomato. Three transgenic lines containing *Rep* sequences truncated at 3′ end showed virus resistance. The three constructs were: 2/5Rep (81 nt of the IR plus 426 nt of the 5′ end of the TYLCV *Rep* gene (coding for 142 amino acids), Δ2/5Rep (85 nt of the IR plus 595 nt of the 5′ end of the TYLCV *Rep* gene in the antisense orientation), and RepΔ2/5Rep (81 nt of the IR plus entire *Rep* gene plus 41 nt of the 3′ *Rep* gene fused with an antisense orientation of 595 nt of the 5′ end of the *Rep* gene plus 85 nt of the IR). Transcripts from the RepΔ2/5Rep were designed to form a hairpin structure. The 2/5Rep constructs terminated within the C4 ORF. The R_1 generation progeny were screened for TYLCV resistance using viruliferous whiteflies. Very few transformants were obtained for three full-length Rep constructs and none of them showed any resistance. No symptoms were observed and no TYLCV genomic DNA was detected by PCR or hybridization in plants transformed with the 2/5Rep, Δ2/5Rep or with RepΔ2/5Rep. These transgenic lines have been screened and continue to be resistant through the R_3 generation. The 2/5 TYLCV *Rep* gene

construct confers high levels of resistance and often immunity in both transformed *N. tabacum* and *S. lycopersicum*, likely through the mechanism of gene silencing (Freitas-Astúa, 2001; Polston et al., 2001). These results suggest that the 2/5 TYLCV *Rep* construct may be a strong inducer of gene silencing (Polston et al., 2007, unpublished data).

Fuentes et al. (2006) demonstrated resistance to TYLCV through the use of the intron–hairpin RNA approach (Smith et al., 2000), using a transformation cassette consisting of 726 nts of the 3-end of the *Rep* (sense and antisense orientation) as the arms of the hairpin, and a functional castor bean catalase as the intron. One out of 11 transformed tomato plants containing a single copy of the transgene showed immunity to TYLCV when young plants (four-leaf stage) were exposed to hundreds of viruliferous whiteflies during a 60-day exposure. No TYLCV DNA was detected in these plants, while both inoculated and non-inoculated plants revealed small interfering RNAs (siRNAs) with homology to the *Rep* gene. A second transgenic line with multiple copies of the insert showed milder symptoms of TYLCV infection and reduced accumulation of TYLCV than the controls. This line showed a lower level of transgene-derived siRNAs than the immune line. No transgene-derived siRNAs were detected in the other fully TYLCV susceptible, transformed lines. Thus these authors have demonstrated engineered resistance/immunity to a DNA plant virus, achieved by the use of the intron–hairpin transcript; a strategy that has been shown successful with RNA viruses (Wang et al., 2000).

Considerable work has been done with the *Rep* coding sequences of other begomoviruses, and resistance has been obtained using partial, full-length or mutated replication-associated (*Rep*) genes of *Bean golden yellow mosaic virus* (BGYMV) (Aragao et al., 1998), *African cassava mosaic virus* (ACMV) (Chellappan et al., 2004), and *Tomato mottle virus* (ToMoV) (Polston & Hiebert, 2001).

2.1.2. Coat protein gene

The begomovirus *CP* gene was one of the first TYLCV genes evaluated for the ability to generate pathogen-derived resistance. The begomovirus coat protein gene was initially thought to offer the best opportunity for broad spectrum resistance to different tomato-infecting begomoviruses. This was based on the high sequence similarity among begomovirus coat protein genes and the important role that this gene's product plays in whitefly transmission, as well as cell to cell and presumably systemic movement (Briddon et al., 1990; Gafni & Epel, 2002).

Kunik et al. (1994) reported that transgenic tomato plants expressing TYLCV *CP* were resistant to the virus. After inoculation of virus with viruliferous whiteflies, resistant tomato plants in the R_1 generation showed a delay in symptoms, a recovery from infection, and resistance upon repeated inoculations. The delayed expression of symptoms and recovery were associated with high levels of expressed TYLCV *CP*. There were no reports of the evaluation for resistance in subsequent generations.

Sinisterra et al. (1999) obtained resistance to ToMoV in tobacco transformed with a ToMoV CP gene that lacked an N terminus. However this resistance was not stablely inherited.

Recently, Zrachya et al. (2006) used transient expression and plant transformation with hairpin constructs containing a 419 nt sequence from the N terminus of the *CP* gene in the sense and antisense orientations ("arms") separated by a 1.2 kb maize ubiquitin intron. This construct was transformed into "Micro-Tom", a short-lived tomato cultivar and plants were evaluated for resistance using whitefly inoculation of TYLCV. Symptomless plants were observed in the R_1 and R_2 generations, while plants with mild symptoms were also observed in the R_2 generation. Resistance was associated with decreased levels of viral DNA, and undetectable levels of *CP*. The mechanism of resistance was proposed to be silencing based on results in transient assays in *N. benthamiana* in which the *CP* construct was able to silence expression of *GFP* from *CP-GFP* constructs.

2.1.3. Other TYLCV coding sequences

To date, studies with *C4* coding sequences have failed to produce transgenic plants when using *C4* in the sense orientation, and a lack of resistance in the antisense orientation (Yang et al. 2004). Few studies have been published on the use of the TYLCV REn, TrAP, V2 coding sequences either partial or full length. However, there are studies using *TrAP* and *REn* sequences of other begomoviruses. Zhang et al., (2005) found that transgenic resistance to ACMV-Kenya in cassava could be achieved expressing antisense RNAs against genes encoding essential proteins (*Rep*, *TrAP*, and *REn*). The lines transgenic for antisense *REn* lines had the highest levels of resistance. They observed the highest levels of short 24 nt, siRNA-like molecules in the resistant transgenic lines.

Abhary et al. (2006) designed an intron–hairpin approach to generate resistance to TYLCV as well as other strains and monopartite begomoviruses by infiltrating cassettes containing segments of conserved sequences of TYLCV into tobacco and tomato. The cassette consisted of TYLCV sequence segments delineated by 350–557 nt –V1V2, 1235–1482 nt –C2C3, and 1566–1632 nt –C1C2 fused together. This sense and antisense chimeric constructs were separated by a chalcone synthase A gene intron and ligated into a binary vector. *A. tumefaciens* containing the binary vector with the silencing trigger cassette (STC) was used to infiltrate leaves. Expression of the STC was confirmed by PCR analysis. The infiltrated plants were challenged by agroinoculation with an infectious clone of TYLCV (TYLCV-[EG]). The STC infiltrated plants were free of symptoms, no accumulation of TYLCV genomic DNA was detected by hybridization or PCR, and a C1C2 probe hybridized with siRNA isolated from silenced plants. They reported a positive correlation of resistance with the accumulation of the TYLCV-specific siRNAs and proposed that PTGS can be used to generate geminivirus-resistance in plants (see Part V, Chapter 5).

2.1.4. Non-coding regions

Although there are few intergenic regions in the TYLCV genome, there are no reports on the use of any intergenic region alone to induce resistance to TYLCV.

Although largely unexplored, intergenic regions may prove useful in the development of resistance. The presence of a short (81 nt) intergenic region increased the frequency and quality of the resistance obtained with a partial TYLCV *Rep* gene (Yang et al., 2004). The intergenic region upstream of the *Rep* gene, if transcribed, would be highly based-paired (Polston 2007, unpublished) and could act as a trigger for PTGS.

Pooggin et al. (2003) bombarded *Vigna mungo yellow mosaic virus* (VMYMV)-infected *V. mungo* with a transient expression cassette containing the bidirectional promoter sequence (209 nt of the intergenic/common region) of VMYMV with a synthetic plant intron between the sense and antisense copy. The transient expression resulted in complete recovery from the infection. The recovery of the whole plant from infection indicated a spreading signal that interfered with virus replication since the bombardment could have only delivered the construct to a few cells. The targeted sequence, the promoter region, is not expected to be transcribed so the interfering mechanism here is proposed to be transcriptional gene silencing (TGS) mediated by the transiently expressed dsRNA which induces methylation of the cognate genomic DNA (Pooggin and Hohn 2004).

2.1.5. The mechanism(s) accounting for pathogen-derived resistance to TYLCV

Although not completely understood yet, PTGS is believed to be responsible in part or totally for several of the pathogen-derived resistances developed to TYLCV and other begomoviruses. These resistances share several characteristics associated with PTGS. However, methylation has also been proposed to play a role in pathogen-derived resistances.

Abhary et al. (2006), using a TYLCV chimera intron–hairpin, detected a strong association between the presence of siRNA and resistance, and concluded that resistance was due to PTGS. Zrachya et al. (2006) designed constructs to produce siRNA against the TYLCV *CP* gene. CP mRNA was not detectable in resistant plants, and the accumulation of siRNA was correlated with the presence of resistance. Possible RNA directed DNA methylation of the construct was proposed to explain cases where siRNAs were detected in transformed but susceptible plants.

The resistance generated by the TYLCV 2/5Rep construct appears to be due to PTGS (Yang et al., 2004). There was a reduction in the transcript level after inoculation with TYLCV when compared pre-inoculated transcript levels (Freitas-Astua, 2001), suggesting co-suppression by siRNA. The resistance was transferred to non-transgenic scions by transmission across a graft union (Polston 2007, unpublished). Finally, the Δ2/5Rep transgene does not code for an obvious protein and tomatoes transformed with this gene were virus resistant, again suggesting an RNA-mediated resistance. In studies in mixed infections with other viruses, the resistance conferred by the TYLCV *Rep* transgene was not suppressed by co-inoculation with the potyvirus, *Potato virus Y* (PVY), but was suppressed by co-inoculation with *Tobacco*

mosaic tobamovirus (TMV) which implies the involvement of a PTGS mechanism (Polston 2007, unpublished), which again suggests the involvement of a PTGS mechanism.

Similar conclusions have been drawn in studies with other begomoviruses. Vanitharani et al. (2004) used transient expression of siRNA targeted to the mRNA of the *Rep* gene of the ACMV to suppress the accumulation of Rep mRNA by 91% and the accumulation of viral DNA by 66% at 36–48 h after transfection. The targeting was sequence specific as indicated by failure to inhibit the replication of *East African cassava mosaic Cameroon begomovirus*. They concluded, "that siRNA can interfere with and suppress accumulation of the economically important ssDNA geminiviruses".

Akbergenov et al. (2006) detected siRNAs of three distinct sizes and of both polarities and with homology to the coding and non-coding, intergenic regions in two distinct bipartite begomoviruses. They concluded that two distinct RNA silencing pathways occur during begomovirus infections. One pathway producing 22 nt siRNAs is associated PTGS and with RNA viruses while the other producing 24 nt siRNAs is associated TGS at repetitive DNA loci. In another study Blevins et al. (2006) tested a panel of *Arabidopsis* mutants in four DICER-like (DCL) genes with two nuclear DNA viruses (geminivirus and pararetrovirus) found virus-derived siRNAs of 21, 22, and 24 nt sizes produced by all four DCLs. They found that the two DNA viruses induced a similar set of siRNA responses as reported for RNA viruses with the only difference noted in a strong accumulation of DCL3-dependent 24 nt siRNAs. They postulate that bidirectional POL II promoters in geminiviruses could generate transcripts that extend beyond their overlapping polyadenylation signals prior to cleavage. These sense and antisense transcripts might form the dsRNA precursors for viral siRNAs. This could explain the origin of siRNAs from both the coding and non-coding regions of the geminivirus genome. Vanitharani et al. (2004) proposed that a strong fold-back structure of geminivirus transcripts as another possibility for templates for DICER cleavage.

In contrast, Bian et al. (2006) postulated that inconsistencies in attempts to develop transgenic resistances against geminiviruses could be due to geminivirus infections causing silencing of the virus-derived transgene through methylation. They studied the fate of *Tomato leaf curl virus* (TLCV)-*C4* transgene with an enhanced CaMV 35S promoter during TLCV infection. The expression of the TLCV-*C4* transgene causes developmental abnormalities in the transformed plants. The TLCV-C4 transcript was readily detected in non-inoculated transgenic plants but the phenotype and transcript disappeared 60 days after TLCV inoculation. In their analysis of DNA methylation during TLCV infections high levels of asymmetric cytosine methylation of the transgene were resolved. They concluded that TLCV as well as other begomoviruses have the means to circumvent host RNA-silencing process initiated in response to DNA virus infection.

2.2. Other approaches

2.2.1. Ribosome-inactivating proteins

Although not demonstrated with TYLCV, one ribosome-inactivating protein, dianthin, has been shown to generate resistance to the begomovirus, ACMV. Dianthin is a ribosome-inactivating protein obtained from *Dianthus caryophyllus* and is one of several known ribosome-inactivating proteins which naturally occur in many plant species. Ribosome-inactivating proteins have been shown to have antiviral activity when applied to virus-inoculated leaves. *N. benthamiana* plants were transformed with an ACMV-inducible cassette consisting of the ACMV AV1 promoter and a dianthin 30 coding sequence (Hong et al., 1996). Upon inoculation with ACMV, resistance plants displayed milder than normal symptoms from which they recovered, and reduced accumulation of viral DNA. Resistance held up against multiple isolates of ACMV but not to four other begomoviruses. Although effective, the safety of this toxic protein is questionable.

2.2.2. Defective-interfering replicons

TYLCV has been reported to produce subgenomic and possibly defective interfering DNAs (Czosnek et al., 1989). Although no reports on the use of these to generate resistance to TYLCV have surfaced, this approach has been used with bipartite begomoviruses. *N. benthamiana* plants of that were transformed with a subgenomic DNA of ACMV showed an attenuation of symptoms when inoculated with normal ACMV (Stanley et al., 1990). This is an inducible resistance response as opposed to the other strategies in which the genes are expressed constitutively. This approach may have an advantage since the subgenomic DNA was only expressed after inoculation with virus.

2.2.3. Zinc finger proteins

Although not demonstrated with TYLCV yet, resistance to the Curtovirus, *Beet severe curly top virus*, was demonstrated in *Arabidopsis thaliana* that expressed artificial zinc finger proteins that were designed to bind to the *Rep* origin (Sera, 2005). Symptoms in inoculated-resistant plants ranged from none to mild, and these corresponded to none to reduced amounts of viral DNA detected. Although this approach generated very good resistance, the applicability is likely to be limited due the sequence variations in replication origins among geminiviruses. Perhaps modifications to this approach could be found which would extend the breadth of resistance.

2.2.4. GroEL

A very recent and novel approach has been to use the expression of a homologue of a *GroEL* gene to induce resistance in tomato (Akad et al., 2007). A GroEL homologue is produced by endosymbiotic bacteria in the whitefly and is essential for the transmission of TYLCV and other begomoviruses (Morin et al., 1999;

Morin et al., 2000). GroEL is associated with the passage of begomoviruses through the whitefly body, and is thought to protect the virus in the insect haemolymph. This GroEL has been shown to readily form complexes with TYLCV both *in vivo* and *in vitro* in a number of studies. Tomatoes transformed with GroEL under a phloem-specific promoter showed milder to no symptoms in plants in the R_0 through R_2 generation (Akad et al., 2007) GroEL/TYLCV complexes were readily detected in resistant plants. It was hypothesized that GroEL/TYLCV complexes formed in transformed plants and that these complexes were interfering in virus movement, thereby reducing the accumulation of TYLCV in leaves produced after inoculation. Symptoms in resistant plants tended to increase with time and then decrease, unlike those of susceptible plants which increased over time and then plateaued. Symptom expression was not correlated with the accumulation of TYLCV DNA, since TYLCV DNA was detected in symptomless plants although at lower amounts than in non-transgenic plants. Whiteflies were able to acquire and transmit TYLCV from resistant plants. Interestingly GroEL/TYLCV complexes were translocated across grafts, but resistance was not. Therefore, this work raises some intriguing questions as to what mechanism(s) are responsible for this resistance.

3. BROAD-SPECTRUM RESISTANCE AGAINST TYLCV AND OTHER BEGOMOVIRUSES

In addition to TYLCV, there are many tomato-infecting begomoviruses (>50 species) and some of these occur in mixed infections with TYLCV and each other. Broad spectrum resistance against TYLCV and other tomato-infecting begomoviruses would be very useful and economically desirable (Freitas-Astua et al., 2002). Some multigenic broad-spectrum resistance has been found in tomato and is in the process of being developed for markets in the tropics. This resistance has the disadvantage of being multigenic, a complex inheritance, and being limited to tomato. Genetic engineering has the potential to create genes for broad spectrum resistance that are simple in inheritance and can be moved into other crops, with the primary limitation being that the crop can be transformed.

3.1. Results with TYLCV sequences

Tomatoes (Fla, 7613) transformed with the 2/5 TYLCV Rep, Δ2/5 TYLCV Rep or with TYLCV RepΔ2/5Rep constructs (Yang et al., 2004) were tested for breadth of resistance by whitefly inoculation with TYLCV, TYLCV-[FL], TYLCV-Mld, and bipartite ToMoV (Polston et al., 2007, unpublished). Transgenic lines that were resistant to TYLCV-[FL] were also resistant to TYLCV, and showed some resistance (recovery) to TYLCV-Mld. Resistant plants showed no symptoms and virus DNA was not detected by PCR. The 2/5*Rep* lines tested initially showed symptoms and then recovered from infection with TYLCV-Mld.

Plants transformed with RepΔ2/5Rep constructs were highly resistant to TYLCV-[FL], TYLCV and TYLCV-Mld in that no viral DNA was detected 3 weeks after inoculation. The 2/5 *Rep* resistance to TYLCV-Mld was unexpected as there is only a 76% sequence identity between the transgene and this strain of TYLCV. None of the lines showed any resistance to ToMoV.

The chimera hairpin approach (Abhary et al., 2006) mentioned previously (section 2.1.3) selected conserved sequences from TYLCV which had a high degree of sequence identity with genome sequences of TYLCV, TYLCV-Mld, *Tomato yellow leaf curl Malaga virus* (TYLCMalV), TYLCSV, and TYLCSV-[ES]). The cassette consisted of TYLCV sequence segments representing the replicase active site, transcription enhancer, promoter, silencing suppressor fused together. This construct was challenged in transient assays by agroinoculation with an infectious clone of TYLCV-[EG] and by whitefly mediated transmission with TYLCV, TYLCV-Mld and TYLCSV-[ES]. After either inoculation method, the infiltrated plants were free of symptoms and no accumulation of TYLCV genomic DNA was detected by either hybridization or PCR. Whitefly inoculated plants showed high frequencies of resistance by 16–20 days after silencing construct infiltration. The transformation of plants with this construct followed by evaluation under field conditions and at different stages of plant development will be important in determining the stability and usefulness of this construct. The approach of using a chimeric construct composed of highly conserved sequence segments from different areas of the begomovirus genome may present a broad-spectrum resistance strategy that could be used to develop resistance to other begomovirus complexes (see Part V, Chapter 5).

3.2. Results with other geminivirus sequences

Zhang et al. (2005) obtained resistance to three strains of ACMV (ACMV-[KE], ACMV-[CM], and ACMV-[NG]) using an antisense construct of the ACMV-[KE] *REn* gene. This was despite of the fact that ACMV-[NG] produces more severe symptoms in susceptible hosts.

4. EVALUATION OF GENETICALLY ENGINEERED RESISTANCE

There are a number of methods that have been used to screen for resistance to TYLCV. Conventionally derived resistances are commonly screened by whitefly inoculation in the greenhouse at the transplant stage and/or inoculation by whiteflies in the field (see Part V, Chapter 1). Transgenic plants have been screened for resistance using a much wider array of techniques. Inoculation techniques include agroinfection using inserts of head to tail dimers, tandem repeats, or 1 + mers of TYLCV in the Ti plasmid, biolistic inoculation with greater than full-length infectious clones, and inoculation of virus by viruliferous whiteflies.

These inoculation methods have been applied to leaf discs as well as whole plants, in either transient expression systems or transformed plants. In addition,

although resistance to TYLCV is needed most acutely in tomato, other plant species which are faster or easier to manipulate have been transformed in studies evaluating various strategies. In many studies *N. benthamiana* has been the plant of choice due to the ease of transformation and inoculation. *N. tabacum* has also been used; however, evaluation in tomato has become more common in the last few years. Recently, Zrachya et al. (2006) demonstrated the usefulness of the miniature tomato cultivar "Micro-Tom" for the rapid evaluation for resistance in transformed tomato. "Micro-Tom" was susceptible to TYLCV showing symptoms in 2 weeks, and seeds in less than 2 months.

Genetically engineered resistance offers us the means to obtain very different types of resistances so more care must be taken in the interpretation of results from various screening methods compared to screening methods used in conventionally derived resistance studies. The "resistance" obtained using one screening approach may not be equivalent to that obtained using another approach. For example, a comparison of the use of leaf discs and whole plants in screening for resistance indicated that although leaf disc assays were able to discriminate between immune and susceptible genotypes, they were not able to "discriminate between sensitive and tolerant plants which support virus replication and cell-to-cell spread but not its long-distance movement" (Czosnek et al., 1993). In addition, another study found that agroinfection overcame natural resistance mechanisms in plants that were immune using whitefly inoculation (Kheyr-Pour et al., 1994). It was proposed that this difference could be due to the introduction of large amounts of viral DNA directly into the vascular system, which bypasses the earliest processes of virus uncoating and synthesis which occur in a natural virus infection (Kheyr-Pour et al., 1994). Since the goal of many studies is to obtain resistance that will hold up in tomato or some other economically significant crop plant, often under high temperatures and light intensities, as well as under high whitefly pressure throughout the life of the plant, great care should be taken to select for resistances that will perform under those conditions.

In addition to the evaluation of plants, transient expression assays have been used to screen constructs and select for those constructs likely to generate resistance. This process is used to avoid the tedious and time-consuming process of producing and evaluating transformed plants. For the most part, constructs identified in transient assays have often proven useful when transformed into plants. Abhary et al. (2006) recently used a screening method that takes advantage of the speed of a transient assay screening system combined with the use of a natural means of virus introduction. Constructs were introduced into tomato by agroinfiltration and then viruliferous whiteflies were used to inoculate TYLCV to the whole plant. High levels of resistance were observed starting 12 days after infiltration of the transgene construct. This approach should enable researchers to rapidly screen different constructs while still using more natural means of establishing virus infections.

5. FUTURE DIRECTIONS

In the field, infection by more than single virus is common in plants in general (Falk & Bruening, 1994), and especially in tomato which is a host to approximately 100 viruses (Jones et al., 2007). Mixed infections can result in a synergistic interaction with higher virus titers (Vance, 1991) or an increase the number or type of infected cells (Ryabov et al., 2001). This type of mixed infection often induces disease symptoms that are more severe than those induced by either single virus (Fondong et al., 2000; Pruss et al., 1997). This type of interaction has implications for both biological and practical uses of transgenic pathogen-derived resistance in agriculture. Synergistic virus interactions can lead to resistance breakage (Maki-Valkama et al., 2000; Wang et al., 2004) or assist in the systemic infection by complementing deficiencies in related or unrelated viruses (Mayo et al., 2000; Saenz et al., 2002). Typically the proteins which mediate these synergistic interactions have been shown to be suppressors of RNA silencing (Brigneti et al., 1998; Li & Ding, 2001; Pruss et al., 1997; Roth et al., 2004). Recent studies have shown that while PVY and several other viruses had no affect on the resistance generated by the TYLCV 2/5Rep transgene, TMV was able to completely suppress this resistance (Polston 2007, unpublished).

However, numerous viruses can infect tomatoes, and it is imperative that in some regions of the world the resistant plants exhibit broad-spectrum resistance. For that reason, gene pyramiding or crosses of lines with different resistance genes are common. Perhaps the pyramiding of transgenes with host-derived genes will be able to address some of the problems. However, there is often a cost to the plant to this use of multiple pathogen resistances that are so common in currently used cultivars (Gemmill & Read, 1998). In addition, there can be unexpected interactions between genes for resistance to one virus with those of another (Griffiths, 1998). Perhaps other approaches will be developed through genetic engineering based on new results from virus-plant genome studies.

PTGS-mediated resistances have one other advantage over conventionally derived resistances; they can be translocated across a graft (Palauqui et al., 1997). This feature has significance for tomatoes, where most of the greenhouse production and some field production employ grafted tomatoes. It is possible to provide protection to susceptible non-transgenic scions by grafting them onto transgenic rootstocks. This has been demonstrated with TYLCV 2/5Rep transgene in tomato (Polston 2007, unpublished). Susceptible scions grafted onto transgenic rootstocks could be cured of established infections of TYLCV. Non-infected scions inoculated after grafting showed reduced symptom expression. Shaharuddin et al. (2006) showed that silenced tomato rootstocks could generate a mobile silencing signal that could move through a graft junction and induce silencing in grafted scions using 1-aminocyclopropane-1-carboxylate oxidase (ACO-1). They found that high levels of ACO-1 mRNA in the scion were necessary for graft-transmitted silencing from a transgenic ACO-1 silenced stock. Antisense ACO-1 trangenic rootstocks also transmit silencing signal into

the scion but the time-course of manifestation of systemic silencing was significantly later (2 weeks vs. 4 weeks). This report and that by other researchers show promise that a tomato rootstock with a strong TYLCV silencing signal could be used to protect grafted non-transgenic tomato scions.

6. CONCLUSIONS

Through transgenic technologies it is possible to produce virus-resistant plants with varied modes of resistance which can be an important factor in determining if the plants should be used in the production of resistant cultivars. Generally, transgenic resistance is thought to be either RNA or protein-mediated. Resistance based on RNA silencing is usually strong, but because of sequence specificity, restricted only to the virus species the transgene was derived from (Prins et al., 1996). Resistance based on protein relies on either a functional protein product being produced at inappropriate time or nonfunctional protein interference. However it can be difficult to distinguish between an RNA- and protein-mediated resistance (Canto & Palukaitis, 2001; Goregaoker et al., 2000). For agricultural use the mechanism of resistance is important because with RNA silencing certain virus interactions can lead to a loss of the resistant phenotype.

Bian et al. (2006) hypothesized that RNA silencing strategy while being an effective defense tool against RNA viruses the "application of this strategy against geminiviruses has produced inconsistent results". Certainly there has been variation in the results obtained with various constructs. However the transgenic-derived resistances to TYLCV described by Fuentes et al. (2006), Antignus et al. (2004), & Yang et al. (2004) showed immunity (no detectable replicating viral DNA) using a screen of high population densities of viruliferous whiteflies at early stages in the plants' development; a standard that is rarely met by conventionally derived resistances. The TYLCV resistance identified by the 2/5 Rep construct in the study by Yang et al. (2004) has been tested in the field during three tomato-growing seasons in Florida (Polston 2007, unpublished). The "inconsistencies" in transgenic derived resistance to geminiviruses, attributed by Bian et al. (2006), may also be due to the use of multicopy transgene plants, heterozygous transgenic plants with duplicated promoters, and resistance screening limited to the first or second generation transgene lines. In order to establish the stability of transgenic resistances, multiple transformed lines and their progenies need to be screened and selected for resistance through several generations. Because of the random insertion of the transgene into a plant genome it is likely that chromosomal insertion site may affect the level of transgene expression. Multiple transgene copies with the same transcription promoters may result in reduced transgene transcription. In fact a phytotoxic gene such as the begomovirus *Rep* may only appear in multiple copies in transformed plants! Shaharuddin et al. (2006) have shown that the level of transgene expression is correlated with the degree of gene silencing.

Until the impasse of the public rejection or fear of transgenic vegetables is overcome research in pathogen-derived resistance will be limited to academic interests only. In fact during the 2006 American Phytopathological Society Annual Meeting there was a marked absence of research reports involving pathogen-derived virus resistance, most likely a reflection of the lack of funding for this area of research. To overcome the barrier to the acceptance of this technology the public needs to be educated about the benefits, such as reduced pesticide use with plants engineered for virus resistance. Perhaps a better understanding of the mechanism(s) involved in pathogen-derived resistance in transgenic plants will reduce the public's concern. The evidence to date indicates that the engineered virus resistance in plants appears to be an activation of normal/natural host defense capabilities against pathogens analogous to the widely accepted immunization of humans against pathogens.

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CHAPTER 5

GENE SILENCING OF TOMATO YELLOW LEAF CURL VIRUS

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1. OVERVIEW

The *Geminiviridae* family has received a great deal of attention in recent years and is becoming one of the most important and studied families of plant viruses. Some reasons why so much effort has been dedicated to their study include the economic and social impact of the diseases they cause (Palmer & Rybicki; 1998; Harrison & Robinson, 1999; Morales & Anderson, 2001) and the emergence of new geminiviruses through recombination or pseudorecombination among strains and/or species in various crops (Zhou et al., 1997; Navas-Castillo et al., 2000; Saunders et al., 2001; see also Part II, Chapter 3).

Geminiviruses are ssDNA viruses that infect a range of weeds and cultivated plants, including both monocots and dicots (Harrison, 1985). For example, economic losses due to geminivirus infections in cassava were estimated to be US$1,300–2,300 million in Africa (Thresh et al., 1998), US$5 billion for cotton in Pakistan between 1992–1997 (Briddon & Markham, 2000), US$300 million for grain legumes in India (Varma et al., 1992), and US$140 million in Florida for tomato (Moffat, 1999). In addition, *tomato yellow leaf curl disease* (TYLCD) was reported to limit tomato production in many regions of the world (Nakhla & Maxwell, 1998).

Begomoviruses have been considered as the most numerous and widespread group of whitefly-transmitted viruses causing severe epidemics in several parts of the world. These epidemics are in connection with some factors like the appearance of efficient vectors, evolution of new variants of the viruses, changing cropping systems, and introduction of susceptible plant varieties (Brown, 1997; Morales & Anderson, 2001; Ribeiro et al., 2003; Varma & Malathi, 2003).

In nature, about 18 species of begomoviruses have been found to infect tomato (Varma & Malathi, 2003; see also Part II, Chapter 2). Most of the new begomoviruses affecting tomato have been identified in the Americas

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where 17 distinct begomoviruses have been isolated from tomato (Polston & Anderson, 1997; Rojas et al., 2000).

The most devastating begomoviruses affecting tomato are those with generic names *Tomato leaf curl virus* and *Tomato yellow leaf curl virus* (TYLCV). So far, 11 viruses associated with TYLCD have been identified. These viruses are widely distributed in Africa, the Americas, Asia, Australia, and parts of Europe (Freitas-Astua et al., 2002; Varma & Malathi, 2003).

The TYLCV-induced disease is reported to cause up to 100% losses in yield in Cyprus, the Dominican Republic, Egypt, Israel, Italy, Jordan, Lebanon, Saudi Arabia, Spain, and Tunisia (Noris et al., 1994; Pico et al., 1996; Czosnek & Laterrot, 1997; Polston & Anderson, 1997; Polston et al., 1999). In many cases TYLCV epidemics lead to abandonment of the crop, particularly in seasons favouring whitefly population build-up (Pico et al., 1996). Morales & Anderson (2001) stated that the introduction of TYLCV to the Dominican Republic was the greatest tragedy in the history of whitefly transmitted geminiviruses affecting economically important crops in the Caribbean, and caused the collapse of the tomato industry in that country.

In this review the application of post-transcriptional gene silencing (PTGS) strategy as a promising tool to control the disease caused by TYLCV will be discussed.

2. MANAGEMENT OF TOMATO YELLOW LEAF CURL DISEASE

Since it has been reported for the first time in Israel in 1930s, several approaches have been used in attempts to control TYLCV. A satisfactory level of disease management can be obtained only through an integrated approach, which combines tomato resistance to the virus, and vector control. Cultural practices such as roguing, avoidance, use of barriers, yellow traps, crop residue disposal, among others, are recommended, but they should be combined with the use of insecticides and resistant varieties in order to be effective (Sharaf et al., 1984; Polston & Anderson, 1997; Antignus et al., 1998; Antignus, 2000; Polston, 2003). In addition, eradication of weeds known to serve as reservoir for TYLCV, like *Solanum nigrum*, *Malva nicaensis*, and *Nicotiana tabacum* contributed in the reduction of TYLCV incidence (Al-Musa, 1986; Bedford et al., 1999;). The use of fine-mesh screens and UV-absorbing plastic sheets has also been used to inhibit the penetration of whiteflies into covered greenhouses. However, these practices create problems of shading, overheating, and poor ventilation (Cohen & Antignus, 1994). In the Dominican Republic enforced host-free periods have been shown to effectively reduce TYLCD (see Part IV, Chapter 4). This practice has great potential in reducing whitefly population once a non-preferred host was used as an alternate crop (Salati et al., 2002). In another study, Liu & Stansly (2000) tested the effects of several surfactants and oils against whitefly nymphs. Although good levels of insect mortality were observed, phytotoxicity was observed on treated tomato plants.

Intercropping tomato with coriander (*Coriandrum sativum*) as a whitefly repellent or cucumber (*Cucmis sativus*), as an immune crop, can be an effective disease control strategy (Ahmed et al., 1996). Nevertheless, this practice may not be possible in all seasons and is not effective unless sown 2 weeks before tomatoes. Ahmed et al. (2001) demonstrated that application of Imidacloprid in combination with integrated pest-management practices can protect tomato from TYLCV infection until 12 weeks after sowing. The rate of TYLCV incidence in Imidacloprid treated plots did not exceed 15.7%. However, there are concerns about chemical control due to the potential of the vector to develop pesticide resistance and the deleterious effect on the environment (Pico et al., 1996). The effect of drip and furrow irrigation systems on whitefly populations and subsequently on TYLCV incidence was investigated by Sharaf et al. (1984). They demonstrated that drip irrigation tended to reduce whitefly population and resulted in the delay of TYLCV incidence for 2 weeks.

Undoubtedly, the use of resistant tomato plants is the best way to control TYLCV, however, all the commercially available hybrids today are tolerant but not immune to the virus. Early infections of these cultivars and high populations of viruliferous whiteflies will overcome the resistance (Polston, 2003).

All these factors together and the unsatisfactory level of disease control obtained using traditional measures led to the search for other control measures that go beyond traditional host genetic resistance, chemical controls and cultural practices.

3. TRANSGENIC APPROACHES TO CONTROL TOMATO YELLOW LEAF CURL VIRUS

In a number of crops, transgenics resistant to economically important plant viruses have been developed by introducing a sequence of the viral genome in the target crop by genetic transformation. Virus-resistant transgenics have been developed in many crops by introducing either viral capsid protein or replicase gene encoding sequences. This concept has been called pathogen-derived resistance (PDR) (Lomonossoff, 1995; Baulcombe, 1996). The coat protein gene, in particular, has been extensively used to engineer resistance to potex-, poty-, tobamo-, cucumo-, tobra-, carla-, and luteoviruses (Beachy, 1993); however, there are fewer reports of engineered resistance to geminiviruses (Bejarano & Lichtenstein, 1994; Kunik et al., 1994; Noris et al., 1996; Sinisterra et al., 1999; Yang et al., 2004; see also Part V, Chapter 4).

Early experiments demonstrated that high level of resistance can be triggered in CP-transformed plants when high levels of the viral capsid protein were expressed, confirming the importance of the actual protein in resistance (Powell et al., 1986; Gonsalves & Slightom, 1993). For example, Kunik et al. (1994) showed that tomato plants expressing the *V1* (CP) gene were resistant to TYLCV infection. The resistance was expressed as a delay in symptoms development and infected plants showed a recovery phenotype. The resistance was

associated with high levels of expressed CP protein. Another gene used for obtaining transgenic resistance to TYLCV is the *Rep* gene (Palukaitis & Zaitlin, 1997). Resistance against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was produced in *N. benthamiana* plants using the TYLCSV *Rep* gene with a deletion of 420 nt (140 amino acid residues) from the 3′ end, however, resistance was transitory and overcome with time (Noris et al., 1996). This truncated gene was also used by another research group to develop TYLCV-resistant tomato plants. It has been demonstrated that transformed plants that expressed high levels of the truncated TYLCSV Rep protein were resistant to TYLCSV but not to the Spanish isolate TYLCSV-ES[1] and plants showed undesired phenotype (Brunetti et al., 1997; Brunetti et al., 2001). Recently, Antignus et al. (2004) provided further evidence on the use of truncated *Rep* gene to confer resistance against TYLCV. They developed a small Rep construct which coded for only the first 129 amino acids of *Rep* gene of TYLCV-Mld. This construct could trigger resistance in transformed tomato plants against TYLCV-Mld when challenged by viruliferous whiteflies. However, challenging the plants by agroinoculation partially overcame the resistance resulting in variable phenotypes ranging from total immunity to susceptibility with intermediate types of resistance. In addition, the construct was not efficient against the Israeli strain (TYLCV).

In contrast to the protein-mediated resistance, RNA-mediated resistance had no direct correlation with the levels of protein produced (Anderson et al., 1992; Audy et al., 1994; Baulcombe, 1994). Bendahmane & Gronenborn (1997) demonstrated that the use of the full-length truncated TYLCSV *Rep* (63-nt leader and 288-nt [5′] *Rep* sequences) conferred moderate resistance to TYLCSV in *N. benthamiana*, and this resistance was inherited in the R2 generation. It was also observed that the level of homology between the antisense RNA and the challenge virus sequence specified the level of resistance obtained. Another group of researchers have shown resistance of *N. benthamiana* to TYLCSV by a double mechanism involving antisense RNA of TYLCSV *Rep* gene and extrachromosomal molecules; however, the plants were not resistant to TYLCV (Franco et al., 2001).

4. POST TRANSCRIPTIONAL GENE SILENCING – AN OVERVIEW

The term PTGS is usually used to describe similar events occurring in diverse biological research fields. Although PTGS was first discovered in plants, similar processes have been described for ciliates (Ruiz et al., 1998), for filamentous fungi, where it is called quelling (Romano & Macino, 1992; Catalanotto et al., 2000), and for animal systems such as nematodes (Fire et al., 1998; Cogoni & Macino, 1999), *Drosophila* (Kennerdell & Carthew, 1998), and mice (Bahramian & Zarbl, 1999) where it is referred to as RNA interference. An RNA silencing-like mechanism was first described in plants following attempts to overexpress gene constructs encoding key enzymes in the anthocyanin biosynthesis pathway, in transgenic petunia (Napoli et al., 1990; van der Krol et al., 1990). Contrary

to expectation, the pigmentation in the flowers of transformed plants was not enhanced. Instead the flowers were depigmented, and significantly, endogenous gene mRNA transcript levels were greatly reduced. Because both the transgene and the endogenous gene were suppressed the observed phenomenon was termed "co-suppression".

In PTGS, high levels of normal mRNA can cause activation of RNA dependent RNA polymerases (*RdRP*) gene, which can synthesize antisense transcripts. Antisense transcripts can also be synthesized when a gene is present in high copy number, especially where tandem-inverted repeated copies are present. Double-stranded RNAs resulting from either RdRP activity or base-pairing between antisense transcripts and mRNAs become targets for type III endoribonuclease Dicer (Waterhouse et al., 1998; Hammond et al., 2000; Zamore et al., 2000), which degrade dsRNAs into small fragments of ~21–25bp called small interfering RNA (siRNA) (Chellappan et al., 2004b). This ribonucleases, also recognizes fold-back, imperfectly base-paired single-stranded RNA (ssRNA) substrates (Hutvagner et al., 2001). After incorporation into the RNA-induced silencing complex (RISC), which contains at least one Argonaute (AGO) protein (Hammond et al., 2000; Martinez et al., 2002), siRNAs act as guides to select mRNA targets for degradation by siRNA/mRNA base-pairing. In addition, siRNA fragments enter the nucleus to guide a methyltransferase complex to sequences for methylation and also spread into other cells to direct the cleavage of homologous ssRNAs. This process appears to be part of the natural defense against viral dsRNAs. Small dsRNAs may serve to target nuclear copies of the gene for methylation, resulting in a feedback mechanism for gene silencing. dsRNAs can also be transmitted intercellularly via plasmodesmata, causing systemic gene silencing (Voinnet & Baulcombe, 1997; Mlotshwa et al., 2002).

Some plant viruses appear to have evolved a counterdefense strategy against PTGS (Brigneti, et al., 1998; Voinnet et al., 1999; Voinnet et al., 2000; Voinnet, 2001; Waterhouse et al., 2001; Baulcombe, 2002). Three lines of evidence support the idea that PTGS is an antiviral defence mechanism in plants. Firstly, virus infection triggers RNA silencing in infected plants that specifically targets the viral and homologous RNAs for degradation. This is best illustrated by the detection of virus-specific siRNAs of both sense and antisense polarities in wild type plants infected with plus-strand RNA viruses (Hamilton & Baulcombe, 1999). The second support is the demonstration that plant viruses encode proteins capable of suppressing RNA silencing (Voinnet et al., 1999; Li & Ding, 2001). A total of 29 suppressor proteins have been identified in plant and animal viruses (Anandalakshmi, et al., 1998; Kasschau & Carrington, 1998; Li, et al., 2002). These proteins are structurally and functionally diverse. For example, potyvirus helper component proteinase (HC-Pro) inhibits the maintenance step of PTGS at or upstream from the production of siRNA (Llave et al., 2000; Mallory et al., 2001) and/or downstream from the production of siRNA (Kasschau, et al., 2003). On the other hand, cucumovirus 2b protein suppresses systemic silencing, possibly after the generation of the silencing signal (Guo & Ding, 2002). *Potato virus*

X (PVX) p25 prevents systemic silencing (Voinnet et al., 2000), through the inhibition of the class of long (~25 nt) siRNAs. Recently, tombusvirus p19 was shown to bind siRNAs in vitro, and so it may prevent the spread of mobile silencing signals (Silhavy et al., 2002). In addition, it has been proposed that the coat protein of *Turnip crinkle virus* probably functions at an earlier step of PTGS, upstream from siRNA production (Qu et al., 2003). Furthermore, the AC4 and C2 of different cassava-infecting geminiviruses have been shown to have the capacity to suppress the induced-PTGS in *N. benthamiana* (Voinnet, et al., 1999; Vanitharani, et al., 2004; Vanitharani et al., 2005). In the monopartite *Tomato yellow leaf curl China virus* (TYLCCNV), the C2 protein, a positional homolog of AC2, was also found to possess silencing suppression activity (Van Wezel et al. 2002). In addition, TYLCCNV-C2 requires the nuclear localization signal, DNA binding and zinc-finger motif for anti-PTGS activity (Dong et al., 2003; Van Wezel et al., 2003).

The third support for a naturally antiviral role of RNA silencing in plants came from the observation that host plants compromised in RNA silencing exhibit enhanced susceptibility to virus infection. In this respect, *GS2*/*SDE1*, *SGS3*, *SDE3*, and *AGO1* genes have been identified in *Arabidopsis thaliana* and are essential for transgene-induced RNA silencing (Mourrain et al., 2000; Chuang & Meyerowitz, 2000; Vance & Vaucheret, 2001). Results of several studies showed that *A. thaliana* mutants defective in either of these genes were all hypersensitive to *Cucumber mosaic virus* (CMV), but were as susceptible as the wild-type plants to the infection of at least five other viruses tested (Dalmay et al., 2000; Mourrain et al., 2000; Morel et al., 2002; Boutet et al., 2003).

5. CONTROL OF TOMATO YELLOW LEAF CURL VIRUS VIA POST TRANSGRIPTIONAL GENE SILENCING

In the last few years several lines of evidence indicated the efficacy of PTGS against RNA and DNA viruses (Guo et al., 1999; Jan et al., 2000; Kalantidis et al., 2002; Marathe et al., 2000; Asad et al., 2003; Pooggin et al., 2003; Seemanpillai et al., 2003; Vanitharani et al., 2003; Chellappan et al., 2004a; Pruss et al., 2004; Jennifer, et al., 2005; Nicola-Negri et al., 2005; Fagoaga et al., 2006). In his early work, Brunetti et al. (1997) showed that tomato plants transformed with the truncated version of *C1* gene of TYLCSV and accumulate high levels of the truncated form of TYLCSV Rep protein (Rep-210) were resistant to TYLCSV. The resistance was due to the partially inhibition of *C1* gene transcription and the formation of dysfunctional Rep-210/Rep complexes. The reduced amount of viral Rep competed with the Rep-210 for the viral sequence required for plus-strand viral replication (Lucioli et al., 2003). Recently, the same research group provided clear evidence that TYLCSV can overcome the resistance in Rep-210 expressing plants via gene silencing (Noris et al., 2004). They found that the reduced but not abolished viral expression triggers, via an RNA-mediated mechanism, down-regulation of Rep-210 and this in turn

releases the transcriptional control of the viral *C1* gene (Lucioli et al., 2003). The Rep-210 expressing plants were found to be resistant to the Portuguese strain of TYLCV (TYLCV-[PT]). This virus strain failed to activate the gene silencing machinery due to the low homology between the transgene and the viral *Rep* gene. On the other hand, the Rep-210 transgene failed to protect plants from the infection with a related virus TYLCSV-ES[1]. This was due to the fact that Rep-210 was unable to repress the transcription of the *C1* gene of TYLCSV-ES[1], resulting in the production of Rep-specific siRNAs, which due to their extensive homology with the Rep-210 transgene, leads to the down-regulation of Rep-210 and prevents the formation of dysfunctional Rep-210/Rep-[ES1] complexes (Lucioli et al., 2003; Brunetti et al., 2001).

Yang et al. (2004) tested the capability of different transgenes obtained from TYLCV genome to trigger resistance in tomato plants against TYLCV. These transgenes were introduced in to plant cells in sense and antisense orientations. The transformed plants were challenge with TYLCV using high number of viruliferous whiteflies and plants were evaluated for resistance under field conditions. Transgenic plants contained one of the following transgenes: 2/5Rep (81 nucleotides of the intergenic region plus 426 nt of the 5′ end of the TYLCV *Rep* gene), Δ2/5Rep(85 nt of the IR plus 595 nt of the 5′ end of the TYLCV *Rep* gene in the antisense orientation), and RepΔ2/5Rep (81 nt of the IR, the entire *Rep* gene, and 41 nt 3′ to the end of the *Rep* gene fused to Δ2/5Rep) showed high level of resistance to TYLCV. Resistant plants were symptomless and TYLCV could not be detected in these plants either by hybridization or PCR. Furthermore, no difference in plant phenotypes could be observed between transformed and non-transformed plants. This is in contrast to the studies of Brunetti et al. (2001), which found a strong correlation between TYLCSV resistance and high levels of Rep protein expression. In his study, Yang et al. (2004) found that expression of the Rep protein in transgenic tomato plants was not necessary to trigger the resistance. Based on the characteristics of the resistance obtained by using constructs that are capable of forming hairpin structures (RepΔ2/5Rep), it has been hypothesized that PTGS might be the mechanism responsible for the resistance. However, further studies are needed to confirm this hypothesis.

Recently, Gafni et al. (personal communication) demonstrated for the first time that transformation of tomato plants with a double-stranded RNA targeted at the TYLCV CP can cause a delay in symptoms development. Transgenic tomato plants expressing the siRNA did not show disease symptoms for 7 weeks post-inoculation, while control non-transformed plants developed TYLCV symptoms two weeks post inoculation. As expected, resistant plants accumulated detectable levels of siRNA specific for the *CP* gene of TYLCV, and the level of CP RNA deceased in resistant plants. These data suggest that PTGS was involved in the mechanism of resistance. Recently Zrachya et al. (2007) showed that the TYLCV *V2* gene acts as a suppressor of RNA silencing, which targets a step in the RNA silencing pathway following the Dicer-mediated cleavage of dsRNA.

In Jordan, TYLCV-Mld and TYLCSV-ES[2] were recently reported for the first time to occur in different locations (Anfoka et al., 2005). In addition, using species and strain-specific primer pairs TYLSCV and TYLCV-Mld could also be detected in Israel and Egypt, respectively (unpublished data). These data together with the fact that cultural practices alone provide unsatisfactory level of TYLCV control, led us to investigate the capability to PTGS to provide acceptable level of resistance against viruses causing TYLCD. To achieve this goal a synthetic silencing construct was designed based on the non-coding conserved regions of TYLCV, TYLCV-Mld, TYLCSV-ES[2], *Tomato yellow leaf curl Malaga virus* (TYLCMalV), and TYLCSV genomes. To design the construct, three regions (C1C2, C2C3 and V1V2) of the virus genome were selected according to their length, gene sharing, stop codons and their priority in viral replication. These fragments were ligated together, and cloned into a binary vector in sense and antisense orientations.

The efficacy of the construct to trigger resistance against TYLCV was first tested in tomato and *N. benthamiana* plants using a transient assay. Promising results were obtained when plants were agro-infiltrated with the construct and few days later were challenge inoculated using an infectious TYLCV clone or viruliferous whiteflies carrying TYLCV, TYLCV-Mld, and TYLCSVES[2]. Results of dot blot hybridization and PCR showed that the best resistance could be obtained when the time interval between construct infiltration and challenge inoculation was between 16–20 days (unpublished data). All resistant plants remained symptomless until the experiment was terminated, whereas, control plants showed severe disease symptoms three weeks post inoculation. No viruses could be detected in resistant plants either by hybridization and PCR. Interestingly, a positive correlation between the resistance and the accumulation of TYLCV-specific siRNAs was also observed. The presence of these siRNAs is a hallmark of the activated RNA silencing process. When the silencing construct

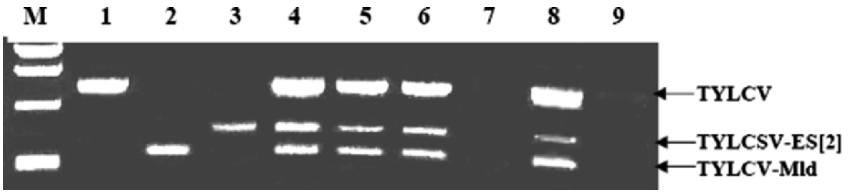

Figure 1. Agarose gel electrophoresis of DNA amplicons obtained by multiplex PCR from tomato plants inoculated with TYLCV, TYLCV-Mld, and TYLCSV-ES[2] using viruliferous whiteflies. The source of the nucleic acid was as follows: lanes 1, 2, and 3, clones of TYLCV, TYLCSV-ES[2] and TYLCV-Mld, respectively; lane 4, tomato plant used as a source for challenge inoculation; lane 5, viruliferous whiteflies used in challenge inoculation; lanes 6 and 8, tomato plants infiltrated with empty vector and 16 and 20 days later were challenge inoculated with viruliferous whiteflies, respectively; lanes 7 and 9, tomato plants infiltrated with a vector harbouring the silencing construct and 16 and 20 days later were challenge inoculated with viruliferous whiteflies, respectively. M, 1 Kb DNA marker.

Figure 2. Gene silencing in controlling *tomato yellow leaf curl disease*. One-month-old non-transformed (left) tomato (*Solanum lycopersicum*) plants line MP-1 and tomato plant expressing the silencing construct (right) were challenge inoculated with TYLCV and TYLCV-Mld.

was used to transform tomato plants, different types of resistance phenotypes were observed in T1 generation. The resistance was expressed as either a delay in symptoms development or immunity. Using multiplex PCR, at least two viruses (TYLCV and TYLCV-Mld) could be detected in control as well as plants showing delay of disease symptoms, however, no virus could be detect in symptomless plants (Figure 1). Furthermore, some plants that showed mild disease symptoms 1 month post-inoculation recovered from the disease and became symptomless (Figure 2). These data together provide compelling evidence that PTGS is a promising strategy to control the disease caused by TYLCV complex.

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PART VI

INTERNATIONAL NETWORKS TO DEAL WITH THE TYLCV DISEASE: THE NEEDS OF THE DEVELOPING COUNTRIES

CHAPTER 1

INTERNATIONAL NETWORKS TO DEAL WITH TOMATO YELLOW LEAF CURL DISEASE: THE MIDDLE EAST REGIONAL COOPERATION PROGRAM

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1. OVERVIEW

The Middle East is a major producer of both processing and fresh market tomatoes (*Solanum lycopersicum*); and tomatoes are a main component of the local cuisines. Since *Tomato yellow leaf curl disease* was first reported in Israel in the early 1950s, it has become one of the major, if not the most important, constraint to production (see historical perspective in Part I, Chapter 1). This disease has been reported in all countries of the Middle East, and the importance of this disease has been associated with the expanding range of vector *Bemisia tabaci* biotype B and of the pathogen, members of the *Tomato yellow leaf curl virus* complex.

Management of this disease has mainly involved methods for reducing the vector population; and in many cases, this was primarily by the application of insecticides. Tomatoes with resistance to *Tomato yellow leaf curl virus* (TYLCV) would effectively reduce losses and reduce the quantity of insecticides needed to obtain satisfactory yields. Several breeding programs were initiated in the 1970s (see Part V, Chapter 3; Nakhla & Maxwell, 1998) and in general, progress was slow. In all cases, resistance to TYLCV was based on introgressions of resistance loci from wild tomato species (e.g., *S. chilense*, *S. habrochaites*, and *S. peruvianum*) (see Part V, Chapter 2). It was not until the 1990s that commercial hybrids with moderate levels of resistance were available.

Because of the seriousness of this disease and the difficulty of managing it, international networks of scientists have been organized to provide solutions. Henri Laterrot from INRA, France, was the first to organize an international project, and it was funded by Commission des Communautés Européennes, in

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 409–415.

the late 1980s (Laterrot, 1995). One goal was to test germplasm in different countries (Israel, Egypt, Jordan, Mali, and Sénégal) and then to combine the resistant plants into a population that could be used in breeding programs (e.g., Pimpertylc, Chiltylc). Subsequently, several international projects have been organized to focus on the management of whiteflies and begomoviruses. This chapter will not attempt to describe them all, but will discuss mainly two international projects that have as their main goal the development of breeding lines resistant to begomoviruses in the Mediterranean Basin and Central America (see www.plantpath.wisc.edu/GeminivirusResistantTomatoes/index.htm).

2. MIDDLE EAST–NORTH AFRICA PROJECT

The Mediterranean project, which is funded by US Aid for International Development, (USAID), as part of The Middle East Regional Cooperation Program involves eight laboratories in the Mediterranean Basin and one in the USA. The main goal of this project is to develop cultivars resistant to members of the TYLCV complex with characteristics suitable for the markets of the collaborating countries of Egypt, Israel, Jordan, Lebanon, Palestinian Authority, Tunisia, and Morocco. Classical breeding approaches using resistant germplasm from Hebrew University of Jerusalem (Vidavsky & Czosnek, 1998) with resistance derived from *S. habrochaites* and from Volcani Center in Israel with resistance derived from *S. peruvianum* (Friedmann et al., 1998) would be the main approach. Additionally, effort would be devoted to developing virus-derived resistance strategies. As a means of pyramiding resistance genes, research would focus on finding breeder-friendly markers for the introgressions from *S. habrochaites*. Initially, the begomoviruses in each country were characterized. TYLCV was known to occur in Jordan, Lebanon, Israel, and Egypt. PCR protocols were developed to detect TYLCV-mild, TYLCV, and TYLCSV (Anfoka et al., 2005; Gorsane et al., 2005), and all three viruses were detected in Jordan and Israel. In Egypt and Lebanon both TYLCV and TYLCV-mild were identified (Anfoka et al., 2007, submitted for publication). Both TYLCV and TYLCSV were associated with tomatoes in Tunisia and Morocco. Thus, it is evident that tomato hybrids need to be resistant to these three monopartite begomoviruses. Hybrids or breeding lines with various sources of resistance genes were initially evaluated in each country, and tomatoes with resistance from *S. habrochaites* (Vidavsky & Czosnek, 1998) or *S. peruvianum* (Friedmann et al., 1998) exhibited resistance in these six countries. Germplasm with *S. chilense* (Agrama & Scott, 2006) resistance genes were evaluated only in Lebanon, Jordan, and Israel and they were found to be resistant. F_1 hybrids were made with local land races or commercial hybrids used in each country and with the different sources of resistance genes. These F_1 plants are being evaluated by scientists in their country and breeding lines developed. The first hybrids from this program have been evaluated in the fall of 2006 in Egypt. The tomato types are determinate growth and round fruit for Jordan and Egypt, indeterminate growth

and round fruit for the open field in Morocco, and semi-determinate growth and roma-shaped fruit for Tunisia, and a semi-determinate growth and large (1 kg) fruit for Lebanon. It is expected that these adapted hybrids will be marketed by local companies in each country. To aid in the breeding effort, marker-assisted selection protocols have been developed for resistance to root-knot nematode (*Mi-1* gene) (El Mehrach et al., 2005) and *Fusarium oxysporum* f. sp. *lycopersici* race 2 (*I2* gene) (Y. Abou Jawdah, 2006 American University of Beirut, personal communication).

In addition to classical breeding, recombinant DNA approaches are being investigated. This required the standardization of a protocol for transformation of tomato and this effort involved five laboratory teams. A workshop was held in Egypt where all parameters associated with transformation were discussed. From this research, a Regeneration and Transformation Protocol booklet was developed (N. Iraki, 2005 Bethlehem University, personal communication). Two different antiviral strategies are being evaluated. One involves the novel approach of using the whitefly protein GroEL which binds to the coat protein of begomoviruses and other viruses (Akad et al., 2004), to engineer plants that express GroEL in their phloem. Evaluation of T_0 to T_3 generation plants by whitefly-mediated inoculation with TYLCV has provided strong evidence that this strategy is effective (Akad et al., 2007). The other approach has involved development of gene-silencing constructs (siRNAs) from TYLCV or TYLCSV, and subsequent evaluation of these constructs in transient assays. Results with this strategy are also very promising (see Part V, Chapter 5; Abhary et al., 2006). The goal is to combine both resistance genes from wild species (Agrama & Scott, 2006; Friedmann et al., 1998; Vidavsky & Czosnek, 1998) and recombinant DNA antiviral constructs into a hybrid, which is expected to be more durable and resistant to the diversity of members of the TYLCV complex. It is expected that these plants would also provide resistance to begomovirus present in other regions of the world.

This project has brought together scientists with diverse backgrounds, such as classical plant breeders, plant pathologists, and molecular biologists, to solve a difficult problem. The focus has been on providing resistant hybrids suitable for the local production, such as salt tolerant tomatoes for Egypt, and large, uniquely shaped tomatoes for Lebanon. These scientists have learned to work together to assist each other in developing and improving technology. Workshops have been an important component of this effort, as well as annual meetings and frequent trips by the team members to assist each other.

3. CENTRAL AMERICAN PROJECT

Begomoviruses have been the most important disease problem on tomatoes in Central American since the 1980s (Morales & Anderson, 2001). Currently, these viruses are bipartite in Central America; however, the monopartite virus, TYLCV, which was introduced from the Eastern Mediterranean region, is the predominant

virus in much of the Caribbean region. In 2001 a 4-year project sponsored by the USAID Cooperative Development Research Program was a collaborative program among scientists in Israel, Guatemala, and USA. One of the objectives was to evaluate tomato germplasm developed in Israel and Florida, USA that had resistance to TYLCV against the diversity of bipartite begomoviruses (Nakhla et al., 2005) in Guatemala. In general, the germplasm with resistance genes from *S. habrochaites* (Vidavsky & Czosnek, 1998), *S. chilense* (Agrama & Scott, 2006), and *S. peruvianum* (Friedmann et al., 1998) was also resistant in Guatemala. Breeding lines were selected primarily with resistance from *S. habrochaites* and *S. chilense*; and hybrids combining both sources of resistance were the most resistant (Mejía et al., 2005). One goal was to license the breeding lines to a Guatemalan company so that prices could be kept low, thereby allowing the majority of small growers to purchase these hybrids. A new seed company, GenTropic Seeds, S.A., was formed by Guatemalan businessmen, and the first hybrids, San Miguel and Llanero, have been being released in the fall of 2006.

4. OTHER INTERNATIONAL PROJECTS

Currently, there are several other large international projects, which are organized primarily to manage the whitefly vector. Tomato breeding for virus resistance is a minor component of these projects; however, there are regional projects in the Caribbean and Southeast Asia, which have a major emphasis on breeding tomatoes for resistance to begomoviruses.

4.1. Tropical whitefly IPM project

This worldwide project recognizes the increasing importance of horticultural crop production in the tropics and the increasing reliance on a pesticide-based control strategy for the whitefly. This project was the first effort to coordinate activities within the International Agricultural Research Centers (IARCs) as part of the Consultative Group for International Agricultural Research (CGIAR). The project is organized around six outputs aimed at reducing whitefly populations and the losses resulting from viruses that they transmit: (1) formation of an international network of researchers on whiteflies and whitefly-transmitted viruses (WTVs); (2) documentation of whitefly projects in prioritized areas; (3) epidemiological studies of whiteflies and viruses in targeted areas; (4) development of IPM strategies; (5) strengthening of national agricultural research services in target countries; and (6) assessment of project impact (Anderson & Morales, 2005; http://www.tropicalwhiteflyipmproject.cgiar.org).

4.2. European whitefly studies network

Initially the project was a mechanism for increasing interaction among scientists, growers, industry personnel, and plant health advisors with the goal of

studying the whitefly and strategies for management of whiteflies. Information is primarily disseminated through a web site (www.whitefly.org), a newsletter, technical publications, and symposia.

4.3. Agricultural biotechnology support project II (USAID)

Biotechnology advances should be available to growers worldwide, and this project is organized to provide information so farmers will have opportunities to make informed choices about using bioengineered plants (http://www.absp2.cornell.edu). Within this effort, two projects focus on developing tomatoes with multiple virus resistance. A project in Southeast Asia will develop *Tomato leaf curl virus*- and *Tomato yellow leaf curl virus*-resistant tomatoes using resistant germplasm from the Asian Vegetable Research and Development Center for the Philippines and Indonesia. It is expected that these virus-resistant tomato hybrids will become available for small-scale farmers. Another project will attempt to combine resistance to TYLCV and potyviruses into locally acceptable tomatoes for Mali.

4.4. South Asia vegetable research network

This project was initiated in 1992 by scientists at Asian Vegetable Research and Development Center to provide research on the management of Tomato leaf curl disease in Bangladesh, Bhutan, India, Nepal, Pakistan, and Sri Lanka (see Part VI, Chapter 2). Progress was made on the evaluation of germplasm with different sources of virus resistance in different locations. Not all sources of resistance responded similarly in all regions, perhaps because of the presence to different begomoviruses or different environments.

4.5. Other projects

The recently funded IPM Collaborative Research Support Program (USAID) has a component on "collaborative assessment and management of insect-transmitted viruses," which will involve begomoviruses and their control (www.oired.vt.edu/ipmcrsp/RFAs/ comp_grant_winners_10–03–05.htm) in the Caribbean and Central America regions. In India, there is a multi-institutional project on sustainable management of *Tomato leaf curl virus* (http://www.tomatoleafcurlandwhitefly.org/#); and tomato varieties Sankranthi, Nandi, and Vybhav with ToLCV-resistance are being commercialized. A project on begomovirus disease management for sustainable production of tomato in the Caribbean was initiated by the Commission of European Communities Research (http://betocarib.cirad.fr) in 2002. Data were collected on epidemiological parameters and a model developed for IPM of tomato production and this IPM package will include the use of begomovirus resistant hybrids.

5. CONCLUSIONS

Diseases of tomato caused by begomoviruses are devastating and have caused considerable hardship for growers in developed countries as well as poor countries of the tropical and subtropical regions of the world. Private seed companies have placed emphasis on the development of tomatoes with begomovirus resistance and excellent horticultural characteristics for the developed countries, and it remains the responsibility of public institutions to develop the tomatoes adapted to local climates and markets with resistance to begomoviruses for the small-scale, poorer farmers of tropical countries. This has best been achieved through the development of international collaborative research teams, which involve the expertise from universities or research centers in the developed countries and the local knowledge and expertise from scientists at public institutions in less developed countries. Through these collaborations, progress is being made towards the development of locally adapted hybrids with excellent resistance to begomoviruses. Once these hybrids are available, innovative approaches for marketing them will be needed. These approaches will vary for each country as issues such as seed production, marketing and distribution, as well as intellectual property rights, will have to be considered.

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CHAPTER 2

AVRDC'S INTERNATIONAL NETWORKS TO DEAL WITH THE TOMATO YELLOW LEAF CURL DISEASE: THE NEEDS OF DEVELOPING COUNTRIES

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1. OVERVIEW

Tomato leaf curl disease severely affects crops in Southeast Asia and South Asia, but networks of researchers facilitated by AVRDC were able to make major progress in overcoming the effects of this disease, which would not have been otherwise possible.

Since its inception in 1971, AVRDC, the World Vegetable Center (AVRDC, 2003), formerly known as the Asian Vegetable Research and Development Center, has had strong links with most countries in the region. The networks created by AVRDC in the late 1980s trace their roots to earlier bilateral agreements and outreach programs, such as the ones in the Philippines, Thailand, Indonesia, and Malaysia initiated in 1975, 1981, 1983, and 1984 respectively. The Asian Development Bank (ADB), one of the founding members of AVRDC provided financial support to establish these bilateral programs. To assess the progress and impact of the outreach programs and to ascertain the research and development needs for the region ADB supported a consultation workshop in 1988 (AVRDC, 1988a). There was a general consensus among the four countries involved that engaging in partnership research offered distinct strategic advantages. Therefore, they wanted to establish a Collaborative Vegetable Research Network for Southeast Asia and requested AVRDC be the facilitator of the network. They also requested ADB to provide the key financial support for such a network. The National Agricultural Research Systems (NARS) were responsible and AVRDC served as a catalyst in the birth of the first AVRDC's vegetable network.

Research networking was one of the key features in the ADB's regional agricultural technical assistance projects at that time. It has proven to be an effective mechanism to promote research to achieve common goals of the participating

H. Czosnek (ed.), Tomato Yellow Leaf Curl Virus Disease, 417–439.

countries, which would not have been possible if the countries had carried out their research individually. The strong partnership of AVRDC with these countries continues until this day. Components of network cooperation included a memorandum of understanding to maintain the commitment of member countries, joint proposal development, the organization of an initial joint planning consultation workshop, training components, infrastructure improvement, monitoring by midterm reviews and final workshops and impact assessment. The success of cooperative networks depended on (1) a clearly defined realistic problem, (2) commonality of the problem, (3) strong self-interest, (4) willingness to commit available resources, (5) availability of external funding, (6) sufficient training and manpower development, and (7) strong leadership. In the following pages the AVRDC's collaborative research into dealing with the *tomato (yellow) leaf curl virus disease* through network modes is described.

2. THE SOUTHEAST ASIA NETWORK (AVNET)

With the creation in 1989 of the Collaborative Vegetable Research Program in South East Asia, known as the Asian Vegetable Network or AVNET, the previous bilateral partnerships attained a new dimension. *Tomato leaf curl disease* was not directly targeted in the two Subnetworks. Subnetwork 1. Disease and Pest Management which focused on Anthracnose and viruses of chili, bacterial wilt of tomato, and IPM of diamond back moth (*Plutella xylostella*), on crucifers, and Subnetwork 2, Varietal Testing (which included yardlong bean, cucumbers, chili, tomato, garlic, and shallot). However, Indonesia and Thailand reported severe leaf-curling symptoms affecting their chili trials (AVRDC, 1993a). Consequently a digoxygenin-labeled nucleic acid probe based on the *tomato yellow leaf curl Thailand virus* (TYLCV-Thai) sequence was developed and deployed by the Thai virologists, who at that time were already researching the tomato leaf curl virus (ToLCV) which caused serious crop losses in Thailand (Attathom et al., 1990). They were consequently able to associate a geminivirus with the leaf curl and yellowing symptoms on chili. Cloning and sequencing of the virus causing chili leaf curl disease in Thailand indicated that it had only 64.5% homology with the bipartite *Tomato yellow leaf curl virus* from Thailand (AVRDC, 1997b, Chiemsombat & Kruapan, 1997). Thus this network provided clear evidence that different distinct geminiviruses affect tomato and peppers.

3. THE SOUTH ASIA VEGETABLE RESEARCH NETWORK (SAVERNET)

This network was initiated in 1992 and consisted of two subnetworks, i.e., Subnetwork 1, Exchange and On-Farm Evaluation of Elite Varieties of Selected Vegetables, including, tomato, eggplant, and *Brassica* sp., and Subnetwork 2, Crop and Pest Management Research, which addressed leaf curl virus and bacterial wilt in tomato and chilies and IPM of eggplant, tomato, and crucifers.

Network partners included Bangladesh, Bhutan, India, Nepal, Pakistan, and Sri Lanka (AVRDC, 1997b). At that time, leaf curl disease of tomato had been observed and considered serious in all countries (Hameed, 1997; Joshi et al., 1997; Begum & Khan, 1997; De Zoysa, 1997; Chatchawankanphanich et al., 1993) with the exception of Bhutan and was already a national research priority in India (Kalloo, 1997; Singh, 1997).

As an initial step a comprehensive review of research on the leaf curl and yellowing viruses of tomato and peppers (Green & Kalloo, 1994) was undertaken by the AVRDC virologist and Dr. G. Kalloo, a well-known vegetable breeder from the Directorate of Vegetable Research in Varanasi, India who at that time had already bred a number of leaf curl virus-resistant tomato lines for North India where tomato leaf curl disease has long been considered one of the most important disease.

Our cooperators in the five NARS received two sets (A and B) of entries with reported resistance/tolerance for testing (AVRDC, 1997b). Some of these had been listed in the review of Green and Kalloo (1994).

Set A consisted of four wild species, (*Solanum habrochaites* LA1777, *S. peruvianum* VL215, and *S. chilense* LA1969 and LA1938), one tomato breeding line from Israel (Ty-52) apparently resistant to TYLCV-IL, and five tomato breeding lines from Jay Scott, University of Florida (FL699sp, FL744, FL736, FL776 and FL505), bred for resistance to *Tomato mottle geminivirus* (ToMoV). H-24, a tomato line developed by G. Kalloo was also included as were the leaf curl virus tolerant commercial hybrids, Tyking and Fiona. Set A was also sent to some of the AVNET collaborators. The results of the multilocation testing of Set A are shown in Table 1.

Set B consisted of populations and lines derived from crosses of various wild tomato species and tomato varieties (Pimhirtylc 91, Pertylc 91, Chepertylc 92, Duotylc 90, Hirseptylc 21, Octotylc 90, Chiltylc 92, Pimpertylc 93, Lignon C 8-6, Progress 7 S68, Roza, Colombian-1988) from H. Laterrot, Institut National de la Recherche Agronomique (INRA, Avignon, France) who at that time had been implementing a resistance breeding network for the *Tomato yellow leaf curl virus* in the Mediterranean region and subtropical Africa, funded by the European Union (Program CEE-DGXII-TS-ZA-0055F) and the private sector. This network relied on multilocation testing of breeding populations developed by H. Laterrot and the use of resistant selections to further advance the populations. Infection of these lines in the SAVERNET countries was consistently lower than that of local checks and ranged from 0–40% in India and Bangladesh. However, Hirseptylc, Pimhirtylc, Pertylc, and Duotylc were found susceptible in Pakistan and Chiltylc in Thailand, under controlled conditions using whitefly inoculations.

These multilocation tests clearly indicated that (1) the virus(es) causing tomato leaf curl disease in the participating countries was not the same, as evidenced by different reactions on the set of tomato lines, and (2) that wild species, particularly LA1932, were offering the best resistance throughout the eight Asian countries

Table 1. Multilocation testing of tomato lines and wild species for resistance to tomato (yellow) leaf curl virus

Entry	Species	Source(s) of resistance	Origin of line[b]	Disease reaction[a]								
				TW[b]	IN	LK	NEP	PAK	BD	PHIL	THAI	US (ToMoV)
TYKING F_1	*lyc*[c]	not available	NL	R	S		R					MS
FIONA F_1	*lyc*	not available	NL	R	MR,MS,S	S	R	S	S	(S)	S	S
TY 52	*lyc*	LA 1969 (*chil*)	ISR	S	R, (S)	S	S	(S)	(MS)	S	S	S
H 24	*lyc*	B 6013 (*hirs* f. *glab*)	IN	R	HR,R	HR	R	HR	MS	S	(MS),S	S
LA 1777	*hirs*		ISR	R	HR	S	HR	HR	R	R	(R)	R
INRA sel.	*per*		F	R	HR	R	R	HR	HR	HR	(R),(MR)	
LA 1969	*chil*		US	HR	HR	HR	HR	HR	R	R,(S)	R	R
LA 1932	*chil*		US	HR	HR,R	R	R	HR	R	R	R	R
FL 699sp	*lyc*	LA 1938 (*chil*) PI 211840 (*pim.*)	US	MS	HR,R	(MS)	HR	S	MR	MR	(MR),S	(MS)
FL 505	*lyc*	Chiltylc (*lyc*) LA 1969 (*chil*) Tyking (*lyc*) Fiona (*lyc*)	US	R	HR,MS	S	HR	S	S	(S)	(MS),S	(S)
FL 744	*lyc*	LA 1932 (*chil*)	US	R	R	(MR)	S	(S)	(MR)	(S)	R	(MS)
FL 736	*lyc*	LA 1938 (*chil*)	US	S	S,MS							(MS)
FL 776	*lyc*	LA 1959 (*chil*)	US	(MS)	S,R							(MS)
TK 70 (SCK)	*lyc*		TW	S	S	S	S	S	S	S	S	

[a] Most tests were conducted under field conditions, where the tomato (yellow) leaf curl disease was endemic. Infection was rated visually. In some instances, especially in case of HR reactions, confirmation of visual ratings was by nucleic acid hybridization tests (NAH). Empty spaces = not tested
HR = highly resistant, 0% infection (visual and NAH)
R = resistant (1–20% infection)
MR = moderately resistant, (21–50% infection)
MS = moderately susceptible (51–75% infection)
S = susceptible (76–100% infection)
( ) = mild symptoms
[b] NL = Netherlands, ISR = Israel, F = France, US = USA, TW = Taiwan, IN = India (Karnataka State), LK = Sri Lanka, NEP = Nepal, PAK = Pakistan, BD = Bangladesh, PHIL = Philippines, THAI = Thailand
[c] *lyc* = *S. lycopersicum, hirs* = *S. habrochaites, per* = *S. peruvianum, chil* = *S. chilense, hirs* f. *glab* = *S. habrochaites* f. *glabratum*

where they were tested. It was also learned that in some locations field testing resulted in variable results. These were attributed to either different levels of virus/whitefly infection pressure or to the presence of geminivirus strains or possibly distinct geminiviruses. The later scenario was confirmed a few years later, when AVRDC virologists detected the presence of several geminiviruses and geminivirus strains in Karnataka, India (AVRDC, 2002). Clearly, this initial multilocation testing suggested that breeding tomato for stable resistance to T(Y)LCV would most likely not be a simple and short process and that it would have to involve multilocation testing in order to be successful.

Through these two networks, an assessment of the importance and distribution of leaf curl virus of tomato in the six participating countries of SAVERNET and the four of AVNET was made possible. Samples were collected from farmers' fields by the NARS and or AVRDC scientists and tested either directly in the country or sent to AVRDC for testing by nucleic acid hybridization (NAH) using various DNA probes developed by AVRDC (1993a). Initially, when reliable diagnostics were not yet available, samples were also sent to D. P. Maxwell (University of Wisconsin, USA) for testing with a mixture of radioactive labeled probes (AVRDC, 1993b). These surveys have in the meantime been completed and cover most of South and Southeast Asian countries (Table 2).

The interest of the NARS in ToLCV research clearly was stimulated through this network and through a 3-week molecular diagnostics workshop at AVRDC in 1993 with the geminivirus research pioneer, Dr. D. P. Maxwell as resource person. Scientists from the NARS were introduced to molecular diagnostic techniques, which for some of them was their first exposure. This included NAH and PCR for the detection of geminiviruses, particularly TYLCV. The development of probes and primers was also discussed. Following the training, and upon return to their respective countries all the researchers from different countries were able to use a common technique for sample collection, detection, and reliable identification of geminiviruses in their respective countries. Shortly after this workshop one of the researchers from Pakistan, Shahid Hameed, decided to pursue a Ph.D. degree at the University of Dundee (UK) on the molecular diversity of begomoviruses of legumes and cucurbits in Pakistan, thus contributing to further scientific manpower development.

AVNET I and II, and SAVERNET I and II were funded by the Asian Development Bank which at that time promoted agricultural diversification in Asia, particularly the growing of vegetables following the traditional rice and wheat crops.

Cognizant of the findings resulting from these early network activities involving TYLCV, AVRDC realized that strong multilateral, bilateral, and institutional partnerships were necessary to address the tomato leaf curl disease problem. These networks, while no longer funded, continue to operate

Table 2. Survey for viruses of tomato in Asia (as of May 2006)

Country	Total No.[a] samples tested	No. (percent) virus positive samples[b]			
		CMV	ToMV	PVY	Begomovirus[c]
Bangladesh	56	1	0	0	412/687
Bhutan	10	NT	NT	NT	0/10
Cambodia	35	2	0	0	32/51
China	67	31	15	17	52/144
Hong Kong	44	22	0	2	0/44
India	72	0	3	0	512/2116
Indonesia	22	4	0	0	13/160
Laos	27	4	0	0	18/27
Malaysia	9	NT	NT	NT	4/9
Myanmar	2	0	0	0	5/5
Nepal	41	19	0	0	177/591
Pakistan	323	NT	NT	NT	24/323
Philippines	141	31	20	28	139/224
Sri Lanka	91	NT	NT	NT	21/91
Taiwan	244	24	30	4	566/1311
Thailand	17	12	0	12	64/209
Turkey	1	0	0	0	1/1
Uzbekistan	5	0	5	2	0/5
Vietnam	68	2	0	0	90/137
Total	**1275**	**152(18)**	**81(10)**	**57(7)**	**2130/6145(35)**

[a] Total no. samples tested for CMV, ToMV, PYV.

[b] samples were tested by DAS-ELISA, using antisera from the German Collection of Microorganisms and Cell cultures (DSMZ) Braunschweig. CMV = cucumber mosaic virus, ToMV = tomato mosaic virus, PVY = potato virus Y.

[c] In some countries more samples were collected and tested for begomoviruses than for the other three viruses. Numbers indicate the number of begomovirus positive samples over the total number of samples collected and tested for geminiviruses by polymerase chain reaction (PCR) using the begomovirus-specific degenerate primer pair PAL1v 978/PAR1c715.

informally – mainly because of the impact they have had on scientific manpower development, the identification and molecular characterization of tomato-infecting geminiviruses in the region (Zeidan et al., 1999; Green et al., 2005) and on the exchange of germplasm, which has led to the release of improved TYLCV-tolerant varieties and lines by the public, as well as private sector in the partner countries (Muniyappa et al., 2002) and AVRDC (Table 3). These networks have clearly established mechanisms for continued cooperation and have strengthened research capacity of the NARS for dealing with the whitefly and geminivirus problems affecting vegetable crops.

Table 3. Selected tomato lines resistant to tomato (yellow) leaf curl disease in Asia bred or distributed by AVRDC

Line	Distribution	Year of release	Resistance gene
Vybhav	India	2002	*Ty-2*/*Ty-2*
Sankranthi	India	2002	*Ty-2*/*Ty-2*
Nandi	India	2002	*Ty-2*/*Ty-2*
CLN 2116 B	International	2000	*Ty-2*/*Ty-2*
CLN 2123 A	International	2000	*Ty-2*/*Ty-2*
CLN 2460 E	International	2003	*Ty-2*/*Ty-2*
CLN 2498 D	International	2003	*Ty-2*/*Ty-2*
CLN 2777 H	International	2007	Unknown

4. THE COLLABORATIVE VEGETABLE RESEARCH AND DEVELOPMENT NETWORK FOR CENTRAL AMERICA, (REDCAHOR)

REDCAHOR, AVRDC's first network in the Americas, Panama, and the Dominican Republic was initiated in 1997 with headquarters at the Inter-American Institute for Cooperation on Agriculture (IICA) in Costa Rica. The Central American Bank for Economic Integration (CABEI), Inter-American Development Bank (IADB), and ROC's International Cooperation and Development Fund (ICDF) provided financial support for REDCAHOR (AVRDC, 2001). The evaluation of tomato and pepper germplasm for geminivirus resistance was a priority target of this network which focused on (1) genetic resources (tomato, sweet pepper, and cucurbitaceae cucurbits) with the aim of finding genes that would offer resistance to the whitefly/geminivirus complex; (2) the validation of commercial cultivars (tomato, peppers, onion, and squash); (3) IPM aspects including studies of the diamond-back moth (*Plutella xylostella*) of crucifers and pepper weevil, and (4) training to increase the critical mass of manpower for vegetable research in general.

Seven hundred accessions of tomato species, accessions, and varieties were evaluated in hotspots throughout the participating countries. Tolerance to the geminivirus complex was identified in the following AVRDC accessions and used for breeding: L167, L170, L176, L623, L667, L772, L944, L951, L1023, L951, L1167, L1247, L1357, L1504, L1684, L1830, L1958, L2094, L5527, L10660, TA 02288, and TA 272802 (Carls, 2000, AVRDC, 2000a). L 667 and L 1501 were *S. pimpinellifolium* and *S. lycopersicum* × *S. pimpinellifolium* respectively, while the rest were *S. lycopersicum*. Most commercial and established local cultivars tested in this network were susceptible to the geminivirus complex.

Training on molecular techniques for the identification of geminiviruses affecting tomato was provided to the pathologists of member countries in 1999 by Dr. R. Rojas of the Laboratorio de Biologia Molecular de Plantas y Virus, Centro de Investigacion en Biologica Cellular y Molecular, Universidad de Costa Rica. The project was discontinued in 2000, due to lack of further funding.

5. COLLABORATIVE NETWORK FOR VEGETABLE RESEARCH AND DEVELOPMENT FOR SOUTHERN AFRICA (CONVERDS)

The establishment of CONVERDS was agreed upon at a regional consultative workshop held in Arusha, Tanzania in 1990, attended by representatives from the Southern African Development Community (SADC) member countries; Botswana, Malawi, Tanzania, Zimbabwe, South Africa, Swaziland, Angola, Mozambique, Mauritius, Lesotho, and Namibia. The German Ministry of Economic Cooperation (BMZ) provided initial financial support for CONVERDS. The network was administered through the AVRDC Africa Regional Program (ARP), managed from Arusha, Tanzania. Major diseases of tomato, such as tomato yellow leaf curl and late blight were targeted. The support of the pathologists and breeders from AVRDC headquarter in creating a facilitating environment through past work was crucial. Molecular diagnostics for tomato geminiviruses had already been developed (AVRDC, 1987, 1992, 1993a), TYLCV-resistant germplasm identified (AVRDC, 1988b, 1999) and interspecific crosses (AVRDC, 1994) with wild species, such as *S. chilense* LA 1969, resistant to the *Tomato leaf curl Taiwan virus* (ToLCTWV), had been initiated.

Several surveys were conducted to assess the distribution and incidence of T(Y)LCV in Tanzania and the SADC countries. An initial survey was conducted in Tanzania in 1993/1994, using NAH with four types of probes, against the Taiwan, Egypt, and India (Ban-1, GenBank Acc. U1746) viruses, and the *Tomato leaf curl Thailand virus*. The Eastern Mediterranean strain of TYLCV appeared to be present in 8% of the samples. However, since many samples with very pronounced leaf curl and yellowing symptoms did not react with any of the four probes (AVRDC, 1996), it was suspected that another geminivirus species may also be present in Tanzania (Nono-Womdim et al., 1996, 2005). CONVERDS enabled AVRDC to establish active contacts with researchers and institutes in Eastern Africa to obtain fresh samples for further investigation of the geminiviruses present on tomato and to release improved tomato cultivars. Since then one new distinct tomato geminivirus has been identified from Tanzania (Genbank accession numbers U73478 and DQ 519575) (Chiang et al., 1992, Shih et al., 2006c).

6. THE CGIAR GLOBAL WHITEFLY PROJECT

In 1997 AVRDC was asked to be a partner in the CGIAR "Global Whitefly IPM Project" (Anderson, 2005). This project adopted an eco-regional problem approach and was structured into six subprojects, each one lead by an International Agricultural Research Center (IARC). (1) *Bemisia tabaci* as a virus vector in cassava and sweet potato in sub-Saharan Africa (led by the International Institute of Tropical Agriculture (IITA); (2) *B. tabaci* as a virus vector in mixed cropping systems of the Caribbean, Mexico, and Central America (led by the International Center for Tropical Agriculture (CIAT); (3) *B. tabaci* as a virus vector in mixed cropping systems of Eastern and

Southern Africa (led by the International Centre of Insect Physiology and Ecology (ICIPE); (4) *B. tabaci* as a virus vector in mixed cropping systems of South East Asia (led by AVRDC); (5) *Trialeurodes vaporariorum* as a direct pest in the tropical highlands of Latin America (led by CIAT); and (6) whiteflies as direct pests on cassava in South America (led by CIAT). The Tropical Whitefly IPM website (www.tropicalwhiteflyipmproject.cgiar.org) provides information on project activities, donors, and partners, and technical information useful to whitefly and geminivirus researchers.

AVRDC was a collaborator in this project from its start until December 2003. It comprised two phases, Phase I (1997–2001) "Sustainable Integrated Management of Whiteflies as Pests and Vectors of Plant Viruses in Asia" (target countries: Bangladesh, Indonesia, Malaysia, Nepal, Sri Lanka, Thailand, The Philippines, Vietnam) and Phase II (2001–2003) "Identification of Components for Inclusion in a Sustainable IPM Strategy for Control of Whitefly-transmitted Geminiviruses in Tomato" (target country: Vietnam). Both phases were funded by the Australian Centre for International Agricultural Research (ACIAR) project number CS2/98/079), with AVRDC, CSIRO, and the University of Western Sydney (UWS) of Australia as the main research providers.

Objectives and research providers in Phase I were:

- Identification of *B. tabaci* biotypes in Asia (CSIRO and NARS).
- Identification of the major host plants of the different biotypes of *B. tabaci* in Asia (NARS, CSIRO).
- Identification of key natural enemies of *B. tabaci* in Asia (CSIRO and NARS).
- Determination of plant hosts affected by geminiviruses and identification of geminiviruses in major crops and development of protocols for detecting viruses in plants (AVRDC, CSIRO, NARS).

Objectives in Phase II were:

- Identification of tomato genotypes resistant to geminiviruses which could be directly released or used in tomato improvement programs (AVRDC, NARS).
- Identification of specific geminiviruses infecting tomato in Vietnam and determination of reactions of resistance sources to specific geminiviruses (AVRDC, NARS).
- Assessment of petroleum spray oil (PSO) in the reduction or delay of geminivirus infection (UWS, NARS).

The project outputs can be accessed via www.aciar.gov.au.

7. PRIVATE SECTOR COLLABORATION

Most of the countries who were partners in the SAVERNET and AVNET are also members of APSA (Asia Pacific Seed Association). Impressed by AVRDC's achievements in breeding tropical tomato for T(Y)LCV resistance

and its record on the molecular characterization of about 27 geminiviruses affecting tomato in South and Southeast Asia, APSA entered into a joint partnership with AVRDC in 2003 on the development of PCR-based molecular markers for the *Ty-2* resistance gene from *S. habrochaites* according to RFLP markers previously developed at AVRDC (1999, 2000a; Hanson et al., 2000). Following the timely delivery of the proposed output, APSA is now contributing to AVRDC's core budget and has become an important stakeholder in our research and development activities that encompass not only tomato but other vegetables, such as *Brassica* spp., *Capsicum* spp, eggplant, and recently also cucurbits. APSA members consider geminiviruses as important production problems of tomato, chili, and cucurbits (AVRDC, 2004–2006).

8. OTHER COLLABORATIVE PROJECTS

AVRDC collaborated in a number of bilateral and regional projects that involved basic research such as the molecular characterization and diversity of geminiviruses, the development of molecular marker techniques, breeding for resistance, vector characterization and management, and postharvest issues (Table 3).

New linkages are continuously being formed as AVRDC has developed into the World Vegetable Center (AVRDC, 2003) and new collaborations, projects, and networks targeting whiteflies and geminiviruses on vegetable crops are emerging, particularly in Central Asia, sub-Saharan Africa, and Central America. The IPM CRSP and the ABSP-II projects in which AVRDC researchers are involved are just two such projects.

In many of these new target countries and regions such as Central Asia and the Caucasus countries the presence T(Y)LCV has already been reported (Czosnek & Laterrot, 1997, Czosnek et al., 1990). AVRDC will make an impact by providing TYLCV tolerant lines for testing and further improvement, improved diagnostics for geminivirus identification and characterization, disease management practices, and training in various aspects of TYLCV research.

9. MAJOR FINDINGS ACCOMPLISHED THROUGH THE NETWORKS AND OTHER COLLABORATIVE PROJECTS

(1) Research on the molecular diversity of tomato begomoviruses has resulted in expanding the knowledge base on tomato begomoviruses in Asia. Following are some of the key findings.

   (a) Information on the host range of certain tomato geminiviruses (Green et al., 1987, 2005).
   (b) Bipartite begomoviruses are present in South India (AVRDC, 2002) in addition to monopartite ones, and monopartite begomoviruses are present in North India (Shih et al., 2003a), in addition to bipartite ones.

(c) More than one begomovirus may be present in individual tomato plants (AVRDC, 2002, Green et al., 2005).

(d) New distinct geminiviruses that were <88% similar in their DNA-A to those previously described were found on tomato in Bangladesh (Genbank Acc. AF 188481) (Shih et al., 1998a, Green et al., 2001), India (Shih et al., 2003a, Chowda Reddy et al., 2005), Indonesia (Genbank Acc. AF 189018) (Tsai et al., 2006), Laos (Genbank Acc. AF 195782) (AVRDC, 2002; Green et al., 2001), Pakistan (Genbank Acc. DQ 116884), Malaysia (Shih et al., 1998b; Green et al., 2001), the Philippines (Genbank Acc. AF 136222) (Shih et al., 1997), Sri Lanka (Genbank Acc. AF 274349), Taiwan (Genbank Acc. U 88692) (Chiang et al., 1992), Tanzania (Genbank Acc. DQ519575) (Shih et al., 2006c), Thailand (Genbank Acc. AF 511529) (Green et al., 2003), Uganda (Genbank Acc. DQ127170) (Shih et al., 2006a), and Vietnam (Genbank Acc. AF 264063) (Green et al., 2001).

(e) Information on the geographic distribution of tomato geminiviruses in Asia and Africa.

- The bipartite tomato geminivirus from New Delhi, North India was also detected in Karnataka and Maharashtra, South India (AVRDC, 2002).
- A strain of the ToLCV from Gujarat, India (Genbank Acc. AF413671) was detected on tomato in Nepal (Shih et al., 2003a).
- A closely related strain (96% sequence homology) of the bipartite *Tomato leaf curl New Delhi virus* (Genbank Acc. U 15015) was found in Pakistan (Genbank Accs. AF 448058, AF 448059) (Khalid et al., 2001; Shih et al., 2003b).
- The tomato begomovirus from Myanmar (Genbank Acc. AF 206674) is a closely related strain (94% sequence homology) of the bipartite *Tomato yellow leaf curl Thailand virus* (TYLCTHV) (Green et al., 2003).
- One of the two begomoviruses identified so far from Vietnam, is a strain of the *Tomato yellow leaf curl Kanchanaburi virus* (Genbank Acc. AF 511529) from Thailand (Green et al., 2005).
- A strain of the tomato geminivirus from Mali is present in Ethiopia (Genbank Acc. DQ 358913) (Shih et al., 2006b).

(2) Several of the networks and projects are listed in Table 4. The major findings are:

(a) The presence of the nonindigenous *B. tabaci* biotype B was found associated with a severe ToLCVD epidemic in the Kolar district in Kolar, India (Banks et al., 2001). This aggressive biotype was also identified in Gujarat, India, more than 1,500 km away.

(b) The whitefly vectors in Asia were found to be highly diverse and to belong to several genetic groups. Using both mitochondrial CO1 and ribosomal ITS1 it was found that they belong to the Asia, Australia, and Bali genetic groups and to an unresolved group of Asian *B. tabaci* (De Barro et al., 2005). However, attempting to assign biotype status was found to be a

Table 4. AVRDC's involvement in bilateral regional and global projects addressing various aspects of tomato leaf curl disease

Project title	Duration	Cooperators	Countries of implementation	Project leader	Funding agency
Strengthening virus research at AVRDC: a) Virus diseases of peppers, tomato, and bulb alliums (onion, shallot, garlic) b) Support of network activities in Southern Africa and South Asia concerning vegetable viruses	5/87–8/94	BBA, Germany	Taiwan, NARS in South and Southeast Asia	Green, S.K. AVRDC	BMZ/GTZ, Germany
Strengthening virus research at AVRDC: a) Virus diseases of peppers, tomato, and bulb alliums (onion, shallot, garlic) b) Support of network activities in Southern Africa and South Asia concerning vegetable viruses	5/87–8/94	BBA, Germany	Taiwan, NARS in South and Southeast Asia	Green, S.K. AVRDC	BMZ/GTZ, Germany
Methodology development of multiple virus resistance in tropical tomato using molecular markers	7/93–6/97	J. Cho, U. Hawaii, USA, S. Tanksley, Cornell U. USA	Taiwan, USA	Green, S.K. AVRDC	COA/OICD, Taiwan
Sustainable management of tomato leaf curl viruses and *B. tabaci* on tomato	1/99–12/01	CSIRO, Australia	Australia, Nepal, India Bangladesh, Vietnam, Sri Lanka, Thailand, Indonesia, Philippines	DeBarro, P. CSIRO	ACIAR, Australia
Tropical Whitefly Project Phase I, Asia component					
Sustainable management and molecular characterisation of *B. tabaci* and ToLCV on tomato	1999–2002	J. Colvin, NRI, UK V. Muniyappa, UASB, India P. Hanson, AVRDC	India	Colvin, J., NRI, UK	Crop Protection Programme, UK (CPP) (A0870) DFID/NRI DFID No. R7460

Sustainable management of *B. tabaci* and tomato leaf curl virus (ToLCV) on tomato	1996–1999	J. Colvin, NRI, UK V. Muniyappa, UASB, India P. Hanson, AVRDC S.K. Green, AVRDC	India	Colvin, J., NRI, UK	Crop Protection Programme (CPP) (A0523) DFID/NRI DFID No. R6627
Tomato leaf curl viruses and *B. tabaci* on tomato	1999–2002	J. Colvin, NRI, UK V. Muniyappa, UASB, India G.B. Valand, AAU, India P. Hanson, AVRDC S.K. Green, AVRDC	India	Hanson, P.	DFID, UK Competitive Research Facility (C1171) DFID No. R7257
Tropical Whitefly IPM Project, Phase II: Sustainable integrated management of whiteflies as pests and vectors of plant viruses in Asia	1/02–12/03	O. Nicetic, U Western Sydney, Australia Nguyen Trong Mai, Research Institute of Fruit and Vegetables (RIFAV), Hanoi Tran Van Hai, University of Cantho, Cantho, Vietnam	Vietnam	Hanson, P. AVRDC	ACIAR, Australia
Tropical Whitefly IPM project Phase III: Whiteflies as vector of viruses in mixed cropping systems in India and Southeast Asia.	4/05–3/08	J. Colvin, NRI, UK V. Muniyappa, UASB, India P. Hanson, AVRDC N.C. Mandal, Visva Bharati University, India	India	Hanson, P. AVRDC	DFID, UK

(continued)

Table 4. (continued)

Project title	Duration	Cooperators	Countries of implementation	Project leader	Funding agency
Control of geminivirus diseases of cotton and tomato in Pakistan and Australia	1/01–12/04	A. Rezaian, CSIRO, Australia Yusifzafar, NIBGE, Pakistan S.K. Green, AVRDC	Australia, Pakistan	Rezaian, A. CSIRO Plant Industry	ACIAR, Australia
On-farm evaluation of tomato leaf curl control measures and monitoring geminivirus diversity in Taiwan	1/03–12/04	J.C. Ko, National Taiwan University	Taiwan	Green, S.K. AVRDC	COA/BAPHIQ, Taiwan
Development of tomato lines combining conventionally bred virus resistance with transgenic resistance	4/04–3/07	Institute of Plant Breeding (IPB), U. of the Philippines at Los Baños Agency for Agricultural Research & Development (AARD) Indonesian Agricultural Biotechnology and Genetic Resources Research Institute	Philippines, Indonesia	Liu, C.A. AVRDC	ABSP II/USAID
Breeding for begomovirus resistance in Guatemala and introduction of marker-assisted selection methods into San Carlos	2002–2005	Mejia, L., San Carlos University, Guatemala City, Guatemala. P. Hanson, J.-F. Wang, E. Graham, and S.K. Green, AVDRC, Taiwan Maxwell, D.P. & C. Allen, University of Wisconsin, USA Scott, J.W. University of Florida, USA	Guatemala	Czosnek, H., The Hebrew University of Jerusalem, Israel	USAID

Development of a PCR-based screening protocol for detection of a gene conditioning geminivirus tolerance in tomato	11/03–present	E. Graham, R. de la Peña, AVRDC	Taiwan	Hughes, J. AVRDC	13 companies of the Asia and Pacific Seed Association (APSA)
Application of molecular markers to broaden the genetic base of tomato for improved tropical adaptation and durable disease resistance	3/04–2/07	J. Chunwongse and Krung Sitadhani, Kasetsart University, Thailand A.T. Sadashiva, Indian Institute Horticultural Research, India (IIHR)	Taiwan, India, Thailand, Germany	Hanson, P. AVRDC	BMZ/GTZ Germany
Sustainable impact generation and technology promotion to manage *B. tabaci* and tomato leaf curl virus disease amongst the poorest South Indian tomato growers	2005–2006	J. Colvin, NRI, UK V. Muniyappa, UASB, India P. Hanson, AVRDC	India	Colvin, J., NRI, UK.	Crop Protection Programme, UK (CPP) (A1162) DFID/NRI DFID No. R8425
Promotion and impact assessment of tomato leaf curl virus disease resistant tomatoes: Phase III	2003–2005	J. Colvin, NRI, UK V. Muniyappa, UASB, India P. Hanson, AVRDC	India	Colvin, J., NRI, UK.	Crop Protection Programme, UK (CPP) (A1077) DFID/NRI DFID No. R8247
Insect transmitted viruses in Central America, the Caribbean and sub-Saharan Africa	2006–2009	NARS and IARCs in the target regions: M. Konton, INERA, Furkina-Faso W. Leke, IRAD, Cameroon J. Brown, U. of Arizona, USA C.M. Deom, U. of Georgia, USA R. Gilbertson, UC Davis	Burkina-Faso, Cameroon, Dominican Republic, Guatemala, Honduras, Jamaica, Mali, Tanzania	Tolin, S. Virginia Tech, USA	IPM-CRSP

(continued)

Table 4. (continued)

Project title	Duration	Cooperators	Countries of implementation	Project leader	Funding agency
		M. Rivera, FHIA Zamorano (Honduras) S. McDonald, MINAG, Jamaica S.K. Green, AVRDC V.J. Legg, IITA			

AAU: Anand Agricultural University, Gujarat, India.
ABSP: The Agricultural Biotechnology Support Project, USA
ACIAR: Australian Centre for International Agricultural Research
APSA: the Asia and Pacific Seed Association
BAPHIQ: Bureau of Animal and Plant Health Inspection & Quarantine, Taiwan, R.O.C.
BBA: The Federal Biological Research Centre for Agriculture and Forestry, Germany
BMZ: Federal Ministry of Economic Cooperation, Germany
COA: Council of Agriculture, Executive Yuan, Taiwan, R.O.C.
CPP: Crop Protection Programme, Natural Resources International, UK.
CSIRO: Commonwealth Scientific and Industrial Research Organization, Australia
DFID: Department for International Development, UK
DFID/NRI: DFID's research program for pre-protection managed by Natural Resources International
GTZ: Gesellschaft für Technische Zusammenarbeit (German Agency for Technical Cooperation), Germany
NARS: National Agricultural Research Systems
NIBGE: National Institute for Biotechnology and Genetic Engineering, Pakistan
NRI: National Resources Institute, University of Greenwich, UK
OICD: Office of International Cooperation & Development, USDA, USA
UASB: University of Agricultural Sciences, Bangalore, India
USAID: United States Agency for International Development, USA
IITA: International Institute of Tropical Agriculture, Nigeria
INERA: Institut de Environnement et de Recherches Agricoles
IRAD: Institut de Recherche Agricole pour le Developpement, Cameroon
FHIA: Honduran Agricultural Research Foundation
MINAG: Ministerio de Agricultura, Jamaica
UWI: University of the West Indies
IDIAF: Instituto Dominicano de Investigaciones Agropecuarias y Forestales, Dominican Republic

largely meaningless exercise as the genetic bounds of most biotypes and their defining biological characteristics have not been defined. Using microsatellites, De Barro (2005) further showed that the genetic structure revealed by both mitochondrial CO1 and ribosomal ITS1 underestimated the underlying genetic structure. The *B. tabaci* of the Asia-Pacific region could be divided into six major genetic groups with little or no gene flow between them. Further, four of the six groups could be further subdivided into two groups again with little gene flow between them.

(3) Knowledge on the epidemiology and on improved or novel sustainable management practices for the reduction of whiteflies and leaf curl virus disease incidence has been generated especially in collaboration with NRI (UK) (Muniyappa et al., 1998; Colvin et al., 1999, 2002). The purpose of NRI's DFID Crop Protection Program funded work was to develop and promote sustainable and cost-effective management practices for two principal researchable constraints, *B. tabaci* and ToLCV, thereby improving both the quantity and quality of Indian tomato production. At the beginning of the project, a socio-economic survey was carried out to assess farmers' perceptions of the problem and 100% of farmers reported ToLCV to be their most serious problem. Research activities involved field experiments into the effect of beneficial insect augmentation, mycopesticides, and ToLCV-resistant varieties on the rate of spread of tomato yellow leaf curl virus disease (ToLCVD) into the tomato crop. The most important findings were as follows.
   (a) Epidemiological data and incorporation into a mathematical model that was used to assess and identify potential novel control techniques. Those that increased and decreased, respectively, the vector emigration and immigration rate had the greatest potential for reducing the spread of the disease (Holt et al., 1999).
   (b) PSO nC 24 applied weekly at concentrations of 1% or 2% reduced geminivirus incidence to 8–18% compared to 25% for the water control. PSO application concentrations of 1% or 2% also increase marketable fruit yields by 50–92% compared to the water control. PSO technology combined with resistant varieties showed excellent potential as a component in an overall geminivirus IPM strategy and a safe substitute for insecticides; furthermore, the large yield increases provide strong evidence that the PSO applications extend additional benefits to tomato crops beyond just geminivirus control.
   (c) A whitefly barrier net was designed for post-transplanted tomato to reduce and delay whitefly immigration into tomato crops in South India. Virus incidence in a susceptible tomato crop surrounded by the net was reduced to 23–50% compared to 100% in the control and resulted in a yield increase of approximately 400%. The benefit to cost ratio for adopting the barrier net was approximately 4.2 to 1 (Hanson, 2002).

(4) Valuable information has been obtained on lines/varieties resistant to the diverse begomoviruses and their strains in different geographic locations

(Table 1) (AVRDC, 1999, 2000a; Muniyappa et al., 2002; Maruthi et al., 2003). This has helped to develop TYLCV-resistant tomato lines at AVRDC, the public sector, the NARS involved in these networks and collaborative projects, and also the private sector (Hanson et al., 2003).

The most successful example is the development of three TYLCV resistant varieties for South India. Three tomato leaf curl virus disease (ToLCVD)-resistant tomato varieties, TLB111, TLB130, and TLB182 were bred at AVRDC and evaluated extensively in on-station and participatory on-farm trials in Karnataka. These varieties performed extremely well and the yield of TLB 182, for instance, was not reduced significantly when 14-day-old seedlings were whitefly-inoculated rigorously with ToLCV. After this, the three ToLCVD-resistant tomato varieties, named Vybhav, Nandi, and Sankranthi, were released successfully and were notified in the Gazette of India for use throughout India. As part of this process, the National Bureau of Plant Genetic Resources, New Delhi, conserved the varieties' seed (Colvin & Muniyappa, 2005).

Demonstration field days were held at the University of Agricultural Sciences, Bangalore, and successful negotiations took place with commercial vegetable seed producers. This resulted in ten of them taking up the rights, on a nonexclusive basis, to multiply and distribute the varieties and/or use them as parental material to develop ToLCVD-resistant hybrids. The latter process is still underway and several of the seed companies have produced ToLCVD-resistant hybrid tomatoes using these lines in the parental crosses.

Data was collected throughout the project on the performance and end-user acceptability of the project's technologies and management recommendations. In particular, data on the horticultural acceptability of the tomato lines to farmers was used in the selection and breeding program. This resulted in the production of tomato varieties that have characteristics desired by farmers and consumers. Of all the options for reduction of TYLCV, the ToLCV-resistant tomato lines showed the most promise in terms of delivering developmental impact and this was the output for which there was the greatest demand from tomato farmers. Throughout most of India, where severe ToLCVD is present, widespread uptake of the project's outputs has the potential to result in a greater than 100% increase in yields compared to susceptible varieties and benefit to cost ratios as great as 6.6 to 1 (Colvin & Muniyappa, 2005). The project's website can be viewed at: http://www.tomatoleafcurlandwhitefly.org/

10. IMPACT AND ECONOMIC INDICATORS OF THE AVRDC NETWORKS

National and international collaborative projects and networks have made significant progress towards improved characterization of the whitefly and begomovirus problem, development of management methods including resistant lines, transfer of technologies and information and manpower development and information.

Clearly these collaborative projects and networks have demonstrated operational efficiency by optimizing financial, physical and human resources, open discussions when the networks were created, identification of priority research areas and by focused efforts that increased return for each invested research dollar compared to numerous small disconnected projects utilizing diverse approaches. The annual budget for AVRDC-the World Vegetable Center is just over US$ 10 million. The most recent External Program and Management Review reported that each dollar the Center spent is returning more than US$5 dollars in increased income streams for vegetable producers and consumers (AVRDC, 2000b). In September 2000 the ADB commissioned an independent evaluation of the networks supported by ADB's Agricultural and Natural Resources Research (ANRR) using external consultants. According to ADB, the vegetable networks coordinated by AVRDC (AVNET-I and II and SAVERNET-I and II) over the period of 9 years have generated an annual economic surplus in excess of US$1.8 billion which represents an internal rate of return of 91% (Asian Development Bank, 2005). TYLCV resistance in tomato is one of the major contributing factors for the above returns. Among the 122 AVRDC tomato cultivars released in 35 countries around the world so far, 14 are tolerant/resistant to the tomato (yellow) leaf curl disease. Some of these lines have already been released (Muniyappa et al., 2002; Colvin & Muniyappa, 2005)) (Table 3). The credit primarily goes to the collaborative networking approach adopted by AVRDC and also to the many bilateral and regional projects listed in Table 3.

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