STUDY OF THE PATHOSYSTEM

BEGOMOVIRUS/BEMISIA TABACI/ TOMATO

ON THE SOUTH WEST ISLANDS OF THE

INDIAN OCEAN

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STUDY OF THE PATHOSYSTEM BEGOMOVIRUS/BEMISIA TABACI/ TOMATO ON THE SOUTH WEST ISLANDS OF THE INDIAN OCEAN

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Scientific adventure is a very hard path that nobody can rob from you

Alain Delatte

To my parents and my brother

CONTENTS

CHAPTER 1

Introduction

Tomato yellow leaf curl virus (TYLCV) belongs to the *Begomovirus* genus within the *Geminiviridae* family (Fig 1-1 A). Begomoviruses are exclusively transmitted by *Bemisia tabaci* (Fig 1-1 B). Tomato yellow leaf curl diseases (TYLCD) are associated to a complex of viral species, including TYLCV, and all inducing rather similar symptoms on tomato (*Lycopersicon esculentum*) plants. The first reports on TYLCV infestations in tomato were from Israel and other countries in the Middle East in the 1930s and since the 1960s the virus has further emerged (Cohen & Antignus, 1994). Since, TYLCV has been reported all over the tropics, subtropics, and the mediterranean (Czosnek & Laterrot, 1997; Jones, 2003) and has recently also been found in areas belonging to the temperate climate zone (Moriones & NavasCastillo, 2000). New introductions of TYLCV have recently been reported (Peterschmitt *et al.*, 1999b; Delatte *et al.*, 2005a) on La Réunion, a small island situated in the south west part of the Indian Ocean. Visual symptoms of the disease consist of curling, yellowing and reduction of the leaflets area together with stunting and flower abortion (Fig1-1 C). When tomato seedlings are infected, production losses can reach 100%. On La Réunion up to 85 % of yield losses have been registered since the first reports of TYLCV in 1997 (Reynaud *et al.*, 2003). To improve the worldwide critical situation, numerous studies have been conducted to find TYLCV resistance genes in tomato and its relatives, to be used in breeding programmes. However, up to now only few virus resistance genes have been identified while so far vector (whitefly) resistance traits have not at all been encountered. Hence, to date TYLCV and its vector have remained a great problem in tomato cultures.

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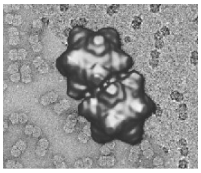

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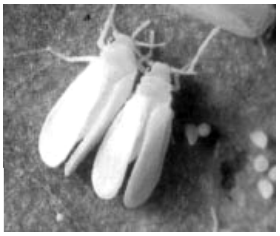

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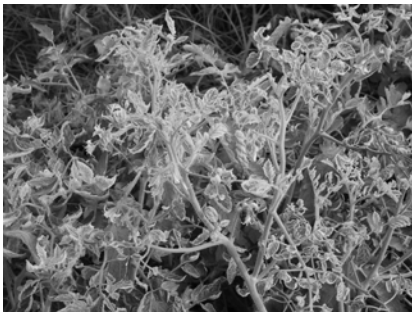

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Fig. 1-1 A: Representation of the TYLCV geminate particles, **B**: *Bemisia tabaci* male (right) and female (left) with eggs on a cabbage leaf, **C**: B: Tomato yellow leaf curl virus symptoms.

GEMINIVIRIDAE

The family Geminiviridae

Geminiviruses are characterised by circular single stranded DNA (ssDNA) genomes encapsidated in twinned quasi isometric particles of about 18 x 30 nm. The *Geminiviridae* family has been divided into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (van Regenmortel *et al.*, 1999).

Cicadulina mbila. Copyright J. Innes Inst.

Circulifer tenellus. Bar represents 1 mm. (Courtesy J. University of California)

Micrutalis malleifera

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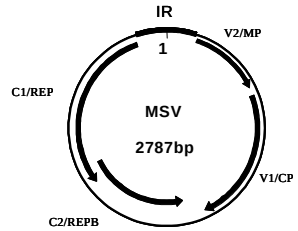
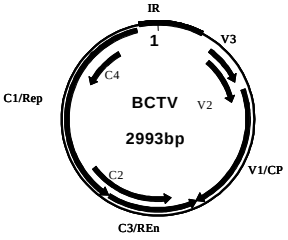
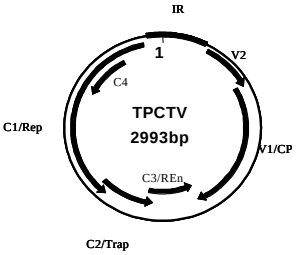
Bemisia tabaci
Ryckwaert, CIRAD



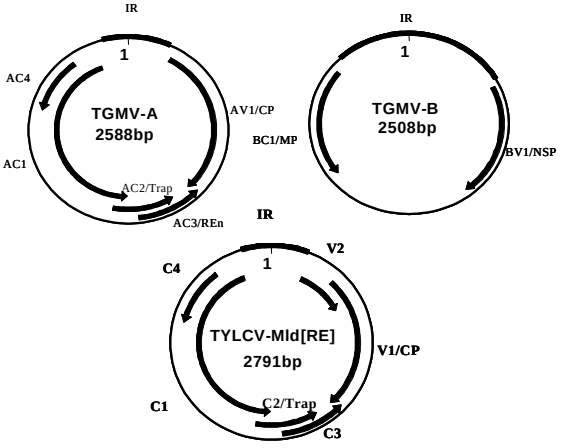
MASTREVIRUS



CURTOVIRUS



TOPOCUVIRUS



Bipartite

BEGOMOVIRUS

Monopartite

BEGOMOVIRUS

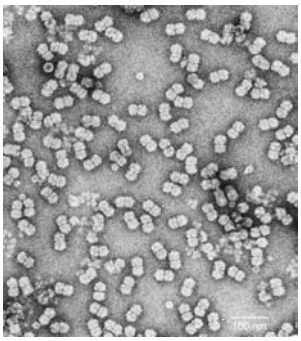
Fig.1-2. Genome representation of Geminiviridae and pictures of their major insect vector species.

Mastrevirus (type species *Maize streak virus*; acronym MSV) have monopartite genomes (2.6-2.8 kilobases) encoding 3 or possibly 4 proteins (ORF: V1, V2 and C1, C2). They are transmitted by leafhoppers (*Hemiptera: Cicadellidae*) in a persistent circulative, and non-propagative manner (Fig. 1-2). They are most often found on monocotyledonous plants, especially on maize. Economically MSV causes the most devastating disease in maize in sub Saharan Africa resulting in up to 100% yield losses (Thottappilly, 1992). MSV is endemic in Africa where wild grasses are its natural hosts. The virus must have moved to maize when the crop was introduced in Africa and neighbouring regions in the 16th century, as maize is also a congenial feeding host of *Cicadulina* spp., the vector of MSV (Varma & Malathi, 2003).

Curtovirus (type species *Beet curly top virus*; acronym BCTV) have monopartite genomes (2.9-3 kb) and are phloem limited. The DNA codes for 6 or 7 proteins depending on the species. They are transmitted by leafhoppers (Hemiptera: *Cicadellidae*) in a circulative, non-propagative manner (Fig. 1-2). The leaves of affected plants develop vein clearing, curling, general malformations and become leathery and brittle; the plants get stunted, turn yellow, and the phloem show necrosis; early infection usually results in early death (Brunt *et al.*, 1996). They infect only dicotyledonous plants, especially sugar beet, tomato and melon. They are found mostly in India, America and Mediterranean countries.

The genus *Topocuvirus* has been created with a former curtovirus, *Tomato pseudo-curly top virus* as type species, on the ground of a different family of vector, treehopper (Hemiptera: *Micrutalis malleifera*) (Fowler) instead of leafhopper (Fig. 1-2), and on the fact that the sole representative has evolved by interspecies recombination between unknown viruses belonging to different genera (Briddon *et al.*, 1996). It was first reported in Florida (Stoner & Hogan, 1950). The TPCTV genome (2861 nts long) encodes 6 proteins (Briddon *et al.*, 1996) which are close to the curtoviruses.

Begomovirus (type species *Bean golden mosaic virus*) is the only genus of the family to be either bipartite with virus genes resident on two different circular ssDNA molecules (DNA A, DNA B) each of about 2.6-2.8 kb, or monopartite with all genes resident on one (DNA A-like) ssDNA of about 2.8 kb (Fig. 1-2). The twinned particles have diameter of 18-20nm, 30 nm long (Fig. 1-3), like most of the geminiviruses.



The begomoviruses are all transmitted by the whitefly *B. tabaci* in a circulative manner and infecting dicotyledonous plants. The work presented in this thesis was focused on monopartite begomoviruses, and therefore an extended review on this genus is provided below.

Fig. 1-3. Electron micrograph of purified, negatively stained TYLCV particles.

Molecular biology of begomoviruses

Genomes of TYLCD-inducing begomovirus species can be monopartite (Mediterranean, Central and North America) or bipartite (Thailand) (Fauquet *et al.*, 2003; Fauquet & Stanley, 2003). TYLCV, as also found on La Réunion, is an example of the larger group of monopartite begomoviruses.

The TYLCV genome encodes 6 partially overlapping open reading frames (ORFs) organised bi-directionally (V1, V2, C1, C2, C3, C4) in two transcriptional units that are separated by an intergenic region (Rybicki *et al.*, 2000). Encoded proteins are:

- The capsid protein (CP): V1 ORF, for encapsidation of the genome and also essential for viral spread (Briddon *et al.*, 1989). The CP and Pre-CP or V2 are both also essential for local and systemic movement, which are supposed to shuttle the viral genome into and out of the host nucleus (Gafni & Epel, 2002). The V1 properties also include binding to and protecting viral ssDNA, self-binding and nuclear membrane. It is also needed for transmission by the whitefly vector. It is necessary for virus transport through the whitefly gut wall into the haemocele, where it binds a GroEL analogue, produced by a bacterial endosymbiont, in a way that may protect virus particles from degradation (Morin *et al.*, 2000).
- The replication-associated protein (Rep): C1 ORF is the only known protein to be involved in viral replication (Desbiez *et al.*, 1995).
- The transcriptional activator protein: C2 ORF is involved in the activation of the transcription from the coat protein promoter. It was found to be localised in the nucleus and contributes to viral pathogenicity (van Wezel *et al.*, 2001).
- The replication enhancer protein: C3 ORF interacts with C1 protein and enhances viral DNA accumulation (HanleyBowdoin *et al.*, 2000).
- The C4 is an important symptom determinant and involved in initiating cell division (Latham *et al.*, 1997; Krake *et al.*, 1998). The C4 may interact with the Rep/C1 ORF and counter the plant defence mechanism (van Wezel *et al.*, 2002).
- Proteins products encoded by the V2 pre-coat (or MP) and C4 ORFs have also been recently hypothesized to be involved in the cell to cell movement of viral DNA (Rojas *et al.*, 2001).

The DNA A of the bipartite species is similar in arrangement to the genome of the monopartite begomoviruses. For New World bipartite begomoviruses, the DNA component lacks the V2 gene. The DNA B component encodes BV1 and BC1, proteins that are essential for cell to cell and systemic movement (Noueiry *et al.*, 1994; Sanderfoot *et al.*, 1996), and can influence host range (Von Arnim, 1992; Ingham *et al.*, 1995). Although not directly involved in interaction with the whitefly vector, DNA B sequences affect the efficiency of virus acquisition by the insect by determining the location of begomoviruses in plant tissues (Liu *et al.*, 1997).

Replication of begomoviruses occurs in the nuclei of the infected cells. Indeed, prior to infecting a plant cell the virus has to cross several barriers such as the cell wall and the plasma membrane, and eventually the DNA should enter the nucleus. This targeting to the host nucleus is supposed to involve host (possibly nuclear shuttle proteins, NSP's, and cytoskeletal elements) and viral proteins, supposedly the coat protein CP (Rojas *et al.*, 2001). Since geminiviruses employ a rolling circle type of genome replication, concatameric double stranded DNA (dsDNA) intermediates are formed, which later are converted to genome sized circular DNA fragments. More precisely, the incoming geminivirus ssDNA is converted by host enzymes into a dsDNA, which serve as a template for the transcription of viral genes. The dsDNA intermediates are transcribed in the nuclei of infected plant cells via a rolling-circle mechanism (Stenger *et al.*, 1991; HanleyBowdoin *et al.*, 2000). As both the replication and the transcription occurs in the nucleus, the import of the viral DNA and /or virions into and out of the host plant cell nucleus is essential for the successful completion of their life cycle. However, a critical step in the begomoviral infection process, i.e. nuclear targeting of the viral genome involving the nuclear pore complex (NPC), is still unknown (Gafni, 2003). When the replication is over, the infectious form of the virus spreads to the adjacent cells via the plasmodesmata (pd). Due to the size of the viral nucleoprotein complex (over 500 kDa) it is impossible to move by free diffusion from the nucleus through the lattice like structure of the cytoplasm to the pd. Indeed in such transport the cytoskeletal elements are expected to be involved, but no data are yet available to confirm this prediction (Gafni & Epel, 2002).

Geographical repartition of TYLCD

Several hypotheses have been suggested for the origin and emergence of monopartite begomoviruses such as TYLCV. Analyses of all known sequences of TYLCV, suggest that the isolates from Israel and Iran may have chimeric genomes that arose by natural recombination between ancestors of the viruses causing TYLCD (tomato yellow leaf curl disease) and ToLCD (tomato leaf curl disease), both diseases originating from the Middle East countries (NavasCastillo *et al.*, 2000). Begomoviruses causing TYLCD are more closely related to each other whereas those causing ToLCD are more diverse (Varma & Malathi, 2003).

Nowadays, despite many similarities in viral properties and in the disease symptoms they induce, the TYLCD can be divided roughly by geographical origin into a number of species (Howarth & Vandemark, 1989; Fauquet & Mayo, 1999; Fauquet *et al.*, 2003; Fauquet & Stanley, 2003). TYLCD was first observed in Israel en 1939-1940 and the appearance of the disease coincided with an increase in the whitefly population. The causal begomovirus was identified in the 90's as TYLCV (Cohen & Antignus, 1994) (Pico *et al.*, 1996). In Africa, TYLCD was first described in the Sudan (Yassin & Nour, 1965; Yassin, 1975), next in Nigeria, but the causal agent was identified as TYLCV only in 1997 (Czosnek & Laterrot, 1997). Since, TYLCD has also been reported from many other African countries: in Cameroon (Czosnek & Laterrot, 1997), Burkina Faso (Konaté *et al.*, 1995), Mali and Senegal (D'Hondt & Russo, 1985), and Egypt (Czosnek *et al.*, 1990). The occurrence of TYLCD was reported from the south of Casablanca in 1996-97 and by 1998 it had

spread to all the tomato growing areas of Morocco (Peterschmitt *et al.*, 1999a; Jebbour & Abaha, 2002).
In the late 1980s, the first reports were made on the occurrence of TYLCD in the Americas and Europe (Accotto *et al.*, 2000). The disease has also spread to the Caribbean islands (MacGlashan *et al.*, 1994; Nakhla *et al.*, 1994; Polston *et al.*, 1994; Ramos *et al.*, 1996; Sinisterra & Patte, 2000; Bird *et al.*, 2001) reaching the French West Indies in 2001 (Urbino *et al.*, 2003). In 1997 the virus has emerged in Florida and one year later (April 1998) up to 100% incidence was recorded in tomato fields (Polston *et al.*, 1999).

Several reasons for the worldwide spread of TYLCD had been put forward. The infection reported in the early 1990's in the Caribbean islands (Dominican Republic) was supposed to be due to an introduction of infected asymptomatic tomato transplants from the Eastern Mediterranean region (Brown & Bird, 1992). For most of the other regions the introduction of TYLCD is supposed to be due to importations of ornamental plants (Polston *et al.*, 1999).

TYLCV host range

Of the several related begomovirus species causing TYLCD, the virus known as TYLCV is by far the most important and the most widely spread. So far, laboratory inoculation by viruliferous whiteflies and field sampling surveys have indicated a potentially wide host range of this virus, spanning 13 plant species in 6 botanical families (Cohen & Antignus, 1994). Host plant species include: *Asclepiadaceae, Asteraceae, Fabaceae, Malvaceae, Solanaceae, Gentianaceae, Cleomaceae, Cucurbitaceae* and *Apiaceae* (Mansour & Al-Musa, 1992; Kegler, 1994; Nakhla & Maxwell, 1998). Examples of host weeds and cultivated crop species are: *Datura stramonium, Hyoscyamus desertorum, L. esculentum, Nicotiana benthamiana, Nicotiana glutinosa, Phaseolus vulgaris, Capsicum annuum, Capsicum chinense, Solanum nigrum, Mercurialis ambigua, Eustoma grandiflorum, Calopogonium spp*. Additional plant species have been shown to be susceptible to TYLCV but do not exhibit any disease symptoms, such as the weeds species *Cleome viscose (Caparidaceae)* and *Croton lobatus (Euphorbiaceae)* (Salati *et al.*, 2002).

Interaction and recombination of begomoviruses

Mixed infection with two or more geminiviruses are common in tropical and subtropical areas (Torres-Pacheco *et al.*, 1996; Harrison, 1997; Sanz *et al.*, 2000; Pita *et al.*, 2001b; Ribeiro *et al.*, 2003). Virus interactions during such mixed infections could play an important role leading to the development of complex diseases (Fondong *et al.*, 2000; Chatchawankanphanich & Maxwell, 2002; Monci *et al.*, 2002). The type of interactions between co-infecting viruses can range from synergism where symptoms are stronger than those observed for the separate viruses (Kamei, 1969; Pruss *et al.*, 1997), to interference, where one virus affects the infection cycle of the second virus (Malinshenko *et al.*, 1989; Fedorkin, 2000; Mendez Lozano *et al.*, 2003).
Possible interactions include also events such as complementation (Berrie *et al.*, 2001) or recombination (Sanz *et al.*, 2000; Pita *et al.*, 2001a; Saunders *et al.*, 2001; Mendez Lozano *et al.*,

2003). An example of virus interactions as host dependent, and interference as antagonism was found between the *Pepper huasteco virus* and *Pepper golden mosaic virus*. Antagonism was found between the two viruses in pepper and in synergism when co-infected in *Nicotiana benthamiana* (Fondong *et al.*, 2000).

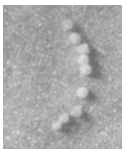
In addition to classical synergism between two geminiviruses new recombinants may arise, with more severe symptoms than induced by either parental virus. An example of this has occurred in Cameroon, where a new double recombinant has been formed between co-infecting *African cassava mosaic virus* isolates, developing more severe symptoms in the field compared to plants infected by either isolate alone (Fondong *et al.*, 2000). Another example of natural recombination of two begomoviruses has been observed with the *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and TYLCV in Spain, the resulting recombinant appearing better fit than the two original isolates (Monci *et al.*, 2002).

Recombination is considered to have a crucial role in virus evolution (Umaharan *et al.*, 1998), especially in geminivirus populations where it strongly contributes to its genetic diversification (Moffat, 1999; Sanz *et al.*, 1999; Sanz *et al.*, 2000; Kirthi *et al.*, 2002; Kirthi & Savithri, 2003). In begomoviruses, recombination is so frequent that it can occur at the strain (Fondong *et al.*, 2000), species (Briddon *et al.*, 1996; Martin *et al.*, 2001; Saunders *et al.*, 2002), genus (Klute *et al.*, 1996; Saunders & Stanley, 1999), and even family levels (Jones, 2003).

THE VECTOR: BEMISIA TABACI (Hemiptera: Aleyrodidae)

Introduction

The absence of historical records relating to indigenous faunae in some countries prevents a systematic study of the introgression of whiteflies into various (continental) areas.The first description of *Bemisia tabaci* or sweetpotato whitefly was in 1889 in Greece (Gennadius, 1889). It was classified in the order Hemiptera, family Aleyrodidae and subfamily Aleyrodinae, and is one of the 1160 aleyrodid species (Gill, 1992). The Indian subcontinent is believed to be the centre of origin of *B. tabaci* because of the numbers and diversity of natural enemies found in that region (Banks, 2001). However, the B biotype (see below, section "biotype") has only recently been recorded in that region (De Barro *et al.*, 2000). The origin of the B biotype might be the northeastern Africa-Middle East-Arabian peninsula region (Pico *et al.*, 1996; Frohlich *et al.*, 1999). Like most whiteflies, *B. tabaci* is arrhenotokous. Females can regulate the sex of their progeny by selective egg fertilisation. Fertilised females lay diploid and haploid eggs (Fig. 1-4) up to 300 per individual, this amount varying greatly, depending on the biotype. The former gives rise to females the latter to males. The unfertilised females only lay haploid eggs. The progeny sex ratio is affected by insect age. Young females lay more female producing eggs than older females (Berlinger, 1986).



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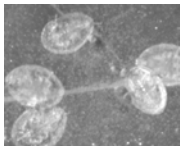

B

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Fig. 1-4. Eggs(A) and larvae (B) of *B. tabaci.*

Feeding Damage and vectoring of viruses

The whitefly *B. tabaci i*s recognised as an important pest on many crops. Three types of damage may be caused by *B. tabaci* (Berlinger, 1986; Pico *et al.*, 1996): i) Direct damage, ii) Indirect damage, and iii) Virus transmission.

Direct feeding damage is caused by the piercing and sucking sap from the foliage of plants. This feeding causes weakening and early wilting of the plant and reduces the plant growth rate and yield (Berlinger, 1986). It may also cause leaf chlorosis, leaf withering, premature dropping of leaves and plant death. Infestations of some whitefly nymphs are associated phytotoxicity disorder, such as the occurrence of irregular ripening of tomatoes and silverleaf of squash (Maynard, 1989; Yokomi *et al.*, 1990). In cole crops, white-stem streaking was also reported. Vein clearing in of foliage on poinsettia and a variety of other ornamentals and vegetables has been observed even when low-level colonisation by the B-biotype (see below) occurs (Brown *et al.*, 1995).

Indirect damage results by the accumulation of honeydew produced by the whiteflies. This honeydew serves as a substrate for the growth of black sooty mould on leaves and fruit. The mould reduces photosynthesis and decreases the yields and market value of the vegetables (Byrne & Bellows, 1991; Jones, 2003).

The third type of damage is caused by the whitefly property to vector various plant viruses. Plant viruses transmitted by whiteflies cause over 114 diseases of vegetable and fibre crops worldwide and 111 of those virus species are vectored by the species *B. tabaci* (Markham *et al.*, 1995; Jones, 2003). In the past decade, whitefly-transmitted plant viruses have increased in prevalence and distribution. The recent impact has been devastating with yield losses ranging from 20 to 100 percent, depending upon the crop, season, and prevalence of the whitefly. As a vector of viruses, *B. tabaci*, has a circulative mode of transmission (Cohen *et al.*, 1989), requiring an average of 6-12h prior to a transmission event (Fargette *et al.*, 1996). Whiteflies feed on the phloem, and in tomato TYLCV shows a phloem tropism in the stem (Byrne, 1990). The phloem tissues are located in the plant leaf in vascular bundles and are the pathway through which sugars and other solutes are rapidly transported throughout the plant, which increase rapidly the virus infection in all the plant parts (Caciagli *et al.*, 1995). In a recent study Morilla et al. (2004) showed that with *in situ* tissue hybridization TYLCSV and TYLCV DNAs are confined to the phloem in tomato or tobacco hosts, irrespective of whether they were inoculated individually or in combination. Indeed to be successfully transmitted by *B. tabaci*, TYLCV particles must be delivered in the phloem sieve tubes.

Females were proved to be more efficient vectors of TYLCV (six-fold) than males (Martin, 1987). Until 1998 TYLCV was not supposed to be transmissible to the progeny, only adults or larvae could acquire the virus. However in 1998 TYLCV-Mld was claimed to be transmitted through the egg for at least two generations (Ghanim *et al.*, 1998). It was also reported (Ghanim *et al.*, 2000) that TYLCV-Mld could be sexually transmitted among whiteflies, from viruliferous males to non viruliferous females, and vice versa. In another report (Bosco *et al.*, 2004) it was shown for the Israel strain of TYLCV that, neither viral DNA nor infectivity were associated with the progeny of viruliferous female whiteflies. In this report it was also demonstrated that only TYLCSV DNA could be transovarially inherited by progeny of whitefly, but not infectivity. These seemingly conflicting results may reflect that different TYLCV strains and whitefly biotypes may interact in very different ways.

Biotypes

The existence of distinct "biotypes" or "host races" of the whitefly *B. tabaci* was proposed in the 1950's after the discovery that morphologically indistinguishable populations of *B. tabaci* exhibited measurably different biological traits with respect to host range, host plant adaptability, and plant virus transmission capabilities (Coats *et al.*, 1994; Brown *et al.*, 1995). Extensive studies have been performed on the systematic of *B. tabaci* (Russell, 1957; Perring *et al.*, 1993a). Differentiation has been mostly based on morphological criteria among the species. Since the 1980's a marked increase of *B. tabaci* populations was observed, being also found on ornamental plants and plant species previously described as non-hosts.

This emergence has led to intensified studies on the systematics of *B. tabaci*. In the United States, a certain strain was found to be more polyphagous, producing silverleaf symptoms (Maynard, 1989; Yokomi *et al.*, 1990) on the genus *Cucurbita* and differed from biological parameters that characterised the initial biotype (denoted biotype A). It was also demonstrated that this newly described strain produces more honeydew, has a higher survival rate on more hosts and lais more eggs than the biotype A (Byrne, 1990). After analysing the honeydew the results showed no chemical differences between the new strain and the A biotype. The conclusions of this study proposed that this strain had access to more phloem sap and that this might be the major difference between the biotype A and the new strain. Then, numerous authors recognised even more differences between the two *B. tabaci* strains and even proposed to separate them as two species (Gill, 1992; Bartlett & Gawel, 1993; Costa *et al.*, 1993b; Perring *et al.*, 1993a; Bellows *et al.*, 1994), i.e. *B. tabaci* versus *Bemisia argentifolii*. This classification was again controverted by others (Campbell *et al.*, 1993) and as a result this strain remained at the biotype level, being denoted biotype B. More recently, studies have been performed using molecular techniques to differentiate *B. tabaci* populations, such as isoelectric focusing electrophoresis (Perring, 1992; Brown, 2000), AFLP (Cervera *et al.*, 2000), mitochondrial marker analysis (Brown & Frohlich, 1995; Frohlich *et al.*, 1999), RAPD-PCR (Gawel & Bartlett, 1993; Perring *et al.*, 1993b; De Barro & Driver, 1997; Guirao *et al.*, 1997; Kirk *et al.*, 2000) and most recently microsatellite studies (De Barro *et al.*, 2003; Tsagkarakou & Roditakis, 2003). Theses analyses plus additional biological tests allowed not only the distinction of just 2 separate biotypes (A and B), but even more, and usually considered as indigenous to a certain part of the world (Bedford *et al.*, 1994b; Frohlich *et al.*, 1999; de Barro & Hart, 2000; Kirk *et al.*, 2000). In a review on *B. tabaci* (Perring, 2001) all reported biotypes have been clustered into 7 groups as follows; group 1: new world biotypes A, C, N, R*;* group 2: cosmopolitan biotypes B, B2*;* group 3: Benin (biotype E) and Spain (biotype S)*;* group 4: India, biotype H*;* group 5: Sudan (biotype L), Egypt, Spain (biotype Q), Nigeria (J)*;* group 6: Turkey (biotype M), Hainan, Korea; group 7: Australia (biotype AN). Up to date 24 distinct populations of *B. tabaci* have been given a biotype designation.

Geographical repartition

Of the many whitefly species in the world, *B. tabaci* is one of the most viruliferous and it has now become globally distributed over all continents except the Antarctics (Martin *et al.*, 2000)(Fig. 1-5). The present-day distribution of *B. tabaci* is presumably related to its close association with agricultural monocrops (Brown *et al.*, 1995).

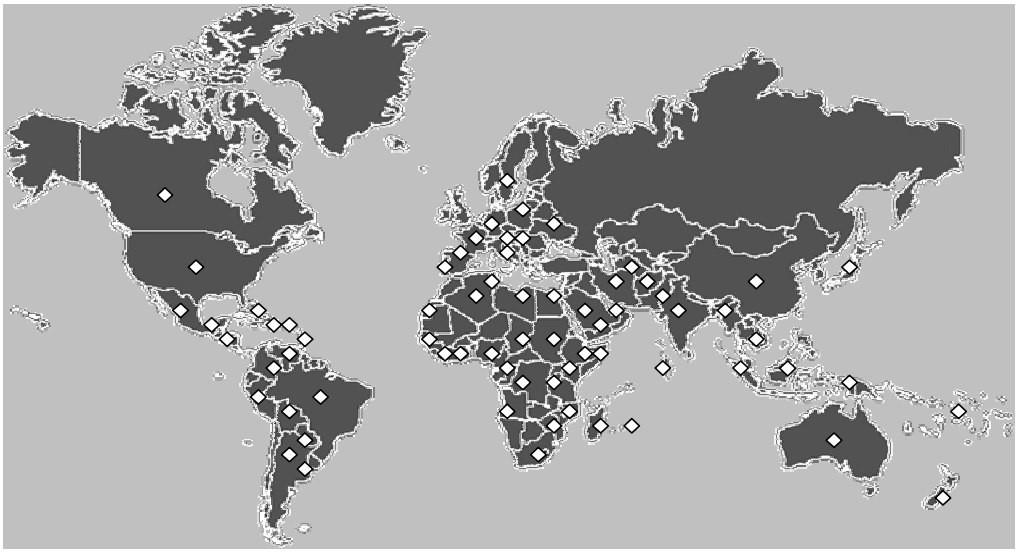


Fig.1-5: World distribution of *B. tabaci* (updated with published data up to 2003)

Host range

B. tabaci is one of the only six whitefly species that have a very broad host range of herbaceous plants (Mound & Halsey, 1978). Indeed *B. tabaci* has an extremely wide host range, despite the substantial differences in the host ranges of the different biotypes of *B. tabaci* (Cock, 1986; Price, 1986). *B. tabaci* can feed on more than 900 plant species (Cock, 1986; Greathead, 1986) belonging to 74 botanical families. A more precise study (Servin *et al.*, 1999) has indicated that 73% of the cultivated plants and 66% of the weeds are host of biotype B. For the other biotypes less information is available. Host plant families reported in the literature often include the following: *Leguminosae, Malvaceae, Solanaceae, Euphorbiaceae, Convolvulaceae, Cucurbitaceae, Labiacae, Verbenaceae, Cruciferae, Amaranthaceae, Rosaceae, Asteraceae* and *Moraceae*. New additions to this list have been reported (Simmons *et al.*, 2000; Oliveira *et al.*, 2001), such as *Cleomaceae, Sterculicaceae, Rubiaceae, Valieranaceae,* and *Hypericacae*. With all these host plants described in the literature and despite the polyphagous nature of *B. tabaci*, outbreaks of *B. tabaci* in various parts of the world during the last decade and apparent differences in host range at

different localities suggest a broad range of genetic variability within and between populations (Basu, 1995). This is quite coherent with the numerous new biotypes with different host ranges found worldwide (Perring, 2001).

Competition

Few studies on competition between biotypes of *B. tabaci* are available. Nevertheless, many competition factors are described throughout the literature, especially regarding to biotype B. The success of invasion of this biotype is apparently linked to its insecticide resistance, which seems to be higher than for other tested biotypes (Costa *et al.*, 1993a; Brown *et al.*, 1995). Furthermore, the B biotype was shown to be able to fly longer distances (Blackmer *et al.*, 1995a; Blackmer *et al.*, 1995b) and had a higher fecundity and shorter life cycle than other biotypes (Bethke *et al.*, 1991). It was shown to have competitive advantages in mix rearing with the Q biotype (Pascual & Callejas, 2004). Nevertheless biotype Q seemed not to be prevalent in field population in Spain, despite its dominance in lab conditions (Guirao, 1997; Moya *et al.*, 2001; Muniz & Nombela, 2001; Nauen *et al.*, 2002).

Competition between insects has been extensively studied. Mac Arthur and Wilson (1967) tried to classify the competitor traits into two categories: life-history strategies and behavioural traits. Competitors were classified either in an r or in a K strategy. In their view, r species display a suite of traits that favour rapid population growth and colonisation of new habitats, while K species were adapted to competition in saturated habitats. Although later studies have shown that the conditions of selection are much more complex, it was shown that species often fall into r or K syndromes (Duyck *et al.*, 2004).

According to all those published data and the classification of Mac Arthur and Wilson (1967) it seemed that the B biotype falls into a mixed *r/K* strategy with higher fecundity and having the competitive advantage in mixed rearing with other biotypes in lab conditions or in fields against some whitefly biotypes (except Q biotype). Successful invasions (almost worldwide) of the B biotype are well document. For example, in 1990-91 this biotype had almost completely replaced the A biotype in California (Gill, 1992), and the same has occurred in the Caribbean basin (Costa *et al.*, 1993a).

RESISTANCE TO BEGOMOVIRUSES

Up to now, almost no efficient measures to control begomoviruses have become available. Several strategies have been investigated and mostly these are either directed towards insect (vector) control or by breeding crops resistant or tolerant to the virus.

Insect control strategies

Potential insect control measures can be divided into 3 main categories: chemical, biologic and physical controls, respectively. Cultural practices for managing *B. tabaci* populations are also being used.

Over the years, chemical or pesticide control measures have only been partially effective, since whitefly populations are polyphagous, can reach very high numbers and because the chemicals were improperly used. As a consequence massive doses have been used in attempts to eradicate the vector. Resistances to several insecticides have been detected within the invasive B biotype (Costa *et al.*, 1993a; Pico *et al.*, 1996). Another drawback of the use of insecticides turned out to be the killing of natural enemies (Gill, 1992).

Natural enemies of whiteflies are numerous, the *Encarsia* and *Eretmocerus* species are the most commonly used to (bio-) control *B. tabaci* populations, even in IPM programs (Gerling, 1990; Gerling & Mayer, 1996; Ellsworth & MartinezCarrillo, 2001). Nevertheless those measures can only regulate the direct feeding damages or delay the progress of the virus disease, but not suppress it, as the threshold of only one or two insects per plant needed for TYLCV transmission is very low (Caciagli *et al.*, 1995).

Physical barriers as fine-mesh screens have been used in the Mediterranean basin to protect crops (Cohen & Antignus, 1994). More recently UV-absorbing plastic screens have been shown to inhibit penetration of whiteflies into greenhouses (Antignus *et al.*, 1996; Antignus *et al.*, 2001). However the use of physical barriers is not the best solution as it is costly and creates problems of shading, overheating and poor ventilation (Lapidot & Friedmann, 2002).

Cultural practices such as crop-free periods, altering dates, crop rotation, and weed and crop residue disposal, high planting densities, floating row cover, mulches, trap crop, or living barriers performed well. But growers may be reluctant to adopt such cultural practices that require significant changes in their conventional practices (Hilje *et al.*, 2001) and might be time consuming.

All these control strategies have shown their drawbacks, and turned out to be not completely efficient in whitefly hence begomovirus control. Therefore the potentially best way to reduce geminivirus incidence still is by breeding crops resistant to the virus (Cohen & Antignus, 1994; Morales, 2001; Lapidot & Friedmann, 2002).

Breeding for resistance to begomovirus

Breeding for natural resistance

All commercial tomato cultivars have been found to be completely susceptible to TYLCV, urging breeders to screen wild tomato accessions for potential resistance traits (Pilowsky & Cohen, 2000). Until now only one major resistance gene to TYLCV had been identified: TY-1 (Zamir *et al.*, 1994) on chromosome 6 of *L. chilense*. Two more resistance modifier genes were mapped to chromosome 3 and 7 (Zamir *et al.*, 1994) of *L. Chilense*. Another TYLCV-resistance gene, originating from *L. pimpinellifolium* had been mapped using RAPD PCR-based markers to chromosome 6, but to a different locus from TY-1 (Chagué *et al.*, 1997). In addition, a resistance gene against the *Tomato leaf curl Taiwan virus* was mapped to chromosomes 8 and 11 of *L. hirsutum* (Hanson *et al.*, 2000). Different breeders' teams used different wild type genetic background to build lines with high levels of resistance, such as: *L. peruvianum* (Lapidot *et al.*, 1997; Friedmann, 1998; Vidavsky & Czosnek, 1998) *L. chilense* (Zamir *et al.*, 1994; Scott *et al.*, 1996) *L. pimpinellifolium* (Vidavsky *et al.*, 1998), and *L. hirsutum* (Vidavsky & Czosnek, 1998; Hanson *et al.*, 2000).

The first TYLCV-resistant commercial cultivar resulting from breeding programmes is TY20, which carries a resistance derived from *L. peruvianum*, showing a delay in symptom development and viral accumulation (Pilowsky & Cohen, 1990; Rom *et al.*, 1993).

In most cases the sources of TYLCV resistance appeared to be controlled by multiple genes (Pico *et al.*, 1996; Pico *et al.*, 1999a). Examples of the different resistant lines studied are given in the review of Lapidot and Friedmann (2002). Nevertheless, after 20 years of breeding programs very few commercial genotypes with increased levels of TYLCV resistance are on the market.

Breeding for resistance for other begomoviruses than TYLCV has also been done on tomato or other host plants, but so far to a lesser extent. For example the TY-1 gene has been successfully used against *Tomato yellow mosaic virus* (ToYMV) (Piven *et al.*, 1995) which is causing high yield losses in Venezuela, and *Tomato mottle virus* (ToMoV) (Scott *et al.*, 1996). Some studies were also carried out on common bean (Velez, 1998; Singh *et al.*, 2000) cassava (Storey, 1936; Hahn, 1980; Fregene, 2000); and cotton (Wilson, 1991).

So far only limited studies have ben carried out with the objective of identifying resistance to bipartite begomoviruses. Most of these studies have been conducted on ToMoV (Scott & Schuster, 1991). A gene mapping study on *L. chilense* accession LA 1932 showed that two additive regions were involved in ToMoV resistance (Griffiths & Scott, 2001). More recently, extensive screening studies to obtain resistance to PYMV have been conducted (Santana *et al.*, 2001; Pietersen & Smith, 2002; Rampersad & Umaharan, 2003a), showing potential sources of resistance to this begomovirus in several wild *Lycopersicon* accessions.

Genetic engineering

The concept of pathogen derived resistance (PDR) was proposed by Sanford and Johnson (1985), who suggested engineering resistance by transforming a susceptible plant with gene sequences derived from the pathogen itself. They suggested that the expression of certain key gene products of a pathogen by the plant either in an inappropriate form or amount, or an inappropriate time during the infection cycle could disturb infection by the invading pathogen (Sanford & Johnson, 1985). Over the years good advances were made with several RNA virus-plant combinations. Successful PDR approaches have also been reported for geminiviruses, although in general, resistance levels reported against DNA viruses seem more difficult to engineer. TYLCV-resistance was attempted using the following five strategies: i) coat protein mediated resistance (Kunik *et al.*, 1994; Bendahmane *et al.*, 1997; Sinisterra *et al.*, 1999), ii) movement protein mediated resistance (Malyshenko *et al.*, 1993; Hou *et al.*, 2000), iii) defective interfering viral DNA, iv) genes in antisense orientation (Day *et al.*, 1991; Bendahmane & Gronenborn, 1997) and v) truncated (Noris *et al.*, 1996) or mutated replicase (rep, C1, AC1) genes (Yang *et al.*, 2004).

The first two approaches involve expression of viral capsid or (mutant) movement proteins in order to inhibit viral proliferation. The last three categories, despite differences in transgene construct used, all aim to inhibit viral replication by disrupting the activity of the Rep gene. For example, Yang *et al.*, (2004) successfully engineered resistance-using *Rep* and *C4* transgene constructs against TYLCV and even after whitefly inoculation, neither viral DNA nor symptoms were observed in the transformed plants.

More recently it was discovered that in most of the (non-begomovirus) cases that PDR was being aimed, the observed transgenic resistance was caused by transcriptional rather than translational expression of the viral transgene sequences (Chellappan *et al.*, 2004a; Vanitharani *et al.*, 2004). The mechanism behind these cases turned out to be RNA silencing or RNA interference (shortly RNAi), a sequence-specific breakdown mechanism in plants which represents a natural antiviral defense mechanism (Voinnet, 2001; Vanitharani *et al.*, 2003; Chellappan *et al.*, 2004b). As begomoviruses have DNA genomes it might be anticipated that, here again, the prospects of using RNAi-based approaches are limited. Indeed so far, only few reports on this issue have been published. Transgene mediated RNA silencing targeted on the *Rep* and *C4* genes were not as successful. The study showed that if the virus reaches a threshold level of expression/replication in the initially infected cells, then virus spreading can no longer be prevented (Noris *et al.*, 2004).

The noncoding begomovirus sequences (IR) have also been tested for the generation engineered virus resistance. Pooggin et al., (Pooggin & Hohn, 2003) demonstrated that transient expression of both sense and antisense *Vigna mungo yellow mosaic virus* (VMYMV) promoter sequences in the IR resulted in complete recovery in infected VMYMV plants.

However, despite all efforts, only few strategies have resulted in important levels of resistance against begomoviruses.

Because of an increasing public concern about their use, the cultivation and consumption of transgenic crops has been more or less abandoned. Another tool to find resistances, which do not

involve transformation, and seemed to be accepted by the European public concern, exists. It consists in using chemical mutagenesis (for example ethylmethanesulfonate (EMS) which can induce point mutations (McCallum *et al.*, 2000) into the plant genome. This technique, combined with rapid mutational screening to discover induced lesions, is named: Targeting Induced Local Lesions IN Genomes (TILLING). Indeed TILLING can be applied to plants without requiring transgenic or sophisticated tissue culture methodology. The drawbacks of this techniques are to detect point mutations which can be challenging, and screening all the plants despite the TILLING method might be expensive (Burch-Smith *et al.*, 2004). Nevertheless TILLING can be automated and is quite rapid compared to other techniques (Colbert *et al.*, 2001; Henikoff *et al.*, 2004). This technique seemed to be very appropriate for RNA viruses, especially with potyviruses, for which it had helped targeting a gene involved in their cycle. Indeed, mutations in the plant gene *eIF4E*, was found to be involved in the cycle of *Lettuce mosaic virus* (LMV; genus *Potyvirus*) and was found to block the viral infection (Nicaise *et al.*, 2003). This type of technique, which had been used for other RNA viruses with success had never been used for DNA viruses, and might be of first interest if it could work for Begomoviruses.

AIM AND SCOPE OF THE STUDY

Since 1997 an epidemic of *Tomato yellow leaf curl virus* (TYLCV, genus: Begomovirus, family *Geminiviridae*) associated with upsurge of whiteflies (*Bemisia tabaci*) on tomato crops has been reported on Réunion, an island situated in the south west part of the Indian Ocean. Yield losses reached 85% during the first years of the epidemic (Reynaud *et al.*, 2003). The virus spread quickly throughout the island, imposing a great problem for tomato cultivation. This PhD research project was initiated by this new problem that Réunion agriculture had to face. The PhD work was aiming to answer several questions regarding to the introduction of the virus on the island, its spread and evolution following the introduction and its control through plant resistance to virus transmission by *B. tabaci*. Introduction of viruses is generally thought to be due to the movement of infected seedlings, ornamental plants or infected vectors when transmission is persistent as in the case of begomoviruses by *B. tabaci*. It is less obvious to consider that fruits collected from infected plants can act as virus inoculum. Considering that this point is of particular interest for tomato because it is one of the legumes most actively traded internationally, the possibility of virus transmission through fruits was investigated for TYLCV (Chapter 2). It was not known if the tomato yellow leaf curl disease that emerged on La Réunion was caused by the introduction of a single or several strains or even species of begomoviruses. Therefore a virus monitoring was organised throughout the island every six months during 4 years (from 2001 to 2004). The results obtained were also compared to samples from 1997 to 2000 to check if any evolution of virus populations would have taken place during the years after the 1997 introduction (Chapter 3). Unlike TYLCV, the vector *B. tabaci* was reported from Réunion many years before the emergence of TYLCV (Bourriquet, 1938; Luziau, 1953; Russell & Etienne, 1985) although it was not observed on vegetable crops in the

recent years preceding the viral epidemics. As the spread of TYLCV is completely dependent on its vector *B. tabaci*, the latter it is one of the major components in the TYLCV/vector/host pathosystem that needs to be studied to allow the design of future control measures. Therefore a first study on the whitefly vector populations was carried out (Chapter 5) on Réunion and some of the other south west islands of the Indian Ocean. The well known invasive B biotype was found on Réunion along with another, supposedly indigenous biotype, named Ms, also found on the other islands of the area. The genetic diversity of both biotypes was investigated using microsatellite markers (Chapter 6). While on Réunion tomato leaf curling was only detected for the first time in 1997, in Madagascar such symptoms were reported earlier (Reckhaus, 1997). Since this report did not specify if these symptoms were due to an imported or indigenous begomovirus, extensive sampling was carried out on TYLCD symptomatic leaves in the neighbouring islands: Madagascar, Mayotte and Seychelles and the presence of two new begomoviruses on Madagascar and Mayotte could be described (Chapters 4). The genomes of these two new begomoviruses were cloned, sequenced and studied in order to test their pathogenicity on tomato. Considering the continuously increasing diversity of begomoviruses, and the fact that they all share the vector *B. tabaci*, a preliminary screening of wild *Lycopersicon* accessions was done focusing on finding resistance that would act on transmission efficiency level, and which may be efficient to all begomoviruses (Chapter 7). Some promising accessions were selected for further analysis and this is reported in Chapter 8. Finally, in the General Discussion (Chapter 9) the major findings and implications of the PhD thesis research are discussed in a broader context, covering aspects such as virus evolution and future control strategies.

PART 1: BEGOMOVIRUS

CHAPTER 2

Tomato yellow leaf curl virus can be Acquired and Transmitted by *Bemisia tabaci* (Gennadius) From Tomato Fruits[1]

ABSTRACT

The whitefly *Bemisia tabaci* is an insect pest causing worldwide economic losses, especially as vector of geminiviruses such as *Tomato yellow leaf curl virus* (TYLCV). Currently, imported and exported tomatoes are not monitored for TYLCV infection because they are not considered to represent a potential risk as virus source for whiteflies. A survey of tomatoes imported into Réunion Island indicated more than 50% of the fruits contained TYLCV as determined by DNA blot analysis. Moreover we showed that TYLCV is present at a high titre in tomato fruits, and demonstrated that it can be acquired by whiteflies and subsequently transmitted to healthy tomato plants. Potential risk of spread TYLCV by tomato fruits in natural conditions needs to be further assessed.

[1]Delatte, H., Dalmon, A., Rist, D., Soustrade, I., Wuster, G., Lett, J. M., Goldbach, R. W., Peterschmitt, M., and Reynaud, B. 2003. *Tomato yellow leaf curl virus* can be acquired and transmitted by *Bemisia tabaci* (Gennadius) from tomato fruits. Plant Dis. 87, p 1297-1300.

INTRODUCTION

In tropical and subtropical climate zones, *Bemisia tabaci* Gennadius (Hemiptera : Aleyrodidae) is an important insect pest. *B. tabaci* provokes direct feeding damages but also causes considerable indirect damage as a vector of numerous geminiviruses (Credi *et al.*, 1989) such as *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*, family *Geminiviridae*), a threatening virus for tomato (Markham *et al.*, 1995; Pico *et al.*, 1996; Moriones & NavasCastillo, 2000). TYLCV was initially described from the eastern Mediterranean regions (Cohen & Harpaz, 1964). In the last decade, due to international transport of plant material and people, TYLCV has spread to the western Mediterranean regions, to the Caribbean Islands, to the American continent, to Réunion island, and to Japan (Kato *et al.*, 1998; Peterschmitt *et al.*, 1999a; Peterschmitt *et al.*, 1999b; Accotto *et al.*, 2000; Salati *et al.*, 2002). Following the spread of TYLCV, the French National Plant Protection Organization (NPPO) is particularly concerned by the risk of any further spread of this virus and more generally by the spread of any other begomovirus. This risk is not only limited to tropical and sub-tropical regions as *B. tabaci* is also a major pest in greenhouses in temperate regions.

Presently, all control measures are focused on the vegetative plant parts and particularly on imported tomato seedlings produced in nurseries of begomovirus infected countries, but were not applied to the imported fruits. A survey conducted in 2000 by the French NPPO has shown that 50% of tomato fruits introduced in France from Mediterranean countries were actually infected with TYLCV (Dalmon, *unpublished results*). In view of this, it has become necessary to repeat this survey and test if tomato fruits can act as a reservoir of viruses from which whiteflies would be able to acquire and spread the virus. In this study, we present the results of a survey conducted by the French NPPO in 2002 on the presence of TYLCV on tomato fruits imported into Réunion from TYLCV infected countries. We showed that TYLCV is present in several parts of vine tomato fruits and that *B. tabaci* can survive for more than 12 hours on fruits. Based on these results we designed a transmission test which demonstrated that *B. tabaci* can acquire TYLCV from tomato fruits and transmit it to healthy tomato plants.

MATERIAL AND METHODS

Insect, plant and virus material

Adults of *B. tabaci* used in this study were from a Réunion population that was started with nymphs collected on cabbage (*Brassica oleracea*) and was subsequently reared on the same species under laboratory conditions: 12h/12h photoperiod (white and red fluorescent tubes), 25 +/- 2° C temperature and 60% +/- 10% relative humidity. The population was identified as belonging to the B biotype using RAPD markers. Fruits of Durinta variety (Western Seeds) of vine tomatoes (*Lycopersicon esculentum*) were collected from TYLCV-infected plants in a greenhouse of Piton Saint Leu (Réunion). Transmission experiments were done on a TYLCV susceptible cultivar Farmer (Know You Seed).

Squash and tissue blot detection of begomoviruses on imported batches of tomato fruits

Twenty-seven batches of tomato fruits imported from different countries (principally Spain and Morocco) into Réunion island during the first quarter of 2002, were assayed for begomovirus presence. Twelve to 50 randomly selected fruits were tested for each batch. Four small pieces (around 0.5 x 0.5 x 0.2 cm) including skin and pulp were sliced with a sterile scalpel near the point of insertion of the peduncle of each fruit and squashed individually onto a nylon membrane. Prints were also made on the membrane with cross sections of the stem for vine tomatoes. Prints of leaf and stem of healthy tomato plants were used as negative control on each membrane. Tomato fruits (skin and pulp) and leaves of infected plants were used as positive control. Virus detection in squash blots and tissue prints were performed by hybridization with a TYLCV-DNA probe, using the DIG high prime DNA labelling and detection starter kit II (Roche, Mannheim), according to the manufacturer recommendations. The TYLCV probe represented a 800 bp DNA fragment of a Réunion TYLCV isolate (GenBank Accession No. AJ010790) including a 3' part of the pre-coat gene and a 5'part of the coat protein gene. A sample was considered positive when a strong signal was revealed on the membrane by comparison to the negative control.

TYLCV distribution in the vine tomato fruits.

Two bunches of vine tomatoes of 8 and 9 fruits (var. Durinta) and 2 others of 5 and 4 fruits (var. Cencara, Vilmorin) collected on TYLCV infected plants were tested for TYLCV detection with triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) using a kit supplied by Adgen, UK. The protocol was according to the manufacturer recommendations (Adgen, UK). Virus distribution was assessed within each fruit by testing separately small pieces of stem, sepals, skin, pulp and peduncle insert (point of insertion of the peduncle inside the fruit) sliced with a sterile scalpel. Plant samples of 0.5g were ground in 3 mL extraction buffer. Positive and negative control were from tomato leaves collected on respectively TYLCV artificially infected and non infected plants of a susceptible tomato cultivar (Farmer) (Know You seed). The detection of the virus was considered to be positive when the absorbance value was higher than the mean absorbance of the healthy control plus three times the standard deviation.

Survival test

Life expectancy of whiteflies feeding on bunches of tomato fruits (var. Durinta) was assessed with 100 B. tabaci, which were introduced in two 1 L beakers (50 whiteflies in each), covered with gauze mesh and containing one small bunch of tomatoes in each. Dead insects were recorded after different time periods: 3, 6, 12, 24 and 48h. The beakers were kept at 25 °C with a 12h photoperiod. The experiment was repeated once.

Transmission test

Bunches of vine Durinta tomatoes used as virus sources for the transmission tests were cut from TYLCV infected plants and one fruit of each bunch was checked for TYLCV infection by TAS-ELISA. Three ELISA-positive bunches, with about 6 to 8 tomatoes each, were placed in a cage in which approximately 1000 non viruliferous B. tabaci were released. Simultaneously, in another cage, about 1000 insects from the same non viruliferous population were placed on a TYLCV infected tomato plant which did not bear fruits. After a 3-hour acquisition access period (AAP), 100 insects were removed from each cage. Fifty of these were caged on 2 leaf stage tomato plants with a plastic tube, 1 whitefly/plant, for a 7-day inoculation access period (IAP). To confirm virus uptake, the 50 remaining B. tabaci were crushed individually on a membrane and analyzed with a TYLCV genome DNA probe (Pico *et al.*, 1999b; Polston *et al.*, 1999). After removing the tubes at the end of the IAP, the plants were treated with an insecticide and placed in an insect-proof cage for 21 days in a greenhouse. The inoculated plants were analyzed for TYLCV detection with TAS-ELISA. The detection of the virus was considered to be positive when the absorbance value was higher than the mean absorbance of the healthy control plus three times the standard deviation. The same test was repeated after a 6-hour and a 12-hour AAP. Three repetitions of the whole experiment were performed with tomato fruits from the same location and the same plants.

Statistic analyses

Analyses of variance (ANOVA) were performed on transformed data (2 arcsinus $\pi x/100$) for homoscedasticity on the mean absorbance values in ELISA tests for the Cencara and Durinta varieties on the different parts of the fruit: sepal, stem, pulp, skin and peduncle insert (Fig. 2-1A-B). The same transformation and ANOVA test were performed on the percentage of ELISA positive plants (Fig. 2-3A), and on the percentage of viruliferous whiteflies (Fig. 2-3B). We used S-plus 6.1 (Insightful Corporation Seattle, Washington, US), which proposes the best test of mean comparison in terms of critical point at level a=0.05.

RESULTS

Begomovirus detection on imported batches of tomato fruits

TYLCV-infected tomatoes were found in 13 out of 27 batches of imported tomato fruits coming from different origins (Table 2-1). On average, 57% of the tomatoes from these 13 batches were infected with TYLCV.

Batch number	Origin	Positive fruits / Total fruits tested
116	Spain	14/24
124	Spain	18/25
146	Spain	21/28
301	Spain	25/30
472	Spain	7/14
486	Spain	5/12
820	Spain	7/25
822	Spain	39/48
111	Unknown	10/24
158	Unknown	31/50
421	Unknown	18/50
390	Morocco	9/29
443	Morocco	11/18

Table 2-1. Detection of *Tomato yellow leaf curl virus* (TYLCV) in 27 batches* of tomato fruits imported into Réunion during the 1st quarter of 2002

*TYLCV was not detected in 14 batches of fruits from several countries (data not shown).

TYLCV distribution in the vine tomato fruits

All the fruits of the bunches of tomatoes, green or mature, collected on TYLCV infected plants of the two cultivars Durinta and Cencara, were shown to be infected by TYLCV using TAS-ELISA. All the fruit components tested for virus detection were positive by ELISA (Fig. 2-1A & B), although the relative distribution was significantly different for the two varieties (Fisher and Tukey tests). The mean absorbance values obtained with peduncle insert is significantly higher than those obtained with skin, sepal and stem for Durinta variety (Fig. 2-1A). Inversely, for Cencara variety, the mean absorbance values obtained with peduncle insert, skin and pulp are significantly lower than those obtained with sepal and stem (Fig. 2-1B).

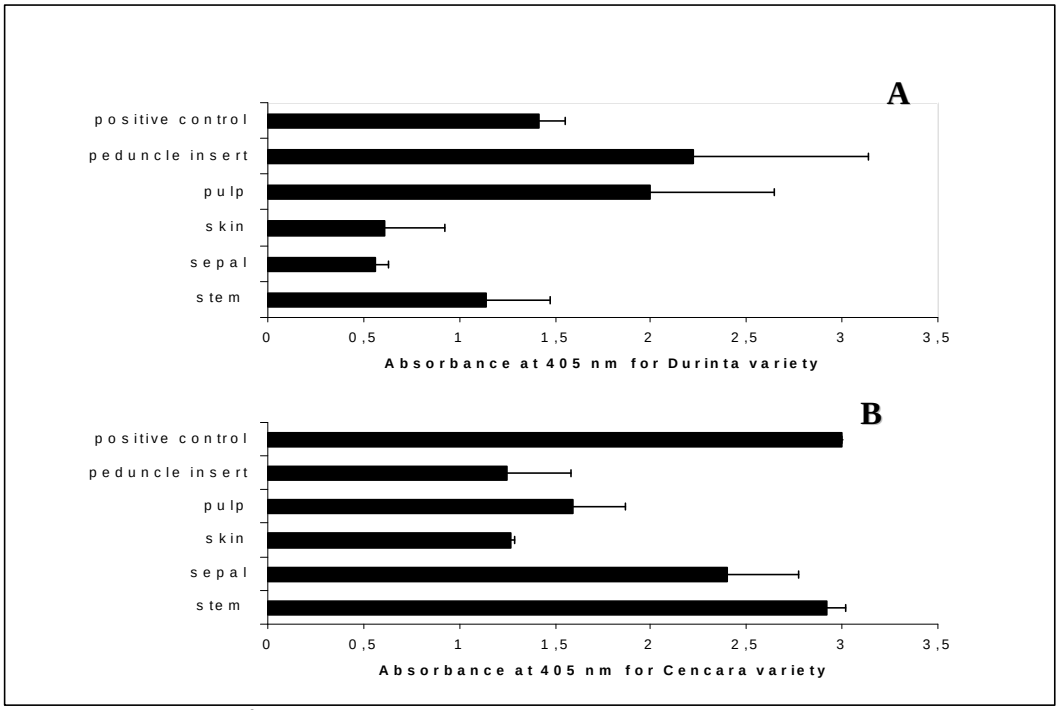


Fig. 2-1. *Tomato yellow leaf curl virus* (TYLCV) detection by triple-antibody sandwich enzyme- linked immunosorbent assay in different parts of 17 vine tomato fruits of 2 infected bunches of Durinta variety (A) and 9 fruits of 2 infected bunches of Cencara variety (B). Positive and negative control were from tomato leaves collected on respectively TYLCV infected and non infected plants of a susceptible tomato cultivar (Farmer). The standard deviation is indicated for each mean absorbance. Fruit parts with the same letter indicate that their mean absorbance values are not significantly different according to the Tukey test (a=0.05). The controls were not included in the ANOVA analyses and in the mean comparison test.

Survival test

Having confirmed that TYLCV is consistently detected in tomato fruits, a second prerequisite for virus transmission from tomato fruits was to show that whiteflies can feed and survive on bunches of tomatoes. We recorded 20% mortality after a 24-hours feeding access on vine tomato fruits and almost 100% after 48 hours (Fig. 2-2).

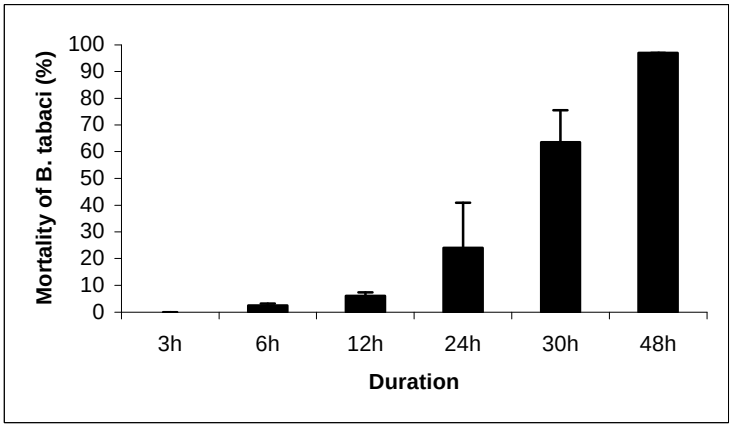


Fig. 2-2. Mortality recorded over time in a population of 200 *Bemisia tabaci* separated in four groups of 50 individuals, each group maintained on a single bunch of tomato fruits (with about 3 to 4 fruits per bunch) within a 1L beaker. The vertical lines represent the standard deviation of the mean mortality of the four groups. The experiment was done twice.

Transmission test

Based on the survival test results, transmission tests were carried out with three acquisition access periods, 3, 6, and 12 hours (Fig. 2-3). Three percent of the insects were shown to transmit the virus to healthy tomato plants after a 3h AAP on TYLCV infected tomato fruits, and up to 8% after a 12h AAP (Fig. 2-3A). The percentage of transmitters after AAP on whole plants was significantly higher (Fisher test and Fisher LSD, P#0.05), with 8% after a 3h AAP, and up to 29% after a 12h AAP. The percentage of transmitters increased significantly (Tukey test) with the increasing duration of the AAP between the 3h and the 12h AAP (Fig. 2-3A).

The percentage of insects that tested positive by TYLCV hybridization was generally higher than the percentage of transmitters (Fig. 2-3 A & B). The percentage of viruliferous individuals increased significantly (Fisher test) with the increasing duration of the AAPs between 3 and 12h AAPs. However no significant difference was found between the two sources of acquisition, fruits or whole plants (Fisher test and simulation based test).

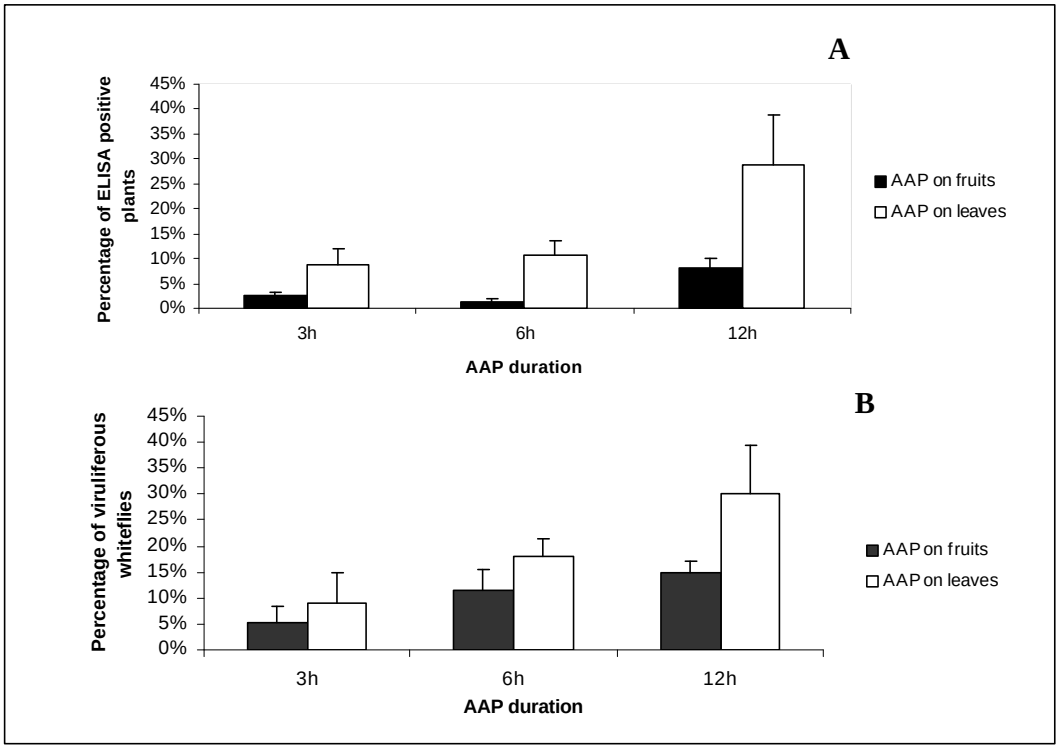


Fig. 2-3. **A.** Transmission of *Tomato yellow leaf curl virus* (TYLCV) from infected tomato fruits and seedlings to healthy tomato plants. These plants were tested by triple-antibody sandwich enzyme- linked immunosorbent assay 3 weeks after the end of a 7-day IAP with *Bemisia tabaci* (1 whitefly per plant) which were given a 3-h, 6-h, and 12-h AAP on TYLCV-infected bunches of tomato fruits or whole plants of the vine tomato cultivar Durinta. Fifty insects were used per AAP. The experiment was repeated 3 times. The standard deviation is indicated for each mean absorbance. **B.** Percentage of viruliferous whiteflies after the 3-h, 6-h, and 12-h AAPs, as determined by analyzing squashed individuals with a TYLCV genome DNA probe. Fifty insects were used per AAP. The experiment was repeated 3 times. The standard deviation is indicated for each mean absorbance.

DISCUSSION

The results presented here are, to our knowledge, the first report of successful TYLCV transmission from fruits of vine tomatoes to tomato plants using *B. tabaci* as vector. This successful transmission is consistent with the ELISA detection of TYLCV in different parts of the vine tomato fruits and the 24 hours survival rate of 80% of a population maintained on such fruits. Although the feeding of the whiteflies does not appear very efficient on fruits regarding to the mortality rate, it seems sustained at least beyond the first 3 hours. Indeed the percentages of viruliferous and infective whiteflies from the 12-hour AAP are significantly higher than those from the 3-hour AAP. A significant increase of the percentage of infective *B. tabaci* was also obtained with increasing AAPs on whole plants as shown previously (Mehta *et al*., 1994).

According to the absorbance values recorded by ELISA, TYLCV concentrations in tomato fruits may be relatively high when compared to the absorbance obtained with an infected tomato leaf (positive control). Since a 3-hour feeding access on TYLCV infected fruits is enough to obtain potentially infective whiteflies, the risk of TYLCV spread through this route cannot be disregarded wherever potentially infected tomatoes are exported or imported to or from foreign countries. Since other begomoviruses are threatening tomato production in various regions of the world, it will be useful to check how far this risk can be expected with other begomoviruses. The risk of virus transmission from fruits was previously emphasized by others in the case of stone fruits, peaches and apricots, with *Plum pox virus* (PPV) (Labonne & Quiot, 2001). However, unlike the stone fruits which bear symptoms due to PPV infection, the risk with TYLCV is emphasized as infected tomato fruits are symptomless.

The 57% TYLCV-infected tomatoes found in average, in samples collected from 13 batches of tomatoes imported into Réunion from TYLCV-infected countries is consistent with the high infection rate detected in 2000 on tomatoes imported into France (Dalmon, *unpublished results*). Unfortunately the use of commercial resistant tomato varieties may not eliminate the risk of importing infected fruits because these varieties do not prevent virus multiplication (Fargette *et al.*, 1996). Screening of resistant cultivars apparently never included TYLCV detection in the fruits (Michelson *et al.*, 1994; Friedmann, 1998). Further studies are required to monitor virus in the fruits of resistant cultivars and also to find stronger resistances which may prevent contamination of the fruits or at least the parts to which the vector can have access. The comparison between cultivars Durinta and Cencara showed indeed that virus distribution within the fruit can be significantly different between two tomato cultivars.

To further assess the risk of TYLCV transmission from fruits in natural conditions, it is suggested to monitor the frequency of TYLCV transmission to healthy tomato plants caged with non viruliferous *B. tabaci* and infected vine tomato fruits. According to observations made during the survival test, it seems that the whiteflies have a preference for the green parts of the tomato bunch, green tomatoes or green parts of further ripened fruit (sepal, stem), red fruit (parts) being less frequented. This needs to be further investigated and substantiated with quantified data since it may help the regulation personnel to design strategies to control the potential risk of virus spread

through fruits. For the time being, although these risks were not yet assessed, it should be recommended to avoid dumping of unmarketable infected tomatoes near tomato fields.

ACKNOWLEDGMENT

We would like to thank M. Grondin for his assistance in the transmission experiment and Dr F. Chiroleu for his statistical assistance. This work was funded by the CIRAD, Le Conseil Régional de la Réunion and le Ministère de l'agriculture (SPV). This study is part of a PhD from Wageningen University of H. Delatte.

CHAPTER 3

Study of the evolution of *Tomato yellow leaf curl virus* since its introduction in a close environment over an 8 year period

ABSTRACT

The evolution of *Tomato yellow leaf curl*-Mild isolate Réunion (family *Geminiviridae*, genus *Begomovirus*) (TYLCV-Mld[RE]) was monitored from 1997 to 2004, just after its first upsurge in the island. Viral DNA fragments comprising part of the C4, C1 open reading frames (ORF), and part of the common region and V1, V2 ORF were amplified by polymerase chain reaction and directly sequenced. The 41 isolates studied had nearly identical sequences. Mutation frequency based on the 2 regions of the genome was estimated at 10×10^{-4}. This result confirmed that DNA plant viruses have an equivalent mutation frequency (number of mutations relative to the consensus, divided by the number of nucleotide sequenced), which are in the same range as those obtained for RNA plant viruses. The IR and the C4 ORF had the highest mutation frequency with the highest percentage of synonymous mutations, compared to the V1 and C1 ORF. The low genetic diversity found in the initial population was consistent with a founder effect associated with a population bottleneck during the introduction of a new virus in an insular environment. It was hypothesized that only one isolate introduction of TYLCV was the result of the epidemic observed in 1997 in La Réunion. The new introduction of another TYLCV strain in 2004 emphasis the risk of new begomovirus introductions and reinforced the importance of monitoring the TYLC disease in La Réunion.

INTRODUCTION

Despite their conditions of obligatory pathogens, viruses are very successful in colonizing new ecological niches, thus frequently creating considerable epidemic outbreaks (Goldbach & Peters, 1994; Polston & Anderson, 1997). Infections of plant viruses are worldwide causing great losses on crops and accounts for million dollars of lost productivity every year. Most plant viruses are RNA viruses, and due to their quasi-species character i.e. a mixture of genetically diverse but related viral genomes, they may evolve rapidly. Their mutation frequencies were associated to the large error rate of the RNA-dependent RNA polymerase. Plant DNA viruses are replicating either through a reverse transcription step like caulimoviruses or are relying on host DNA polymerase like geminiviruses. As expected from the high error rate of reverse transcriptases, a high mutation rate was detected with *Cauliflower mosaic virus* (Blanc, 2003). It was more unexpected to detect a high mutation rate with geminiviruses because they rely on host DNA polymerases which are supposed to be more accurate. The mutation rate detected with artificially selected *Maize streak virus* populations showed that it is in the same range as the mutation rate of RNA viruses (Isnard *et al.*, 1998) A high genetic variability was also detected, but at a regional level, with *Beet curly top virus* using restriction endonuclease profiles and partial sequencing (Stenger & Ostrow, 1996; Stenger & McMahon, 1997) and with *Cotton leaf curl virus* using sequencing (Sanz *et al.*, 1999).

It is now clear that the genetic variability of DNA viruses can be similar to the variability of RNA viruses. At this stage, it would be very interesting to evaluate how fast this variability is appearing in natural conditions from a population with a narrow genetic base.

The recent introduction of TYLCV into Réunion (Peterschmitt *et al.*, 1999c) is providing an excellent opportunity of not only studying the genetic variability of a geminivirus at a particular time, but to monitor its evolution over time in natural conditions following a population bottleneck. This may confirm that the genetic diversity of a geminivirus can increase rapidly from a population of a small size as from the population that was contained in the one or the several infected plants and/or the few viruliferous whiteflies that were accidentally introduced into Réunion a few years ago.

Geminiviruses are the most damageable viruses, especially within the begomovirus genus, which are one of the major group infecting dicotyledonous plants with more than 100 begomoviruses described up to now (Jones, 2003). Within these numerous begomoviruses the *Tomato yellow leaf curl virus* (TYLCV) vectored by *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in a circulative way (Cohen & Harpaz, 1964) is one of the most spread and one of the most serious constrain to tomato production in many parts of the world (Moriones & NavasCastillo, 2000). The TYLCV, was for the first time found in La Réunion (situated in the south west part of the Indian Ocean, 700 km east of Madagascar) in 1997 (Peterschmitt *et al.*, 1999c), no other begomovirus were detected before on this volcanic island. This situation provided a unique opportunity to analyse the evolution of a plant DNA virus in space and time, as we approximately knew when TYLCV had been introduced on La Réunion. In the present study, field populations of TYLCV-Mld[RE] isolates have been collected since its introduction over a sampling period of 8

years, and molecular evolution was monitored by sequence determination of two selected parts of its genome. The TYLCV-Mld[RE] has a genome of 2791 bp and an organisation of inferred genes and intergenic regions typical of that observed in begomoviruses which have two virion senses and four complementary senses open reading frames (ORFs) (Delatte *et al.*, 2005b). The two selected parts of the genome to be studied were according to the different ORF properties and mutations probabilities. The V1 properties include binding to and protecting viral ssDNA, self binding, nuclear targeting, nuclear export and mediating systemic movement in plants. The V1/CP is also needed for transmission by the whitefly vector. It controls virus transport through the whitefly gut wall into the haemocoele, where it binds a GroEL analogue, produced by a bacterial endosymbionte, in a way that may protect virus particles from degradation (Harrison *et al.*, 2002). The V2 transcription product has also been recently hypothesized to be involved together with the protein product of the C4 into the cell to cell movement of viral DNA (Rojas *et al.*, 2001). The C4 is an important symptom determinant and involved in initiating cell division (Latham *et al.*, 1997; Krake *et al.*, 1998). The C4 may interact with the Rep/C1 ORF and counter the plant defence mechanism (VanWezel, 2002). The C1 encodes for a protein, the only known protein to be involved in viral replication (Desbiez *et al.*, 1995). The C3 has been hypothesized to function as a replication enhancer protein (HanleyBowdoin *et al.*, 2000).

Since our aim was to detect an increase of the genetic variability of the TYLCV population in La Réunion, the fragments that were used for sequencing were selected in regions that were known to be variable, i.e. regions for which the selection pressure is low. Moreover, since the origin of replication (ori) was described as a major recombination site (Sanz et al 1999), two regions were selected for sequencing, one at each side of the ori. This should increase our chance to detect recombinant genomes and particularly the recombinant TYLCV strain detected in Israel, whose recombinant sequence is located at the 5'-end of the C1 ORF (Navas Castillo et al., 2000). The 5' part of the V1 ORF, also chosen, is the most variable part of this ORF (Padidam *et al.*, 1995), however it is quite a conserved ORF, and the V2, which is also very conserved. The second region was chosen on the C1/C4 ORFs, knowing that the C4 is the most variable region of TYLCV, when aligning all the TYLCV isolates available on GenBank, however the C1, coding for the replicase is conserved.

In this study we report, to our knowledge, for the first time the monitoring of a TYLCV-Mld isolate over an 8-years period in a close environment, following a recent introduction.

MATERIAL AND METHODS

Field sampling

Samples exhibiting TYLCV-Mld[RE] symptoms were collected from 11 sites situated in 7 regions of the west part of La Réunion, in the tomato growing area (Fig. 3-1). Samplings were realised every 6 months during the high infestation periods (in mid summer, February/March and the beginning of spring, September/October) from 2001 to 2004. From 1997 to 2000, surveys were realised once a year starting from the first outbreak of TYLCV-Mld[RE] in La Réunion (Peterschmitt *et al.*, 1999c). La Réunion is a subtropical area where there is no cold winter and tomato production all over the year in the low lands, enabling the permanent presence of virus source. Samples were cut and stored lyophilised by anhydrous calcium chloride (Bos, 1977).

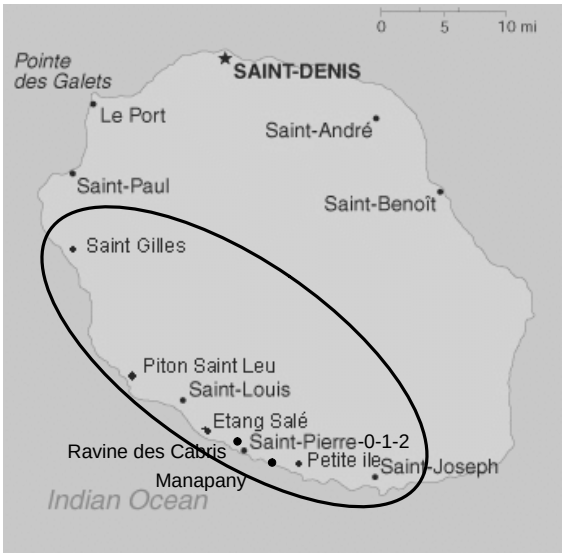


Fig. 3-1. Map of La Réunion showing the repartition of the sampling sites, which are surrounded by the ellipse.

Genetic characterisation of virus isolates

Variations of TYLCV-Mld[RE] were monitored in two selected regions of the genome. The first region (region V; 460 nucleotides long) was amplified between the conserved nonanucleotide of the intergenic region (IR) and the first 195 nucleotides of the V1 ORF (Fig. 3-2). The second region (region C; 445 nucleotides long) was spanning most of the C4 ORF and a part of the C1 ORF (Fig. 3-2). Total DNA was extracted from the dried samples with Qiagen kit (Paris, Fr) according to the manufacturer's instructions.

Two polymerase chain reactions (PCR) were performed on the extracted DNA, using primers V2790/C837 and TY2460/TY1944 designed on the TYLCV-Mld[RE] isolate (Delatte *et al.*, 2005a). PCR reactions were carried out in 25 µl volumes with the following programme: a first cycle of 5 min at 94 °C, then 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final cycle at 72 °C for 5 min. PCR products were visualized with ethidium bromide stained agarose gels, the specific bands were extracted from gels (kit: geneclean Turbo, Q-biogene, Carlsbad, USA) quantified and sequenced. A 460 bp fragment was sequenced for the V region and a 436 bp for the C region, which combined represent 32% of the total genome. In these two fragments, the complete V2 ORF was present, a 195 bp of the beginning of the V1 ORF, three quarter of the C4 ORF sequence (285 bp) and 460 bp of the C1 ORF.

The error rate of the polymerase used was 10-7. It will be compared to the frequency of mutation detected on the sequenced region of TYLCV isolates to determine if the error rate of the polymerase can be neglected in the sequence analysis.

Sequences obtained were aligned and analysed with DNAMAN software package version 5.2.2 (Lynnon BioSoft, Quebec, Canada).

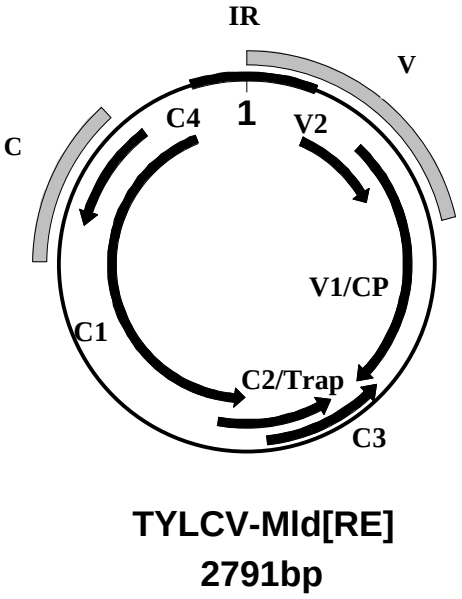


Fig. 3-2. Schematic representation of the genome organisation of *Tomato yellow leaf curl virus*-Mild isolate Réunion. Arrows show the open reading frames, grey boxes represent the genomic regions examined in this study.

The DNA partial sequences of the isolates were arranged so that the first nucleotide in the sequence corresponds to the first base (A) of virion strand replication (Laufs *et al.*, 1995).

Table 3-1. Number of samples analysed per year for the V1/V2 and C1/C4 region of the genome of the *Tomato yellow leaf curl*-Mild isolate Réunion.

	1997	1998	2000	2001	2002	2003	2004	Total
No of samples (MP/CP)	3	4	5	10	9	7	3	**41**
No of samples (C1/C4)	3	4	5	8	4	5	3	**34**

RESULTS

Genetic diversity of TYLCV isolates

As a result of surveys conducted during 1997 to 2004 in the main tomato growing area in the west of La Réunion (Fig 3-1), a total of 41 samples were obtained. All the collected samples presenting symptoms gave a positive amplification by PCR, the number of sequenced regions is presented in table 1. The diversity TYLCV isolates was analysed for regions V (IR, V1, V2) and C (C1, C4) (Fig. 3-2).

The use of PCR, followed by a direct step of sequencing allowed us to estimate the exact frequencies of mutations and follow each mutation over time on the different sequenced regions A or B. Two mutations were observedin more than one isolate (frequent mutations), whereas 34 mutations were observed in only one isolate each (rare mutations) (Fig. 3-3). The recombinant TYLCV strain previously described was not detected in any of our samples.

One of the two frequent mutations was at position 2181 (a T instead of a G) within the C1 and C4 ORFs. It is a non synonymous mutation in the C4 ORF but synonymous in the C1 ORF. This mutation detected for the first time in an isolate collected in 1998 and subsequently every year until the last sampling in 2004. It was detected with 15 samples out of the 34 samples analysed. This mutation was detected for the first time within an isolate collected near St Pierre in 1998 and was subsequently detected at least once in the following sampling locations: Petite île (2000, 2003), Saint Pierre (2000, 2002), Etang Salé (2001, 2002, 2003), Piton Saint Leu (2001), Saint Louis (2002, 2004), Saint Gilles (2003) (Fig. 3-1).

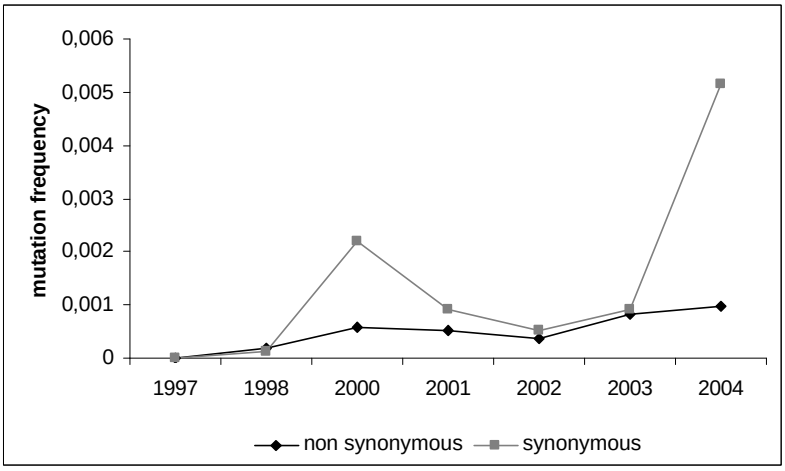


Fig. 3-3. Frequences of mutations of non synonymous and synonymous from 1997 to 2004.

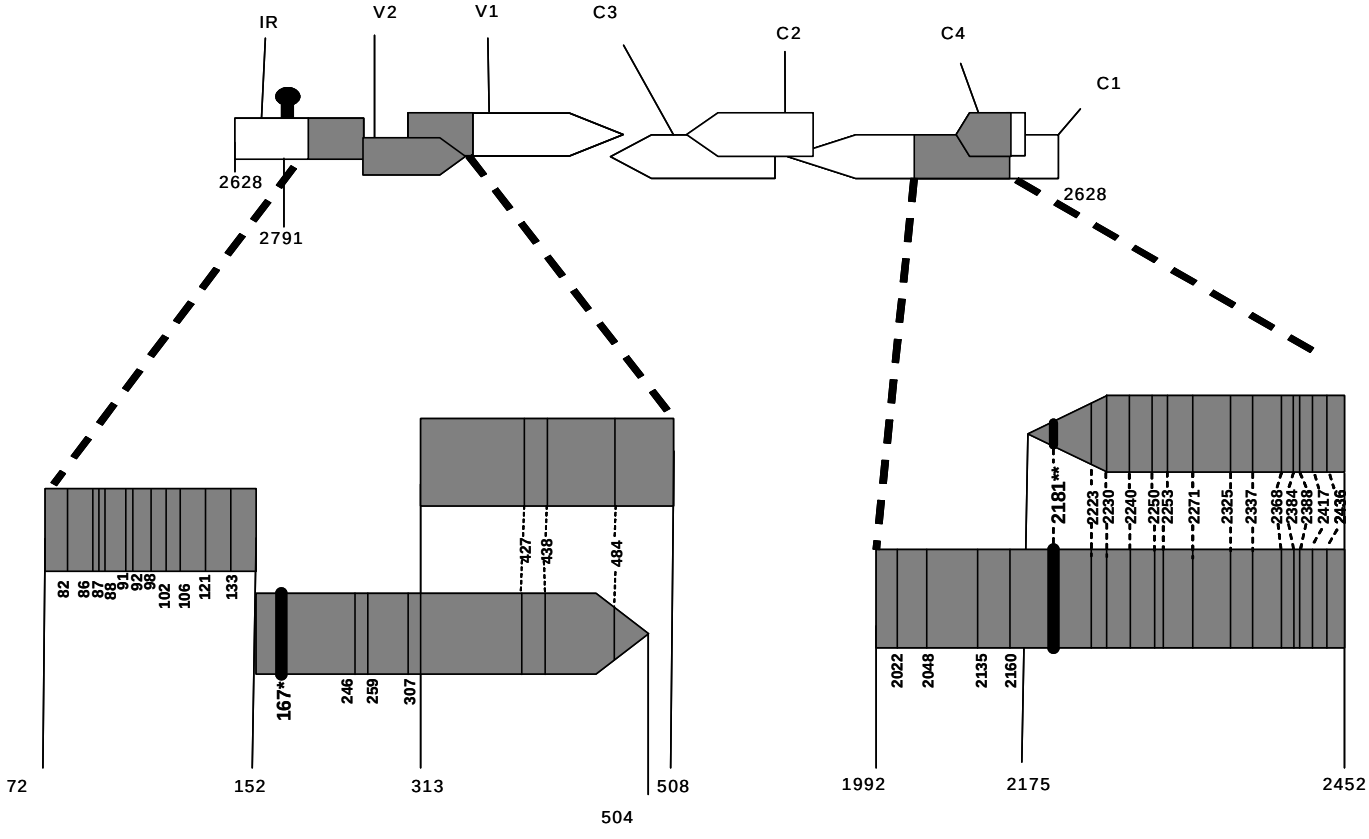


Fig. 3-4. The 2791 nucleotide sequence consensus of *Tomato yellow leaf curl virus* isolate La Réunion is represented with ORFs and intergenic region (IR). The grey parts of the figure are the sequenced regions with a zoom representing all the mutations. The thick bars represents the position of the 2 lasting mutations over the year, and the * represent the position of the non synonymous mutation which is sustained over the year. The ** represent the position of the synonymous mutation which is sustained over the year.

The other frequent mutation was at position 167 (T instead of a C) within V2 ORF. It is a synonymous mutation that was detected with 5 samples out of the 41 analysed) It was detected for the first time in 2001 and was still detected in 2004. Isolates carrying this mutation were collected in Etang Salé (2001, 2003), Petite île (2002), Bassin Martin (2002) and Saint Louis (2004). (Fig. 3-1).

No mutations were observed in the 3 sequences of 1997, and only 1 non synonymous and 1 synonymous mutation in 1998 over the 4 sequences.

Taking all the mutations together there are more synonymous mutations than non synonymous one (Fig. 3-4). We can also observe an increase of mutations in time, despite the low number of sequences in 2004, the highest frequencies of mutation for non synonymous and synonymous mutations were observed in 2004.

Table 3-2. Mutation frequency for the different open reading frames and the intergenic region (IR) and the global mutation frequency for all the sequences studied.

	IR	V2	V1	C1	C4	Global
Mutation frequency	33×10^{-4}	3.6×10^{-4}	6.7×10^{-4}	15×10^{-4}	21×10^{-4}	10×10^{-4}
Number of nucleotide sequenced	3360	8190	12784	15180	9141	33516

Distribution of mutations in C3, C4, V1, V2 ORFs and IR, of TYLCV isolates

The mutation frequency was calculated as: the number of mutations relative to the consensus, divided by the number of nucleotide sequenced. The highest frequency of mutations was found for the IR, C1 and C4 ORFs, with the lowest observed for V1 and V2 ORFs (table 3-2). The global frequency of mutation was of 1×10^{-3}.

The highest number of synonymous mutations was found in the C1 ORF with 18 mutations, and the highest number of non synonymous mutations was in the C4, with 17 mutations (table 3-3). Nevertheless, the number of non synonymous mutations was lower for the C1 ORF compare to the C4. The lowest number of non synonymous mutation was found in the V1 and V2 ORF. This suggests that relatively few nucleotide substitutions occurred in the V1 and V2 regions of the analysed population, and that a limited number of these resulted in amino acid replacements.

Table 3-3. Synonymous and non synonymous mutations for the different sequenced regions, isolate sampled region names (Fig. 3-1) and mutation positions (Fig. 3-4) are included. Acronyms were given for some of the sampled regions: Ravine des Cabris (RC), Piton St Leu (PSL) and St Pierre (SP).

	1997	1998	2000	2001	2002	2003	2004
No of samples (MP/CP)	3	4	5	10	9	7	3
No of samples (C1/C4)	3	4	5	8	4	5	3
IR	0	0	SP-1-106 RC 102 SP-2-88	St Gilles 98 SP-1-92	0	RC 91	St Louis-1-87 St Louis-1-102 Etang salé 82 Etang salé 121 St Louis-2-86 St Louis-2-133
V1 synonymous	0	0	0	0	0	RC 427	0
V1 non synonymous	0	0	0	0	0	RC 484	St Louis-1-438
V2 synonymous	0	0	SP-2-246	Etang Salé 167	Bassin Martin 167 Petit île 167 RC 259 Etang salé 307	Etang Salé 167 RC 427	St Louis-2-167 St Louis-1-438
V2 non synonymous	0	0	0	0	0	RC 484	0
C1 synonymous	0	SP-0-2181	SP-0-2181 Petite île 2181 SP-2-2325 SP-2-2337 SP-0-2388 RC 2384	Etang Salé 2181 PSL-1-2181 PSL -1-2436 PSL -2-2271 SP-1-2250 SP-1-2417	St Louis 2181 Etang Salé 2181 SP-1-2181 RC 2181 SP-1-2223	Petit île 2135 St Gilles 2181 Etang Salé-1-2181 Etang Salé-2-2181 RC 2181 Petit île 2181 St Gilles 2253	St Louis 2181 Etang salé 2022 Etang Salé 2160
C1 non synonymous	0	0	0	PSL-2-2240	0	RC 2368 Etang Salé-2-2368	Etang salé 2048 Etang salé 2230
C4 synonymous	0	0	SP-2-2325	SP-1-2250	0	0	Etang salé 2022 Etang Salé 2160
C4 non synonymous	0	SP-0-2181	SP-0-2181 Petite île 2181 SP-2-2337 SP-0-2388 RC 2384	Etang Salé 2181 PSL -1-2181 PSL -1-2436 PSL -2-2240 PSL -2-2271 SP-1-2417	St Louis 2181 Etang Salé 2181 SP-1-2181 RC 2181 SP-1-2223	St Gilles 2181 Etang Salé-1-2181 Etang Salé-2-2181 RC 2181 Petit île 2181 Petit île 2135 St Gilles 2253 RC 2368 Etang Salé-2-2368	St Louis 2181 Etang salé 2048 Etang salé 2230

DISCUSSION

Prior to 1997 few constrains over tomato production were observed in La Réunion. Since TYLCV-Mld [RE] was detected, up to 80% of yield losses were observed in open field area on susceptible cultivars (Reynaud *et al.*, 2003). A survey was realised since this date to monitor the disease incidence over the island. Indeed samplings from 1997 up to 2004 were closely studied here in order to evaluate the molecular evolution and mutation frequencies of this newly introduced virus.

Over the studied samples, few mutations were found at the beginning of the outbreak of the disease, showing a very low variability in the isolate sequences. This low genetic diversity found in the initial population is consistent with a founder effect associated with a population bottleneck during the introduction of this virus into Réunion. Since we only detected three consensus sequences with few point mutations, which appeared later than 1998 we have no evidence of the introduction of two strains of TYLCV.

The global mutation frequency of 10×10^{-4} obtained was similar to the one observed by Isnard *et al.*, (1998) for the mastrevirus *Maize streak virus*. Therefore, these results confirmed that begomoviruses and mastreviruses of the geminivirus family have an equivalent mutation frequency, which are in the same range as those obtained for RNA plant viruses. Indeed, several mutation frequencies of plant RNA viruses were studied: a 10.2×10^{-4} was observed for tobacco mosaic virus (TMV) (Rodriguez-Cerezo & Garcia-Arenal, 1989), a 11.3×10^{-4} was observed for satellite of TMV (Kurath *et al.*, 1992) and a $3.9\text{-}22 \times 10^{-4}$ was observed for foreign sequences replicating in TMV (Kearney *et al.*, 1993). Although geminiviruses are replicated by host DNA polymerase(s) which are presumed to have a high fidelity of replication, their mutation frequency is in the same range as viruses which encode their own polymerases. As proposed by Sanz et *al.*, (1999), the high mutation frequency of geminiviruses may be explained by a lack if postreplication repair.

It is not known if these two mutations established because of drift or due to a positive selection. Further tests comparing the fitness of isogenic clones differeing only by the two lasting mutations observed, are needed to know how these mutations have established in Réunion. More exhaustive samplings should also be done to confirm the lasting of these two different genotypes over time. When those sequences comprising the macroheterogeneity sites were blasted in Genbank, no other sequences presented these mutated sites. This emphasised the hypothesis of bottleneck population which has evolve in isolation.

The IR and the C4 ORF had the highest umber and frequency of mutation whereas the V1, C1 and V2 ORFs seemed less variable, with the highest number of synonymous mutations for the C1. These results are consistent with the literature, knowing that the C1, is the only ORF coding for the replicase (Desbiez *et al.*, 1995) and V1 ORF for the infection process in cell to cell movement. Therefore, non synonymous mutations in the different sequenced parts tend to accumulate at position where no structural or functional motifs are found and needed for virus replication or movement.

The mutations observed on La Réunion isolates of TYLCV-Mld[RE] are consistent with the results obtained by Sanchez-Campo et al., (Sanchez-Campos *et al.*, 2002) on TYLCSV which are in favour of the theory that begomoviruses might be evolving due to recombination events (Hou & Gilbertson, 1996; Padidam *et al.*, 1999; Pita *et al.*, 2001b). However, an important number of mutations was obtained in la Réunion on much fewer sequences in sampling sites very close to each other. This might be due to the close environment of the island and its smaller area, but also as this population result from a bottleneck, it is in the process of stabilisation.

Being in an insular environment, with close watch on imported material, with no other islands of the region having the TYLCV, it allowed us to have a more reliable evaluation of the mutation frequency. Other studies which had been carried on begomoviruses had also indicated that in absence of recombination with other strains or species, the level of intra-strain variability in the geminiviruses was limited, with some diversity recorded (Gilbertson *et al.*, 1991; Stenger & McMahon, 1997; Faria & Maxwell, 1999). Nevertheless these field isolates diversity reported might be due to that described begomovirus were present for a longer time or were even indigenous in the sampled area.

Unfortunately, despite all the measures employed to prevent new virus introduction in La Réunion, they are still possible. In April 2004 a new strain of TYLCV was recoded in Saint Gilles, in an open field tomato production area which is not too far from the port and the airport (Delatte *et al.*, 2005a). The overlapping, or recombination or disappearing or even *statu quo* of these two new strains of TYLCV should be further studied in La Réunion over time, in carrying on the monitoring over the island. Furthermore, to continue the monitoring in La Réunion will allow us to detect any other introductions of begomoviruses.

CHAPTER 4

South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group[1]

ABSTRACT

Biological and molecular properties of Tomato leaf curl Madagascar virus isolates from Morondova and Toliary (ToLCMGV-[Tol], -[Mor]), Tomato leaf curl Mayotte virus isolates from Dembeni and Kahani (ToLCYTV-[Dem], -[Kah]) and a Tomato yellow leaf curl virus isolate from Réunion (TYLCV-Mld[RE]) were determined. Full-length DNA components of the five isolates from Madagascar, Mayotte, and Réunion were cloned and sequenced and, with the exception of ToLCMG-[Tol], were shown to be both infectious in tomato and transmissible by *Bemisia tabaci*. Sequence analysis revealed that these viruses had genome organisations of monopartite begomoviruses and that both ToLCMGV and ToLCYTV belong to the African begomovirus but represent a distinct monophyletic group that we have tentatively named the South West islands of the Indian Ocean (SWIO). All of the SWIO isolates examined are 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.

[1]Delatte, H., Martin, D. P., Naze, F., Golbach, R. W., Reynaud, B., Peterschmitt, M. & Lett, J. M. (2005). South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. *Journal of General Virolog*, **86**, 1533-1542.

INTRODUCTION

The genus *Begomovirus* contains dicotyledonous infecting whitefly-transmitted viruses in the family *Geminiviridae*. Most described begomoviruses have bipartite genomes encapsidated as circular single stranded DNA (ssDNA) molecules within twin icosahedral (or geminate) particles. Whereas bipartite begomoviruses usually require both a DNA A and DNA B component to produce symptomatic infections, monopartite begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV), require only a DNA A-like component for infectivity (Navot *et al.*, 1991).

TYLCV is an important tomato pathogen that, following its emergence from the Mediterranean Basin in recent years (Lett *et al.*, 2004) is progressively spreading throughout the world (Cohen & Antignus, 1994; Pico *et al.*, 1996; Czosnek & Laterrot, 1997; Polston *et al.*, 1999; Moriones & NavasCastillo, 2000). In 1997, a severe outbreak of tomato yellow leaf curl disease occurred on Réunion, one of the South West islands of the Indian Ocean (SWIO). Yield losses reached 85% the first year of the epidemic on the most susceptible cultivars (Reynaud *et al.*, 2003) and the disease has become the primary factor limiting both open field and protected greenhouse tomato production on the island. No begomoviruses had been detected in Réunion tomatoes prior to 1997 and it has now been determined that two strains of an exotic virus, the “Israel” and the “Mild” strains of TYLCV, are the causal agents of the disease (Peterschmitt *et al.*, 1999b). There is precedent for human spread of TYLCV into new habitats, i. e. the Carribean and Florida (Polston et al., 1999), and the finding that whiteflies can acquire the virus from fruits demonstrates yet another route of potential dissemination (Delatte *et al.*, 2003a). The influx of exotic viruses onto SWIO is also not restricted to tomato begomoviruses. Other begomovirus of cassava such as the *African cassava mosaic virus* (ACMV) (Fauquet & Fargette, 1990), *East African cassava mosaic virus* (EACMV) (Swanson, 1994) and *South African cassava mosaic virus* (SACMV) (Berrie *et al.*, 2001) have also been detected in Madagascar (Ranomenjanahary, 2002).

In 2001, a tomato virus symptom survey on the islands of Madagascar and Mayotte identified both the association of the begomovirus vector species, *Bemisia tabaci*, with tomato plants and the presence of plants displaying leaf curling and plant stunting symptoms characteristic of begomoviruses. Analysis of partial viral genome fragments isolated from leaf samples collected during this survey indicated the presence of two potentially new *Begomovirus* species (Delatte *et al.*, 2003b; Lett *et al.*, 2004).

In this study we report the construction of agroinfectious viral clones, symptom evaluation, whitefly transmission tests, and analysis of the full length DNA sequences of TYLCV-Mld[RE] and two isolates from two new monopartite begomovirus species. The new species, tentatively named Tomato leaf curl Madagascar virus (ToLCMGV) and Tomato leaf curl Mayotte virus (ToLCYTV), belong to the African begomoviruses but represent a distinctly unique monophyletic group that we refer to as the SWIO group. We report that the SWIO isolates appear to have been actively recombining amongst themselves.

METHODS

Plant material

Agroinoculation and transmission experiments were carried out on the TYLCV susceptible tomato (*Lycopersicon esculentum*) genotype, Farmer (Know You Seed) in a growth chamber maintained at 25 °C with a 12/12 h photoperiod.

Sampling and DNA extraction

Tomato leaves presenting leaf-curling symptoms were collected from individual plants in Saint Pierre (Réunion), Morondova and Toliary (Madagascar), and Dembeni and Kahani (Mayotte). The leaves were preserved by dehydratation with calcium chloride ($CaCl_2$) (Bos, 1977). Total DNA was extracted from dried samples using the DNeasy Plant Mini Prep kit (Qiagen, Paris, France) according to the manufacturer's instructions.

Table 4-1. PCR Primers used in this study

Primer names	Primer sequences	Expected size
DNA-A (detection)		
VD360	5'-AGRCTGAACTTCGACAGC-3'	906 nt
CD1266	5'-TCTCAACTTCARGGTCTG-3'	
V196	5'-CGGATTTCGTTGTATGTTAGC-3'	804 nt
C1000	5'-AAGGGGTTTTCAGTATGGTT-3'	
AV494	5'-GCCYATRTAYAGRAAGCCMAG-3'	552 nt
AC1048	5'-GGRTTDGARGCATGHGTACAT-3'	
(Wyatt & Brown, 1996)		
MP16	5'-CCTCTAGATAATATTACCKRWKGRCC-3'	480 nt
MP82	5'-CGGAATTCYTGNACYTTRCANGGNCCYTCRCA-3'	
(Umaharan *et al.*, 1998)		
DNA-A (cloning)*		
RéunionV160	5'-GCACAGGATCCACTTCTAAATGAATTTCC-3';	Full sequence
RéunionC164	5'-TCGTAGGATCCCACATATTGCAAGACAAAC-3'	
MadagascarV148Q	5'-GGTGTGGATCCATTGTTAAATGAGTTCCC-3'	Full sequence
MadagascarC153Q	5'-GTGCGGGATCCCACATTGTGACAGGCCC-3'	
DembeniVQ	5'-TAGTGAAGCTTAGATAATCGTTTTTGTC-3	Full sequence
DembeniCQ	5'-CGCAGAAGCTTTGACGCGCGATTCTTATTG-3'	
KahaniVQ	5'-AAGAGAAGCTTAGATAATTGTTTTTGTC-3'	Full sequence
KahaniCQ	5'-CGCAGAAGCTTTGACGCGCGATTCTGATTG-3'	
DNA-B (detection)		
PBL1v2040	5'-GCCTCTGCAGCARTGRTCKATCTTCATACA-3'	600 nt
PCRc1	5'-CTAGCTGCAGCATATTTACRARWATGCCA-3'	
(Rojas *et al.*, 1993b)		
DNA-Beta (detection)		
Beta 01	5'-GGTACCACTACGCTACGCAGCAGCC-3'	600-700 nt and 1350 nt
Beta 02	5'-GGTACCTACCCTCCCAGGGGTACAC-3'	
(Briddon *et al.*, 2002)		

*Cloning sites are underlined

PCR detection

A first polymerase chain reaction (PCR) was used to amplify two fragments from the extracted DNA of all samples using two degenerate primer sets: MP16-MP82 (Umaharan *et al.*, 1998) and AV494-AC1048 (Wyatt & Brown, 1996). A less degenerate primer set was designed from previously obtained SWIO begomovirus sequences and used to amplify 904 nts of the core region of the coat protein (CP) gene of DNA (VD360-CD1266; Table 4-1; (Brown *et al.*, 2001)). PCR reactions were carried out in 25 µl volumes with the following programme: a first cycle of 5 min at 94 °C, then 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final cycle at 72 °C for 5 min. The presence/absence of a DNA B genome component was also determined for each of the isolates using the PCR primers: PBL1v2040 and PCRc1 (Rojas *et al.*, 1993a); Table 4-1). The presence/absence of DNA β molecules was determined for each of the isolates using the primers Beta 1 and Beta 2 ((Briddon *et al.*, 2002); Table 4-1).

Cloning strategies

Abutting primers with non-homologous tails were designed for each isolate in order to obtain a full length DNA product (Patel *et al.*, 1993). Abutting primers were designed over a *Bam*HI restriction site for samples from Réunion (RéunionV160-RéunionC164; Table 4-1), and Madagascar (MadagascarV148Q-MadagascarC153Q; Table 4-1). For Mayotte samples, abutting primers were designed over the *Hind*III restriction site (Dembeni: DembeniVQ-DembeniCQ; Kahani: KahaniVQ-KahaniCQ; Table 1). The PCR conditions used were: 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final elongation of 10 min at 72 °C. Amplicons of approximately full genome size (~2800 bp) were isolated using the Geneclean Turbo kit (Qbiogene, Paris, France) from 1 % agarose gels and cloned using the pGEM-T Easy Vector System (Promega, Lyon, France). The complete DNA components of five clones corresponding to the isolates from Dembeni, Kahani, Toliary, Morondova and Réunion were sequenced by gene walking by Sequentia (Clermont Ferrand, France).

Agroinoculation

While the infectivity of the cloned DNA components of isolates from Réunion and Madagascar were tested using full head-to-tail DNA dimers (constructed at *Bam*HI restriction sites), partial DNA head-to-tail dimers were constructed (at *Hind* III restriction sites) for the viruses from Mayotte. Both full and partial dimers were inserted into the binary vector, pCAMBIA 2300 (Cambia, Australia). Recombinant plasmids were mobilised from *Escherichia coli* JM-109 cells (Promega, WI, USA) into *Agrobacterium tumefaciens* (strain C58) using triparental mating using *E. coli* HMB101 containing the plasmid helper pRK 2013 (Ditta, 1980). The identity of all clones was verified by restriction endonuclease analysis. Ten day old susceptible tomato seedlings were agro-inoculated with the five constructs using a needle (Paximadis & Rey, 2001) and infection symptoms were evaluated between fifteen and twenty days post-inoculation. All the agro-inoculated plants showing symptoms were tested for the presence of viral DNA using either specific degenerate

primers designed to amplify the isolates from the two new species (V360-CD1266; Table 4-1) or a specific non-degenerate primer designed to amplify TYLCV DNA (V196-C1000; Table 4-1).

Transmission tests

B. tabaci transmissibility of the viruses was evaluated by determining whether whiteflies could successfully transmit them from symptomatic PCR positive agroinoculated plants to healthy tomato plants. *B. tabaci* adults used for the transmission tests were from a cabbage reared B biotype population initiated from whiteflies initially collected on cabbage in Réunion (Delatte *et al.*, 2005c). In each transmission test fifteen whiteflies were permitted a 3-day acquisition access period (AAP) on PCR positive symptomatic agroinoculated tomato plants. These insects were then transferred onto healthy tomato plants and allowed an inoculation access period (IAP) of three days. Twenty one days following the IAP, symptoms were evaluated and symptomatic plants tested for the presence of DNA by PCR, using the specific primers described above (V360-CD1266; V196-C1000; Table 4-1).

Sequence analysis

The full DNA sequences of the five isolates were arranged that the first nucleotide in the sequence corresponds to the first base (A) of virion strand replication (Laufs *et al.*, 1995). Potential open reading frames in each of the isolate sequences were identified using DNAMAN (version 5.2.2, Lynnon Biosoft, Quebec, Canada). Full DNA A-like and A sequences of related viruses used in phylogenetic analyses were obtained from public sequence databases using BLASTN (NCBI-URL). Two outgroup sequences were used during phylogenetic analyses, an isolate from Australia of the monopartite species *Tomato leaf curl virus* isolate (S53251; (Stonor *et al.*, 2003)) and an isolate from Florida of the bipartite species *Tomato mottle virus* isolate from Florida (NC001938; (Polston *et al.*, 1993). Multiple sequence alignments were performed using the optimal alignment method of DNAMAN. Phylogenetic trees were generated using PHYLIP (Felsenstein, 1989) using the neighbour joining method or the Jukes Cantor corrected distances, 2000 bootstrap replicates were performed.

Detection of potential recombinant sequences, identification of likely parental sequences, and localization of possible recombination breakpoints in multiple sequence alignments was carried out using the RDP (Martin, 2000), GENECONV (Padidam *et al.*, 1999), MAXIMUM χ^2 (Maynard, 1992), CHIMAERA (Martin, 2004), RECSCAN (Martin, 2005) and SISTER SCAN (Gibbs, 2000) methods as implemented in RDP2 (Martin, 2004). The analysis was performed with default settings for the different detection methods and a Bonferroni corrected *P*-value cutoff of 0·05.

RESULTS

Cloning and infectivity of isolates

While PCR amplification and cloning of apparently full length DNA A-like components was possible from all symptomatic leaf samples, DNA B and DNA β specific PCR yielded no amplification products for any of the examined leaf samples. TYLCV susceptible tomato plants developed symptoms typical of those observed in the field, when agroinfected with cloned DNA of all isolates except for ToLCMGV-[Tol] which was non-infectious (Fig. 4-1). In all cases the presence of viral DNA could be confirmed in all symptomatic plants by PCR. Relative to healthy controls, symptomatic plants agroinoculated with ToLCYTV-[Dem] and -[Kah], and ToLCMGV-[Mor] had a stunted bushy appearance with severely shortened rachis, curled petioles and curled leaves (Fig. 4-1). Plants agroinoculated with TYLCV-Mld[RE] were stunted with yellow, curled leaves. All of the isolates that produced symptoms in agroinoculated tomato could also be transmitted by whiteflies into TYLCV susceptible healthy tomato plants. Again, symptoms in the whitefly-inoculated plants resembled those observed in the field for the different isolates and could be confirmed by PCR detection of the viral genome. The ability of cloned DNA components to cause symptomatic infections of tomato resembling those observed in the field, coupled with our inability to confirm the presence of either DNA B or DNA β in field samples, indicated that TYLCV-Mld[RE], ToLCYTV-[Dem], ToLCYTV-[Kah], and ToLCMGV-[Mor] most likely have monopartite genomes.

Fig. 4-1. Symptoms twenty days after agroinoculation of the TYLCV susceptible tomato genotype, Farmer, with the various begomoviruses characterised in this study. Numbers 1, 2, 3 and 4 correspond respectively to a non inoculated control (1), ToLCYTV-[Kah] (2), ToLCMGV-[Mor] (3), and TYLCV-Mld[RE] (4).

Table 4-2. GenBank and EMBL accession numbers of complete Begomovirus DNA A-like and A sequences used in this study.

Begomovirus Name	***Acronym***	***Accession No***
Tomato leaf curl Mayotte virus - [Dembeni]*	ToLCYTV-[Dem]	AJ865341
Tomato leaf curl Mayotte virus - [Kahani]*	ToLCYTV-[Kah]	AJ865340
Tomato leaf curl Madagascar virus - [Morondova]*	ToLCMGV-[Mor]	AJ865338
Tomato leaf curl Madagascar virus - [Toliary]*	ToLCMGV-[Tol]	AJ865339
Tomato yellow leaf curl virus - Mild [Réunion]	TYLCV-Mld-[RE]	AJ865337
South African cassava mosaic virus	SACMV	AF155806
South African cassava mosaic virus - M12	SACMV-[M12]	AJ422132
Tomato yellow leaf curl Sardinia virus	TYLCSV	X61153
Tomato yellow leaf curl virus - Mild	TYLCV-Mld	X76319
Tomato yellow leaf curl virus	TYLCV	X15656
Tomato leaf curl virus	ToLCV	S53251
African cassava mosaic virus - [Kenya]	ACMV-[KE]	J02057
African cassava mosaic virus - Uganda Severe	ACMV-[UGSvr]	AF26806
East African cassava mosaic virus - [Tanzania]	EACMV-[TZ]	Z83256
Tobacco leaf curl Zimbabwe virus	TbLCZV	AF350330
Tomato yellow leaf curl Malaga virus	TYLCMalV	AF271234
Tomato yellow leaf curl Sudan virus - [Gezira]	ToLCSDV-[Gez]	AY044137
Tomato mottle virus - [Florida]	ToMoV-[FL]	NC001938
African cassava mosaic virus - [Cameroon-DO2]	ACMV-[CM/DO2]	AF366902
African cassava mosaic virus - [Ivory coast]	ACMV-[CI]	AF259894
African cassava mosaic virus - [Nigeria]	ACMV-[NG]	X17095
African cassava mosaic virus - [Nigeria-Ogo]	ACMV-[NG-Ogo]	AJ427910
African cassava mosaic virus - Uganda Mild	ACMV-UGMld	AF126800
African cassava mosaic virus - Uganda Severe	ACMV-UGSvr	AF126803
East African cassava mosaic virus - Uganda2 Mild	EACMV-UG2Mld	AF126804
East African cassava mosaic virus - Uganda2 Severe	EACMV-UG2Svr	AF126806
East African cassava mosaic Zanzibar virus	EACMZV	AF422174
East African cassava mosaic virus - [Kenya]	EACMV-[KE]	AJ516003
East African cassava mosaic virus - [Malawi]	EACMV-[MW]	AJ006461
East African cassava Malawi mosaic virus - [K]	EACMMV-[K]	AJ006460
Ageratum yellow vein virus	AYVV	X74516
Bhendi yellow vein mosaic virus - [301]	BYVMV-[301]	AJ002453
Ageratum yellow vein virus	AYVV	X74516
Bhendi yellow vein mosaic virus - [Madurai]	BYVMV-[Mad]	AF241479
Cotton leaf curl Gezira virus - [Sida]	CLCuGV-[Sida]	AY036007
Eupatorium yellow vein virus - [MNS2]	EpYVV-[MNS2]	AJ438936
Indian cassava mosaic virus	ICMV	Z24758
Okra yellow vein mosaic virus - [201]	OYVMV-[201]	AJ002451
Soybean crinkle leaf virus - [Japan]	SCLV-[JA]	AB050781
Tobacco curly shoot virus - [Y41]	TbCSV-[Y41]	AJ457986
Tomato leaf curl Gujarat virus - [Vadodara]	ToLCGV-[Vad]	AF413671
Tomato leaf curl Malaysia virus	ToLCMV	AF327436
Tomato leaf curl Laos virus	ToLCLV	AF195782
Tomato yellow leaf curl China virus	TYLCCNV	AF311734
Tomato yellow leaf curl virus - [Gezira]	TYLCV-[Gez]	AY044138
Tomato yellow leaf curl Sudan virus - [Shambat]	ToLCSDV-[Sha]	AY044139
Tomato yellow leaf curl Sardinia virus - [Sicily]	TYLCSV-[Sic]	Z28390
Tomato yellow leaf curl Sardinia virus - [Spain1]	TYLCSV-[ES1]	Z25751
Tomato yellow leaf curl Sardinia virus - [Spain2]	TYLCSV-[ES2]	L27708
Tomato yellow leaf curl Thailand virus - [Y72]	TYLCTHV-[Y72]	AJ495812
Tomato yellow leaf curl virus - [Aichi]	TYLCV-[Aic]	AB014347
Tomato yellow leaf curl virus - [Almeria]	TYLCV-[Alm]	AJ489258
Tomato yellow leaf curl virus - [Cuba]	TYLCV-[CU]	AJ223505
Tomato yellow leaf curl virus - [Dominican Republic]	TYLCV-[DO]	AF024715
Tomato yellow leaf curl virus - [Iran]	YLCV-[IR]	AJ132711
Tomato yellow leaf curl virus - [Portugal]	TYLCV-[PT]	AF105975
Tomato yellow leaf curl virus - [Puerto Rico]	TYLCV-[PR]	AY134494
Tomato yellow leaf curl virus - [Shizuokua]	TYLCV-[Shi]	AB014346
Tomato yellow leaf curl virus - [Spain7297]	TYLCV-[ES7297]	AF071228
Tomato yellow leaf curl Thailand virus - [1]	TYLCTHV-[1]	X63015
Watermelon chlorotic stunt virus	WmCSV	AJ012081
Watermelon chlorotic stunt virus - [IR]	WmCSV-[IR]	AJ245652

*Begomoviruses are named according to the ICTV guidelines (Fauquet *et al.*, 2003)

Genome organisation and molecular comparison with other begomoviruses

The lengths of the complete TYLCV-Mld[RE], ToLCMGV-[Mor], ToLCMGV-[Tol], and ToLCYTV-[Dem], and ToLCYTV-[Kah] DNA sequences are 2791, 2777, 2775, 2765 and 2768 nucleotides, respectively. The organisation of inferred genes and intergenic regions for all five viruses is typical of that observed in begomoviruses which characteristically have two virion senses and four complementary senses open reading frames (ORFs).

We detected three anomalies in the nucleotide sequence of ToLCMGV-[Tol] that may explain lack of infectivity of its clone. The first, and potentially most serious, is a single nucleotide frame-shift mutation near the beginning of the V2 ORF. The other anomalies were two unusual termination codons in the C4 ORF. For purposes of comparing the putative ToLCMGV-[Tol] V2 and C4 amino acid sequences with those of other viruses in (Tables 4-2 and 4-3) we "corrected" the sequence by inserting a T nucleotide at position 282, and changing an A at position 2163 and an A at position 2376 to a G and a C respectively.

	ToLCMGV-[Tol]	ToLCMGV-[Mor]	ToLCYTV-[Dem]	ToLCYTV-[Kah]	TYLCV-Mld[RE]	TYLCV-Mld	TYLCV	TYLCSV	TYLCMalV	ToLCSDV-[Gez]	ToLCV	EACMV-[TZ]	EACMZV	SACMV-[M12]	SACMV-[ZA]	ACMV-[KE]	ACMV-UGSvr	TbLCZWV	ToMoV-[FL]
ToLCMGV-[Tol]	**																		
ToLCMGV-[Mor]	94	**																	
ToLCYTV-[Dem]	**86**	**85**	**																
ToLCYTV-[Kah]	**82**	**83**	90	**															
TYLCV-Mld[RE]	**81**	**82**	**81**	**80**	**														
TYLCV-Mld	**81**	**82**	**81**	**80**	98	**													
TYLCV	**80**	**81**	79	78	92	93	**												
TYLCSV	**80**	79	**80**	76	78	78	77	**											
TYLCMalV	**80**	**81**	**80**	79	91	90	**85**	**83**	**										
ToLCSDV-[Gez]	79	**80**	78	77	86	86	84	76	**84**	**									
ToLCV	75	75	76	75	74	74	74	73	74	74	**								
EACMV-[TZ]	76	74	76	74	72	72	72	73	72	70	69	**							
EACMZV	78	78	78	77	76	75	75	74	76	74	71	**86**	**						
SACMV-[M12]	**83**	**81**	**83**	79	79	78	77	78	78	76	72	**80**	**82**	**					
SACMV	**83**	**81**	**82**	79	79	79	78	78	78	76	73	**80**	**82**	93	**				
ACMV-[KE]	75	76	76	75	74	74	72	71	73	73	70	70	73	74	74	**			
ACMV-UGSvr	76	76	76	75	74	74	73	71	73	73	70	70	73	74	74	97	**		
TbLCZWV	76	77	76	75	77	77	75	75	77	75	73	71	74	74	75	71	71	**	
ToMoV-[FL]	65	66	64	64	64	64	64	64	64	65	64	62	65	63	63	61	61	64	**

Fig. 4-2. Matrix of pairwise identity percentages of A component sequences of 19 begomoviruses. The matrix was generated using the full optimal alignment method and the observed divergency distance method options of DNAMAN software (Lynnon, Biosoft). Abbreviations and accession numbers of viruses are provided in table 4-2. Percentages of identity above 90% are underlined, percentages of identity between 80 and 90% are in bold.

BLAST searches with the entire sequences of ToLCMGV-[Mor], -[Tol] and ToLCYTV-[Dem], -[Kah] indicated that these isolates were most closely related to *Tomato yellow leaf curl virus* (TYLCV), *South African cassava mosaic virus* (SACMV) and *East African cassava mosaic virus* (EACMV). As expected, a BLAST search with the entire sequence of TYLCV-Mld[RE] indicated that it was very closely related to TYLCV-Mld. Accordingly, seven African and five

Mediterranean begomovirus sequences were chosen for detailed comparison with the five sequences described in this study (Table 4-3, Fig. 4-2).

ToLCMGV-[Mor] and ToLCMGV-[Tol], the two isolates from Madagascar, share 94% genome sequence identity. ToLCYTV-[Kah] and ToLCYTV-[Dem], the two isolates from Mayotte, share 90% identity which is close to the 89% taxonomic threshold commonly used for begomovirus species distinction (Fauquet *et al.*, 2003). TYLCV-Mld[RE] is clearly a member of the Mediterranean and African tomato begomoviruses with its genome sequence sharing 98% identity with that of TYLCV-Mld (Fig. 4-3).

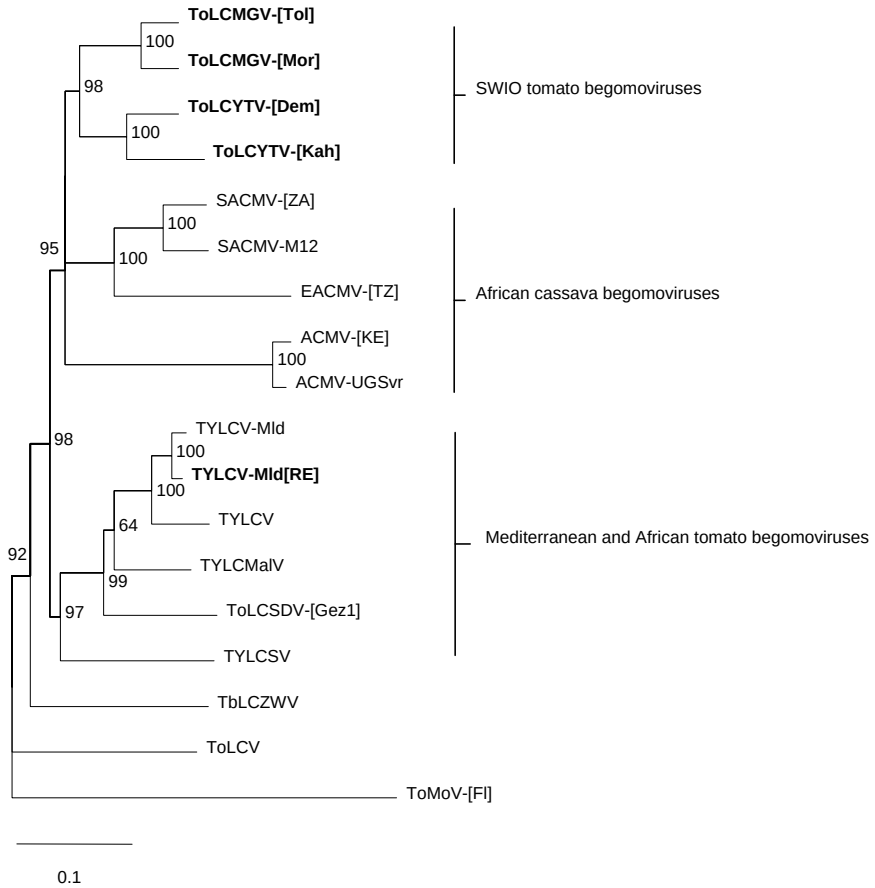


Fig. 4-3. Neighbour joining tree indicating the phylogenetic relationships between the DNA sequences of ToLCMGV, ToLCYTV and TYLCV-Mld[RE] isolates and those of a representative sampling of publicly available African and Mediterranean begomovirus sequences. The tree was constructed using Jukes-Cantor corrected distances and rooted using ToMoV-[FL] as an outlyer. Numbers associated with nodes indicate the percentage support for those nodes in 2000 bootstrap replicates. Whereas horizontal distances represent genetic distances as indicated by the scale bar, vertical distances are arbitrary. Abbreviations and accession numbers of viruses are provided in table 4-1.

Table 4-3. Percentage nucleotide and deduced amino acid sequence identities shared between full DNA-A, ORF and intergenic region sequences of either **A** Tomato leaf curl Mayotte virus isolate Dembeni (ToLCYTV-[Dem]), or **B**

Tomato leaf curl Madagascar virus isolate Morondova (ToLCMGV-[Mor]), and those of selected begomoviruses originating from Africa or, in the case of *Tomato leaf curl virus* (ToLCV) and *Tomato mottle virus* (ToMoV-[Fl]), from Australia and the USA respectively. Abbreviations and accession numbers of viruses are provided in table 4-2.

ToLCYTV-[Dem]

A	DNA-A	CP/V1		MP/V2		C1		C2		C3		C4		IR
	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt
ToLCYTV-[Kah]	90	97	99	95	97	86	87	95	90	93	90	88	75	77
ToLCMGV-[Mor]	86	86	91	88	89	87	90	85	79	87	81	90	79	66
ToLCMGV-[Tol]	85	86	90	88	89	87	90	85	78	86	80	91	82	77
SACMV	82	84	90	86	85	84	88	83	73	83	75	86	75	75
SACMV-[M12]	83	86	91	86	83	83	88	83	73	83	77	87	75	75
TYLCSV	80	81	90	82	84	82	85	77	67	76	64	85	69	72
TYLCV-Mld	81	86	92	84	84	83	84	83	73	82	75	86	69	57
TYLCV-Mld-[RE]	81	86	92	84	85	83	84	83	71	83	77	85	69	57
TYLCV	79	86	90	84	85	79	79	82	71	82	75	74	46	54
TYLCMalV	80	81	88	82	83	83	85	86	71	83	78	85	68	59
ToLCSDV-[Gez]	78	74	76	85	85	84	86	83	73	82	72	87	72	61
ToLCV	76	76	78	74	64	80	83	74	65	75	67	84	67	68
ACMV-[KE]	76	78	83	78	74	77	79	80	69	79	70	80	56	57
TbLCZV	76	76	83	85	81	78	78	78	67	77	70	81	60	58
EACMV-[TZ]	76	81	89	71	65	73	77	82	72	82	77	66	37	74
ACMV-[UGSvr]	75	80	85	71	64	74	76	83	75	82	75	67	42	70

ToLCMGV- [Mor]

B	DNA-A	CP/V1		MP/V2		C1		C2		C3		C4		IR
	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt
ToLCMGV-[Tol]	94	99	100	99	87	92	95	99	99	98	96	90	77	79
ToLCYTV-[Dem]	86	86	91	88	89	87	90	85	79	87	81	90	79	66
ToLCMYV-[Kah]	83	87	91	87	89	85	86	85	79	85	81	91	82	62
SACMV	81	84	89	87	89	82	87	85	77	84	81	83	65	63
SACMV-[M12]	81	84	88	86	85	81	86	86	77	85	82	83	66	66
TYLCSV	79	82	88	84	87	81	84	77	68	77	64	84	66	62
TYLCV-Mld	82	84	88	88	89	84	86	85	80	83	79	87	70	61
TYLCV-Mld-[RE]	82	84	88	87	88	84	86	85	79	84	81	87	71	61
TYLCV	81	84	87	88	89	81	82	85	79	83	78	78	52	60
TYLCMalV	81	81	87	85	86	84	86	85	79	85	82	87	70	64
ToLCSDV-[Gez]	80	76	77	88	87	84	88	83	79	84	78	88	72	63
ToLCV	75	74	75	71	62	80	83	73	65	75	68	87	72	64
ACMV-[KE]	76	78	83	79	74	76	79	81	71	81	73	77	54	58
TbLCZV	77	75	81	89	89	78	80	79	67	80	73	82	60	64
EACMV-[TZ]	74	80	89	71	64	72	76	85	75	84	82	70	41	64
ACMV-[UGSvr]	76	81	87	71	62	73	76	86	76	84	82	70	42	65

Phylogenetic analyis of the sequences determined in this study and all other publicly available full length African and Mediterranean isolate sequences indicated that ToLCMGV-[Mor], -[Tol] and ToLCYTV-[Dem], -[Kah] formed a distinct monophyletic sub-group within the African group that we have named the South West islands of the Indian Ocean (SWIO) (Fig. 4-3). The highest nucleotide identity of DNA detected between isolates of ToLCMGV and ToLCYTV, was 86 % when comparing ToLCMG-[Mor] and ToLCYTV-[Dem]. The greatest degree of genome-wide sequence identity shared by ToLCMGV and ToLCYTV isolates with other currently described full length sequences were 82% for ToLCMGV-[Mor] with TYLCV-Mld and 83% for ToLCYTV-[Dem] with SACMV (Fig. 4-2). We therefore propose that, according to the ICTV criteria for begomovirus species demarcation using DNA complete sequence (Fauquet *et al.*, 2003; Fauquet & Stanley, 2003), both ToLCMGV and ToLCYTV be considered new species as their nucleotide identities with other begomovirus are below 89%.

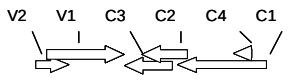
Analysis of recombination

We analysed a 62-sequence alignment of full length SWIO (Table 4-2), African and Mediterranean begomovirus DNA sequences for evidence that the SWIO isolates had undergone recombination. We initially screened the alignment looking for all evidence of recombination involving the SWIO isolates either as potential recombinants (i.e. as acceptors of sequence) or as parental donors of sequence in non-SWIO recombinants. Six different detection methods identified an enormous amount of evidence for recombination involving the SWIO sequences as either donors or acceptors of sequences (at least 130 unique events identified by RDP2). We analysed each of the identified events individually and used a phylogenetic approach to verify the parental/donor identifications made by RDP2. This involved construction and comparison of bootstrapped neighbour joining trees from the two portions of the alignment corresponding to regions of potential recombinants originating from different parental sequences. Wherever there was good phylogenetic evidence that an inferred recombination event involved a SWIO isolate as a donor sequence (i.e. there was little or no evidence that the SWIO isolate was the recombinant), we marked the "recombinant region" in the non-SWIO recombinant sequence for later removal. Having examined all events with associated *P*-values $< 1{\cdot}0 \times 10^{-6}$ (i.e. the most obvious events), we removed all the identified evidence of non-SWIO isolate recombination from the alignment. This was carried out by treating the identified "recombinant region" in the recombinant sequence as missing data in subsequent analyses. We scanned the four SWIO isolates in pairs (i.e. 6 pairs in total) against the rest of the sequences in the alignment. Following identification of the more obvious recombination events (events identified with multiple comparison corrected *P*-values $< 1{\cdot}0 \times 10^{-5}$) that involved SWIO isolates as acceptors of sequence (determined phylogenetically as described above) and removal of the identified recombinant regions from the alignment (also as described above), the six SWIO isolate pairs were screened one last time against the rest of the alignment for the least obvious detectable events.

It was apparent from this analysis that all of the SWIO isolates together bear detectable evidence of at least fifteen past recombination events (Fig. 4). In all isolates other than ToLCMGV-[Tol] we

detected a complex mosaic of sequences in an ~350 nucleotide region spanning sequences encoding the N-terminal portion of Rep. Whereas there is statistically significant evidence that this region of the ToLCYTV-[Dem] sequence has three distinct origins, it has at least four distinct origins in both ToLCYTV-[Kah] and ToLCMGV-(Mor *et al.*). Importantly, in all cases the parental sequences identified were one of the SWIO isolates and a sequence only distantly related to previously characterised mainland African begomovirus isolates (either listed as "unknown" or with a "~" prefix in Fig. 4-4).

Fig. 4-4 Recombinant regions detected within SWIO virus sequences: Dem = ToLCYTV-[Dem], Kah = ToLCYTV-[Kah], Mor = ToLCMGV-[Mor], and Tol = ToLCMGV-[Tol]. The genome at the top of the figure corresponds with the schematic representation of sequences given below it. Region coordinates are nucleotide positions of detected recombination breakpoints in the multiple sequence alignment used to detect recombination. Wherever possible, parental sequences are identified. "Major" and "Minor" parents are sequences that were used, along with the indicated recombinant sequence, to identify recombination. Whereas for each identified event the minor parent is apparently the contributor of the sequence within the indicated region, the major parent is the apparent contributor of the rest of the sequence. Note that the identified "parental sequences" are not the actual parents but are simply those sequences most similar to the actual parents in the analysed dataset. Whenever a "~" prefix is included before a parental sequence name, the isolate named is only a distant relative of the parental virus of that region. Recombinant regions and parental viruses were identified using the RDP (R), GENECONV (G), BOOTSCAN (B), and MAXIMUM CHI SQUARE (M), CHIMAERA (C) and SISTER SCAN (S) methods. The reported P-value is for the method in bold type and is the best P-value calculated for the region in question. Whereas upper case letters imply a method detected recombination with a multiple comparison corrected P-value <0·01, lower case letters imply the method detected recombination with a multiple comparison corrected P-value <0·05 but larger than or equal to 0·01.



			Parental sequences			
Event	Region	Minor	Major	Detected by	*P*-value
ToLCMYV – [Dem]						
a	1055 - 1192	~ToLCV	~Tol	rb**C**	7.5×10^{-3}
b	2529 - 2599	Tol	Kah	**R**BCS	5.1×10^{-4}
c	2602 - 2724	Mor	Kah	**R**GBMCS	1.7×10^{-10}
ToLCMYV – [Kah]						
a	1055 -1192	~ToLCV	~Tol	rb**C**	7.5×10^{-3}
b	1748 - 1777	Unknown	Dem	**R**g**B**	9.4×10^{-4}
c	2524 - 2848	Unknown	Dem	RGBM**C**S	6.0×10^{-15}
d	2620 - 2653	~ACMV	Dem	**R**GBMCS	3.9×10^{-10}
e	2654 - 2684	Mor	Dem	R**G**BS	4.3×10^{-4}
ToLCMGV – [Mor]						
a	1661 - 2068	Unknown	~Dem	M**C**	1.2×10^{-4}
b	2288 - 2370	Unknown	Tol	R**G**BM	1.2×10^{-4}
c	2529 - 2599	Unknown	Tol	**R**S	7.5×10^{-3}
d	2650 - 2710	~ChaYMV	Tol	**R**GBs	1.1×10^{-12}
e	2716 - 2854	Unknown	Tol	**R**GBMCS	2.9×10^{-27}
ToLCMGV – [Tol]						
a	2074 - 155	Dem	Mor	GB**M**S	2.3×10^{-8}
b	164 - 574	Unknown	~Dem	rbM**C**	1.6×10^{-3}
c	1504 - 2149	Unknown	Kah	**M**C	1.8×10^{-5}
d	2377 - 2440	~ToLCV	Mor	**R**GB	2.0×10^{-4}

DISCUSSION

We have isolated and characterised what appear to be isolates of two new begomovirus species that are the causal agents of tomato diseases on the SWIO of Madagascar and Mayotte. On the basis of the complete DNA sequences of two isolates from Toliary and Morondova, and in accordance with the ICTV *Geminiviridae* Study Group guidelines (Fauquet *et al.*, 2003; Fauquet & Stanley, 2003) the isolates should be considered members of the new species, Tomato leaf curl Madagascar (ToLCMGV) with the two isolates designated the names ToLCMGV-[Tol], and –[Mor], respectively. Similarly we propose that the isolates from Dembeni and Kahani be considered members of a second new species, Tomato leaf curl Mayotte virus (ToLCYTV) with the two isolates designated the names ToLCYTV-[Dem], and -[Kah], respectively. We also proposed that the monophyletic group to which these four isolates belong should be named the South West islands of the Indian Ocean (SWIO) group.

We have demonstrated the infectivity and whitefly transmissibility of cloned DNA sequences for three of the four SWIO isolates. Our inability to detect either DNA B or DNA β in source leaf material, and the induction of leaf curling and stunting symptoms in agroinoculated tomato plants similar to those observed in the field in the absence of these other genome components, indicates that the SWIO viruses are most likely monopartite.

Recently a new biotype (Ms) of *B. tabaci* has been identified on Madagascar and other SWIO (Delatte *et al.*, 2005c). Although biotype Ms is genetically closely related to the *B. tabaci* B and Q biotypes, it has been estimated that biotype Ms diverged from biotype B and Q as long as 3 (± 0.3) million years ago. It is possible that the SWIO viruses have evolved in relative isolation for a similar period and it will be interesting to determine whether the SWIO isolates have any transmission advantage relative to mainland African and Mediterranean isolates in biotype Ms.

That the SWIO viruses may have been evolving in relative isolation for a prolonged period is supported by the results of our recombination analysis. Had there been substantial influx of mainland begomovirus isolates onto the islands it would be expected that genetic exchange between mainland and island isolates would be detectable. Such exchanges are, for example, easily detectable both amongst and between divergent African and Mediterranean isolates (Padidam *et al.*, 1999). None of the sequences within the recombinant regions identified in the SWIO isolates closely resembled that of any known non-SWIO begomovirus, indicating that genetic exchange in these viruses has most likely been limited to that occurring between relatively unique island isolates. It is important to note, however, that the recombination analysis does not preclude the possibility of genetic exchange between viruses on different islands. In fact, there is highly significant evidence that, firstly, a 856 bp fragment of the Madagascar isolate, ToLCMGV-[Tol], originated from a virus closely resembling the Mayotte isolate, ToLCYTV-[Dem] (*P*-value = 2·3 X 10^{-8}) (Fig. 4), and, secondly, that a 122 bp fragment of ToLCYTV-[Dem] originated from a virus closely resembling the Madagascar isolate, ToLCMGV-[Mor] (*P*-value = 1·7 X 10^{-10}). When and where these potential recombination events occurred is an open question but it cannot be discounted that both ToLCYTV and ToLCMGV isolates might occur on both islands.

This study highlights the need for further sampling and monitoring of begomovirus diversity in both tomato and non-tomato hosts on the SWIO such as Madagascar, Mayotte and the Comoros archipelago. Such activities would almost certainly lead to the identification of more new species and provide early warning of the presence of newly imported and potentially dangerous exotic begomoviruses. Many of the SWIO are small enough that repetitive and reasonably exhaustive begomovirus surveys on them are feasible. Isolated begomovirus populations on the smaller, remote SWIO such as Mayotte could provide one of the last and best remaining opportunities to non-destructively test begomovirus evolutionary hypotheses and population genetic models. Continuous maintenance of sampling projects on these islands might also provide opportunities for testing begomovirus epidemiological models whenever importation of exotic viruses to these islands does occur.

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PART II: THE INSECT VECTOR *BEMISIA TABACI*

CHAPTER 5

A new silverleaf inducing biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) Ms, indigenous to the islands of the South West Indian Ocean[1]

ABSTRACT

Following the first detection of *Tomato yellow leaf curl virus* (TYLCV) from Réunion (700kms east of Madagascar) in 1997 and the upsurge of *Bemisia tabaci* (Gennadius) on vegetable crops, two genetic types of *B. tabaci* were distinguished using RAPD-PCR and cytochrome oxidase I (COI) gene sequence comparisons. One type was assigned to biotype B and the other was genetically dissimilar to the populations described elsewhere and was called Ms after the name of the Mascarenes Archipelago. This new genetic type forms a distinct group that is sister to two other groups, one to which the B biotype is a member and one to which the Q biotype belongs. The Ms genetic type is thought to be indigenous to this region as it was also detected in Mauritius, Seychelles, and Madagascar. Both B and Ms populations of *B. tabaci* induced silverleaf symptoms on *Cucurbita* sp., and were able to acquire and transmit TYLCV. Taken together these results indicate that the Ms genetic type should be considered a new biotype of *B. tabaci*.

[1]Delatte, H., Reynaud, B., Granier, M., Thornary, L., Lett, J. M., Goldbach, R. & Peterschmitt, M. (2005). A new silverleaf-inducing biotype Ms of *Bemisia tabaci* (Hemiptera: Aleyrodidae) indigenous of the islands of the south-west Indian Ocean. *Bulletin of Entomological Research* 95, 29-35.

INTRODUCTION

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a widely distributed species throughout the tropical and subtropical regions of the world. Morphologically indistinguishable populations of *B. tabaci* exhibiting measurably different biological traits such as host range have been described and these are referred to as host races or biotypes (Bird & Maramorosch, 1978). In the past two decades, *B. tabaci* has become a major problem both as a pest and as vector of plant viruses, especially geminiviruses (Markham *et al.*, 1995). During this period one particularly polyphagous and highly fecund population, named biotype B, was encountered in the USA (Costa *et al.*, 1991). This biotype is characterised by both induction of silverleaf symptoms on *Cucurbita spp*. (Cucurbitaceae) and biochemically by a unique and homogeneous esterase profile (Bedford *et al.*, 1994a). Further analyses using Random amplified polymorphism DNA- polymerase chain reaction (RAPD PCR) (De Barro & Driver, 1997; Guirao *et al.*, 1997; Banks, 2001), or specific gene sequences such as cytochrome oxydase I (COI), mitochondrial 16S rDNA (Frohlich *et al.*, 1999) or ribosomal intergenic transcribed spacer 1 (De Barro *et al.*, 2000), showed that biotype B is not restricted to Northern America, but is also present in South America, Africa, Asia, Australia, and Europe. Though biotype B appears to have originated from the North East Africa/Middle East/Arabian peninsular region, international trade of ornamental plants has apparently favoured its current worldwide dispersal (Brown & Bird, 1995). Interestingly, wherever biotype B was introduced, at least one other biotype was detected that is generally considered to be indigenous (De Barro, 1998; Frohlich *et al.*, 1999; Banks, 2001; Moya *et al.*, 2001).

Réunion is a French island situated in the Mascarenes archipelago, in the South West part of the Indian Ocean (700kms east of Madagascar). *Bemisia tabaci* was first described from Réunion in 1938 (Bourriquet, 1938) and later by Luziau (Luziau, 1953), there were no further reports until 1997, when TYLCV infections of tomato crop were detected for the first time (Peterschmitt *et al.*, 1999b). Thereafter, *B. tabaci* has been found on most vegetable crops, ornamentals, and weeds in all the tomato-producing areas of the island. It was not known if the emergence or re-emergence of *B. tabaci* in Réunion was due to the introduction of a new strain of *B. tabaci* or to the upsurge of a local population. Tomato is the first vegetable crop in Réunion with for example 9000 tons production in 2002. Yield losses due to TYLCV reached 85% on the most susceptible cultivars (Reynaud *et al.*, 2003). The aim of this work was to identify the populations of *B. tabaci* in Réunion.

EXPERIMENTAL PROCEDURE

Insect and plant material

Bemisia tabaci individuals were collected from annual poinsettia *Euphorbia heterophylla* L. (Euphorbiaceae), tomato *Lycopersicon esculentum* Mill. (Solanaceae), eggplant *Solanum melongena* L. (Solanaceae), cabbage *Brassica oleracea* L. (Brassicaceae), sweet potato *Ipomea batatas* L. (Convolvulaceae), cucumber *Cucumis sp*. L. (Cucurbitaceae), cotton-leaved jatropha *Jatropha gossypiifolia* L. (Euphorbiaceae), bean *Vigna sp.* L. (Papillonaceae), *Chromolaena odorata* King & H.E. Robins. (Asteraceae), and cotton *Gossypium sp.* L. (Malvaceae) (table 5-1). On Réunion, adults were allowed to emerge from whitefly pupae (generation 0) following observation under stereomicroscope to only select whiteflies of the *B. tabaci* species using the key in Martin (Martin, 1987). Except for whitefly sample 18 (table 5-1) collected on sweet potato and tested only at generation 0, isofemale lines were established from the other samples as follows. Single adult females (generation 0) were caged onto an uninfested plant using insect-proof cages. A single female progeny (generation 1) was then collected and used to establish generation 2 and likewise for generation 3. Each of these lines was reared on plants of the same species as the host plant on which the nymphs were collected originally, except for lines 4, 5 and 6 (table 5-1), which were reared on tomato. Adult whiteflies were collected at generations 1, 2, or 3 for DNA analysis. They were placed in micro tubes filled with ethanol (90%) and stored at –20°C until processing. After selection, lines 2, 4, 6, 11, 12, 15 and 17 were maintained without selection for biological tests. Insects were reared in a climate chamber with a 12:12h photoperiod, a temperature of 25 +/- 2° C and relative humidity of 60 +/- 10%. Adult *B. tabaci* were also collected from nearby islands, Madagascar, Mauritius, Seychelles, and Mayotte and preserved in ethanol. Ethanol-preserved reference biotype A individuals from Colombia and biotype B from France were supplied by Nicolas Sauvion (Institut National de Recherche Agronomique). Adults of *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) were obtained from Réunion following the approach described above for *B. tabaci*.

DNA extraction and RAPD-PCR analyses

Insects were individually ground for 20 s in 5µl of extraction buffer, containing: 50mM KCl, 10mM Tris-base, pH 8, 0.45% Nonidet P-40, 0.45% Tween 20, and 500 µg/ml proteinase K. Extraction buffer (20µl) was added to the crude extract and was briefly centrifuged. The extract was incubated at 65 °C for 1 h, submitted to a short centrifugation, incubated at 100 °C for 15 min, and again briefly centrifuged. A volume of 35µl of water was added to this extract which was then stored at –20 °C until use. The RAPD-PCR reactions were carried out in a volume of 25µl consisting of 5µl of DNA and 20µl of PCR mix. The PCR reaction mix contained 2.5mM of each dNTPs, 80mM $MgCl_2$, 5u of *Taq* DNA polymerase and 20µM of primer. Amplification was done using a Perkin-Elmer thermocycler using the following parameters: 5 min at 94 °C, 2 min at 40 °C, and 3 min at 72 °C, followed by 29 cycles of 1 min at 94 °C, 1.5 min at 40 °C, and 2 min at 72 °C.

Quantities of 10 µl of the amplification products were loaded on a 1.4% agarose gel, migrated for 3 h at 120 volts and stained with a solution of 1µg/ml ethidium bromide. Eight 10-mer oligonucleotides were used as primers. Three of these (F12, H9, H16) were according to De Barro & Driver (1997) while the others were chosen at random: B1: CAGAACTCGG, B2: ACCATCGGCA, B3: ATCCCTGAAG, G1: ATTGTTCCTT and P1: TGACACATAG. Three sets of whiteflies, sets 1, 2 and 3, were amplified with these RAPD primers (table 5-1). For each set of whiteflies and each primer, the RAPD-PCR products were generated in the same experiment and resolved on the same gel. Individuals of biotype A from Colombia, biotype B from France as well as *T. vaporariorum* from Réunion were used as controls in each set. For whiteflies of set 2 (table 5-1, Fig. 5-1), the presence or absence of bands in the RAPD patterns was recorded and a matrix of distances was constructed using the coefficient of Sokal and Michener (1958). Two programs were used for the construction of a Neighbour Joining tree (Saitou & Nei, 1987): Numerical Taxonomy and Multivariate Analysis System (NTSYS) and Darwin (Perrier *et al.*, 1999). Bootstraps were generated using Darwin.

Analyses of molecular variance, F-statistics of genetic differentiation

Genetic variation and the F-statistics for the different host plant lines of *B. tabaci* within set 2 were carried out by AMOVA using Arlequin (Excoffier *et al.*, 1992). The run was performed without the data of the control individuals for biotype A and *T. vaporariorum.* Males and females were used, as no differences have been shown between the two (Moya *et al.*, 2001). Differences between the B and the non-B group (see below) (F_{CT}), among host plant populations within groups (F_{SC}) and within host plants (F_{ST}) were assessed. The genetic distance was estimated as the number of fragment differences between haplotypes. The contribution of the three partitions to the total variance, as well as the three F-statistics were tested statistically by randomisation tests based on 3024 permutations. The population pairwise F_{ST} was then estimated.

COI gene sequencing

An 817-nucleotide fragment of the mitochondrial COI gene was amplified for individuals of *B. tabaci* selected from lines representative of the different RAPD types and from non-Réunion populations (table 5-1), using the following primers: C1-J-2195 and L2-N-3014 (Frohlich *et al.*, 1999). PCR assays were conducted using 2µl of template DNA extracted as above, in a total reaction volume of 25µl. The PCR reaction mix contained 2.5mM of each dNTPs, 50mM $MgCl_2$, 5u of *Taq* DNA polymerase and 20µM of each primer. Amplification was done with the following parameters: 2 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C for 30 cycles. PCR products were fractionated on 0.9 % agarose gels, and bands were visualised as described above.

Table 5-1: Geographic origin and host plant of *Bemisia tabaci* populations investigated in this study and summary of the results of the molecular and biological tests

Code [(1)]	Geographic origin	Host Plant	RAPD[(2)] Set 1	Set 2	Set 3	COI (EMBL accession no.)[(3)]	Transmission rate (no. of repetitions)[(4)]	SSL[(5)]
Bemisia tabaci								
1	Réunion/St Gilles les Hauts	Eggplant	1B[(2)]	2B				
2	Réunion/St Gilles les Hauts	Eggplant		2B	6B	B (AJ550176) B (AJ550177)	48 (290)	
3	Réunion/St Gilles les Hauts	Eggplant		1B				
4	Réunion/Petite Ile	Eggplant		1B			36 (100)	
5	Réunion/Etang Salé les Hauts	Eggplant		1B				
6	Réunion/Ravine des Cabris	Cucumber		1B			18 (139)	
7	Réunion/St Gilles les Hauts	Tomato		1B				
8	Réunion/St Gilles les Hauts	Tomato	1B					
9	Réunion/St Gilles les Hauts	Cabbage	1B	1B				
10	Réunion/Bassin Martin	Cabbage	1B					
11	Réunion/Bassin Martin	Cabbage		2B	4B		53 (325)	SSL
12	Réunion/Ligne Paradis	Cotton	1B	1B	4B	B (AJ550174) B (AJ550175)	14 (176)	SSL
13	Réunion/Ligne Paradis	Cotton	1B	2B				
14	Réunion/Petite Ile	Annual poinsettia	1B	1B				
15	Réunion/Gol les Hauts	Annual poinsettia		2Ms	4Ms	Ms (AJ550178) Ms (AJ550179)	43 (205)	SSL
16	Réunion /Saint Joseph	Cotton- leaved Jatropha	1B					
17	Réunion/Bois d'Olive	Sweet potato	1Ms	1Ms		Ms (AJ550180)	37 (235)	SSL
18	Réunion/Bois d'Olive	Sweet potato	1Ms			Ms (AJ550181)		
19	France/Antibes Control B biotype	Eggplant	*1B*	*1B*	*1B*	B (AJ550169) B (AJ550170)		
20	Madagascar/Tulear	Tomato				Ms (AJ550171)		
21	Mauritius/Mahebourg	Bean				Ms (AJ550172)		
22	Mayotte/Cambani	Tomato				B (AJ550173)		
23	Seychelles/Mahe	Jatropha				Ms (AJ550182)		
24	Colombia/Cali Control A biotype	*Chromolaena odorata*	*1A*	*1A*	*1A*	A (AJ550167) A (AJ550168)		
Trialeurodes vaporariorum								
25	Réunion/Bassin Martin	Tomato	1Tv	1Tv	1Tv			
26	Réunion/Piton Hyacinthe	Tomato				0 (AJ550183)		

(1) Codes 1- 17 correspond to *Bemisia tabaci* isofemale lines derived from a series of field and greenhouse collections. Codes 18, 25, 26, correspond to whiteflies obtained from field collected 3rd or 4th instar nymphs. Codes 20-23 correspond to adults collected in the field during this study. Codes 19 and 24 were adults received from Institut National de Recherche Agronomique and used as controls for biotypes B and A, respectively.

(2) Number of whiteflies tested followed by the biotype identification according to RAPD patterns. Biotype identification are in bold for individuals used as control for B and A biotypes (populations 19 and 24, respectively). Tv, RAPD pattern obtained with a *T. vaporariorum* population.

(3) Biotype identification based on a 370-nucleotide fragment of the *B. tabaci* mitochondrial COI gene.

(4) Mean percentages of the number of tomato plants infected with *Tomato yellow leaf curl virus* following transmission with different *B. tabaci* populations from Réunion.

(5) Symptoms of silverleaf.

PCR products were cloned into a pGEMT-Easy vector (Promega, WI, USA) and sequenced with plasmid specific primers. In addition, sequences for *B. tabaci* from a range of countries were obtained from Genbank (Fig. 5-2). Sequences were aligned with the Optimal Alignment method of DNAMAN (version 5.0; Lynnon BioSoft, Quebec, Canada). The phylogenetic tree was set up with a Jukes and Cantor distance matrix using the Neighbour Joining method of DNAMAN (Saitou & Nei, 1987). Thousand bootstrap iterations were performed. The molecular clock hypothesis was tested with the relative rate test (Takezaki *et al.*, 1995).

Silverleaf test

Cucurbita moschata seedlings at the one leaf stage with removed cotyledons were individually caged under a net in which one pair of whiteflies was introduced. Plants were observed for symptoms of silverleaf (SSL) 21 days later. Fifteen pairs of each of the following *B. tabaci* populations were tested: lines 11 and 12 (B group) and lines 15 and 17 (non-B group) (table 5-1). Fifteen plants grown under the same conditions, without insects were used as controls. The SSL was rated according to Yokomi *et al.* (1990).

Transmission test

Transmission tests were carried out with the following *B. tabaci* lines derived from different host species: lines 2, 4, 6, 11 and 12 of the B group and lines 15 and 17 of the non-B group (see below) (table 5-1). For each line, a group of individuals was placed on one TYLCV-infected tomato plant of the cultivar Farmer (Know You Seed) for a 3-day acquisition access period (AAP). After the AAP, each whitefly was caged with one healthy tomato plant (cv. Farmer) under a plastic cylinder (diameter: 2 cm, height: 8.5 cm) for a five-day inoculation access period (IAP). The top of the cylinders was covered with a thin net to allow evaporation and airflow. Whiteflies were introduced into the cage through a 3 mm hole in the wall of the cylinder, which was subsequently closed with a piece of filter paper to help absorb humidity. At the end of the IAP, insects were removed and plants were treated with an insecticide (imidacloprid, Confidor®, Bayer, Basel, Switzerland) and placed in an insect-proof cage for symptom observations for 18 to 21 days. By adding all the transmission tests performed during different experiments, a total of 200 insects were tested for each line. The inoculated plants were finally analyzed using triple antibody sandwich – enzyme linked immunosorbent assay (TAS-ELISA, Adgen Ltd, UK) for virus detection. The protocol followed manufacturer recommendations. Absorbance values were recorded at 405 nm with a spectrophotometer every 15 min for 2 h. A transmission was considered to be successful when the absorbance (A) obtained from the inoculated plant was higher than the mean A of the negative controls (crude extracts of 10 plant samples from non inoculated plants) + 3 times their standard deviation.

RESULTS

RAPD-PCR analysis

B. tabaci whiteflies were tested with the 8 RAPD primers except individuals of set 2 (table 5-1) which were not tested with primer B2. A total of 67 polymorphic bands were scored with the individuals of set 2. A Neighbour-Joining tree (Fig. 5-1) was derived from the Sokal and Michener distance matrix using the RAPD patterns obtained for set 2 minus line 7 which only amplified with primers B1, B3, and F12. A B-group including a control biotype B individual from France (population 19, table 5-1) and most of the lines from Réunion was supported with a 100% bootstrap value (Fig. 5-1). A non-B group consisting of the annual poinsettia and sweet potato lines (code 15 and 17 respectively) was supported with a 71% bootstrap value. Individuals tested in sets 1 and 3 exhibited either the B or non B patterns that were distinguished with individuals of set 2 (table 5-1).
The results of the hierarchical analyses, including the results of the AMOVA test and their corresponding F-statistics of genetic differentiation are presented in table 2. Three sources of variation were taken into account: difference between B and non-B groups (F_{CT}), among host plants within groups (F_{SC}), and within host plants (F_{ST}). Most of the haplotype diversity was found between B and non-B groups (57.3%). Diversity within the host plants was also high (36.3%). The difference among host plants within groups was small (6.4%). The high value obtained for the F_{CT} (0.57), showed a high level of genetic differentiation between the B and non-B group. The F_{ST} value (0.64) was quite high, which revealed a very high genetic differentiation for insect populations within host plants. A lower value of 0.15 was observed for the FSC, meaning that individuals among hosts plants and within the same group were genetically more homogeneous.

Table 5-2: Analysis of molecular variance, and F-statistics of genetic differentiation when *Bemisia tabaci* set 2 haplotypes are grouped hierarchically according to B and Ms groups, among host plant populations within groups and within host plants

Source of variation	d.f	Sum of squares	Variance	%	F-statistics
Between B and Ms groups	1	39.12	6.66	57.3	F_{CT}: 0.57
Among host plants within groups	5	29.86	0.75	6.4	F_{SC}: 0.15
Within host plants	11	46.41	4.22	36.3	F_{ST}: 0.64
Total	17	115.39	11.62		

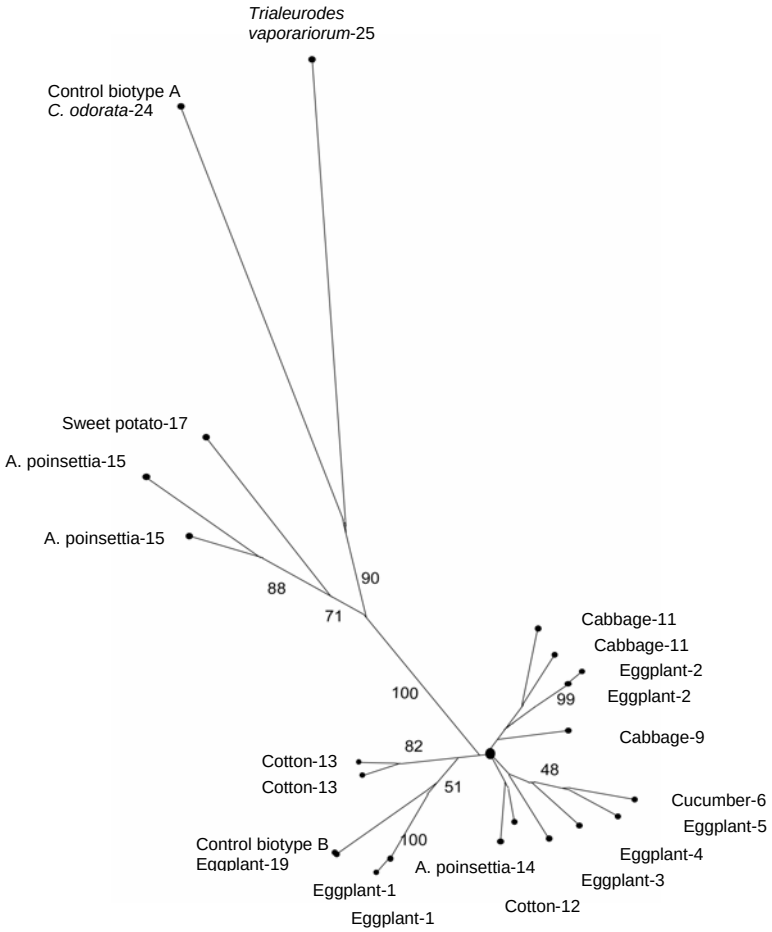


Fig. 5-1: Unrooted neighbour joining tree of 16 populations of whiteflies based on the similarity values of Sokal and Michener inferred from 67 RAPD polymorphic fragments amplified with seven primers, B1, B3, G1, P1, F12, H9, H16. The codes given for each individual refer to Table 5-1. Individuals labelled with the same names were repeats within the same line. Bootstrap values based on 1000 random replicates are indicated on branches for values over 40%.

CO1 gene sequence comparisons

The phylogenetic tree was estimated using a 370bp portion of the CO1 sequence (Fig. 5-2). Individuals from Réunion previously identified as belonging to the B biotype using RAPD, clustered (99% bootstrap support) with other B individuals obtained from Genbank. The individual from Mayotte also belonged to the B group. Individuals belonging to the Q and related biotypes were sister to this group (83% bootstrap support). Non-B lines from Réunion (lines 15, 17 and 18) together with individuals from Madagascar, Mauritius and Seychelles, formed a third sister group with 100% bootstrap support. This new group detected in the South West of the Indian Ocean was named Ms from the Mascarenes archipelago. One of the two individuals of the B-type eggplant line 2 (AJ550177) is divergent from the other individuals of the B/B2 group with a mean distance of

0.031 ± 0.003. All the individuals analyzed here from the islands of the South West Indian Ocean cluster in a large African- Mediterranean- Arabian group (86% bootstrap value) containing Ms genetic type, B biotype, Q biotype and Q-related African individuals. The mean distance between B group and the Q group (0.047 ± 0.005) is lower than the distance between the Ms group and the group formed by B and Q biotypes (0.069 ± 0.007). The molecular clock hypothesis is not rejected by the relative rate test (Takezaki *et al.*, 1995) and therefore a linearized tree (Nei & Kumar, 2000) can be built. This allows for inferring nodes for which divergence time can be estimated based on Browers's estimation, 2.3% pairwise sequence divergence per million years (Brower 1994). The divergence between the Ms group and the group formed by B and Q biotypes was estimated at 3 ± 0.3 million years.

The remaining individuals form the same Asian, American and African clusters that have been identified in previous studies (Frohlich et al., 1999; De Barro et al. 2000; Legg et al., 2002). As expected the 2 individuals sequenced as A biotype control clustered with the America group, and the *T. vaporariorum* individual from Réunion clusters with the *T. vaporariorum* from which the sequence was available in Genbank.

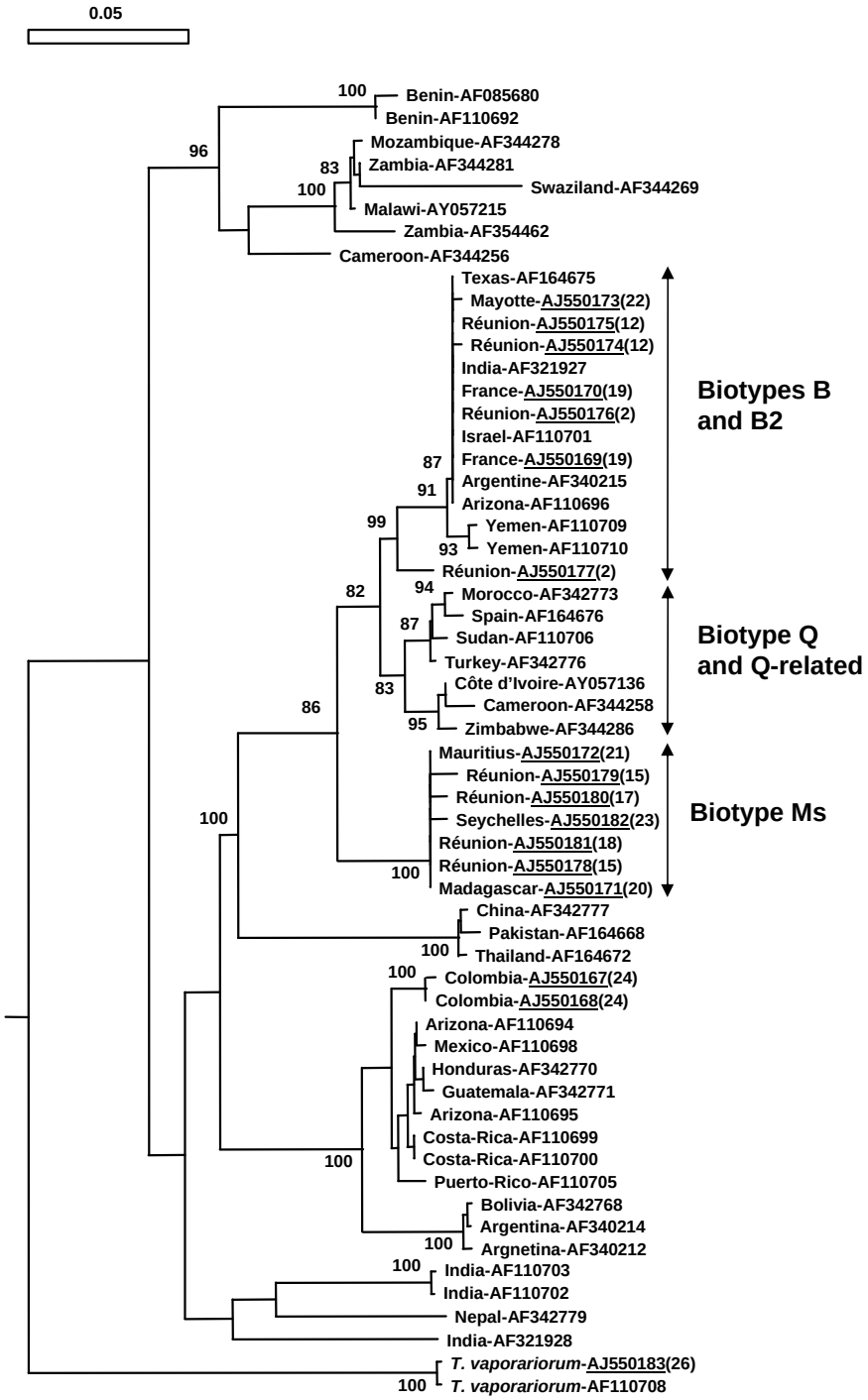
Silverleaf test

All the tested *B. tabaci* lines from the B and the Ms groups induced SSL on *Cucurbita moschata* (table 5-1). No significant differentiation of symptoms was noticed between lines.

TYLCV transmission test

The TYLCV was transmitted by all the *B. tabaci* lines tested (table 5-1). The B and non-B lines could not be discriminated using transmission rates which are ranging from 14 to 53%.

Fig. 5-2: Rooted neighbour joining tree showing the genetic distance among 370 nt cytochrome oxydase I fragments of *Bemisia tabaci*, either sequenced in this study or selected from GenBank. Sequences are identified with their accession number and the geographic origin of the tested whitefly. The accession numbers of the sequences generated in this study are underlined and followed by their code number (in brackets) given in Table 5-1. Two *Trialeurodes vaporariorum* sequences were added to this comparison, one was obtained in this study and the other one was from Genbank. The scale measures the Jukes and Cantor distance between sequences. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes; only percentages = 81% are indicated.


0.05
Benin-AF085680
Benin-AF110692
Mozambique-AF344278
Zambia-AF344281
Swaziland-AF344269
Malawi-AY057215
Zambia-AF354462
Cameroon-AF344256
Texas-AF164675
Mayotte-AJ550173(22)
Réunion-AJ550175(12)
Réunion-AJ550174(12)
India-AF321927
France-AJ550170(19)
Réunion-AJ550176(2)
Israel-AF110701
France-AJ550169(19)
Argentine-AF340215
Arizona-AF110696
Yemen-AF110709
Yemen-AF110710
Réunion-AJ550177(2)
Biotypes B and B2
Morocco-AF342773
Spain-AF164676
Sudan-AF110706
Turkey-AF342776
Côte d'Ivoire-AY057136
Cameroon-AF344258
Zimbabwe-AF344286
Biotype Q and Q-related
Mauritius-AJ550172(21)
Réunion-AJ550179(15)
Réunion-AJ550180(17)
Seychelles-AJ550182(23)
Réunion-AJ550181(18)
Réunion-AJ550178(15)
Madagascar-AJ550171(20)
Biotype Ms
China-AF342777
Pakistan-AF164668
Thailand-AF164672
Colombia-AJ550167(24)
Colombia-AJ550168(24)
Arizona-AF110694
Mexico-AF110698
Honduras-AF342770
Guatemala-AF342771
Arizona-AF110695
Costa-Rica-AF110699
Costa-Rica-AF110700
Puerto-Rico-AF110705
Bolivia-AF342768
Argentina-AF340214
Argnetina-AF340212
India-AF110703
India-AF110702
Nepal-AF342779
India-AF321928
T. vaporariorum-AJ550183(26)
T. vaporariorum-AF110708

DISCUSSION

Two genetic types of *B. tabaci* were identified in Réunion with RAPD markers and COI sequences, the cosmopolitan B biotype, and a so far undescribed genetic type we have named Ms after the Mascarenes Archipelago. The Ms genetic type was also detected from Mauritius as well as from the neighbouring islands of Seychelles and Madagascar. This genetic type was therefore considered to be indigenous to these islands. The phylogenetic tree comparing the COI sequences of the Ms genetic type to those of a panel of *B. tabaci* originating from a wide geographical range showed that the Ms genetic type belongs to a Mediterranean/Arabian Peninsular/African group of genotypes to which biotypes Q and B also belong (group MsQB, 86% bootstrap value). This is consistent with the link showed by others between the geographical origin and the phylogeny of *B. tabaci* (De Barro & Hart, 2000; Perring, 2001; Viscarret *et al.*, 2003). The genetic distinction between the MsQB group of populations mainly detected on non-cassava species, and an African group of populations detected on cassava and *Asystasia gangetica* (96% bootstrap value) illustrates that both geographic isolation and host plant species may have contributed, in this part of the world, to the population structure of *B. tabaci* (Brown & Frohlich, 1995; Abdullahi *et al.*, 2004).

Since Réunion is a volcanic island that appeared relatively recently, around 2 million years ago, the Ms genetic type has probably not arisen in Réunion because its divergence from the group formed by B and Q biotypes is estimated to be older, 3 ± 0.3 million years. The divergence could have occurred in Madagascar where Ms genetic type was also detected, because it is estimated that Madagascar drifted away from the continent about 165 million years ago. Extensive samplings in South East African countries are needed to further study the origin of the Ms genetic type which has so far not been reported from Africa.

As expected, the B biotype populations induced the typical SSL. Ms populations were able to induce the same symptoms confirming that non-B populations are quite able to induce SSL (De Barro *et al.*, 2000) and therefore indicating that the SSL test was not sufficiently diagnostic to identify the B biotype of *B. tabaci* (Secker *et al.*, 1998). All the whitefly lines transmitted TYLCV. As the tested populations were collected and reared on different host species, host plant adaptation may have influenced transmission efficiency (Bedford *et al.*, 1994a; Brown *et al.*, 1995) more than the genetic background of B and Ms populations. Biological differences between B and Ms genetic types are however suspected because biotype B was detected in most of our samplings (table 5-1) whereas the Ms genetic type was only found at two out of ten sampling sites and on two out of eight host species. Moreover, the visual observations made while maintaining a B and an Ms population on cabbage under the same rearing conditions, always showed that the B population reached a high density whereas the Ms population remained at a low density.

Taken together, these data indicate that the Ms genetic type detected on the islands of the South West Indian Ocean should be considered as a new biotype: (i) Ms genetic type was detected in a specific and rather isolated geographic region of the world, (ii) the genetic distance between Ms

genetic type and its two closest biotypes B and Q was higher than the distance between B and Q, (iii) Ms genetic type is producing SSL unlike the reported non-B biotypes.

The absence of *B. tabaci* populations on vegetable crops before the first detection of TYLCV in 1997 suggests that the polyphagous biotype B was introduced simultaneously with TYLCV or shortly before. Moreover, the absence of any recent detection of infestations of *B. tabaci* prior 1997 on Réunion, although it was reported there much before, suggest a low fecundity of the indigenous Ms biotype and/or a restricted host range. However, Ms biotype was not found to be monophagous since it was collected on two host species in our samplings, it produced progeny on *Cucurbita moschata* and could be reared on cabbage.

Future work will focus on the detection of the natural host range of Ms biotype, its fecundity on these host plants, its ability to interbreed with biotype B, its geographical distribution within the islands on which it was detected, and its possible presence in South East Africa. The insularity of Réunion emphasis the interest of further studies on ecological, genetic and spatial interactions between the two biotypes.

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CHAPTER 6

Microsatellites reveal the coexistence and genetic relationships between invasive and indigenous whitefly biotypes in an insular environment[1]

ABSTRACT

Introduced exotic species are responsible for important biodiversity and agronomic losses. However when morphologically similar resident species are present, it may be very difficult to assess the current extension of the invasion, as well as the relationships between invasive and resident species. We here illustrate the use of microsatellite data together with bayesian clustering methods to document invasions in a group of major tropical pests: *Bemisia tabaci*, which comprises several morphologically indistinguishable biotypes with different agronomical impacts. We focused on the invasion of the B biotype in the Réunion island previously occupied by the indigenous Ms biotype. We showed (i) that, without prior information on genetic variation, clustering methods identify two groups of individuals that can safely be identified as the B and Ms biotypes; (ii) that the B biotype has invaded all regions of the island, and showed no signs of genetic founder effect relative to the Ms biotype (iii) that the B and Ms biotypes coexist throughout most of their ranges, although they are specialised to different ranges of host plants and finally (iv) that introgression may occur between the two biotypes when they are in syntopy.

[1]Delatte, H., David, P., Granier, M., Lett, J. M., Goldbach, R., Peterschmitt, M. & Reynaud, B. (2005). Microsatellites reveal the coexistence and genetic relationships between invasive and indigenous whitefly biotypes in an insular environment. *Molecular ecology* Submitted in a slightly modified version.

INTRODUCTION

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a widely distributed polyphagous species throughout the tropical and subtropical regions worldwide (Oliveira *et al.*, 2001). Morphologically indistinguishable populations of *B. tabaci* exhibiting measurably different biological traits, such as host range, have been described and these were referred to as host races or biotypes (Bird & Maramorosch, 1978). In the past two decades, *B. tabaci* has become a major problem both as a pest and as vector of plant viruses, especially geminiviruses (Markham *et al.*, 1995). During this period a particularly polyphagous and highly fecund population, named biotype B, was discovered in the USA (Costa & Brown, 1991), and displaced the indigenous whitefly populations, named biotype A, in the 1980's, (Brown *et al.*, 1995). This occurred over large geographic areas, from Florida, through Texas, Arizona, to California in a short period of time (Perring, 1991; Gill, 1992; Bellows *et al.*, 1994). Several hypothesis for the displacement of the A biotype are known, such as the B biotype high reproduction rate, broad host range (Bellows *et al.*, 1994) or better resistance to insecticide (Costa *et al.*, 1993c). Indeed the increase of the B biotype might have been enhanced due to a massive spray of insecticides during the first years of the epidemic, killing all the natural enemies, and leaving the area free for whitefly invasions (Gill, 1992). Nevertheless, the same situation has not always been observed. In some cases some biotype like the Q biotype in Spain has remained predominant, whereas it has been demonstrated that the B biotype prevails in artificial mixed laboratory populations (Pascual & Callejas, 2004). However, in most of the cases, whether the B biotype displaces or simply coexists with indigenous ones, new begomovirus epidemics are reported (Polston & Anderson, 1997; Ribeiro *et al.*, 2003).

Since 1997 a severe outbreak of *Tomato yellow leaf curl virus* (TYLCV) (Peterschmitt *et al.*, 1999b) has been observed in La Réunion, an island of the Indian Ocean, 700kms east of Madagascar. The viral epidemic was associated with a raise of its vector population *B. tabaci*. Prior to 1997 no *B. tabaci* infestations of similar importance had ever been reported in La Réunion. A preliminary study on *B. tabaci* realised by RAPD-PCR and sequencing of a part of the cytochrome oxidase I gene has revealed the presence of the B biotype together with an indigenous biotype named Ms (Delatte *et al.*, 2005c). The introduction of the B biotype in the insular environment of La Réunion, provides a great opportunity to analyse a colonisation process from the beginning. However, because the biotypes cannot be distinguished morphologically, it has always been very difficult to follow the progress of biotype B invasions in the field. We have developed microsatellite markers to this end. Microsatellites were chosen as they are co-dominant and highly polymorphic markers, distributed throughout the nuclear genome and generally neutral unless linked to loci under strong selection. These characteristics make them good candidates for the study of population differentiation (Tautz, 1989) and the colonisation process.

The current study objectives were (i) to develop enough polymorphic microsatellite markers to reveal spatial and temporal variation in genetic composition of *B. tabaci* populations (ii) to test whether microsatellites allow to recognise the presence of two distinct sub-populations,

corresponding to biotype Ms and B, in La Réunion populations and (iii) to assess the present state and temporal dynamics of the joint spatial distributions of biotypes Ms and B in La Réunion.

MATERIAL AND METHODS

Whitefly sampling

Three samplings of adult whiteflies were realised during the high infestation periods of *B. tabaci* (February-March 2001: **S1;** September-October 2001: **S2;** February-March 2002: **S3**), which are respectively correlated with the high temperature period (February-March) and the end of the low temperature period (September-October). Adults were collected on 10 different host plants whenever possible (see below) in the whole island (Fig. 6-3) on 19 different sites characterised by their agro-geographic situation (housing zone, sugar cane plantation, vegetable cropping area, fallow area, forest, natural zones) and their altitude (below 300m). Samples were collected below 300m as above this altitude, *Trialeurodes vaporariorum* were relatively more abundant than *B. tabaci*. The south and east part of La Réunion are the wettest part of the island, with the east mostly an area of sugar cane. The West and North are the vegetable area and the dryer part of the island. Insects were preserved in tubes with ethanol (90%). The different host plants sampled were: annual poinsettia (*Euphorbia heterophylla*), poinsettia (*Euphorbia pulcherrima*), tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena)*, cabbage (*Brassica oleracea*), ipomea (*Ipomea maxima*), watermelon (*Cucurbita sp*.), bean (*Vigna sp.*), lantana (*Lantana camara*), apple of Peru (*Nicandra physaloides)*, and cassava (*Manihot esculenta*).

Table 6-1: Microsatellite sequences, motives, predicted sizes and accession numbers registered in EMBL.

Name /Primer sequence (5'-3') and fluorochrome	Repeat	Predicted size	Accession number
Group 1			
53a / TTCGTAACGTCTTTAAATTTTTGC -FAM 53b / **TTG**TGGAGCATATAGCCTTTTTGG*	20(GT)	148 bp	AJ866707
7a / AGGGTGTCAGGTCAGGTAGC -HEX 7b / **TTT G** CGTAATGGTAACATGTTTAGAAAA*	8(GT)	176 bp	AJ866710
5a / ATTAGCCTTGCTTGGGTCCT -NED 5b /**TTT G** CAAAAACAAAAGCATGTGTCAAA*	8(GT)	207 bp	AJ866711
62a /CTTCCTTAGCACGGCAGAAT -FAM 62b / **TTT G** GCGCAATTTTTAGCGTCTGT*	8(GT)	209 bp	AJ866714
Group 2			
41a / AATCTTAGTGCTAAAGTTTTCTT- FAM 41b / **TT G** TGTTAGGATGATAGGCTTGGA*	**7(AC)**	**165 bp**	**AJ866709**
11a / CCAGAAAAGTGGACTTAAGA - HEX 11b / **TTT G** GATCTGGGTGTTTTCTTCTA*	9(GT)	180 bp	AJ866708
32a / GCTCAAAATTGTTGGCTCTGA -NED 32b/ **TTTG**AGCTCCGCCCTTAAATTGTT*	9(CA)	199 bp	AJ866713
59 a / CGGCGTTTCTCGTTTTCTT -FAM 59 b / **TTTG**CCAACTGAAGCACATCAATCA*	44(T) 18(G)	208 bp	AJ866712

* Parts of the sequences in bold represent the "Pig tail" added to the original sequence.

Microsatellite-enriched library building procedure

A partially modified protocol of Billote *et al.* (1999) was used in order to build the enriched library. Total genomic DNA extraction was done with the DNA plant tissue kit (Qiagen, S.A., Courtaboeuf, Fr.) from 25 mg of *B. tabaci* (ethanol conserved) from a laboratory strain of the B biotype from La Réunion. The total DNA was sheared with SAU 3AI enzyme. The cohesive ends of DNA fragments were polished by the action of the Kleenow polymerase (Gibco BRL). The screening was realised over fragments containing AC repetitions by a biotine-labelled probe of 8 repetitions of a TG dinucleotide.

Several microsatellite sequences were recovered, and then tested with DNA coming from different *B. tabaci* biotypes. Only 8 sequences were kept for the present analysis (Table 6-1).

DNA extraction, amplification and detection of microsatellites

Whiteflies have haploid males and diploid females. Each field-captured whitefly was therefore sexed before DNA extraction (Delatte *et al.* 2005). One primer of each locus was fluorescently end-labelled with 6-Fam, Hex or Ned (Table 6-1). In order to facilitate an accurate genotyping a "Pig tail" was added at the 5' end of each primer (Brownstein *et al.*, 1996). The polymerase chain reaction (PCR) was conducted in a 20 µl reaction volume with three different PCR mix. For primers P7-11-53 10x PCR buffer, 0.15 mM dNTP, 0.25 pmol per primer 1.2 mM $MgCl_2$, 0.2 units Taq DNA polymerase (Goldstar, Eurogentec, Seraing, Belgium) 2 µl of each DNA. For primer P59, the same concentrations were used in the mix except for $MgCl_2$, where a 2 mM concentration was used. For primers P5-32-41-62, the same concentrations were used except for $MgCl_2$ and dNTP, with 3 mM and 0.2 mM, respectively. Two different PCR programs were used. A single soak at 94 °C for 5 min, followed by 5 cycles at 94 °C for 30 s, 65 °C for 1 min, 72 °C for 1 min, then 30 cycles at 94 °C for 30 s, 64.5 °C (-0.5 °C/cycle) for 1 min, 72 °C for 1 min, then 5 min at 72 °C. This program was used with primers P 5-32-41-62. The second program consists in a single step at 94 °C for 5 min followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and then 10 min at 72 °C. After visualization of the product in an agarose gel 2 µl of the amplification product were mixed with 10 µl of ultra pure formamide and 0.3 µl of size marker (GeneScan ROX, Foster city, CA, USA), and loaded on ABI PRISM 700 automated sequencer. Allele sizes were calculated using the software program GeneScan ver. 3.1, and genotyper ver. 2.5 available from Plant Research International (PRI) in Wageningen (Nl).

Data analyses

Only females were used for the genetic diversity analyses due to their diploid state. To differentiate the B from the Ms biotype, we used the software Structure ver. 2.1 of Pritchard *et al.* (2000). This software differentiates mixed populations based on allele frequency at each locus. The software was run with the option no admixture. 2 Ms and 3 B insect controls from our lab rearing were included in the analysis. In order to check the biotype classification (B vs Ms) provided by the software *Structure* a fragment of the mitochondrial cytochrome oxidase I (COI) gene was amplified for 6 individuals using primers: C1-J2195 and L2-N3014 (Frohlich *et al.*, 1999) and sequenced.

Two of the six insects were classified as Ms according to Structure: Saint Paul S1 / Cambuston S3, four classified as B: Manapany S1 / Petite Ile S1 / Petite Ile S2 / Etang Salé S2. Sequences obtained were aligned with the Optimal Alignment method of DNAMAN (version 5.0; Lynnon Biosoft, Quebec, Canada). The phylogenetic tree was set up with a maximum likelihood distance matrix using the Neighbour Joining method of DNAMAN (Saitou & Nei, 1987). An AFC analysis was also performed using Genetix 4.01 (Belkhir *et al.*, 1996-2004) in order to visualize the major axes of genetic variation within the sample, and the position of the groups defined by the *Structure* software along these axes.

Genetic diversity of each biotype was quantified by the mean number of alleles per locus, the observed heterozygosity (H_o) and the Hardy-Weinberg expected heterozygosity (H_e). For the two biotypes separately, Weir and Cokerham (1984) estimates of the fixation index (F_{is}) within localities and of the F_{st} among localities were calculated using the Genepop software ver. 3.3 (Raymond, 1995). The null hypotheses of Hardy-Weinberg frequencies within populations, and lack of population structure, were tested using exact tests at each locus using the Markov chain algorithm of Guo and Thompson (1992), implemented in Genepop 3.3. A hierarchical AMOVA was obtained with the Arlequin software (Excoffier *et al.*, 1992), partitioning the genetic variance into three components: (i) within-site within-biotype, (ii) among-sites within biotype, and (iii) between biotypes.

Using raw data, large heterozygote deficiencies were present at some loci within most sites and both biotypes. In order to obtain robust results, we repeated all analyses using pooled alleles that grouped together alleles of similar size (see results for details on the pooling procedure). Most results (with the exception of heterozygote deficiencies) were similar to the non pooled dataset. Unless otherwise stated, we present only the more robust results obtained after pooling alleles.

Once all individuals were classified as B or Ms we were in position to analyse the variation in relative frequency of the two biotypes in space and time. This was performed using an analysis of deviance model for binary data (y=1 for B, 0 for Ms), incorporating the effects of (i) sampling period (S1, S2, S3), (ii) sampling location described as quadrant (East, West, North, South) and site within quadrant (4 to 6 sites) (iii) host plant described as type (cultivated *vs* weed) and species within type (see above for the list of plant species). Tests of significance of particular effects were performed using standard model simplification procedures (Crawley, 1993). Accordingly, the change in deviance due to the removal of a given term was compared to a X^2 distribution. The residual deviance consistently showed hints of under-dispersion, rather than over-dispersion, making the X^2 tests relatively conservative in this case.

RESULTS

Microsatellite variability and allele size classes

From the total 662 whiteflies collected and tested, 34 were unsuccessfully amplified and 61 which were amplified for less than 6 of the loci were discarded. The eight microsatellite loci were highly polymorphic and had 9 to 33 alleles per locus. Many alleles scored were distant of less than two nucleotides, and after a first genetic analysis a strong heterozygote deficiency had been observed at many loci. Especially heterozygotes between two alleles with small size difference (2 bp or less) were lacking. We suspected that this was due to scoring artefacts and the difficulty to assess the true allele size with accuracy of 1 bp using an automated sequencer. In order to obtain conservative estimates of population structure and F-statistics, we decided to pool our alleles into allele-length intervals. To this end, allele frequencies were calculated for each locus and plots of frequency *versus* allele size were generated. Each plot typically displayed several peaks spanning over several bp. Our pooling procedure was such that each peak corresponds to a synthetic allele; the limit classes between two peaks were conservatively pooled with the highest peak. After transformation the eight microsatellite loci had between 4 to 11 synthetic alleles per locus. The most polymorphic marker was P59 with 11 synthetic alleles. The least polymorphic markers were P41 and P32 with respectively 4 and 5 synthetic alleles. In the following, we present only the results obtained with the synthetic alleles, which are consistent with those based on raw alleles (except for within-population heterozygote deficiencies, see below), though probably more robust to scoring artefacts. The heterozygote deficiencies largely decreased upon pooling alleles, even in loci that remained very polymorphic, suggesting that *f* values in the original dataset were largely artefactual (Part B of Table 6-2).

Table 6-2: Genetic diversity of the B and Ms populations within and among sites (all periods pooled). Results are presented after (A) and before pooling alleles (B, see text for details on allele pooling). The observed heterozygosity (Ho), the expected heterozygosity (He) and the fixation indices (Fis and Fst) of Weir and Cockerham (1984) were given by the software Genepop. Hardy-Weinberg tests are indicated together with Fis values, and tests of homogeneity among populations together with Fst values. *: p<0.05, **: p<0.01, ***: p<0.001. Non significant p-values are not indicated. The p-values were combined over all loci using Fisher's method.

	After (A)				**Before (B)**			
Locus / biotype	***Ho***	***He***	***Fis W&C***	***Fst W&C***	***Ho***	***He***	***Fis W&C***	***Fst W&C***
Ms								
P 53	0.5	0.7	0.29***	0.05***	0.58	0.93	0.36***	0.04***
P 62	0.5	0.5	-0.21*	0.02*	0.66	0.67	-0.02***	0.03***
P 7	0.4	0.4	-0.06***	0.04***	0.41	0.72	0.37***	0.07***
P 5	0.3	0.4	0.27***	0.06***	0.43	0.67	0.33***	0.05***
P 41	0	0.1	0.25***	0.28***	0.07	0.43	0.83***	0.10***
P 59	0.3	0.5	0.33***	0.10***	0.36	0.86	0.56***	0.05***
P 11	0.1	0.1	0.05	0.01	0.16	0.62	0.72***	0.06***
P 32	0.3	0.4	0.35***	0.10***	0.36	0.73	0.50***	0.05***
Average	**0.3**	**0.4**	**0.17*** **	**0.06*** **	**0.38**	**0.7**	**0.43*** **	**0.05*** **
B								
P 53	0.7	0.7	0.04***	0.03***	0.71	0.91	0.19***	0.04***
P 62	0.2	0.4	0.44***	0.16***	0.32	0.74	0.52***	0.11***
P 7	0.2	0.2	-0.14*	0.04***	0.35	0.78	0.52***	0.11***
P 5	0.1	0.4	0.57***	0.17***	0.23	0.63	0.57***	0.14***
P 41	0.2	0.2	-0.07	0.03	0.28	0.58	0.45***	0.10***
P 59	0.5	0.7	0.26***	0.05***	0.59	0.94	0.36***	0.04***
P 11	0	0	0.24	0.01	0.11	0.75	0.85***	0.08***
P 32	0.6	0.6	-0.02	0.01	0.62	0.86	0.26***	0.02***
Average	**0.3**	**0.4**	**0.18*** **	**0.07*** **	**0.4**	**0.77**	**0.45*** **	**0.08*** **

Biotype differentiation and phylogenetic analyses

Five hundred and sixty seven genotypes (females and males), together with 3 laboratory whitefly controls of the B strain and 2 laboratory whitefly controls from the Ms strain, were given to *Structure* . The whitefly population was separated into two groups (group 1: 308 individuals; group 2: 259 individuals), based on the variation of allele frequencies for the whitefly tested (Fig. 6-1. A). The individual assignation probabilities were all close to either 0 or 1, with no whiteflies in the [0.20; 0.80] interval, indicating the rarity of intermediate genotypes that combine alleles typical of both groups. Whiteflies present in group 1 clustered with the B biotype controls, while individuals present in the group 2 clustered with the Ms controls.

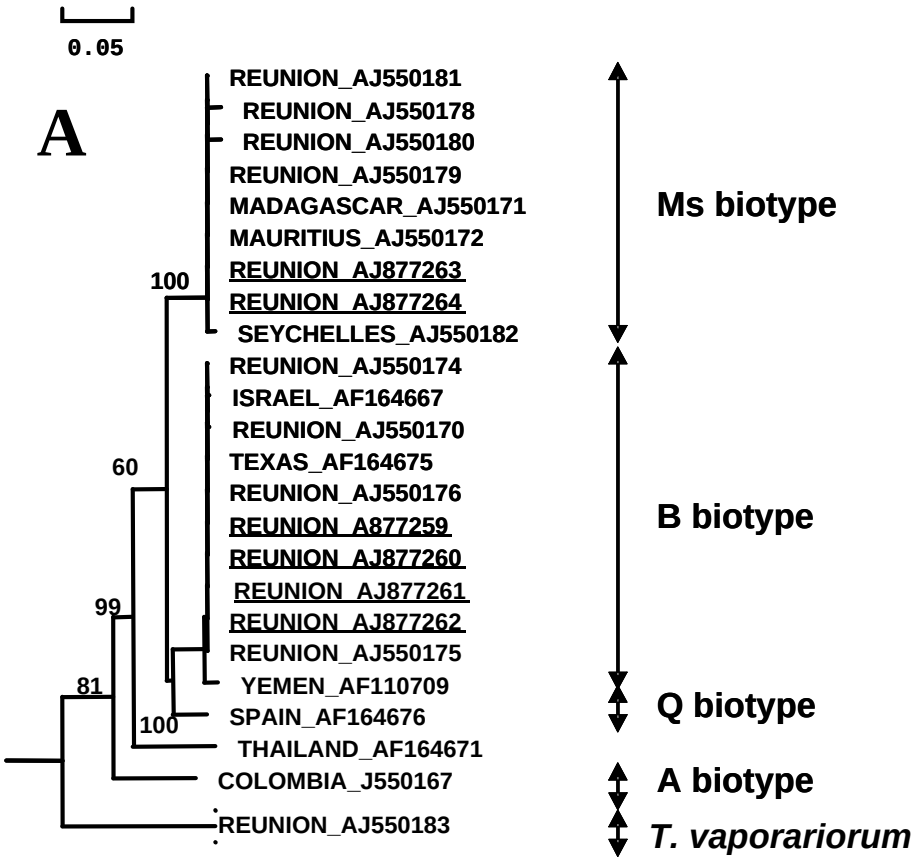

Fig. 6-1A and B. Identification of biotypes in the sample by molecular data.

1.A: Rooted neighbour joining tree showing the genetic distance among 370 nt cytochrome oxydase I fragments of *Bemisia tabaci*, either sequenced in this study or selected from GenBank. Sequences are identified with their accession number and the geographic origin of the tested whitefly. The sequences generated in this study are underlined and followed by their code number. One *Trialeurodes vaporariorum* sequence was added to this comparison, as an out-group. The scale measures the maximum likelihood distance between sequences. Numbers associated with nodes represent the percentage of 2000 bootstrap iterations supporting the nodes; only percentages = 60% are indicated.

To confirm the identity of the B and Ms groups obtained with *Structure*, partial COI sequences were generated from 6 of the 657 insects sampled. They were compared to each other and to GenBank sequences. Nucleotide sequence comparison was carried out on a fragment of 370 nts. Out of the 6 insects tested with COI 2 whiteflies: Saint Paul S1 (AJ877263) / Cambuston (S3 AJ877264) had been classified by *Structure* as Ms and 4 as B: Manapany S1 (AJ877259) / Petite Ile S1 (AJ877260) / Petite Ile S2 (AJ877261) / Etang Salé S2 (AJ877261), registered in EMBL. In the

phylogenetic tree derived from these alignments, these two groups belong to different well-supported clades (Fig. 6-1. B). The first one contains only sequences from the Indian Ocean region (including La Réunion) while the second also contains other sequences known to belong to invasive populations of the B biotype from Israel or USA. Thus, mitochondrial phylogeny is consistent with the B and Ms biotype classification obtained by *Structure* software.

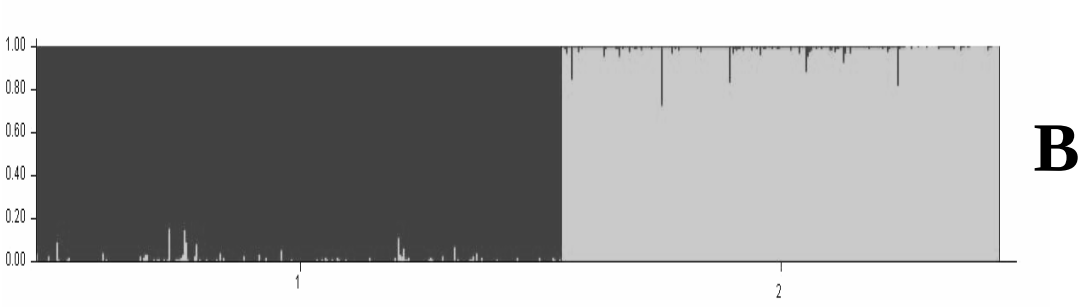

Fig. 6-1. B: Summary plot of estimates of Q (the estimated membership coefficient for each individual in each cluster) given by the software *Structure* ver. 2.1. Each of the 562 individual is represented by a single vertical line broken into k populations (k=2 in our case), with length proportional to each of the k inferred. The numbers 1 and 2 correspond respectively to B and Ms biotypes.

Genetic variation within and between the B and Ms biotypes

Genetic diversity was similar between the two biotypes (Table 6-2), whether synthetic alleles were used or not. Synthetic allele frequencies are given in appendix. For four loci of biotype Ms and B, the average observed heterozygosity (Ho) was lower than the average expected heterozygosity (He). The average within-population heterozygote deficiencies were similar for the B and Ms biotype (0.18 and 0.17, respectively) (Table 6-2 A). Both biotypes also show genetic substructure among populations (i.e. sampling sites), as attested by significant values of Fst (Table 6-2 A). The Fst values are comparable in both biotypes.

The AMOVA test showed most variation to be contained within population (66%) and among biotypes (31%) (Table 6-3). The genetic differences between the B and Ms biotypes account for much more genetic variance (31%, Fct=0.32, $p<0.001$) than those among populations within the same biotype (5%, Fsc=0.04, $p<0.001$). As mentioned above, this differentiation among populations is present both within the B and within the Ms biotypes.

The genetic AFC allows a graphical representation of the major axes of genetic differentiation within the sample. Only diploid females were included in the AFC. Although this analysis did not make use of the groupings identified by the Structure software, there is a striking correspondence between *Structure* groups and the position of individuals along the first two axes of the AFC, which together represent 12.03% of total genetic variation (see Fig. 6-2A).

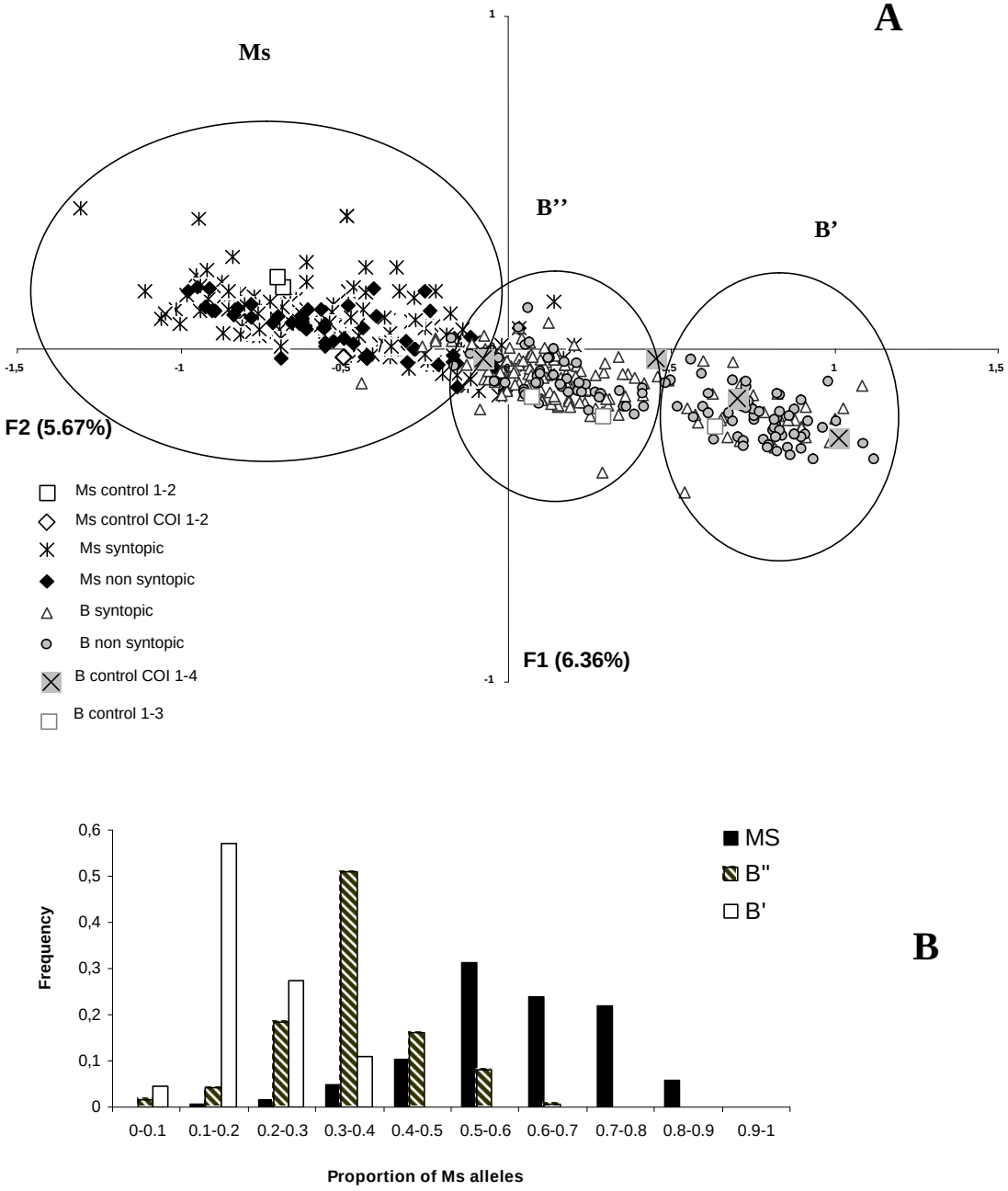

Fig. 6-2 Repartition of genotypes of *Bemisia tabaci* sampled over La Réunion and proportion of biotype Ms alleles per group of genotype. **A** First factorial plane of the genetic AFC. Biotypes B and Ms as identified by *Structure* are represented by different symbols; we also distinguish two subgroups within the B biotype, B' and B'' (see text). **B:** Distribution of the proportion of alleles of the Ms type within individuals of each of the three groups Ms, B' and B''.

Table 6-3: Analysis of molecular variance, and F-statistics of genetic differentiation between B and Ms biotypes and among sampling sites of *Bemisia tabaci* (populations) computed by the method of Excoffier *et al*, (1992). The P value is of: $P<10^{-5}$ for the three F-statistics.

Source of variation								
Among biotypes			Among sites within biotypes			Within sites within biotypes		
V	%	F_{CT}	V	%	F_{SC}	V	%	F_{ST}
0.60	31.37	0.32***	0.05	2.54	0.04***	1.25	65.74	0.34***

Despite a large dispersion of the data within each biotype, individuals from the two biotypes identified by *Structure* form well-separated clusters in the first factorial plane, especially along axis 1. However, in the factorial plane, the B group seems to contain two subgroups, one close (B’’), the other far (B’) from the Ms group (Fig. 6-2). Indeed, the distribution of the first principal coordinates (F1) within the B biotype is strongly bimodal, with one mode centred on 0.2 (subgroup B', 91 individuals) and the other on 0.8 (subgroup B" 173 individuals) (see Fig. 6-2).

However this separation was not found by *Structure*, even when allowing it to define more than two groups (results not shown). In order to investigate in more details the genetic constitution of the B' and B" groups, we first defined as "Ms alleles" all alleles with higher frequency in the Ms than in the B biotype (including both B' and B"). Then, as "B alleles" all other alleles, then evaluated for each individual the relative proportions of each category over all scored loci.

The results are presented in Fig. 6-2B, where the histograms make apparent that the subgroups B' and B" differ with respect to the distribution of this proportion (Non-parametric Kruskal-Wallis ANOVA, $X^2=114.0$, $P<0.001$). This confirms that the B" group is genetically intermediate between B' and Ms, as suggested by its position on the factorial plane. However not all loci participate equally in this effect. At most loci the frequency of the Ms allele in the B" group is near (though slightly above) that in the B' group (see appendix); the exception is locus P62, at which the frequency of the Ms allele in B" (0.917) is very high, equal to that in the Ms biotype (0.899), and very different from the B' group (0.069) (see appendix).

Other subgroups can be defined within the B cluster, on the basis of coexistence or not with the Ms biotype in the field. Within the B cluster, some individuals (hereafter *syntopic individuals*) belong to samples in which Ms individuals were also captured (i.e. in the same site, same plant and at the same time), while others (*non syntopic*) belong to samples in which no Ms individuals were caught (Fig. 6-2A). The syntopic B and non syntopic B subsamples were genetically differentiated (Fst=0.07, p<0.001 over all loci). Specifically, although syntopic and non-syntopic individuals were present in the two subclusters B' and B", non-syntopic individuals tended to be more concentrated in the B' (61 B' and 48 B") and syntopic individuals in the B" cluster (29 B' and 123 B"; Kruskal-Wallis non parametric ANOVA on the first principal coordinate, syntopic versus non syntopic: X^2=33.05, P<0.001, see Fig. 6-2A). On the other hand, no symmetrical difference was observed between syntopic Ms and nonsyntopic Ms individuals (Fig 6-2A, Kruskal-Wallis ANOVA, X^2=0.006, NS).

Frequency of the B biotype as a function of spatial, temporal, and host plant variables

Once non-significant interactions were simplified out, the final statistical model included the effects of host plant type (cultivated or weed) and species within type; sampling season; geographical sector and population within sector; as well as a significant interaction between sector and sampling season (Table 6-4).

Table 6-4. Deviance analysis on the effect of space, time and host plant on relative proportions of the B and Ms biotypes. Interactions between factors are denoted by dots (x.y), while nested factors are denoted by parentheses (x(y)). Only significant factors have been retained in the final model. Each factor or interaction is tested using changes in deviance between two models: with and without the factor. The model without the factor is denoted "reference model".

Factors	*df*	*Changes in deviance relative to reference model*	*p (X^2)*	*Reference model*	*Deviance of reference model*
Crop or weed (c)	1	18.67	<0.001	y+p(s)+y.s	464.57
Host plant (h(c))	9	24.92	0.003	y+p(s)+y.s+c	464.53
Year (y)	2	6.49	0.039	h(c)+p(s)	455.19
Sector (s)	3	20.02	<0.001	h(c)+y	619.49
Population (sector) (p(s))	15	150.8	<0.001	h(c)+y+s+y.s	546.41
Year. sector (y.s)	6	27.72	<0.001	h(c)+p(s)+y	448.7
Residual	525	420.98			
Global deviance	561				774.99

Biotype B tended to be concentrated in crops while biotype Ms was predominant on weeds (Fig. 6-3-Table 6-4). Over the total amount of *B. tabaci* biotype B, 68% of individuals identified as biotype B, but only 26% identified as biotype Ms were collected on vegetable crops (respectively 206 insects for B and 67 for Ms). The B population was found in all the sampled host plants whereas the Ms was absent from cotton and bean (Fig. 6-3). With regard to host plant species, eggplant and cabbage favoured the B biotype, while annual poinsettia and lantana favoured the Ms biotype. (Table 6-4, Fig. 6-3).

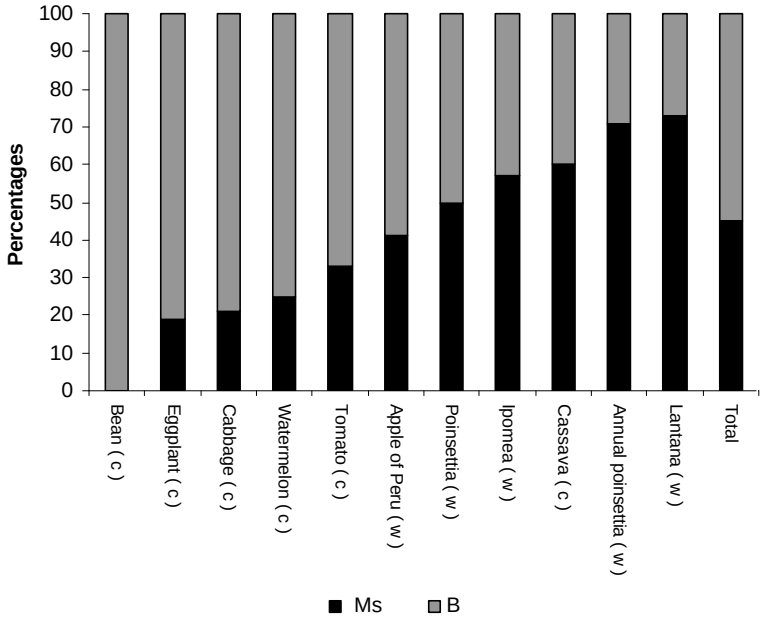

Fig. 6-3 Percentages of biotypes B (in grey) and Ms (in black) in different host plants, summed over all sites and times, Host plants were separated into two categories: weed (w) or cultivated (c).

Biotype B was present almost at each sampling site and at every sampling period except in the east part during the last samplings. Biotype B was dominant throughout the island except in the eastern region (Fig. 6-4). The deviance analysis showed that there were variations at the temporal, spatial and ecological levels (Table 6-4). Globally the east and north sectors had more Ms biotypes than the west and south. An increase in the Ms population was observed in the east over time, whereas in the three other sectors a decrease in number over time was observed.

DISCUSSION

(i) Posterior biotype discrimination on the basis of microsatellite data

The objectives of this study were to analyse the genetic variability of an indigenous and an exotic biotype of *Bemisia tabaci* present in La Réunion and their field distributions using newly developed microsatellite markers. Identification of biotypes and assessment of their relative proportions in the field is of primary importance in agronomic terms, because biotypes largely differ in their ecological requirements as well as agronomical impact (reviewed in: Perring 2001; Oliveira *et al.* 2001). Many invasions by biotypes of *B. tabaci* may remain cryptic because established biotypes, morphologically indistinguishable from invasive ones, are already present (Bird & Maramorosch, 1978). In this respect, *B. tabaci* is not an isolated example (Genner *et al.*, 2004) and there is a growing need of reliable genetic screens to evaluate the impact of cryptic invasions in many different groups. Up to now, the main available techniques to distinguish biotypes involved laboratory assays of biological traits, plus molecular tests such as sequencing of mitochondrial genes (Frohlich *et al.*, 1996). Neither method is adapted to large-scale assessment of biotype frequency at the regional scale (especially if previously undescribed biotypes are involved). Our results show that microsatellite analysis, combined with bayesian statistics (*Structure* software) can efficiently fill this gap. The validity of the groups given by *Structure* is supported by several lines of evidence. First, the assignation probabilities given by Structure are always very close to 0 or 1, suggesting that it has efficiently converged towards well-defined group limits. Second, the differenciation (Fct) between the two groups assumed to be biotypes B and Ms is very high (0.32) relative to geographic variation within biotypes (Fsc=0.04). Third, the two groups are well-separated in the first factorial plane of the AFC, an independent type of analysis that makes no assumptions on sample subdivision. Fourth, all the controls, be they laboratory or COI-sequenced individuals, have been correctly classified. This consistency suggests that *Structure* classification is relatively robust to the violation of one of its basic hypothesis: Hardy-Weinberg frequencies within each group. Here, both groups are characterized by (i) significant, if low, geographic substructure, and (ii) large heterozygote deficiencies, a part of which might be of artefactual origin.

Two other microsatellite banks were recently published (De Barro *et al.*, 2003; Tsagkarakou & Roditakis, 2003), which are providing more microsatellite markers which might be very interesting for other genetic studies on large scale experiments on different biotypes.

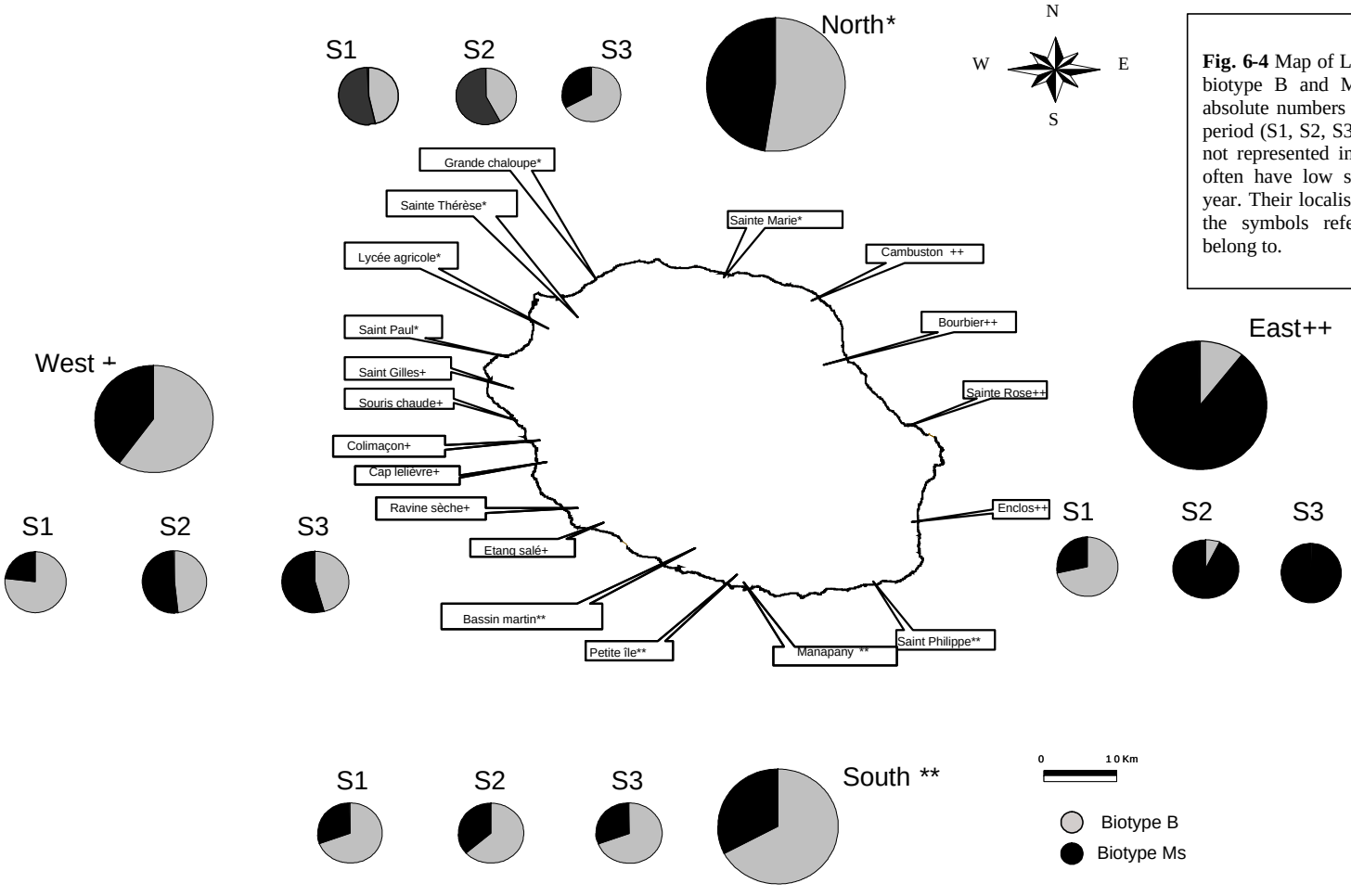

Fig. 6-4 Map of La Réunion with whitefly biotype B and Ms global repartition in absolute numbers per sector and sampling period (S1, S2, S3). The different sites are not represented individually because they often have low sample sizes in a given year. Their localisation is represented, and the symbols refer to the sectors they belong to.

(ii) Genetic variation within Biotypes

Both biotypes displayed considerable genetic variation at the microsatellite loci analysed (up to He=0.9). As we have mentioned in the Results section, we recognise that one part of this variation may be artefactual. However, even with a very conservative pooling procedure, the remaining variation is important and retains all the main information of the dataset in terms of genetic structure (within-biotype Fst, *Structure* group identities, AFC). Biotype Ms has already been found in other islands of the region (Delatte *et al.*, 2005c), and its area of origin could be on another island, especially knowing that La Réunion is one of the youngest islands in the area (2.2 million years). Genetic diversities within the biotypes Ms and B, one ancient indigenous, the other a recently arrived invader, are comparable. This suggests that the introduction of biotype B was not associated with a severe genetic bottleneck. More than one introduction might be responsible for this invasion and this might have occurred several years ago, even some years before the first TYLCV upsurge observed in 1997. Interestingly, in a few years, the introduced biotype has reached the same levels of geographic structuration (Fst) across the island as the local biotype.

(iii) Ecological, spatial and temporal determinants of biotype distributions

Genetic screening provides for the first time the opportunity to analyse the details of biotype distribution. Our first main result is to document a rapid spread of the invasive B biotype towards all parts of the island, although it has not wiped out the resident biotype in any sector. However the proportions of the two biotypes are far from constant and vary according to geographic or ecological factors. The B biotype is found predominantly in the north, west and south part, while Ms predominates in the eastern region, although it is present in all the sampled sites. This is consistent with field observations. Indeed the first *B. tabaci* upsurge (1991) was observed in the south and so were the first TYLCV symptoms (at Saint Pierre in 1997). According to epidemiological surveys in 1997-1999, TYLCV spread from Saint Joseph (south, close to Manapany) to La Possession (north west, close to St Paul) in two years, and no symptoms of TYLCV have been reported so far from the eastern part of La Réunion (Reynaud *et al.*, 2003). A general scheme of the invasion process of the B biotype seems to emerge, with a starting point somewhere in the southwest and an invasion flow down south and up north.

Although the relative frequencies of biotypes may not have reached equilibrium in all parts of the island yet, ecological differentiation is already visible between the two biotypes. The low frequency of the B biotype in the eastern regions could potentially be explained by a better adaptation of the Ms biotype to their very humid climate. However, the distribution of host plants can also be responsible. Indeed the B biotype is dominant on vegetable crops, whereas the Ms biotype is found on weeds, which are abundant in the east, where there are relatively few vegetable crops.

We are not in position to predict the outcome of interactions between both biotypes in the field. In their review Reitz and Trumble (Reitz & Trumble, 2002) provide compelling evidence that supports the importance of interspecific competition as a mechanism structuring insect species

communities despite assumption that food is not limited for these species. As de Bach (De Bach, 1966) points out, the displacement of an established species is more likely to occur than its complete exclusion, which might be the case of the Ms biotype. However we cannot exclude the possibility that the Ms biotype be in the process of being almost completely excluded by B as happened to the A biotype in the USA (Brown *et al.*, 1995).

Another potential outcome to consider is inter-biotype mating and emergence of a hybrid swarm. The AFC results suggests two distinct subgroups within biotype B, one of which (B') is genetically far from the Ms biotype, while the other (B") has a higher frequency of alleles typical of the Ms group, especially at one locus (P62). Two hypotheses may account for these two groups: (i) either there are two biotypes, not one, within the cluster identified by *Structure* and identified as B. (ii) Or there is some local introgression of Ms alleles within B genotypes, and B' and B" represent pure and introgressed forms of the B biotype. We think the second hypothesis is more likely for several reasons. First, if B' and B" were independent biotypes, there would be no reason why the genetic differences between them bear mainly on the frequency of alleles typical of the Ms biotype. We would therefore not expect B" to be exactly intermediate between B' and Ms in the first factorial plane of the AFC analysis. Second, COI sequences are available in both B' and B", and they are both identical to reference B sequences. Third, we observed more B" individuals in syntopic populations of the B biotype and more B' individuals in non-syntopic populations. This is consistent with the idea that introgressed individuals should be found more frequently when the B and Ms biotypes are in close contact. Note that unequal introgression across loci (e.g. the much larger introgression rate at locus P62 than at other loci) is a quite regular phenomenon in the hybridization literature (Barton & Hewitt, 1985; Bierne *et al.*, 2003). The assumption that *B. tabaci* biotypes are genetically isolated prevails in the literature, though actual tests are rare (Frohlich *et al.*, 1996; Frohlich *et al.*, 1999; DeBarro *et al.*, 2000). Biotypes are ecologically separated by their distinctive host ranges (see above for the case of B and Ms). However this is only a partial isolation, as attested by the many individuals found in syntopy in the present study. More data are needed to definitely document the ability of both biotypes to interbreed when present in the same plant and more evolutionary time is needed to confirm, and know the long-term outcome, of introgression in the field.

ACKNOWLEDGEMENTS

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Appendix. Allele frequencies at each of the 8 locus of groups Ms, B'', B' and B (total B'+B"), respectively. Alleles classified as typical of Ms (see text) are in italics. Alleles were conservatively grouped into size classes (see text), so each allele represents a range of sizes.

P53					P62					P7					P5				
allele size	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**
130-133	0.266	0.085	0.022	0.064	***180***	0.01	0	0	0.000	**131**	0	0.003	0	0.002	**181**	0	0.011	0	0.008
137-140	0.128	0.418	0.467	0.434	**201**	0	0	0.017	0.006	**154**	0	0.018	0.034	0.023	**202-204**	0.008	0.034	0.183	0.085
141-144	0.007	0.06	0.093	0.071	**203-205**	0.033	0.025	0.075	0.041	***160***	0.035	0.016	0	0.011	**207-210**	0.038	0.14	0.783	0.358
148-151	0.021	0.016	0.011	0.015	***206-208***	0.247	0.105	0.063	0.091	**173-176**	0.022	0.068	0.18	0.103	**211**	0.102	0	0.006	0.002
152-154	0.312	0.197	0.165	0.186	**209-210**	0.062	0.066	0.822	0.312	**178-183**	0.75	0.878	0.787	0.849	***212-214***	0.565	0.815	0.028	0.548
155-158	0.05	0.063	0.093	0.073	***211-214***	0.643	0.793	0.006	0.537	***184-188***	0.193	0.013	0	0.009	***215-219***	0.281	0	0	0.000
159-162	0.209	0.123	0.143	0.130	**215-220**	0.005	0.006	0.011	0.007	**207**	0	0.003	0	0.002	***227***	0.005	0	0	0.000
163-169	0.007	0.038	0.005	0.027	**226**	0	0.006	0.006	0.006	**214**	0	0.003	0	0.002					

P41					P59					P11					P32				
allele size	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**	**allele size**	**Ms**	**B''**	**B'**	**B**
132	0.007	0	0	0.000	***118***	0.005	0	0	0.000	***169***	0.007	0.008	0	0.005	**192-196**	0.109	0.457	0.542	0.486
152	0.017	0.011	0	0.008	***171***	0.017	0	0.022	0.008	***174-176***	0.069	0.005	0.006	0.005	***200-203***	0.635	0.387	0.333	0.369
158-162	0.03	0.122	0.207	0.149	**175-178**	0.072	0.214	0.124	0.183	**178-182**	0.92	0.982	0.994	0.986	***204-206***	0.121	0.034	0.042	0.036
163-167	0.947	0.866	0.793	0.843	***179-182***	0.636	0.074	0.022	0.056	***186***	0.002	0	0	0.000	***207-209***	0.112	0.015	0.024	0.018
					183-186	0.069	0.335	0.455	0.377	**194**	0	0.005	0	0.004	**210-214**	0.023	0.107	0.06	0.091
					193-198	0.17	0.03	0.017	0.025	***208***	0.002	0	0	0.000					
					200-204	0	0.033	0.073	0.047										
					205-209	0.002	0.163	0.202	0.177										
					210-212	0	0.003	0.051	0.019										
					213-215	0.024	0.128	0.022	0.091										
					216-219	0.005	0.021	0.011	0.017										

PART III: RESISTANCE TO BEGOMOVIRUSES

CHAPTER 7

Vector and graft-inoculation of mono and bipartite begomoviruses reveals both broad and specific resistant genotypes in *Lycopersicon* species[1]

ABSTRACT

Since the early nineties, *Potato yellow mosaic virus* (PYMV, bipartite begomovirus) has caused yield losses in tomato crops in the Caribbean. Recently, *Tomato yellow leaf curl virus* (TYLCV, monopartite begomovirus) overlaps the occurrence of PYMV. Until now, very few tropical tomato cultivars resistant to TYLCV, and none resistant to PYMV or both viruses are available. This study was carried out with the objective of identifying resistance to both viruses. Artificial infections of 22 wild *Lycopersicon* accessions were conducted for PYMV and TYLCV using vector and graft-inoculation; incidence and severity were rated. Although none of the accessions were completely immune, we observed resistance to both viruses in a single genotype. In *L. pimpinellifolium* INRA Hiruste and LA 1478 partial resistance to both viruses was observed after vector-inoculation and was overcome after graft-inoculation. It was hypothesized to concern vector resistance, which could be efficient against other begomoviruses. A high level of resistance was observed in *L. chilense* LA 1969 to both viruses after vector inoculation and was not overcome after graft-inoculation of TYCLV. In contrast, *L. pimpinellifolium* LA2187-5 was found highly resistant to PYMV and susceptible to TYLCV. The use of different types of resistance in breeding programs is discussed.

[1]Boissot, N., Delatte, H., Urbino, C., Dintinger, J., Reynaud, R. & Pavis, C. (2005). Vector and graft-inoculation of mono and bipartite begomoviruses reveals both broad and specific resistant genotypes in Lycopersicon species. *Plant Disease* Submitted in a slightly modified version.

INTRODUCTION

Begomoviruses (family *Geminiviridae*) have become one of the major limiting factors in worldwide tomato production (Polston & Anderson, 1997), their rapid expansion being due to intensified international trading and global spread of both vector and infected plant material, including tomato fruits (Delatte *et al.*, 2003a). More than 100 begomoviruses are exclusively transmitted by the whitefly *Bemisia tabaci* (Jones, 2003). Most begomoviruses have bipartite genomes, nevertheless some begomoviruses, originated from the Old World have monopartite genome. Since the late eighties, *Potato yellow mosaic virus* (PYMV), belonging to the bipartite group of begomoviruses, has spread over the Caribbean. On tomato, PYMV was first reported in Venezuela (Guzman *et al.*, 1997). It was then identified in Guadeloupe, Martinique and in the Dominican Republic (Polston *et al.*, 1998). A recombinant virus between PYMV and a virus closed to the *Sida Golden Mosaic Virus* from Honduras, so called now PYMTV, was reported in Trinidad (Umaharan *et al.*, 1998). At least five other bipartite begomoviruses infected tomato in the Caribbean Islands (Jones, 2003). *Tomato yellow leaf curl virus* (TYLCV), representative of the monopartite group, was known as a major disease on tomato in Middle East countries for decades. From the late eighties, TYLCV has spread to many countries of the Old and New World, even reaching Réunion Island (Indian Ocean) in 1997 (Peterschmitt *et al.*, 1999b). TYLCV has also spread to the Caribbean reaching the French West Indies in 2001 (Jones, 2003). The TYLCV isolate occurring in Guadeloupe has a strong similarity with TYLCV isolates from Cuba and the Dominican Republic (Urbino *et al.*, 2004).

The emergence and rapid spread of begomoviruses such as TYLCV and PYMV, sometimes causing mix-infections in regions of overlap, represent a major threat for tomato production. Because insecticide used for managing vector activity is ineffective (Costa *et al.*, 1993a) and damageable for environment, genetic host resistance is of crucial importance in the sustainable control of these diseases. Many wild *Lycopersicon* species have been screened for their response to TYLCV (Pico *et al.*, 1996). In most cases, resistance to TYLCV identified in several accessions of wild origin appeared to be oligogenically inherited with resistance factors ranging from recessive to dominant (Morales, 2001). A main factor of resistance was mapped on chromosome 6, for both resistances derived from *L. chilense* (Zamir *et al.*, 1994) and *L. pimpinellifolium* (Chagué *et al.*, 1997) with two modifier genes present on chromosome 3 and 7 for *L. chilense*. Two genes acting epistatically were mapped in a population derived from *L. hirsutum* (Hanson *et al.*, 2000). In contrast, very few studies were carried out with the objective of identifying resistance to bipartite begomoviruses. Most of these studies have been conducted on *Tomato mottle virus* (ToMoV) (Scott & Schuster, 1991). A gene mapping study showed that two additive regions were involved in ToMoV resistance from *L. chilense* accession LA 1932 (Griffiths & Scott, 2001). In the Caribbean islands, the likelihood of mixed infections by TYLCV and PYMV argue for a strategy of breeding tomato resistant to both diseases.

The objectives of this study were to evaluate and compare levels of resistance to PYMV and TYLCV in a wide range of *Lycopersicon* accessions, focusing on resistance against both viruses. To

do this, we worked on 6 *Lycopersicon* species, and PYMV and TYLCV incidence and severity were quantified after vector and graft-inoculation. The study was carried out in Guadeloupe, for PYMV, and in Réunion Island, for TYLCV.

MATERIALS AND METHODS

Plant material

Seeds from several wild types of *Lycopersicon* were obtained from the Institut National de la Recherche Agronomique, Monfavet (France), from Vilmorin Ltd. (France), and from the Tomato Genetics Resources Centre (University of California, Davis, USA). Caraïbo from INRA, Guadeloupe (France) and Farmer from Know You Seed, are commercial cultivars, they were used as susceptible controls. Seeds were germinated under insect-proof greenhouse conditions and the whole experiment carried out in greenhouses with day-temperature ranging from 20 to 35°C.

Insect material

B. tabaci B biotype populations used for screening in Réunion island originated from nymphs collected on cabbage (*Brassica oleracea*), whereas, in Guadeloupe, they originated from nymphs collected on Cucurbitaceae. Insects were mass-reared under laboratory conditions (12h/12h photoperiod, 25 ± 2° C) on cabbage in Réunion and on tomato plants (cv Lanaï) in Guadeloupe. Cabbage plants with nymphs at L4 stage were transferred to cages containing TYLCV-infected tomato plants of Farmer. After emergence of the adults the cabbage plants were removed, and the adults were kept feeding for a 72 hr-acquisition access period (AAP) on the tomato plants, before being used as viruliferous vectors for tests of resistance to TYLCV. In Guadeloupe, adults from mass rearing were transferred to PYMV-infected tomato plants of Caraïbo for 48hr-AAP, before being used as viruliferous vectors for testing the resistance to PYMV.

Vector-inoculation

As transmission rates are higher in TYLCV than in PYMV (C. Pavis, C. Urbino, R. Guillaume, and N. Boissot, *unpublished*), the experimental conditions differed for both viruses. Fifteen whiteflies for TYLCV inoculation and 50 for PYMV inoculation were caged under plastic cylinders (diameter: 7 cm, height: 10 cm) containing one plant at the two-leaf stage for 72-hr inoculation access period (IAP) for TYLCV and 48-hr IAP for PYMV. The top of the cylinders was made of thin netting to allow evaporation and airflow. Whiteflies were introduced into the cage through a 3mm hole of the cylinder, which was closed with a piece of filter paper to absorb extra humidity. At the end of the IAP, insects were removed, and plants were treated with imidaclopride (Confidor®, Bayer, Basel, Switzerland) and placed into insect proof cages.

Graft-inoculation

A piece of TYLCV-infected stem of susceptible cultivar Farmer or PYMV-infected stem of susceptible cultivar Caraïbo, was used as a scion onto three-weeks old seedlings from the tomato accessions screened in the experiment.

Disease assessment

Several incomplete experiments, each including susceptible controls, were carried out for vector and graft-inoculation tests. Since these experiments were carried out under the same optimal conditions for virus inoculation, the data obtained for each accession were pooled for analysis. For both viruses, disease incidence (proportion of infected plants in an accession) was estimated 3 weeks after inoculation, by recording symptoms. The presence/absence of begomovirus was confirmed by triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) using a kit supplied by Adgen (Ayr, Scotland, UK). The protocol was according to the manufacturer's recommendations (Adgen). Leaf-extracts prepared from TYLCV or PYMV-infected plants of Farmer or Caraïbo were used as positive controls and healthy non-infected tomato leaf extracts as negative controls. Virus was declared to be present in a plant when the absorbance value was higher than twice the mean absorbance of the negative control. Intervals of confidence for disease incidence (P = 0.05) were determined following Saporta (Saporta, 1990). Symptom severity was assessed on diseased plants using a rating scale from 0 (no symptoms) to +++ (severe symptoms) similar to the one used by Maruthi *et al.* (Maruthi *et al.*, 2003).

RESULTS

Infection pressure in vector and graft-inoculation studies

One hundred percent of the plants of the susceptible control Caraïbo showed symptoms after graft-inoculation with PYMV while 20% were symptomless and tested negative (TAS-ELISA) after vector-inoculation. As heavy inoculation was used (50 whiteflies caged per plant), these plants were supposed to represent the rate of escape from infection in PYMV experiments. We used 2 to 15 control plants for each PYMV experiment that allowed to discard unsuccessful inoculation with at least P = 0.96). TYLCV-inoculated plants of the susceptible control Farmer showed an infection rate of 94% after vector inoculation and 100% after graft inoculation. Therefore, we considered that very few plants escaped in whitefly-mediated TYLCV inoculation experiments. Generally, disease incidence was higher after graft inoculation than after vector inoculation, while symptom severity was not affected by the inoculation procedure.

Table 7-1. Number of diseased plants showing symptoms or TAS-ELISA positive (incidence) and symptoms on diseased plants (severity) in *Lycopersicon* accessions after vector or graft-inoculation of PYMV.

Species	Accessions	Vector inoculation			Graft inoculation		
		Severity[a]	Incidence and Confidence interval (%)[b]		Severity[a]	Incidence and Confidence interval (%)[b]	
L. esculentum							
	Caraïbo	++	92/115	71-87%	++	50/50	93-100%
	CRA66	+++	8/9	54-100%	++	1/1	
	Atatürk	0	15/36	26-58%	0	6/21	11-49%
L. pimpinellifolium							
	INRA Hirsute	++	28/71	28-50%	++	29/33	71-97%
	LA 121	+++	8/8	63-100%			
	LA 1478	+	27/56	35-62%	+	28/30	72-99%
	LA 1582	+++	13/13	75-100%			
	LA 722	++	35/41	68-94%	++	2/2	
	LA 2187-5	0	2/27	1-23%	0	7/39	7-35%
	Typique site 10	+++	22/30	54-88%			
	Vil Oseil	++	48/59	68-91%	++	19/22	63-97%
	Wva 106	+++	30/30	88-100%			
L. chilense							
	LA 1969	0	8/44	8-34%			
L. peruvianum							
	CMV INRA		36/41	72-96%			
L. hirsutum							
	PI 134 417		9/9	69-100%			
	LA 1777		7/10	44-93%			
L. pennellii							
	LA 716		4/9	14-72%			

[a] Severity: 0 : no symptoms, + mild symptoms (light yellowing, no curling), ++ moderate symptoms (light yellowing, slight curling) +++ severe symptoms (stunting and curling, severe mosaic).

[b] $p = 0.05$

Screening for PYMV resistance

Incidence and severity of PYMV infection in tested tomato accessions are summarized in Table 7-1. *L. esculentum* accession CRA 66 was at least as susceptible as Caraïbo after PYMV inoculation. In contrast, *L. esculentum* accession Atatürk showed only 30 to 40% of PYMV infected plants after both inoculation procedures. The Atatürk infected plants remained symptomless indicating high resistance to PYMV.

Among the nine *L. pimpinellifolium* accessions tested, five were susceptible to PYMV with incidence and symptom severity comparable to those of the control (LA 121, LA 1582, LA 722, Typique site 10, Vil Oseil, and Wva 106). LA 2187-5 was found to be highly resistant to PYMV using either inoculation method. This accession was obtained in Guadeloupe by selfing one resistant plant free of PYMV (TAS ELISA test) after vector inoculation among ten plants of the accession LA 2187. Symptoms were never observed on LA 2187-5 after PYMV inoculation, except for some mild symptoms on one plant after graft inoculation. Nevertheless, PYMV was detected by TAS-ELISA in 7% of the plants after vector inoculation, and 18% after graft inoculation. Fifteen plants of the F_1 generation from the cross CRA 66 X LA 2187-5 and 13 plants of the F_1 generation from

the cross Vil Oseil X LA 2187-5 were vector-inoculated with PYMV. Only one plant of each F_1 was free of virus, all the other plants exhibited severe PYMV symptoms (76-100% of susceptible F1 plants). Then, both F1 were as susceptible as the control Caraïbo, demonstrating that this resistance to PYMV is recessive. For accessions LA 1478 and INRA Hirsute, an incidence of about 40% lower than on the control Caraïbo was obtained after vector inoculation. For both accessions, this partial resistance tended to be overcome at high inoculation pressure by graft inoculation. Symptoms on diseased plants were mild (LA1478) to moderate (INRA Hirsute).

Accessions belonging to four other species of *Lycopersicon* were vector-inoculated with PYMV. *L. chilense* LA 1969 was highly resistant with few plants infected but remained symptomless. Incidence on *L. pennelli* LA 716 was lower than on the control Caraïbo. In contrast, incidence was as high as on the control Caraïbo after vector inoculation on *L. hirsutum* accessions PI 134 417 and LA 1777, and *L. peruvianum* CMV INRA. Symptom scoring using our standard scale was unreliable on *L. pennelli*, *L. hirsutum* and *L. peruvianum*. Nevertheless all the infected plants exhibited symptoms that were severe on PI 134 417.

Screening for TYLCV resistance

Incidence and severity of TYLCV infection in tested tomato accessions are summarized in Table 7-2. *L. esculentum* CRA66 was slightly less susceptible to TYLCV than Farmer, whereas Atatürk showed partial resistance with 40 to 60% of the plants exhibiting mild/moderate symptoms using either inoculation method.

Among the eleven *L. pimpinellifolium* accessions tested, partial resistance to TYLCV, effective for incidence but not for severity, was observed in INRA Hirsute, LA 121, LA 1478, and in a lesser extent in LA 2187-5 after vector inoculation. Resistance in these accessions was overcome when plants were graft-inoculated.

L. chilense LA 1969 showed the highest level of resistance to TYLCV with less than 15% of infected plant after vector inoculation. This proportion increased when we used graft inoculation but TAS-ELISA positive plants still remaining symptomless. Incidence of TYLCV was only reduced after vector inoculation on *L. peruvianum* CMV INRA, *L. hirsutum* LA 1777 and *L. pennelli* LA 716 compared to Farmer, but not after graft inoculation. Symptoms were mild to moderate on these three genotypes.

Table 7-2. Number of diseased plants showing symptoms or TAS-ELISA positive (incidence) and symptoms on diseased plants (severity) in *Lycopersicon* accessions after vector or graft-inoculation of TYLCV

Species	**Accessions**	**Vector inoculation**			**Graft inoculation**		
		Severity[a]	Incidence and Confidence interval (%)[b]		Severity[a]	Incidence and Confidence interval (%)[b]	
L. esculentum							
	Farmer	+++	30/32	78-99%	+++	20/20	84-100%
	CRA66	+++	20/29	39-86%	+++	17/22	54-92%
	Atatürk	+	11/27	23-60%	+	19/31	42-79%
L. pimpinellifolium							
	64316		19/20	75-100%			
	66063	+++	39/44	68-96%			
	INRA Hirsute	+++	67/143	32-53%	+++	75/77	92-100%
	L3708	++	37/41	81-97%			
	LA 121	++	11/33	17-52%	+++	12/16	48-93%
	LA 1478	+++	63/123	42-60%	+++	38/49	64-86%
	LA 2187-5	++	49/79	48-75%	++	11/14	50-94%
	PI 126461	+++	22/26	62-85%			
	Typique site 10	+++	56/64	76-94%	+++	11/13	51-96%
	Vil Oseil	++	20/30	46-82%	+++	24/24	80-100%
	Wva 106	+++	62/65	86-98%	+++	75/75	94-100%
L. chilense							
	LA 1969	+	3/20	4-38%	0	10/15	38-85%
L. peruvianum							
	CMV INRA	++	15/56	15-41%	++	38/46	68-92%
L. hirsutum							
	LA 1777	++	20/60	21-47%	++	35/49	57-83%
L. pennellii							
	LA 716	+	2/13	1-44%	++	26/30	68-96%

[a] Severity: 0 : no symptoms, + mild symptoms (light yellowing, no curling), ++ moderate symptoms (light yellowing, slight curling) +++ severe symptoms (stunting and curling, severe mosaic).

[b] **p = 0.05**

Comparison of resistance between PYMV and TYLCV

LA 1969 was the only accession, which was highly resistant to both viruses when vector-inoculated. A partial resistance in Atatürk and LA 716 was effective against both viruses by reducing incidence and severity when plants were vector-inoculated; this resistance was partially overcome when plants were graft-inoculated by TYLCV. Partial resistance in accessions INRA Hirsute and LA 1478, which was effective against both viruses, only reduced disease incidence and was overcome by graft inoculation with both viruses. The most PYMV resistant accession LA2187-5 was only slightly resistant to TYLCV. Incidence of TYLCV was significantly lower on *L. hirsutum* LA 1777 and *L. peruvianum* CMV INRA than on Farmer. LA 1777 and CMV INRA were susceptible to PYMV.

DISCUSSION

Graft mediated transmission was 100% successful on the susceptible controls (Caraïbo for PYMV and Farmer for TYLCV). Cage inoculation technique was described as the most suitable whitefly-mediated inoculation technique to screen wild *Lycopersicon* (Pico *et al.*, 1998), and was used in this study. We inoculated individually two-leaf old plantlets with 50 PYMV-viruliferous insects that allowed reaching 80% transmission rate for this virus on the control. Because of the small size of the plantlets, we did not increase the number of viruliferous insects used that could allow improving this rate. TYLCV transmission rate (94%) on the control was higher to PYMV transmission rate in accordance with previous studies (Delatte *et al.*, 2005c) (C. Pavis, C. Urbino, R. Guillaume, and N. Boissot, *unpublished*). Despite the lower efficiency of PYMV transmission enough viruliferous insects were used to compare the levels of resistance to TYLCV and PYMV in our accessions.

Potential sources of natural resistance to begomoviruses are present in wild types of *Lycopersicon* (Pico *et al.*, 1996; Morales, 2001). With respect to bipartite begomoviruses, resistance has been investigated for viruses from Florida (Scott & Schuster, 1991), Brazil (Santana *et al.*, 2001), Trinidad (Rampersad & Umaharan, 2003b) or India (Maruthi *et al.*, 2003) for example. Some resistant accessions to these begomoviruses were previously described as being resistant to TYLCV. Nevertheless because resistance evaluation procedures differed, broad resistance was not clearly evaluated. When comparing incidence and severity for TYLCV and PYMV in the genotypes tested in this study, it appeared that both broad-spectrum forms of resistance and virus-specific were available in wild germplasm.

Because viruses transmitted by *B. tabaci* are deposited within the phloem through salivation, altered feeding behaviour could result in a significant decrease in incidence of several begomoviruses. This type of vector resistance has been reported in studies with *Rice ragged stunt virus* transmitted by planthopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) on rice (Parejarearn *et al.*, 1984) and MMV/MStV transmitted by the planthopper *Peregrinus maidis* (Ashmead) (Hemiptera: Delphacidae) on maize (Dintinger *et al.*, 2005). We used graft plus vector-

inoculation of two begomoviruses to track this vector resistance. It is tempting to assume that vector resistance should be efficient against PYMV and TYLCV when plantlets were vector-inoculated (mainly lower incidence than on control) but inefficient when plantlets were graft inoculated. As, in most cases, we observed for both viruses that incidence was higher after graft inoculation than after vector inoculation, partial resistance to the vector might be frequent in wild *Lycopersicon*. *L. pimpinellifolium* INRA Hirsute had the clearest phenotype of vector resistance, partially resistant to TYLCV and PYMV upon vector inoculation, susceptible to TYLCV and PYMV upon graft inoculation. The resistance to both viruses after vector inoculation found in the *L. pennelli* accession LA 716 (overcame after graft inoculation by TYLCV) was in accordance to its acyl sugar contents, known to be a whitefly repellent (Liedl *et al.*, 1995). Five genomic regions were detected as being associated with acyl sugar production (Mutschler *et al.*, 1996). Nevertheless, to our knowledge, the transfer of these factors in a cultivated *L. esculentum* has not been successful, probably due to unfavorable linkages. Then, vector resistance should be verified by studying incidence of disease after vector inoculation by other begomoviruses in *L. pimpinellifolium* INRA Hirsute.

L. chilense LA 1969 was highly partially resistant to both PYMV and TYLCV, with plants becoming infected remaining symptomless or showing only mild symptoms. LA 1969 was first reported to be resistant to TYLCV by graft inoculation (Fargette *et al.*, 1996) and vector inoculation (Zakay *et al.*, 1991). It was reported resistant to TMoV (Scott & Schuster, 1991) and PYMTV (Rampersad & Umaharan, 2003b) in field screenings and to a Brazilian bipartite begomovirus after controlled vector inoculation (Santana *et al.*, 2001). Piven *et al.* (Piven *et al.*, 1995) observed only slight symptoms on this accession after vector inoculation with ToYMV from Venezuela, a begomovirus very closed to PYMV (Urbino *et al.*, 2004). The presence in LA 1969 of a broad-spectrum resistance against begomoviruses, both from the New World and the Old World, is supported by our results. Nevertheless, Santana et al (Santana *et al.*, 2001) observed the line TY52, homozygous for TY-1 (Michelson *et al.*, 1994) susceptible to the Brazilian begomovirus. In accordance with this result, *L. esculentum* populations or lines derived from LA 1969 and selected for TYLCV resistance were only partially resistant to the Brazilian begomovirus (Santana *et al.*, 2001), to PYMTV (Rampersad & Umaharan, 2003b), TMoV (Scott *et al.*, 1996) and to PYMV (data not shown). The major gene TY-1 that controls resistance to TYLCV in LA 1969 may not control the resistance to other begomoviruses. Then, the resistance factors to other begomoviruses than TYLCV in LA 1969 may have been lost during introgression process of TY-1 in *L. esculentum*. Because interspecific crossability barriers are strong between *L. chilense* and *L. esculentum*, introgression of several resistance factors from LA 1969 in *L. esculentum* will be hazardous and then the used of LA 1969 broad resistance will be difficult.

Although being only moderately resistant to TYLCV, *L. pimpinellifolium* LA2187-5 showed the highest level of resistance to PYMV among all accessions tested. This is the first resistance to PYMV described. This resistance appeared as recessive in our preliminary study on F_1 generations but further genetic analyses are needed to evaluate the number and the role of factors involved. Otherwise, oligogenic recessive resistance to TYLCV has been reported in *L. peruvianum* LA 111 (Pilowsky & Cohen, 1990). Recently, natural recessive resistances to potyviruses were shown to

correspond to a mutation in a host factor required by the virus to complete its cycle (Lellis *et al.*, 2002; Ruffel *et al.*, 2002; Nicaise *et al.*, 2003). To investigate if recessive resistance to other viruses fits with this framework, new groups of crop expressing recessive resistance to virus (other than potyvirus) may be investigated (Diaz-Pendon *et al.*, 2004). Because genetic studies are easier in *L. pimpinellifolium* than in other wild *Lycopersicon*, recessive resistance to PYMV in *L. pimpinellifolium* might be a candidate to study recessive resistance to begomoviruses.

Faced with the rapid spread of begomoviruses throughout tomato cultivation areas, broad resistance to begomoviruses is urgently needed. Vector resistance could be used as a first barrier that would prevent massive infection by any new begomovirus. According to begomovirus occurrence, resistance to virus *sensu stricto s*hould be associated to vector resistance. For the Caribbean islands we propose to combine vector resistance from *L. pimpinellifolium* INRA Hirsute, and resistance to PYMV from LA 2187-5. Those factors of resistance should be introgressed in tropic-adapted genotypes resistant to TYLCV as those developed in Cuba (Gomez *et al.*, 2004). Nevertheless more detailed genetic studies have to be carried to use the factors of resistance from *L. pimpinellifolium*. As crosses of *L. pimpinellifolium* with *L. esculentum* are compatible, and progenies polymorphic, genetic studies of resistance in LA 2187-5 could be quickly conducted on an interspecific cross such as CRA 66 X LA 2187-5. Another interspecific cross is needed to study potential vector resistance *mpinellifolium* INRA Hirsute. Thus, QTL mapping with examination of virus resistance under graft inoculation parallel to vector inoculation might clarify the different genetic factors which may be involved in these lines.

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CHAPTER 8

Characterisation of a quantitative resistance to transmission of *Tomato yellow leaf curl virus* in a *Lycopersicon pimpinellifolium*[1]

ABSTRACT

Two wild genotypes of the same species, *L. pimpinellifolium* were tested for resistance to *Tomato yellow leaf curl virus*. Plants were inoculated using both vector inoculation and graft inoculation. Presence and quantification of viral DNA were assessed by dot blot hybridization. The incidence and severity were scored. The wild accession *L. pimpinellifolium* INRA-hirsute showed a moderate resistance overcame by a high inoculation pressure. Nevertheless, regarding to the disease progression in term of cumulative number of viruliferous whiteflies this partial resistance overcame by a high inoculation pressure expressed a typical quantitative resistance pattern. When graft inoculated, all the susceptible and resistant cultivars expressed symptoms. This accession had already been tested against a bipartite begomovirus and found to have the same level of resistance for both viruses. It confirmed that this partial resistance might affect the virus transmission efficiency of the vector. This quantitative resistance appears to be a good candidate for further use in breeding programmes against other begomovirus.

[1]Delatte, H., Holota, H., Reynaud, B., Dintinger, J. (2005). Characterisation of a quantitative resistance to transmission of *Tomato yellow leaf curl virus* in a *Lycopersicon pimpinellifolium*. *European Journal of plant pathology* Submitted in a slightly modified version.

INTRODUCTION

In tropical and subtropical tomato production zones, *Bemisia tabaci* Gennadius (*Hemiptera: Aleyrodidae*) is an important insect pest. *B. tabaci* provokes direct feeding damages but above all causes considerable indirect damages as a vector of numerous geminiviruses. In recent years, the Old World monopartite begomovirus *Tomato yellow leaf curl virus* (TYLCV) (*Geminiviridae* family) has spread worldwide (Nakhla *et al.*, 1994; Pico *et al.*, 1996; Momol *et al.*, 1999; Navas-Castillo *et al.*, 1999; Moriones & NavasCastillo, 2000). It has also reached La Réunion (Peterschmitt *et al.*, 1999b), an island situated in the south west part of the Indian ocean, where it became the first limiting factor for production both in the fields and in protected greenhouses. Since few viruliferous whiteflies may transmit the virus to a large number of plants, chemical controls as well as IPM strategies employed for controlling the vector revealed unsuccessful to decrease the TYLCV incidence on cultivated tomatoes (Reynaud *et al.*, 2003). Cultivation of tomatoes with resistance to the virus and/or the vector is a more effective solution for a sustained control of TYLCV. No resistance to TYLCV was found in cultivated *Lycopersicon esculentum* (Laterrot, 1989; Pico *et al.*, 1999b; Pilowsky & Cohen, 2000) and, during last decades, considerable efforts has been done to develop TYLCV resistant cultivars by transferring resistance from wild types of *Lycopersicon* into cultivated tomatoes. Nevertheless, progress in breeding for TYLCV resistance has been slow because of the complex genetics of resistance, which probably explain why the cultivars and breeding lines most often are not as resistant as wild species. For progress in breeding for resistance to TYLCV in tomato, see Lapidot and Friedmann (2002). Still, until now few resistant cultivars are available, the vast majority of those cultivars are resistant to TYLCV and to no other begomovirus (Lapidot & Friedmann, 2002).

Therefore, a first screening over several wild type tomatoes was performed (Boissot *et al.*, 2005) with the objective of identifying a resistance to mono- and bipartite begomoviruses. Out of this screening study the accession *L. pimpinellifolium* INRA-hirsute appeared to be a good candidate, being partially resistant to *Potato yellow mosaic virus*, a bipartite begomovirus and to TYLCV upon vector inoculation, susceptible to TYLCV and PYMV upon graft inoculation. Therefore, *L. pimpinellifolium* INRA-hirsute was chosen to be further tested for TYLCV resistance. An accession of the same species was chosen to be the susceptible control: WVA 106. To characterise this resistance, vector inoculation with increasing numbers of viruliferous whiteflies and graft-inoculation were carried out. Presence and quantification of viral DNA, then incidence and severity were scored for the two wild accessions.

MATERIAL AND METHODS

Insect culture

Adults of *B. tabaci* used in this study were from a population of La Réunion that was started with nymphs collected on cabbage (*Brassica oleracea*) and was subsequently reared on the same species under laboratory conditions: 12h/12h photoperiod (white and red fluorescent tubes), 25 +/- 2° C temperature and 60% +/- 10% relative humidity. The population was identified as belonging to the B biotype using cytochrome oxidase (COI) markers (Delatte *et al.*, 2005c). A part of those *B. tabaci* nymphs were transferred at the stage L4 from cabbage plants to 1.0 x 1.0 x 1.5 m insect proof-cages filled with the TYLCV-infected tomato susceptible cultivar Farmer (Know You Seed) under an insect proof greenhouse. The whiteflies were kept feeding on tomato infected plants until their use for transmission tests.

Virus culture

The TYLCV used in this study was from the isolate of La Réunion: TYLCV-Mld[RE] (Peterschmitt *et al.*, 1999b; Delatte *et al.*, 2005a). This field isolate was maintained in insect proof-cages inside an insect-proof greenhouse and propagated by whitefly generation to generation in the susceptible tomato cultivar Farmer.

Plant material

The wild accession *Lycopersicon pimpinellifolium* 'INRA-hirsute' was compared to the susceptible control *L. pimpinellifolium* 'WVA 106'. Seeds of both varieties were obtained through INRA-URPV of Guadeloupe and further re-conducted in La Réunion.

Vector inoculation

Three trials were conducted during different seasons in 2003 and 2004 for vector inoculation. For each trial, a split plot design was laid out with three blocks. Three main plots per block corresponded to three inoculation access period (IAP 6-h, 24-h, and 96-h), and 2 subplots per main plot corresponded to the 2 tested accessions INRA-hirsute and WVA106. Within each trial, one block was a 1.0 x 1.0 x 1.5 m insect proof-cage inside the insect-proof greenhouse. Inside the insect proof-cage, each subplot consisted of 20 to 30 two-leaf stage plantlets for each accession (in split plot design). Each insect proof-cages contained a large amount of insects previously feeding on infected tomato plants. Three durations of the IAP were obtained in the same cage by exposing the plantlets to whiteflies for IAP of 6, 24 or 96 hours. As a whole 120 to 160 plants per accession were tested per trail.

Before removing the plantlets from the cages, the mean number of feeding insects was estimated for each IAP to access efficiency of inoculation (see *cniwh* latter). The transmission efficiency (proportion of inoculative insects) was determined on a sample of 100 individuals collected from the insect-proof cages and representing the whole population. Single whitefly were each confined in small PVC tube cages containing a susceptible tomato seedling at the two-leaf stage, and were

maintained in a greenhouse. After a 72 h IAP, plants were sprayed with an insecticide (Confidor®, Bayer, Basel, Switzerland), and placed in an insect-proof greenhouse to allow symptoms development. The occurence of symptoms on the leaves was recorded 21 days later and a TAS-ELISA (Adgen, UK) was performed to assess the virus presence.

After the estimation of insects, the plantlets were removed after each IAP, sprayed with an insecticide (Confidor®, Bayer, Basel, Switzerland), repotted, then placed in an insect-proof greenhouse. Plants were sprayed with an insecticide (Confidor®, Bayer, Basel, Switzerland) once a week to avoid secondary infestations, and provided with fertilisation. Symptoms were recorded every 3.5 days on individual plants for up to 31.5 days postinoculation (dpi). Viral DNA accumulation was quantified every 3.5 days during the 14 dpi in the third upper leaf of the fifteen first plants of trials 2 and 3 by dot blot hybridization.

Graft inoculation

Thirty three-week old seedlings of each accession were side graft-inoculated with a stem of TYLCV-infected susceptible cultivar Farmer as a scion. To be consistent with the IAP-96h of the vector inoculation, day 0 was taken into account 96 hours after grafting. Symptom severities were recorded every 3.5 days on individual plants for up to 31.5 days dpi. Viral DNA accumulation was quantified in the third upper leaf of the plants by dot blot hybridization every 3.5 days during the 14 dpi as described below.

Symptoms assessment

Symptoms were evaluated on the 3 last fully expanded leaves of each plant using a scale established for rating TYLCV symptoms. The scale ranged from 1 (no symptom) to 10 (dead plant) with numbers respectiveley corresponding to the 0 to 4 scale scale of Lapidot et al. (2002). This choice of scale was done to simplify the statistic analysis in avoiding 0.

Dot-blot hybridization

A small amount of leaves (0.05 g) was used at each sampling (dpi+3.5; dpi +7; dpi +10.5; dpi +14) and ground in 0.5 ml of 0.4 M NaOH, then 10 µl were dotted on nylon membrane (Hybond N+; Amersham Pharmacia, Freiburg, Germany) (Lapidot *et al.*, 1997). Dots of respectively 5, 10, 25 and 50 ng of full length TYLCV-Mld[RE] (amplified from the clone (Delatte *et al.*, 2005b) plus two negative controls (non inoculated plants) and one positive plant were added on each membrane, in order to quantify the viral DNA. A chemiluminescent probe of the full length TYLCV-Mld[RE] was labelled with peroxydase according to the manufacturer's instructions (Amersham Pharmacia, Freiburg, Germany). Pictures of the membranes were analysed with the software Koadarray 2.2 (Koada Technology, UK) in order to quantify viral DNA.

Variable description

The variables, calculated on a subplot basis, were disease incidence (inc_u), defined as the percentage of plants exhibiting symptoms at the u^{th} dpi, and, disease severity (sev_u), defined as the

mean disease score of the plants exhibiting symptoms at the uth dpi. To integrate these variables over time, we calculated the area under the disease progress curve (AUDPC) (Jeger & Viljanen-Robinson, 2001), called inc_a for the disease incidence, and sev_a for the disease severity. $inc_a = \sum_{i=1}^{n-1} \{([inc_i + inc_{i+1}]/2)(t_{i+1} - t_i)\}/(t_n - t_1)$ in which inc_i was the percentage of plants exhibiting symptoms at the ith rating date and $sev_a = \sum_{i=1}^{n-1} \{([sev_i + sev_{i+1}]/2)(t_{i+1} - t_i)\}/(t_n - t_1)$ in which sev_i was the mean disease score of the plants exhibiting symptoms at the ith rating date; t_i was the time post-inoculation at the ith observation; n was the number of dates at which disease was recorded.

For estimating the inoculation pressure, we used the cumulative number of inoculative whitefies *cniwh*. The *cniwh* was according to the formula: $cniwh_{ijk} = r_i \sum_{n=1}^{k} \bar{x}_{ijn}.t_n$. With $cniwh_{ijk}$ as the cumulative number of inoculative whiteflies x hours at the k^{th} IAP (k = 1 for 6 hr, 2 for 24 hr, and 3 for 96 hr), in the j^{th} cage of the i^{th} trial. With $\bar{x}_{ijn}$, the mean number of whiteflies per plant (visually estimated) in the j^{th} cage of the i^{th} trial during t_n hours With the t_n, the number of hours corresponding to 1st IAP (6 hr, n = 1), the difference between 2nd and 1st IAP (18 hr, n = 2), or the difference between 3rd and 2nd IAP (72 hr, n = 3). With r_i, the proportion of inoculative whiteflies overall estimated on the i^{th} trial.

Statistics

An ANOVA was performed to determine the effects of trial, IAP, and genotype on inoculation pressure estimated by *cniwh.* The effect of genotype was determined for both TYLCV incidence and severity. ANOVAs were performed for each trial individually and across trials, taking into account IAP and cage effects. The variables analysed were inc_a and sev_a. Means of genotype for each IAP were separated by SNK multiple-range test (α = 0.05). ANOVAs were also performed for the grafting trial taking into account the effect of repetitions. The effect of genotype was determined for virus concentration at 3.5, 7, 10.5, and 14 DPI, after vector-inoculation (ANOVA performed across trials 2 and 3) as well as graft-inoculation. All analyses were conducted using PROC GLM (SAS 8.01, SAS Institute, Inc., Cary, NC).

RESULTS

Vector and graft inoculations

Whitefly-mediated inoculation were successful with ~100% of the plants infected on susceptible genotype WVA106 at 14 days post-inoculation for IAP-24 h or 96 h, and ~ 90% for IAP-6 h. No plant escaped when graft-inoculated as shown by 100% of the plants infected on WVA106 at 14 days post-inoculation. ANOVA on *cniwh* showed a significant effect of trial ($P < .0001$), IAP ($P < .0001$), cage ($P = 0.0076$), and interaction trial x IAP ($P < .0001$), but none on the genotype or on inoculation pressure.

The proportion of whiteflies transmitting the virus (proportion of inoculative whiteflies estimated on a sample of 100 individuals from the insect population in each trial) ranged from 28 to 35%, depending of the trial. Nevertheless, the *cniwh* estimated values were on average five times lower in trials 2 and 3 than in trial 1, due to a much larger number of whiteflies mass-released in this last one (table 8-1).

Table 8-1. Estimation of the cumulative number of inoculative whiteflies x hours (*cniwh*) in *Tomato yellow leaf curl virus* (TYLCV) trials.

VWA106	**IAP (h)** [a]			
	6	**24**	**96**	**Trial mean**
Trial 1	61 (27) [b]	225 (53)	1802 (524)	696 (317)
Trial 2	31 (6)	111 (19)	255 (29)	132 (34)
Trial 3	22 (12)	101 (35)	314 (132)	145 (59)
IAP mean	38 (11)	146 (28)	790 (297)	
Genotype mean				325 (116)
INRA-hirsute				
Trial 1	53 (7)	312 (93)	1125 (323)	497 (188)
Trial 2	24 (6)	83 (24)	234 (15)	114 (32)
Trial 3	19 (5)	85 (11)	219 (82)	108 (38)
IAP mean	32 (6)	160 (47)	526 (178)	
Genotype mean				239 (72)
General mean				282 (68)

[a] IAP: Inoculation Access period (total time in hours, during which the seedlings were in the cages with viruliferous insects)

[b] Means over all cages (standard error).

Temporal analyses

AUDPC values of incidence (inc_a) and severity (sev_a) were used to analyse TYLCV progression in relation to time and to quantify disease resistance in INRA-hirsute after vector- and graft-inoculation (Fig. 6-1). After vector-inoculation, ANOVA across the three trials showed a significant effect of genotype ($P<.0001$) and IAP ($P<.0001$) for incidence as well as severity. Significant effects of the trial ($P=.007$ for inc_a, $P=.027$ for sev_a), of the cage ($P=.008$ for inc_a), and of the interaction trial x genotype ($P=.016$ for inc_a) also were found. Mean values of incidence for INRA-hirsute were found to increase significantly with IAP (table 8-2, Fig. 8-1 A, C and E), suggesting a quantitatively inherited type of resistance to TYLCV in this genotype. Therefore, the efficiency of this resistance was strongly reduced by an intense inoculation pressure, corresponding to 96-h IAP (Fig. 8-1E and F). This was confirmed by the graft-inoculation trial, which showed 100% of infected plants in genotype INRA-hirsute not differing from the susceptible WVA106 (Fig. 8-1G). In contrast, mean values of severity were found to be high for both genotypes whatever the inoculation pressure (Fig. 8-1B, D, and F). Mean value levels of incidence for INRA-hirsute at IAP-96h was equivalent at the mean value of WVA106 after an IAP-6h (table 8-2).

Although significant differences were observed between the two genotypes (table 8-2), this quantitative resistance was shown to be above all effective by reducing the TYLCV disease incidence. Moreover, no significant difference between WVA106 and INRA-hirsute was found after graft-inoculation (Fig. 8-1H).

Table 8-2. Means of AUDPC values per genotype and per Inoculation Access Period (IAP) for the three trials after vector inoculation. Values followed by the same letter are not significantly different at level 0.005 according to SNK test on means.

IAP	Genotype	Incidence	Severity
6 h	**Hirsute INRA**	0.44 [c]	3.63 [c]
	WVA 106	0.68 [b]	4.07 [b]
24 h	**Hirsute INRA**	0.58 [d]	3.99 [b]
	WVA 106	0.82 [a]	4.68 [a]
96 h	**Hirsute INRA**	0.71 [b]	3.93 [b]
	WVA 106	0.84 [a]	4.47 [a]

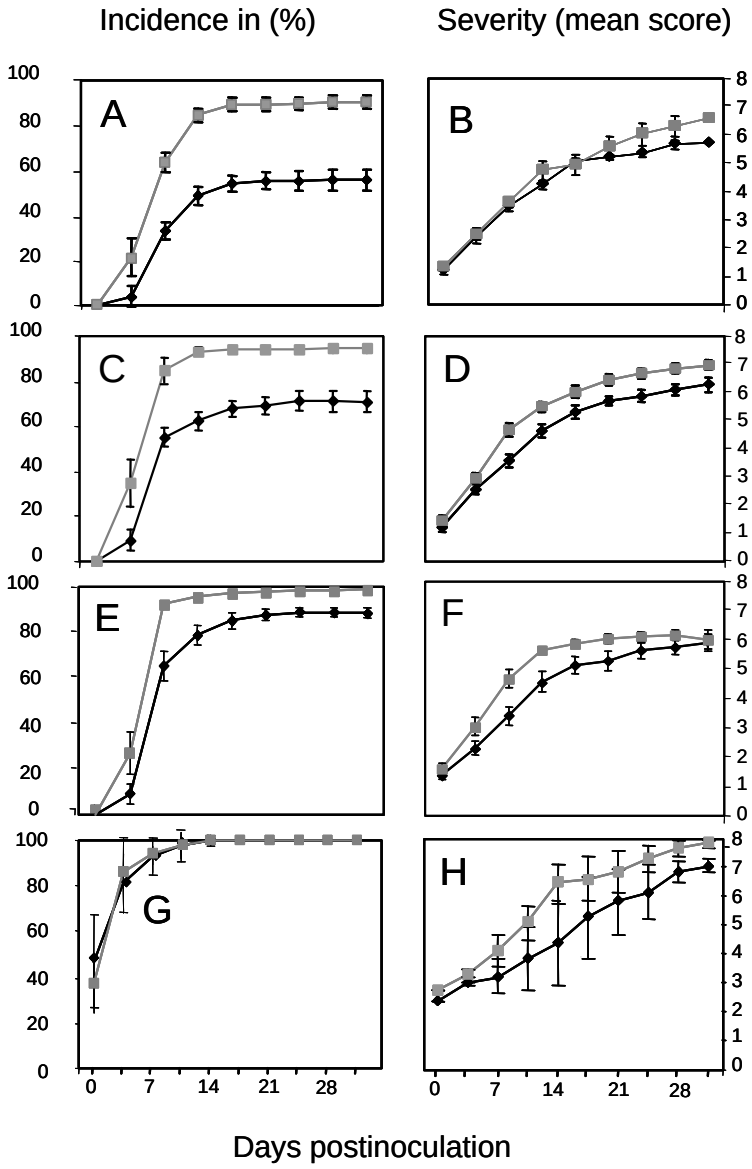

Fig. 8-1. Mean values of disease incidence and severity after vector inoculation (A, C, E and B, D, F, respectively) for trials 1-2-3, and graft inoculation (G and H, respectively) of *Tomato yellow leaf curl virus* (TYLCV) versus days postinoculation (dpi). Wild accessions of *Pimpinellifolium* INRA-hirsute (♦) and WVA106 (■) were tested. The mean incidences in A, C and E and severities in B, D, F are corresponding to plants exposed to a 6h inoculation access period of viruliferous whiteflies (IAP), 24h IAP and 96h IAP, respectively. Vertical bars are presenting the SE.

Dot blot hybridization

The average TYLCV DNA concentration at 3.5 dpi was almost 4 times higher in grafted-plants than in vector-inoculated plants with 96-hr IAP (Fig. 8-2). Then, this difference decreased with time and reversed, indicating that accumulation of virus was delayed when plants were vector-inoculated. TYLCV DNA accumulation detected at 3.5, 7, 10.5, and 14 dpi in INRA-hirsute and WVA106 was not significantly different for both techniques of inoculation.

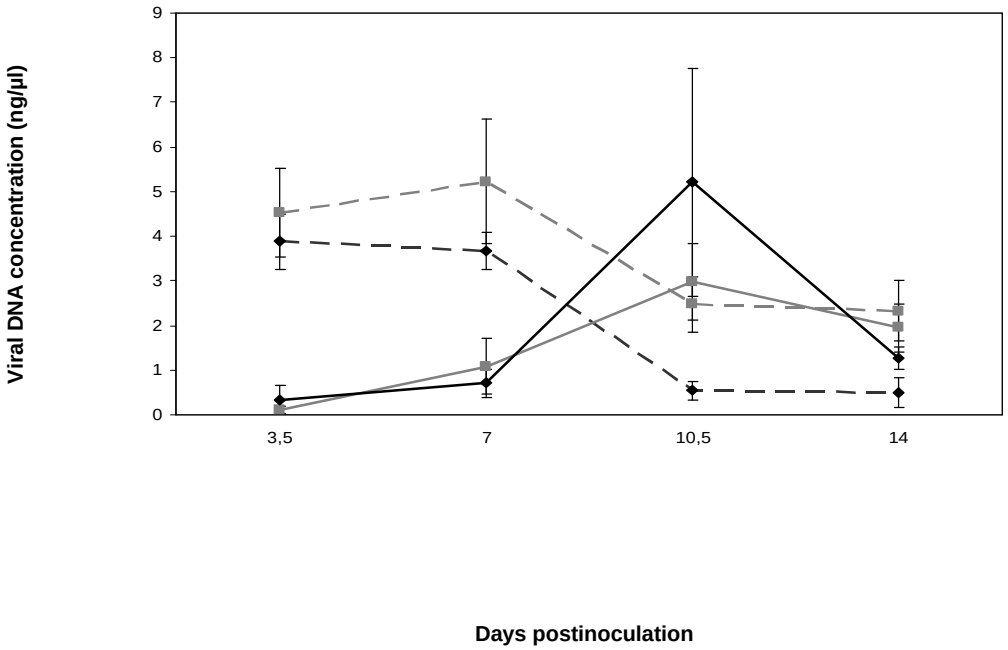

Fig. 8-2. *Tomato yellow leaf curl virus* concentration in relation to days postinoculation. Gray lines are presenting concentrations in *Pimpinellifolium* Wva106 (■) by dot blot hybridization. Dark lines are presenting concentrations of *Pimpinellifolium* INRA-hirsute (♦). The dotted lines are presenting the average obtained after graft inoculation and the plain one the average concentration after massive whitefly inoculation (on plants after the inoculation access period of 96h). Vertical bars are presenting the SE.

DISCUSSION

As all cultivars of tomato *Lycopersicon esculentum* are susceptible to TYLCV, breeding programmes have been based on the transfer of resistance genes from wild type accessions. One of the difficulties in selecting a source for resistance to TYLCV in wild *Lycopersicon* accessions stands for the fact that the disease might be expressed with varying degrees of incidence as well as of severity. Variation in disease expression is often related with the difficulties in visual scoring for resistance, but also with the method of TYLCV detection, which does not always provide accurate quantification of viral DNA (Pico *et al.*, 1998; Pico *et al.*, 1999a). Discrepancies observed between studies also may be due to the varying responses against the different TYLCV isolates used in the experiments and differences in method of inoculation (Pico *et al.*, 2001). Thus, combined methods of TYLCV inoculations are necessary to screen wild accessions to TYLCV resistance. Furthermore, multiple criteria such as visual scoring (incidence and severity) and viral quantification are necessary to evaluate this resistance.

Several studies reported the accession *L. pimpinellifolium* INRA-hirsute as partially resistant to both the vector and the virus but no comparison between inoculation with and without vector supported this hypothesis (Zakay *et al.*, 1991; Kasrawi & Mansour, 1994; Pico *et al.*, 2001). In our study, we tried to characterize this partial resistance to TYLCV-Mld[RE] by graft- and vector-inoculation in an attempt to dissociate the two types of resistance. We also quantified more accurately the response in INRA-hirsute to TYLCV-Mld[RE] infection by assessing the effect of inoculum pressure on disease incidence and severity, and virus content with increasing numbers of viruliferous whiteflies.

When compared to the susceptible accession WVA106, INRA-hirsute shows a partial resistance to TYLCV-Mld[RE] incidence that may be overcome by an intense inoculation pressure corresponding to more than five inoculative whiteflies feeding on a single plant during 96 hours. In contrast, the symptom severity seemed not to be reduced as shown by disease progress curves, which are more or less similar for the two genotypes. These results are consistent with those obtained on plants graft-inoculated with TYLCV-Mld[RE], in which final TYLCV-Mld[RE] disease incidence was 100% with severe symptoms, whatever the genotype.

No significant difference was observed between both studied accessions when quantified by dot blot hybridization. Nevertheless a very high value of virus concentration was observed after grafting (compared to vector inoculation). The high level of DNA accumulation at the beginning of the infection (3.5 DPI) confirmed that graft inoculation permit a large amount of virus inoculation than vector inoculation. Furthermore, it suggests that partial resistance in INRA-hirsute had no influence on virus accumulation in the plant. These results also showed that this resistance was inefficient when a massive quantity of virus was inoculated into the phloem, and suggested the presence in INRA-hirsute of resistance mechanisms to the vector, which reduce transmission and initial entry of the virus into plant cells. No difference for whitefly preference between WVA106 and INRA-hirsute was found, as no different numbers of whiteflies were counted on WVA106 and INRA-hirsute (no significant effect of genotype on *cniwh* throughout the three experiments), this

partial resistance might result from a modified feeding behaviour of the vector on host plants in the phloem cells. In a previous study with comparative tests of resistance against TYLCV-Mld[RE] and PYMV-GA on 6 wild *Lycopersicon* species, INRA-hirsute was found partially resistant to both viruses when inoculated by viruliferous whiteflies while it developed symptoms when graft-inoculated (Boissot *et al.*, 2005). This strongly supports the hypothesis of a virus-non specific resistance to the vector reducing final disease incidence in resistant genotype.

In their study, Kasrawi *et al.* (1988) reported INRA-hirsute as partially resistant to the virus with only 50% of grafted plants presenting moderate symptoms. However we found in our study 100% of infected plants with severe symptoms. These results are also in contradiction with the ones of Pico et al. (2001). These discrepancies might be due to the environmental conditions and grafting procedures.

Our results differ slightly from the findings of Pico *et al.* (2001) who assumed the presence of resistance to the virus in INRA-hirsute. These authors reported 100% of the plants detected positive in this accession after stem-agroinoculation, similarly to what was found in our study after graft-inoculation. In contrast, average symptom rating at 30 dpi scored by these authors was twice lower than that scored in our conditions and reduced TYLCV DNA accumulation. However, they compared the symptoms of the wild accessions tested to *Lycopersicon esculentum* accessions. As symptoms in wild species are generally much weaker than in cultivated tomatoes, comparison of this partial resistance to *L. pimpinellifolium* WVA106 was a better estimator to evaluate the real value of INRA-hirsute as a possible source of resistance for breeding.

In our study, viral DNA accumulation was quantified, and at 7 dpi a very high DNA accumulation was quantified, showing the virulence of the virus isolate used. Lapidot et al. (1997) found a positive correlation between the resistance level, as evidenced by relative yield loss, and viral DNA accumulation for all higher resistant accessions tested. TYLCV DNA accumulation in INRA-hirsute and WVA106 was not significantly different whatever the inoculation techniques and the time after inoculation, this confirmed that INRA-hirsute had no resistance to virus multiplication.

In breeding programs for resistance to begomoviruses, host genotypes that can be infected but exhibit reduced disease incidence may be discarded without full consideration for their epidemiological effects at the population level in the field. In this study, quantification of TYLCV-Mld[RE] showed that accession INRA-hirsute, which possesses a moderate level of resistance, was effective in reducing the apparent infection rate of TYLCV epidemics. In other disease pathosystems, this type of resistance that reduces the rate of epidemics has been reported to be effective against several pathotypes.

As a whole the partial resistance found in INRA-hirsute, might affect the virus transmission efficiency of the vector. Nevertheless further experiments should be done, such as using EPG technique to record the insect feeding behaviour and better characterize this resistance. This resistance should also be tested against other begomoviruses than TYLCV and PYMV. If this partial resistance is confirmed as affecting the virus transmission efficiency of the vector, this might be very interesting to reduce the virus impact of other begomovirus. Especially as partial resistances

are known to be polygenic (Parlevliet, 1989) and more durable (Lindhout, 2002). If combine with other strong resistance to the virus this partial and quantitative resistance might improve the level of resistance of tomato lines.

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CHAPTER 9

GENERAL DISCUSSION

Begomoviruses rank among the most devastating pathogens of tomato worldwide, with reported yield losses of up to 90% (Polston, 2001). To date, with sensitive diagnostic tools being available, many different begomoviruses have been distinguished and described, and most of these appear to have rapidly emerged due to the unintentional spread of a potent *Bemisia tabaci* vector biotype, the B biotype. This epidemiological situation reinforces the importance of studying introduced *B. tabaci* biotypes and their role in settling new begomovirus diseases in insular environments, and this precisely represented the aim of the current Ph D study.

Bemisia tabaci invasive biotypes and emergence of begomoviruses: a threat to agriculture

Over the past 20 years, new biotypes of *B. tabaci* have been discovered in many parts of the World (Perring, 2001). In most cases, whiteflies are only studied after begomovirus outbreaks resulting in crop devastation (Polston & Anderson, 1997; Ribeiro & al., 1998; Peterschmitt *et al.*, 1999c) or, when an upsurge of the insect leads to dramatic feeding damage (Gill, 1992). These events are usually coupled with the introduction of an invasive biotype such as the biotype B (Ribeiro *et al.*, 2003). The situation described in this thesis for La Réunion is completely in line with these earlier findings. As described in Chapters 3 and 4 the emergence of begomovirus diseases in tomato cultivations on La Réunion seems to coincide with the appearance of the B biotype in this close environment. This biotype appears very successful in invasion, apparently as it is more insecticide resistant (Costa *et al.*, 1993a; Brown *et al.*, 1995), can fly longer distances (Blackmer *et al.*, 1995a; Blackmer *et al.*, 1995b) and has a higher fecundity and shorter life cycle than other tested biotypes (Bethke *et al.*, 1991). Moreover, the B biotype has been shown to possess competitive advantages over other biotypes (Pascual & Callejas, 2004) except for biotype Q (Muniz & Nombela, 2001); (Guirao, 1997; Moya *et al.*, 2001) (Nauen *et al.*, 2002). According to the so far published studies, biotype B seems to have a kind of mixed *r/k* strategy (MacArthur & Wilson, 1967), with higher fecundity and a competitive advantage in mixed rearing with other biotypes under both laboratory and field conditions. Successful invasions (almost worldwide) of the B biotype are well documented. For example, in 1990-91 this biotype had almost completely replaced the A biotype in California (Gill, 1992), and the same has occurred in the Caribbean basin (Costa *et al.*, 1993a).

In Chapter 5 the presence of the biotype B on La Réunion along with another biotype has been described. The latter appeared genetically dissimilar to populations described elsewhere and is thought to be indigenous to this region as it was also detected on Mauritius, Seychelles, and Madagascar. Therefore, it was called Ms after the name of the Mascarenes Archipelago. The Ms

biotype forms a distinct group that is sister to two other groups, one to which the B biotype is a member and one to which the Q biotype belongs. According to the results of Chapter 5 the biotype B is supposedly introduced on La Réunion in the 90's, from that time on co-existing with the indigenous Ms biotype which probably has been present on the island for many years in low numbers (Bourriquet, 1938; Luziau, 1953; Russell & Etienne, 1985). To distinguish the different populations of *B. tabaci* present on La Réunion, RAPD-PCR analysis and sequencing of the cytochrome oxidase I gene were done. The first technique is known as not being very reliable and not reproducible. The second technique is very reliable but too expensive for extensive screenings.

As described in Chapter 6, a new bank of molecular markers (microsatellite markers) was developed and this set of markers allowed rapid distinction of the invasive biotype from the indigenous, and also provided a good tool for studying interspecific competition of populations. Using these microsatellite markers a survey over time showed that in 2002 biotype B was dominant throughout the island except for the eastern region. The genetic data also showed that some syntopic B individuals were genetically very close to Ms syntopic individuals. Experimentations on inter-mating biotypes B and Ms are currently going on at the CIRAD laboratory. The results obtained so far indicate that crossings between a B female and an Ms male are giving hybrids (in 11% of the crosses), whereas crossings between B male and Ms female gave no hybrids. Of course these preliminary data need to be substantiated and the F2 generation to be tested. Nonetheless these first biological data combined with the molecular data obtained from field populations, suggest that crossings between the two biotypes should be possible. The fact that crossings are only possible in one way with biotype B females is of first interest, especially knowing that the common biotype determination is currently done by analysing the COI gene, which are inherited from the female. In Chapter 6, the use of microsatellite data allowed us to divide the B biotype population of La Réunion into 2 separate groups. One seemed genetically very distinct from the Ms biotype and was named B' and the other was much closer to the Ms biotype, and was named B'' (most often found in syntopy with individuals from Ms biotype). As B'' has approximately 30-40% of its loci in common with Ms this group indicates the introgression of Ms genes into the B biotype, the biotype B thus tending to replace or completely introgress the indigenous biotype. This is coherent with the results obtained so far in our recent intermating experiments. Although inter-biotype mating studies have been carried out in several countries, this strategy has not yet been described for the B biotype in field populations. Mating interactions between biotype B and indigenous biotypes from eastern Australia revealed no mating compatibility with any of the biotypes tested, leading to parapatric population (De Barro & Hart, 2000). This was suggested to be due to cytoplasmic incompatibility, distracting male behaviour or to a single locus complementary sex determination model. The same situation was observed for the A biotype in the United States, where no mating compatibility was found with the B biotype (Perring *et al.*, 1993a; Perring *et al.*, 1993b).

The biotypes situation on La Réunion appears 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 to the introgression of one population into another. This might lead to the complete disappearance of the Ms biotype and the appearance of a fitter hybrid group of whitefly in

La Réunion. This is a very interesting phenomenon that should be further studied, especially as it represents a case of intraspecific competition occurring in an isolated environment. It would also be important to understand the optimal developmental conditions of the invasive biotype, especially regarding abiotic factors and host plant preference. These factors could possibly be used as determinant to predict new invasions. Especially in knowing that the indigenous biotype Ms was found on most of the islands of the south west part of the Indian Ocean (Mauritius, Madagascar, Seychelles and Mayotte) and no upsurge of whitefly population was ever described in Madagascar and Seychelles. The B biotype was found in Mauritius (1998)(Ganeshan & Abeeluck, 2000), La Réunion (1997) and Mayotte (2002). The invasion of the B biotype was coupled with an upsurge of the TYLCV in Réunion island (Peterschmitt *et al.*, 1999c), whereas in Mayotte there is no reports yet of new begomovirus introductions or upsurge of whitefly populations. Nevertheless, indigenous begomoviruses transmitted by *B. tabaci* are present on Mayotte (Lett *et al.*, 2004) and Madagascar (Delatte *et al.*, 2003b) as described in Chapter 4. These begomoviruses, were found to infect tomato crops at a low rate compared to countries where the B biotype is present (Brown & Bird, 1995). Although the indigenous biotype Ms is genetically closely related to the B and Q biotypes, it has been estimated (on basis of mitochondrial COI markers) that this biotype diverged from B and Q as long as 3 (± 0.3) million years ago (Chapter 5). This estimate, though, is not compatible with the time of the continental separation of Madagascar from the African continent (about 160 million years). It might be that the (ancestral) biotype Ms has been introduced in Madagascar after becoming an island, a couple of million years ago and has evolved separately since, but this is still an open question.

Nevertheless, as being vectored by *B. tabaci*, it is possible that the begomoviruses of the south west islands of the Indian Ocean (SWIO), which are apparently complex recombinants with none of the sequences within the recombinant regions closely resembling that of any known non-SWIO begomovirus, have evolved in relative isolation for a similar period as the biotype Ms. It will be interesting to determine whether the SWIO isolates have any transmission advantages relative to mainland African and Mediterranean isolates with biotype Ms, especially as host-interactions are a decisive factor in the spread of geminiviruses (Bedford *et al.*, 1994a; Brown *et al.*, 1995), and *B. tabaci* plus begomoviruses might have both co-evolved with their host plants.

A host plant survey should be done to assess the host range of these indigenous SWIO begomoviruses. Both SWIO viruses described in Chapter 4 were found to infect members of the *Solanacea*. a tropical family of which the oldest fossils have been found in the Cretaceous layer (65 million years ago), with its major centre of diversity found in Central and South America, and minor centres in Australia and Eurasia (D'Arcy, 1991). This implies that most of the *Solanaceae* found in the SWIO area have been introduced, and, consequently, that the indigenous SWIO begomoviruses should have other reservoir host plants.

The genomic sequences of the two SWIO tomato begomoviruses described in Chapter 4 show that these viruses are closely related to the African cassava begomoviruses with bootstrap values of 100 (SACM, and EACMV) (Fig. 9-1). Three cassava-infecting begomoviruses (ACMV, SACMV and EACMV) which are all three found in continental Africa have recently also been found on

Madagascar (Ranomenjanahary, 2002), Cassava is also not indigenous to the area. Recent genetic studies have indicated that cassava (*Manihot esculenta* subsp. *esculenta)* originates from wild populations at the southern border of the Amazon basin, indicating this region as the likely site of its domestication (Olsen & Schaal, 1999; Olsen & Schaal, 2001). In all, it seems likely that the current tomato- and cassava-infecting begomoviruses as found on Madagascar have been derived from ancestral begomoviruses of other host plant species and which have apparently been able to adapt very well to their new hosts. This suggests that they might have evolved from common ancestors and later diverged on different hosts. Indeed more studies should be done to understand the phylogeny of these begomoviruses and on their indigenous host ranges. Furthermore, the possibility of interviral recombination should be assesses, for instance by mixed infection studies in the laboratory, in order to evaluate the risk of emerging recombinants, which might cause more severe disease symptoms (Fauquet, 1999), especially if one realises that SWIO begomoviruses often have common host plants. TYLCV has already reached La Réunion, and is a potential recombinational partner for the indigenous viruses.
Also the introduction of ToLCMGV, from Madagascar into Mayotte or the introduction of ToLCYTV originating from Mayotte into Madagascar represent potential risks to find mix begomovirus infections. Hence, it might be of interest to test for possible recombination events between TYLCV and these 2 new begomovirus species, or between ToLCMGV and ToLCYTV, under laboratory conditions.

The worldwide spread of begomoviral diseases is mostly due to importation of infected ornamentals or seedlings (Polston & Anderson, 1997), but in Chapter 2 it is shown that also tomato fruits may serve as virus inoculum. This finding is of prime importance in view of the intensive trading between the islands of the SWIO area. Begomoviruses found in Madagascar and Mayotte have most probably a common ancestor, and movement of infected material is the first cause of the introduction of the disease. Actually both TYLCV and *B. tabaci* are quarantine pathogens, and most plants susceptible to both pathogens are carefully checked by the National Plant Protection services at the custom. Nonetheless,, in April 2004, for the first time the “Israel” strain of TYLCV has been recorded on La Réunion (Delatte *et al.*, 2005a), despite all efforts to prevent new introduction of such pathogens. This new introduction reinforced the fact that infected tomato fruits can be an important way to import tomato begomoviruses if all the required conditions are present.

Evolution is the process by which the genetic structure of the population of an organism changes with time. Most studies on viral evolution have been done on RNA viruses, the predominant type among plant viruses (Zaccomer *et al.*, 1995).

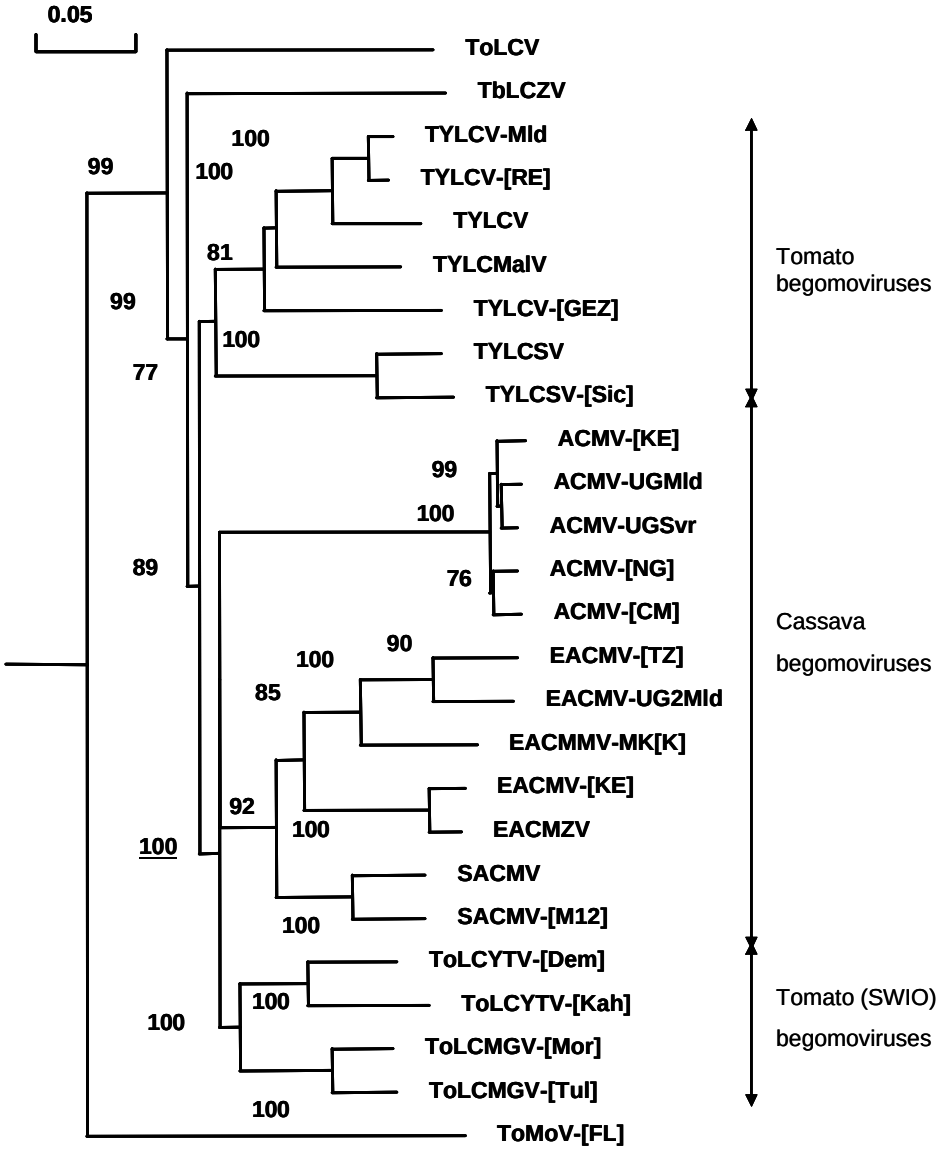

Fig. 9-1. Neighbour joining tree indicating the phylogenetic relationships between the DNA sequences of ToLCMGV, ToLCYTV and TYLCV-Mld[RE] isolates and those of a representative sampling of publicly available African and Mediterranean begomovirus sequences (GenBank). The tree was constructed using Jukes-Cantor corrected distances and rooted using ToMoV-[FL] as an outlyer. Numbers associated with nodes indicate the percentage support for those nodes in 2000 bootstrap replicates. Whereas horizontal distances represent genetic distances as indicated by the scale bar, vertical distances are arbitrary.

Various studies and computer-aided comparisons have indicated that “modular evolution” (Botstein *et al.*, 1980) and recombination are dominant features of viral evolution, in addition to divergence based on point mutagenesis. Most probably the simpliest form of functional evolution shown by viruses is their ability to switch hosts. Indeed some analyses have shown that one single nucleotide change may permit host switching (Boulton *et al.*, 1991). In the last 15 years extended sequencing of full length begomoviral genomes provided the opportunity to study their evolution and try to assemble their phylogeny. Despite this no mutation rate has been determined for begomoviruses, as it had been done for tobamoviruses (Gibbs, 1980) and *Maize streak virus* (Isnard *et al.*, 1998) a geminivirus belonging to the genus *Mastrevirus*. Although many examples of recombination between begomoviruses have been reported (Fondong *et al.*, 2000; Chatchawankanphanich & Maxwell, 2002; Monci *et al.*, 2002), only few studies focussed on the evolution (by mutation and selection) of these viruses over time (Chapter 3 and (Sanchez-Campos *et al.*, 2002). In the study of Sanchez-Campo *et al.* (2002) only few mutations over an 8-year period were recorded in a TYLCSV population present in southern Spain. It was concluded that the low genetic variability found in the TYLCSV population studied might be the result of a founder effect with subsequent selection against less fit variants arising by mutations. However a higher number of mutations was observed in our TYLCV-Mld survey on La Réunion (Chapter 3) based on fewer sequences in sampling sites which were much closer to each other, and the global mutation frequency evaluated at 10×10^{-4}, based on all available sequences of the study. This might be due to the close environment of the island and its smaller area. The subtropical climate of La Réunion with overlapping field and greenhouse cropping seasons might also be an important factor for the appearance of mutants, as the climate does not provide an annual (winter) bottleneck effect.

Being in an insular environment, with close watch on imported material, and with no other islands of the region having the TYLCV, allowed us to have a more reliable evaluation of the mutation frequency. Previous studies carried out on begomoviruses indicated that in the absence of recombination with other strains or species, the level of intra-strain variability in geminiviruses is limited, with some diversity recorded (Gilbertson *et al.*, 1991; Stenger & McMahon, 1997; Faria & Maxwell, 1999). Nevertheless these field isolates diversity reported in the referred publications might be due to that described begomovirus were present for a longer time or were even indigenous from the sampled area.

A continued analysis should be carried out to try to evaluate the rate of “field” mutations in a TYLCV population within a close environment such as Réunion, with fewer climatic constrains, and try to compare it with “continental” and temperate evolution of TYLCV. With such studies the global mutation frequencies of begomoviruses might be estimated.

Host resistance: a solution to control begomovirus infections?

The emergence and rapid spread of begomoviruses is at least partially caused by the intensified international trading of unintentional infected plant material and the introduction of their vectors. This has lead to the current situation that begomoviruses represent worldwide a major limiting factor in tomato production areas. Control measures in begomovirus-infested regions have traditionally been emphasised on vector control (Hilje *et al.*, 2001; Palumbo *et al.*, 2001), mainly using pesticides or physical barriers. Chemical control measures have only been partially effective as whitefly populations can reach very high numbers, leading to heavy pesticides use. This practice has in turn led to the development of pesticide resistance in whiteflies (Palumbo *et al.*, 2001). Faced with the rapid spread of begomoviruses throughout tomato cultivated areas, broad resistance to begomoviruses is urgently needed. Vector resistance could be used as a first barrier that would prevent massive attack by any new begomovirus.

Since all cultivated tomatoes are extremely susceptible to begomoviruses, wild *Lycopersicon* species have been screened for possible resistance traits against TYLCV (Laterrot, 1992; Pilowsky & Cohen, 2000) or bipartite begomoviruses (Santana *et al.*, 2001; Pietersen & Smith, 2002; Rampersad & Umaharan, 2003b). In most cases, resistance to TYLCV identified in accessions of wild origin appeared to be oligogenically inherited with resistance factors ranging from recessive to dominant (reviewed in Lapidot & Friedmann, 2002). Among these resistance factors, only one major partial resistance gene to TYLCV has been identified: TY-1 (Zamir *et al.*, 1994) on chromosome 6 of *L. chilense*. Two more resistance modifier genes were mapped to chromosome 3 and 7 (Zamir *et al.*, 1994) of *L. Chilense*. Another TYLCV-resistance gene, originating from *L. pimpinellifolium* has been mapped using RAPD PCR-based markers to chromosome 6, but to a different locus from TY-1 (Chagué *et al.*, 1997). In addition, a resistance gene against the *Tomato leaf curl Taiwan virus* was mapped to chromosomes 8 and 11 of *L. hirsutum* (Hanson *et al.*, 2000). Despite all the efforts to screen wild type tomatoes, no studies have been done to find a broader resistance to mono and bipartite begomoviruses. In Chapter 7, a screening study was dedicated to find resistance to both types of begomoviruses infecting tomato: monopartite (TYLCV) and bipartite (PYMV) begomoviruses. Unfortunately, among the 20 accessions screened, few appeared to be good candidates for resistance. Although none of the accessions were completely resistant, it was shown that a single phenotype could show an enhanced resistance level to both viruses. In *L. pimpinellifolium* INRA Hiruste and LA 1478 partial resistance to both viruses was observed after vector-inoculation and which was overcome after graft-inoculation. Therefore it was hypothesized to concern resistance to begomovirus transmission, which could be efficient against more than one species of begomovirus. A high level of resistance to both viruses was observed in *L. chilense* LA 1969 after vector inoculation and which was not overcome after graft-inoculation of TYCLV. This would imply that this accession contains a broad-spectrum resistance against begomoviruses from both the New World and the Old World. In LA 1969 the major gene TY-1, described by Zamir *et al.* (1994), controls resistance to TYLCV but it is not clear whether it is also involved in the now observed resistance to bipartite begomoviruses.

In contrast, *L. pimpinellifolium* LA2187-5 was found highly resistant to PYMV and susceptible to TYLCV. To our knowledge, this is the first resistance to PYMV described. The resistance appeared recessive in our preliminary study on F_1 generations but further genetic analyses are needed to evaluate the number and the role of factors involved.

As crosses of *L. pimpinellifolium* with *L. esculentum* are compatible, and progenies polymorphic, genetic studies of resistance in LA 2187-5 could be quickly conducted on an interspecific crossing such as CRA 66 x LA 2187-5 (Chapter 7). Another interspecific crossing is needed to study potential vector resistance in *L. pimpinellifolium* INRA Hirsute.

The accession showing the most quantitative resistant to vector transmission of both viruses was selected for further studies (Chapter 8). The idea was to test if this accession had really a broad and interesting level of resistance against vector transmission. This was indeed found for the accession INRA hirsute, and the hypothesis of a quantitative resistance to vector transmission could be confirmed. It would be interesting to map this resistance genes, and compare its map localisation with the other resistance loci found in chromosomes 3, 6 and 7. This accession appeared a good candidate to use in breeding for a broader resistance than what has already been done. Nevertheless, introgression of quantitative resistances, which are supposed to be polygenic (Parlevliet, 1979; Parlevliet, 2002) into *Lycopersicon esculentum* is a long and difficult job, as components contributing to the resistance might get lost during crossings for introgression. Therefore, other approaches than conventional breeding, such as transgenesis (based on RNAi) or chemical mutagenesis (McCallum *et al.*, 2000) should also be considered as these may lead more rapidly to the desired phenotypes.

SUMMARY

Tomato yellow leaf curl virus (TYLCV) belongs to the genus *Begomovirus* within the *Geminiviridae* family, and is exclusively transmitted by the whitefly species *Bemisia tabaci* (Hemiptera: *Aleyrodidae*) (Gennadius). It is an emerging virus which since the 1980's has globally spread over many tropical, subtropical, and Mediterranean countries. By now it has even reached such isolated places as La Réunion, an island situated in the south-western part of the Indian Ocean. Since its first introduction in 1997, the virus has spread quickly throughout this island, becoming a great constraint for tomato cultivation. At the same time an upsurge of whiteflies was reported on vegetable crops.

For future control and prevention of begomovirus infestations on La Réunion further information on the identity and genetic properties of the introduced begomoviruses and on the whiteflies involved is urgently needed. The aim of the studies presented in this thesis was, therefore, to investigate how new begomoviruses can potentially be introduced in La Réunion, which begomoviruses are currently present and which whitefly biotypes are involved in their spread. Also a start was made to search for useful resistance sources in a range of available wild *Lycopersicon* accessions.

In Chapter 2, a potential way of unintentional introduction of new begomoviruses into an area free of begomoviruses was investigated. In La Réunion, and other areas, imported seedlings, ornamentals and all plant material are carefully checked at the custom for the presence of TYLCV or *B. tabaci*, due to their quarantine status, but not imported tomato fruits, as these were regarded not to represent a great risk. In view of the possibility that a potential import route had escaped attention, a survey of tomatoes imported into La Réunion had been made and it was demonstrated (Chapter 2) that more than 50% of the fruits contained TYLCV as determined by DNA blot analysis. Furthermore it was demonstrated that TYLCV can be acquired from this infected fruit by whiteflies and subsequently transmitted to healthy tomato plants. This successful transmission is consistent with the ELISA detection of TYLCV in different parts of vine tomato fruits and the 24 hours survival rate of 80% of a whitefly population maintained on such fruits.

Prior to 1997 hardly any virus problem was encountered in the tomato production in La Réunion. Since that year, the begomovirus TYLCV-Mld was detected, causing yield losses of up to 80% in open field cultivation of susceptible cultivars. Since that year, surveys have been performed to monitor the disease incidence over the island. In Chapter 3 samplings from 1997 up to 2004 were studied in order to evaluate the mutation rate of this newly introduced virus. The mutation frequency (number of mutations relative to the consensus, divided by the number of nucleotides sequenced), based on 2 regions of the genome, was estimated at 10×10^{-4}. This result confirmed that DNA plant viruses may have a mutation frequency equivalent to those obtained for plant RNA viruses. The low genetic diversity found in the initial population was consistent with a founder effect associated with a population bottleneck during the introduction of a new virus in an insular

environment. It was hypothesized that the epidemic observed in 1997 in La Réunion was the result of only a single TYLCV introduction.

The introduction of TYLCV on La Réunion illustrates that plant viruses are very successful in colonising new ecological niches. Realising the intensive trade of vegetables and fruits between all the South western islands of the Indian Ocean (SWIO), and that in 2001, a tomato virus symptom survey on the islands of Madagascar and Mayotte identified an association between *B. tabaci* and tomato plants displaying symptoms characteristic of begomoviruses, the survey for tomato begomoviruses was extended to these islands (Chapter 4). A first analysis of leaf samples collected during this survey indicated the presence of two potentially new *Begomovirus* species, which were called *Tomato leaf curl Madagascar virus* (ToLCMGV) and *Tomato leaf curl Mayotte virus* (ToLCYTV). Both biological and molecular properties of ToLCMGV isolates from Morondova and Toliary (ToLCMGV-[Tol], -(Mor *et al.*)), ToLCYTV isolates from Dembeni and Kahani (ToLCYTV-[Dem], -[Kah]) and of a TYLCV isolate from La Réunion (TYLCV-Mld[RE]) were determined (Chapter 4). Full-length DNA components of the five isolates from Madagascar, Mayotte, and La Réunion were cloned and sequenced and, with the exception of ToLCMG-[Tol], were shown to be both infectious in tomato resulting in virus transmissible by *B. tabaci*. Sequence analysis revealed that these viruses had genome organisations typical of monopartite begomoviruses and that both ToLCMGV and ToLCYTV belong to the African begomovirus but represent a distinct monophyletic group tentatively named the SWIO begomovirus group (Chapter 4). All of the SWIO isolates examined are 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.

The upsurge of TYLCV observed in La Réunion in 1997 coincided with an increase of its insect vector on vegetable crops. As no such increase of *B. tabaci* populations had ever been recorded on this island prior to this event, the arrival of a new biotype of *B. tabaci* was highly suspected. Therefore, the *B. tabaci* populations of La Réunion were more closely investigated (Chapter 5). Using RAPD-PCR and cytochrome oxidase I (COI) gene sequence comparisons, two genetic types of *B. tabaci* were distinguished. One type was assigned to biotype B and the other was genetically dissimilar to the populations described elsewhere and was called Ms after the name of the Mascarenes Archipelago. This new biotype formed a distinct group that is sister to the B biotype group to which also the Q biotype belongs. The Ms biotype is thought to be indigenous to the region as it was also detected on Mauritius, Seychelles, and Madagascar. The divergence from the group formed by the B and Q biotypes was estimated to date back more than 3 ± 0.3 million years ago. Both B and Ms populations of *B. tabaci* induced silverleaf symptoms on *Cucurbita* sp., and were able to acquire and transmit TYLCV.

The recent colonisation of the *B. tabaci* biotype B on La Réunion provided a great opportunity to analyse its colonisation process (Chapter 6). As biotypes of *B. tabaci* are morphologically indistinguishable, a set of 8 microsatellite markers was developed and used to survey the level of genetic variability and the population dynamics of both the indigenous biotype

Ms and the invasive biotype B. The results showed that microsatellite analysis, combined with bayesian statistics can efficiently distinguish both biotypes. Genetic diversity within the biotypes Ms and B were comparable, suggesting that the introduction of biotype B was not associated with a severe genetic bottleneck. More than a single introduction might have been responsible for its invasion and these might have occurred several years ago. Interestingly, within a few years the introduced biotype has reached the same level of geographic structuration (Fst) across the island as the local biotype. Biotype B has spread towards all parts of the island, although it has not wiped out the resident biotype in any sector. However, the relative proportions of the two biotypes are far from constant and vary according to geographic or ecological factors. Possibly the distribution of their host plants is of importance here. Indeed the B biotype is dominant on vegetable crops, whereas the Ms biotype is predominant on weeds. Nevertheless, some B populations tend to be genetically more similar to Ms genotypes than other isolated B populations and this is suggestive for some introgression. However, more data are needed (and, perhaps more evolutionary time) to definitely document the presence or absence of hybrids and the ability of both biotypes to interbreed.

As a first step towards begomovirus control in cultivated tomatoes, in Chapter 7 a series of accessions of six *Lycopersicon* species were tested and compared for their potential levels of resistance to TYLCV and to another, Caribbean begomovirus, *Potato yellow mosaic virus* (PYMV). PYMV and TYLCV incidence and severity were quantified after vector and graft inoculation in separate experiments. The results obtained indicate that resistance to both viruses may exist in a single genotype. A high level of resistance to both viruses was observed in *L. chilense* LA 1969 after vector inoculation and which was not overcome after graft-inoculation of TYCLV. In two accessions of *Lycopersicon pimpinellifolium* (INRA-hirsute and La 1478) a partial resistance to both viruses was observed after vector inoculation that was overcome after graft inoculation. It was hypothesized that this resistance represented vector resistance to begomovirus transmission. Additionally, *L. pimpinellifolium* LA2187-5 was found highly resistant to PYMV but susceptible to TYLCV.

After this first screening for begomovirus resistance, the resistance to TYLCV as observed in two wild genotypes of *L. pimpinellifolium* (INRA-hirsute and WVA 106) was studied into some further detail. TYLCV incidence, symptom severity, and virus content were assessed after massive viruliferous whiteflies inoculation or grafting (Chapter 8). A moderate resistance, which was overcome at high inoculation pressure, was observed for INRA-hirsute. This partial resistance showed a typical quantitative resistance pattern. Based on Chapter 7 plus the graft and vector inoculation results obtained in Chapter 8, the partial resistance in INRA-hirsute was concluded to act on vector transmission efficiency. This resistance could therefore be effective against other begomoviruses as well.

Finally, in Chapter 9 the results obtained in the experimental Chapters are discussed with respect virus evolution, *B. tabaci* population dynamics, and potential options to control begomovirus disease in tomato.

RESUME

Le *Tomato yellow leaf curl virus* (TYLCV) appartient au genre *Bégomovirus* au sein de la famille des *Geminiviridae*, il est exclusivement transmis par une espèce d'aleurode : *Bemisia tabaci* Gennadius (*Hemiptera: Aleyrodidae*). Virus émergeant, le TYLCV a largement été disséminé depuis les années 80 en zones tropicales, subtropicales et méditerranéennes. Il est actuellement présent dans de très nombreuses régions parfois très isolées telle que l'île de la Réunion, située dans le sud ouest de l'océan Indien. Depuis sa première introduction en 1997, le TYLCV a été observé dans les principales zones de productions maraîchères de l'île, provoquant d'importants dégâts sur la tomate. Simultanément, une pullulation d'aleurodes a été également observée sur les plantes maraîchères. Une lutte efficace et durable contre le TYLCV à La Réunion ne peut être menée sans connaissances approfondies sur les bégomovirus introduits et les aleurodes vecteurs. Par conséquent, les objectifs de cette thèse étaient de mettre en évidence les sources d'introductions potentielles de nouveaux bégomovirus à La Réunion, de faire l'inventaire des bégomovirus actuellement présents dans la sous-région et des biotypes d'aleurodes responsables de leur propagation. La recherche de nouvelles sources de résistance dans une gamme d'accessions de *Lycopersicon* sauvages a également été menée.

Dans le chapitre 2 de la thèse, une nouvelle voie accidentelle d'introduction de nouveaux bégomovirus dans des zones au préalable sans bégomovirus a été mise en évidence. A La Réunion comme dans d'autres pays, les importations de plantules, de plantes ornementales ou de tout matériel végétal sont strictement contrôlées par les services phytosanitaires pour la présence, entre autre, du TYLCV ou d'aleurodes, du fait de leur statut d'organismes de quarantaine. Néanmoins, les importations commerciales de tomates (fruits) n'ayant jamais été considérées comme un risque potentiel, ne sont jamais contrôlées. Ce risque d'introduction de bégomovirus n'ayant jamais été testé, une enquête sur les lots de tomates importés à La Réunion a été conduite. Il a été démontré (chapitre 2) par analyse moléculaire (dot blot) que 50% des lots de tomates importés testés contenaient du TYLCV. Il a aussi été démontré expérimentalement que le TYLCV pouvait être acquis à partir de fruits infectés par des aleurodes et retransmis à des plants de tomates sains. La détection par ELISA du TYLCV dans toutes les différentes parties du fruit et le taux de survie de 80% des insectes maintenus sur de tels fruits durant 24h rendent possible un tel mode de transmission.

Avant 1997, peu de maladies virales étaient responsables de pertes sur la production maraîchère de tomate à La Réunion. Depuis la détection du TYLCV-Mld[RE] en 1997, de fortes pertes de rendement allant jusqu à 80% ont été enregistrées en plein champ sur tomates sensibles. Dès 1997, une campagne de prélèvements a débuté dans les différents foyers de la maladie sur toute l'île dans le but de suivre l'évolution du virus en milieu insulaire (Chapitre 3). Les échantillons prélevés de 1997 à 2004 ont été étudiés afin de connaître la diversité virale du TYLCV-Mld [RE] et

d'évaluer la fréquence d'apparition des mutations de cette nouvelle souche virale introduite. La fréquence de mutation (nombre de mutations relatives au consensus, divisées par le nombre total de nucléotides séquencés), basée sur 2 régions du génome du TYLCV a été estimée à 10×10^{-4}. Ce résultat confirme le fait que les virus phytopathogènes à ADN auraient une fréquence de mutations équivalente à celle des virus phytopathogènes à ARN. La faible diversité génétique virale trouvée dans la population initiale est en accord avec un effet de fondation associé à un goulot d'étranglement, lors de l'introduction d'un nouveau virus dans un nouveau milieu. L'hypothèse d'une introduction unique de TYLCV a donc été émise.

L'introduction du TYLCV à La Réunion illustre bien le fait que les virus phytopathogènes sont de très bons colonisateurs de nouvelles niches écologiques. Connaissant les échanges commerciaux existant entre les îles du sud ouest de l'océan Indien (SWIO) et le fait qu'en 2001et en 2003 une enquête menée respectivement à Madagascar et Mayotte a révélé des symptômes de maladies à bégomovirus associés à la présence de leur vecteur (*B. tabaci*), une campagne de prélèvements a été réalisée sur ces îles (Chapitre 4). Une première analyse de feuilles portant des symptômes caractéristiques des bégomovirus a montré la présence de deux nouvelles espèces de *Bégomovirus* qui ont été nommées le Tomato leaf curl Madagascar virus (ToLCMGV) et le Tomato leaf curl Mayotte virus (ToLCYTV). Les propriétés biologiques et moléculaires de deux isolats du ToLCMGV de Morondova et de Toliary (ToLCMGV-[Tol], -(Mor *et al.*)), et de deux isolats du ToLCYTV de Dembeni et de Kahani (ToLCYTV-[Dem], -[Kah]) ainsi qu'un isolat du TYLCV de La Réunion (TYLCV-Mld[RE]) ont été déterminées (Chapitre 4). Les génomes à ADN viraux complets de chacun des isolats de Madagascar, Mayotte et La Réunion ont été clonés, séquencés et leur pouvoir pathogène vérifié, à l'exception du ToLCMG-[Tol]. Leurs capacités de transmission par *B. tabaci* ont également été vérifiées. L'analyse de ces nouvelles séquences de bégomovirus a montré qu'ils avaient une organisation typique de bégomovirus monopartite et que le ToLCMGV ainsi que le ToLCYTV appartenaient au groupe des bégomovirus africains. Cependant, ils représentent un groupe monophylétique distinct qui a été nommé le groupe des bégomovirus du SWIO (chapitre 4). Tous les isolats examinés du SWIO se sont révélés être des recombinants complexes. Toutefois, aucune des séquences au sein des régions recombinantes ne ressemble à celles d'un bégomovirus connu n'appartenant pas au groupe SWIO, suggérant une évolution en isolement de cette population virale.

L'apparition de la maladie du TYLC en 1997 à La Réunion a coïncidé avec l'augmentation des populations de son insecte vecteur *B. tabaci* sur les cultures maraîchères. Comme aucune pullulation de *B. tabaci* n'avait été décrite auparavant sur cette île, l'arrivée d'un nouveau biotype d'aleurode a été fortement suspectée. L'utilisation de la technique RAPD-PCR et du séquençage du gène du cytochrome oxidase, a permis de distinguer au sein des populations réunionnaises de *B. tabaci* deux types génétiques (Chapitre 5). L'un a été assimilé au biotype B et l'autre, différent génétiquement des populations décrites de par le monde a été nommé : biotype Ms, en raison de sa provenance (l'archipel des Mascareignes). Ce nouveau biotype forme un nouveau groupe frère du groupe déjà formé par les biotypes B et Q. Le biotype Ms semble être originaire de la zone, car il a

été retrouvé à Madagascar, à l'île Maurice et aux Seychelles. La divergence entre le groupe formé par les biotypes B et Q et le groupe Ms a été estimé à plus de 3 +/- 0.3 millions d'années. Les biotype B et Ms induisent des symptômes d'argenture sur feuilles de *Cucurbita* sp. et sont capables d'acquérir et de transmettre le TYLCV.

La récente introduction du biotype B de *B. tabaci* à La Réunion nous a permis d'analyser son processus de colonisation (Chapitre 6). Comme les biotypes de *B. tabaci* sont indistincts morphologiquement, un jeu de huit marqueurs microsatellites a été caractérisé et utilisé pour mesurer la variabilité génétique et la dynamique des populations des deux biotypes, l'un indigène le biotype Ms et l'autre exotique le biotype B. L'utilisation de marqueurs combinés à une analyse de statistique bayesienne, a permis de différencier aisément les deux biotypes. La diversité génétique des deux biotypes est comparable, et suggère que l'introduction du biotype B n'a pas été associée à un fort goulot d'étranglement. Ainsi, plus qu'une seule introduction du biotype B pourrait être responsable de l'invasion, qui pourrait dater de plusieurs années. En l'espace de quelques années, le biotype introduit a atteint le même niveau de structuration géographique sur l'île que le biotype indigène. Le biotype B a colonisé toutes les régions favorables de l'île, mais n'a jamais supplanté totalement le biotype indigène et ce, dans aucun secteur de l'île. Néanmoins, la proportion relative des deux biotypes varie en fonction de facteurs écologiques ou géographiques. La répartition des plantes hôtes pourrait être un facteur important dans cette répartition. En effet, le biotype B est prédominant sur cultures maraîchères alors que le biotype Ms l'est plutôt sur des dicotylédones adventices. Certaines populations de biotype B tendraient à être génétiquement plus proches des populations de Ms, que d'autres populations de B plus isolées, suggèrent fortement l'échange de matériel génétique. Néanmoins, plus de données sont nécessaires (et peut-être plus de temps d'évolution) pour prouver la présence ou l'absence d'hybrides, et ainsi la possibilité d'intercroisement en conditions naturelles des deux biotypes.

Dans le Chapitre 7, la résistance au TYLCV et à un autre bégomovirus des Caraïbes, le *Potato yellow mosaic virus* (PYMV) sur 6 espèces d'accessions de *Lycopersicon* sauvages a été évaluée. L'incidence et la sévérité au PYMV et au TYLCV ont été quantifiées après inoculation par vecteur et par greffage dans des expérimentations séparées. Bien qu'aucune des accessions testées n'ait été complètement résistante, les résultats obtenus suggèrent l'existence de résistance aux deux virus dans un seul génotype. Un très fort niveau de résistance aux deux virus a été observé chez *L. chilense* LA 1969 après inoculation par vecteur et ne fut pas surmonté par le greffage de greffons infectés par le TYLCV. Dans deux accessions de *L. pimpinellifolium* (INRA-hirsute et La 1478) une résistance partielle aux deux virus a été observée après inoculation par vecteur, mais fut surmontée par le greffage. Une première hypothèse serait que cette résistance devrait être basée sur de la résistance à la transmission des bégomovirus par l'insecte vecteur. Enfin l'accession *L. pimpinellifolium* LA2187-5 s'est montrée résistante au PYMV et sensible au TYLCV.

Après ce premier criblage d'accessions de *Lycopersicon* pour la résistance aux bégomovirus (Chapitre 7), la résistance au TYLCV a été étudiée plus en détail dans 2 génotypes de *L. pimpinellifolium* (INRA-hirsute and WVA 106) dans le chapitre 8. L'incidence, la sévérité et la quantité de virus ont été suivies après une inoculation de masse par aleurodes virulifères et après greffage. Une résistance modérée, surmontée par une forte pression d'inoculation a été observée pour l'accession INRA-hirsute. Cette résistance partielle a montré un comportement typique d'une résistance quantitative. Les résultats obtenus dans le Chapitre 7 ainsi que ceux obtenus après greffage et inoculation par vecteur permettent d'affirmer que la résistance présente dans l'accession INRA-hirsute agit comme une résistance à la transmission virale par l'insecte. Cette résistance pourrait ainsi être efficace contre d'autres espèces de bégomovirus.

En conclusion de cette thèse, les résultats obtenus dans les chapitres expérimentaux sont discutés aux regards de l'évolution des virus, de la dynamique et génétique des populations de *B. tabaci*, et des perspectives de lutte contre les maladies à bégomovirus chez la tomate (Chapitre 9).

SAMENVATTING

Tomato yellow leafcurl virus (TYLCV) behoort tot het geslacht Begomovirus binnen de familie *Geminiviridae*, een grote familie van plant-infecterende DNA virussen. Net als alle andere begomovirussen wordt TYLCV uitsluitend verspreid door de wittevlieg *Bemisia tabaci* (Heminoptera: *Aleyrodidae*) (Gennadius). Sinds de jaren tachtig heeft het TYLCV zijn areaal aanzienlijk uitgebreid naar nieuwe gebieden in de tropische, subtropische en mediterrane klimaatzones. De laatste jaren heeft het virus zelfs een aantal geïsoleerde gebieden bereikt, waaronder La Réunion, een eiland gelegen in het zuidwestelijk deel van de Indische Oceaan. Na introductie in 1997 heeft het virus zich in snel tempo over dit eiland verspreid en vormt hier thans een belangrijke beperkende factor in de tomatenteelt. Tegelijkertijd nam ook het aantal meldingen van wittevliegen op groentegewassen toe.
Om beheersing en bestrijding van begomovirusinfecties op La Réunion in de toekomst mogelijk te maken, is het belangrijk om de identiteit en de genetische eigenschappen van de betrokken virussen en de *B. tabaci* biotypen te kennen. Dit vormde dan ook een voorname doelstelling van het onderzoek dat beschreven is in dit proefschrift. Daarnaast werd ook een start gemaakt met het zoeken en beschrijven van mogelijke resistenties tegen deze begomovirussen.

In Hoofdstuk 2 werd allereerst nagegaan in hoeverre TYLCV via geïnfecteerde tomaten (vruchten) geïntroduceerd kan worden. Zowel het virus als de betrokken vector zijn geclassificeerd als quarantaine pathogenen, en dientengevolge wordt geïmporteerd plantmateriaal aan de grens zorgvuldig gecontroleerd op het voorkomen van virus en vector. Echter geïmporteerde tomaten worden niet gecontroleerd, omdat er altijd van uit gegaan is dat deze geen potentiële besmettingsbron vormen.
De resultaten van Hoofdstuk 2 laten zien dat deze aanname niet juist was. Terwijl in meer dan 50% van de geteste geïmporteerde tomaten TYLCV aangetroffen werd, kon bovendien worden vastgesteld dat *B. tabaci* in principe in staat is om TYLCV uit deze geïnfecteerde vruchten op te nemen en hiermee gezonde tomatenplanten te infecteren.

In 1997 werd het TYLCV (de milde stam, aangeduid als TYLCV-Mld) voor het eerst in de tomatenteelt op La Réunion gevonden en sinds dat jaar wordt door middel van regelmatige inspecties en bemonstering van planten de verspreiding van de virusziekte gevolgd. In Hoofdstuk 3 zijn virusmonsters, verzameld tussen 1997 en 2004, nader onderzocht om de mutatiefrequentie in het virale DNA genoom sinds de eerste introductie te bepalen. Op basis van de nucleotidenvolgorde van 2 genomische regio's kon een mutatiefrequentie worden vastgesteld van 10×10^{-4}, wat in ongeveer dezelfde orde van grootte ligt als de mutatiefrequentie van virale RNA genomen. De beperkte genetische diversiteit van de initiële viruspopulatie wijst op een "founder" effect dat samenhangt met een "bottleneck" effect tijdens de introductie op het eiland. Uit de verkregen

gegevens kan worden afgeleid dat TYLCV zich hoogstwaarschijnlijk middels een eenmalige introductie op het eiland gevestigd heeft.
De introductie en vervolgens vestiging van TYLCV op La Réunion illustreert opnieuw het vermogen van plantenvirussen om zich succesvol te kunnen nestelen in nieuwe ecologische niches. In het licht van de intensieve groente- en fruithandel tussen de eilanden van het zuidwestelijke deel van de Indische Oceaan, en vanwege het feit dat er in 2001 op zowel Madagascar als op Mayotte tomaten werden aangetroffen met virusziektesymptomen die bovendien verband leken te houden met het voorkomen van *B. tabaci*, werd het onderzoek naar het voorkomen van begomovirussen in tomaat uitgebreid naar deze eilanden (Hoofdstuk 4). Een eerste analyse van bladmateriaal duidde op de aanwezigheid van 2 soorten begomovirussen, die *Tomato leaf curl Madagascar virus* (ToLCMGV) en *Tomato leaf curl Mayotte virus* (ToLCYTV) werden genoemd. Zowel de biologische als de moleculaire eigenschappen van deze 2 virussen werden in Hoofdstuk 4 nader onderzocht, samen met een Réunion isolaat van het TYLCV (TYLCV-Mld[Re]). Van deze virussen werden volledige infectieuze kloons verkregen. Sequentieanalyse van de drie soorten virussen leerde dat ToLCMGV en ToLCYTV behoren tot de groep van Afrikaanse begomovirussen, maar dat zij daarbinnen een aparte fylogenetische cluster vormen, die de voorlopige naam van SWIO (South West Islands of the Indian Ocean) begomovirus cluster werd gegeven.

Het verschijnen van TYLCV op La Réunion in 1997 ging gepaard met een opvallende toename van *B. tabaci* op groentegewassen. Omdat voor die datum nooit enige toename van wittevlieg was opgemerkt, werd vermoed dat het hier om kolonisatie door een nieuw geïntroduceerd biotype ging. Dit werd nader uitgezocht in Hoofdstuk 5. Met behulp van RAPD-PCR en cytochroom oxidase I (COI) gensequenties werden diverse populaties vergeleken en kon het naast elkaar voorkomen van twee verschillende biotypen worden vastgesteld, te weten het kennelijk ingevoerde biotype B en een biotype dat nog niet eerder beschreven was. Aangenomen werd dat laatstgenoemde het van oorsprong op het eiland voorkomende biotype was en deze werd derhalve Ms genoemd, naar het Mascarenen eilandenrijk waartoe ook La Réunion behoort. Op basis van hun genetische afstand werd geschat dat de divergentie tussen de biotypen Ms en B meer dan 3 (+/- 0.3) miljoen jaren geleden plaatsvond. Beide biotypen veroorzaakten zilverbladschade op *Cucurbita sp*. en beiden bleken in staat om TYLCV op te nemen en over te dragen.

In Hoofdstuk 6 werd de recente kolonisatie van La Réunion door het biotype B verder onderzocht. Door middel van microsatelliet analyse werd de genetische variabiliteit en populatiedynamica van zowel het originele Ms als het invasieve B biotype onderzocht. De genetische diversiteit binnen beide biotypes bleek vergelijkbaar, hetgeen er op kan wijzen dat de introductie van biotype B niet gepaard ging met een groot genetisch “bottleneck” effect en dat er sprake geweest kan zijn van meer dan slechts een enkele introductie. Binnen slechts enkele jaren heeft biotype B eenzelfde spatiële populatiestructuur (Fst) bereikt als het oorspronkelijke biotype. Ondanks dat dit invasieve biotype zich verspreid heeft over alle delen van het eiland, is nergens het Ms biotype totaal verdrongen. De verhouding waarin zij beiden naast elkaar voorkomen bleek

behoorlijk te kunnen variëren, en was kennelijk afhankelijk van geografische en ecologische factoren, waarbij de verspreiding van geschikte waardplanten wellicht ook een rol speelt. Terwijl biotype B domineert op groentegewassen werd biotype Ms vooral op de wilde vegetatie gevonden. Sommige biotype B populaties bleken genetisch nauwer verwant te zijn aan Ms dan andere geïsoleerde B populaties, en dit zou kunnen wijzen op introgressie. Echter meer gegevens en wellicht ook een langere evolutionaire tijdspanne zijn nodig om hybridisatie tussen de beide biotypes definitief vast te stellen.

Begomovirussen vormen steeds vaker een beperkende factor in de tomatenteelt, en dit geldt nog het meest voor het TYLCV dat zich inmiddels naar vele landen in de Oude en Nieuwe Wereld heeft weten te verspreiden. Terwijl TYLCV een voorbeeld is van een begomovirus met een enkelvoudig, ongedeeld DNA genoom, is *het Potato yellow mosaic virus* (PYMV) een voorbeeld van een begomovirus met een tweeledig DNA genoom. Dit virus heeft zich vooral verspreid over het Caribische gebied. In Hoofdstuk 7 is nagegaan of het mogelijk is tegelijkertijd waardplantresistentie tegen het monopartite TYLCV en het bipartite PYMV te verkrijgen. Hiertoe is een reeks beschikbare wilde *Lycopersicon* accessies getoetst voor mogelijke resistentie tegen de beide virussen. Daarbij werden de planten op twee manieren geïnoculeerd, nl. met behulp van wittevliegen (de natuurlijke wijze) en d.m.v. enting. In *L. chilense* (LA 1969) werd een hoog niveau van resistentie gevonden tegen beide virussen, zowel bij vector-inoculatie als bij ent-inoculatie. In twee accessies van *Lycopersicon pimpinellifolium* (INRA-hirsute en LA 1478) werd partiële resistentie tegen beide virussen waargenomen indien vector-inoculatie werd toegepast, maar niet bij ent-inoculatie. Daarom werd verondersteld dat hier sprake kon zijn van vectorresistentie resulterend in verminderde virusoverdracht. Van *L. pimpinellifolium* LA 2187-5 kon worden vastgesteld dat deze zeer resistent was tegen PYMV maar vatbaar voor TYLCV.
Na dit eerste onderzoek naar bruikbare begomovirusresistenties werd de TYLCV resistentie zoals aangetroffen in twee *L. pimpinellifolium* accessies (INRA-Hirsute en WVA 106) in Hoofdstuk 8 verder onderzocht. Gebaseerd op de vergaarde gegevens kon vastgesteld worden dat de partiële resistentie van de accessie INRA - Hirsute gebaseerd is op verlaging van virusoverdrachtefficiëntie en daarom wellicht ook inzetbaar is tegen andere begomovirussen.

Tenslotte worden in Hoofdstuk 9 de resultaten van het promotie-onderzoek in een bredere context bediscussieerd waarbij aspecten als virale evolutie, populatiedynamica van *B. tabaci* en mogelijke strategieën om begomovirusziekten in tomaat te bestrijden de revue passeren.

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About the author

Hélène Delatte was born on December 23rd, 1977, in Fort de France, Martinique. After finishing her academic high school in Martinique, she studied agronomic engineering at ISTOM, in Paris, and graduated in 1999. During her studies at ISTOM she did a 3-months training in *in vitro* culture in Larenstein School in the Netherlands and got a Degree of tropical crop production. She also did 2 long training periods, one as a production supervisor in Madagascar (1997) and one of 6 months in Vietnam on citrus diseases during her ingeneering studies at ISTOM.

Subsequently she followed an Msc degree in crop protection in order to specialise in virology in Wageningen University (Nl, 1999-2001). She did 2 Msc thesis, the minor one dealt with the evaluation of thrips resistance in pepper to control *Tomato spotted wilt virus*. The major thesis was partly realised between the CIRAD-Montpellier and CIRAD-Réunion on *Tomato yellow leaf curl virus* and *Bemisia tabaci* its insect vector. She received her Msc diploma in January 2001, and from February 2001 till June 2001 she was employed as a guest searcher in the laboratory of CIRAD-Réunion. In June 2001 she was awarded of a sandwich PhD fellowship from Wageningen University and financial help from CIRAD to carry on her researches between the virology laboratory of Wageningen, of CIRAD-Montpellier and CIRAD-Réunion, under the supervision of Pr. R. Goldbach, HDR Dr. B. Reynaud and Dr. M. Peterschmitt, within the research project that resulted in this thesis.

ACCOUNT

Delatte, H., Reynaud, B., Lett, J. M., Peterschmitt, M., Granier, M., Ravololonandrianina, J. & Goldbach, R. (2003). First Molecular Identification of a Begomovirus Isolated from Tomato in Madagascar. *Plant Disease* **86**, 1404.

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PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (ECTS 4)

- Study of the pathosystem begomovirus/ *Bemisia tabaci*/ tomato in the South West part of the Indian Ocean' (2002)

Writing of project proposal (ECTS 4)

- Study of the pathosystem begomovirus/ *Bemisia tabaci*/ tomato in the South West part of the Indian Ocean' (2001)

Post-Graduate Courses (ECTS 3)

- Spatial modelling in ecology (2002) Course on Access and Visual Basic. CIRAD (2004)
- Statistics (R software). CIRAD (2005)

Deficiency, Refresh, Brush-up and General Courses (ECTS 6)

- Scientific and Professional communication in English. CIRAD (2003)
- Population Genetics. University of La Réunion (2003)

PhD discussion groups (ECTS 8)

- CIRAD Réunion weekly discussion groups including PhD students (2002-2005)
- WU Laboratory of Virology research discussion and PhD students progress meetings (2002-2005)
- CIRAD annual "PhD students day" (2002-2005)
- Organising the CIRAD annual "PhD student day" edition 2005 (2005)

International symposia, workshops and conferences (ECTS 6)

- International Congress of Virology (Paris). JDC Conseil/ IUMS (2002) (poster)
- International Workshop of *Bemisia tabaci* (Spain). University of Barcelona (2003) (2 posters)
- International Congress of Geminiviruses (South Africa). University of Cape Town (2004) (oral presentation)
- Virology Congress of Aussois (France). CNRS and IBMP-Strasbourg (2005) (oral presentation)

Laboratory training and working visits (ECTS 5)

- CIRAD-Montpellier. Virus Cloning and Agroinfection (2003)
- PRI-Wageningen. Microsatellite analysis of whitefly DNA (2002)
- CIRAD and CEFE-Montpellier. Analysis of whitefly data (2004)