TABLE DES MATIERES

Introduction

Introduction

1. Contexte général

Depuis les premières descriptions du SIDA au début des années 1980, de nombreuses études ont été réalisées sur les causes et les conséquences de l'émergence ou de la ré-émergence des maladies virales. Les virus émergents sont définis comme ceux qui sont récemment apparus ou ceux dont les populations ont récemment augmenté en prévalence, en pathogénicité et/ou en répartition géographique (Holmes and Rambaut 2004 ; Jones 2009). Ce phénomène a été mis en avant ces dernières années par les épidémies virales telles que celles associées aux maladies émergentes de l'Homme (SRAS, Holmes and Rambaut 2004 ; chikungunya, Schuffenecker et al. 2006 ; grippe HIN1, Smith et al. 2009), des animaux (fièvre aphteuse, Grubman and Baxt 2004) et des plantes (mosaïque du manioc, Patil and Fauquet 2009 ; panachure jaune du riz, Fargette et al. 2006 ; enroulement foliaire et jaunissement de la tomate, Moriones et al. 2011).

Même s'il est toujours difficile de prédire quel virus représente la plus grande menace d'émergence, quand et où il va survenir, des progrès importants ont été réalisés dans l'identification des facteurs écologiques et moléculaires associés à l'émergence (Fargette et al. 2006; Holmes and Rambaut 2004; Jones 2009; Moriones et al. 2011; Smith et al. 2009; Woolhouse et al. 2001).

Alors que les maladies émergentes chez l'Homme et les animaux sont généralement causées par des virus à ARN, la plupart des émergences virales chez les plantes sont liées à des virus ADN du genre *Begomovirus* transmis par l'aleurode *Bemisia tabaci* (Seal et al. 2006). Le potentiel important d'évolution de ces virus à ADN, leur prévalence en augmentation permanente et l'existence de biotypes invasifs de leur insecte vecteur *B. tabaci,* font de ces virus un modèle idéal pour la description et la compréhension de l'émergence virale et des mécanismes sous-jacents. Les récentes avancées sur les moteurs de l'évolution des bégomovirus et les facteurs écologiques associés à leur émergence seront autant d'atouts pour la compréhension des dynamiques des populations virales et pour le développement de stratégies de contrôle.

2. Les acteurs : Begomovirus et *Bemisia tabaci*

a. La famille des Geminiviridae

La famille des géminivirus présente une très grande diversité en termes d'organisation génomique, de gamme d'hôtes et d'insectes vecteurs. Sur la base de ces propriétés, les géminivirus ont été divisés en quatre genres dont le nom dérive du membre type : *Begomovirus*, *Curtovirus*, *Mastrevirus* et *Topocuvirus.* Les *Mastrevirus* (infectant principalement des plantes monocotylédones et transmis par cicadelle) et les *Begomovirus* (infectant des dicotylédones et transmis par aleurode) sont les genres les mieux caractérisés (Pour revue Rojas et al. 2005). L'espèce type du genre *Begomovirus* est le *Bean golden mosaic virus*, virus de la mosaïque dorée du haricot (Van Regenmortel et al. 2000). Les bégomovirus sont responsables de viroses sur de nombreuses cultures d'importance économique et sociale dans le monde entier et particulièrement en zones tropicales, notamment sur le manioc (*Manihot esculenta*), le haricot (*Phaseolus vulgaris*), le coton (*Gossypium hirsutum*) et la tomate (*Solanum lycopersicum*).

b. Organisation génomique et fonction des différentes protéines

Les bégomovirus sont monopartites ou bipartites, c'est-à-dire que l'information génétique est portée par un ou deux ADNs circulaires distincts de 2,5 à 2,8 kb (ADN A-*like* ou ADN A et ADN B ; Briddon et al. 2010; Fauquet et al. 2008). Une grande majorité des bégomovirus originaires de l'Ancien Monde sont monopartites. C'est le cas notamment du *Tomato yellow leaf curl virus* (TYLCV; Antignus and Cohen 1994) et des bégomovirus indigènes des îles du sud ouest de l'océan Indien, comme le *Tomato leaf curl Comoros virus* (ToLCKMV; Delatte et al. 2005b; Lefeuvre et al. 2007c). Plus récemment, des ADN satellites ont été mis en évidence en association avec certains bégomovirus (Briddon and Stanley 2006; Dry et al. 1997; Nawaz-ul-Rehman and Fauquet 2009). Ces ADNs d'environs 1300 bases participent à l'infection virale, sans être pour autant nécessaires à celle-ci. Deux types majeurs sont pour le moment décrits, les ADN Beta (Briddon et al. 2008) et les ADN-Alpha (anciennement nommés ADN-1 ; Briddon et al. 2004). Les ADN-Beta ont été décrits comme des activateurs de la pathogénicité (Saunders et al. 2004), avec un possible rôle de suppression du

post-transcriptional gene silencing (PTGS ; Briddon and Stanley 2006; Cui et al. 2005; Vanitharani et al. 2005) tandis que les ADN-Alpha, qui portent un gène codant pour une protéine associée à la réplication (protéine Rep) semblent avoir un rôle dans la modulation de l'accumulation du virus (Briddon and Stanley 2006). L'implication des ADN-Alpha dans la pathogénicité a été récemment mise en évidence avec une activité de suppression du PTGS (Nawaz-Ul-Rehman et al. 2010).

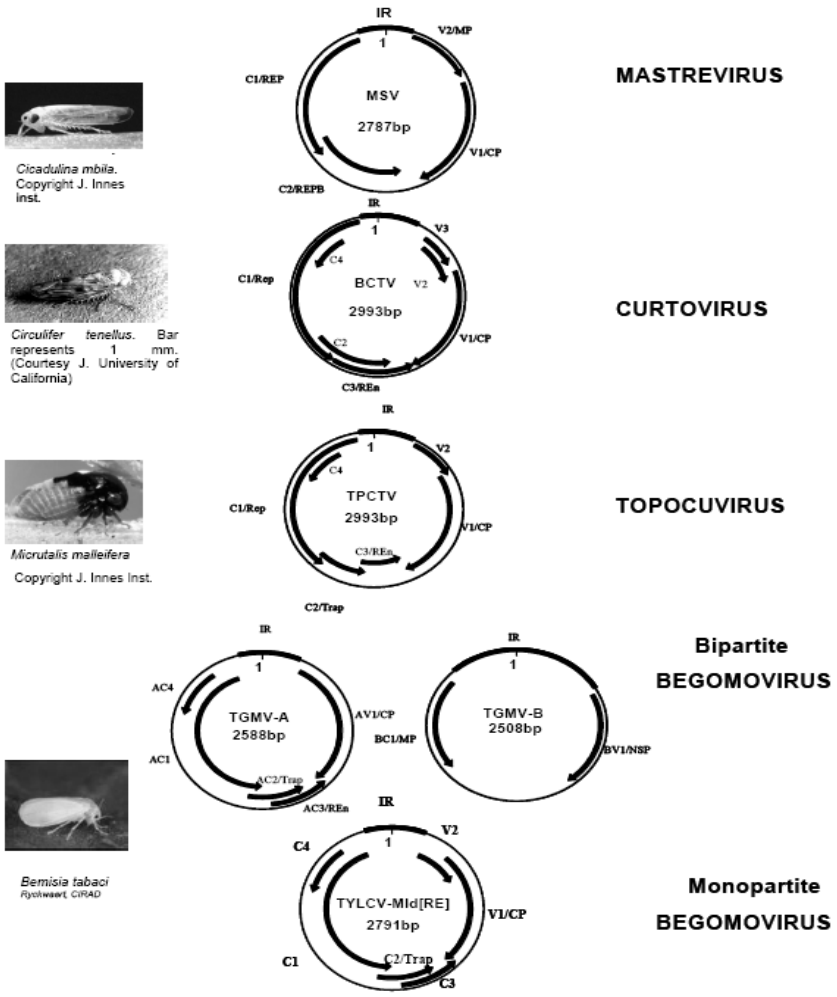


Figure 1 : Représentation des génomes d'un exemple type des différents genres de la famille des *Geminiviridae* et photos de leurs insectes vecteurs respectifs.

L'ADN A des bégomovirus monopartites présente six régions codantes ou ORFs pour « open reading frame » dont certaines sont chevauchantes et une zone intergénique (*intergenic region* ou IR). Les ORFs V1 et V2 sont codés par le brin viral alors que les ORFs C1, C2, C3 et C4 sont codés par le brin complémentaire (Figure 3). La tige boucle (5'-TAATATTAC-3') est une région conservée de l'IR

chez tous les bégomovirus. Elle représente le point d'initiation de la réplication (Orozco and Hanley-Bowdoin 1996).
L'ORF CP (V1) code pour la protéine de capside qui représente l'unité de base dans la constitution de la particule virale en doublet des géminivirus. La CP associée à la protéine de mouvement MP (V2, absente chez les bégomovirus bipartites) intervient dans les mécanismes de diffusion du virus dans la plante hôte. Lors de l'infection et de la réplication, l'ADN viral migre dans le noyau des cellules. Il doit traverser plusieurs barrières cellulaires avant d'accéder à l'intérieur du noyau, pour s'y répliquer. C'est lors de cette migration que les protéines virales, notamment la protéine CP, sont indispensables pour protéger le matériel génétique (Rojas et al. 2001). Par ailleurs, la CP a un rôle essentiel dans la reconnaissance et la transmission par son insecte vecteur (Briddon et al. 1990; Caciagli et al. 2009; Czosnek 2007).

La protéine associée à la réplication (Rep ; ORF C1) intervient lors de la multiplication virale. En association avec la protéine activatrice de la réplication (REn ; ORF C3), elle permet l'accumulation de l'ADN viral dans les cellules végétales (Hanley-Bowdoin et al. 2000). Ces deux protéines sont également connues pour leur implication dans la dérégulation et le détournement de voies métaboliques cellulaires de l'hôte comme : contrôle du cycle cellulaire, réplication de l'ADN, différentiation cellulaire et expression des gènes (Arguello-astorga et al. 2004; Castillo et al. 2004; Gutierrez 2002; Settlage et al. 2001). La protéine activatrice de la transcription (TrAP ; ORF C2) contribue au pouvoir pathogène du virus et à l'activation de la transcription des ORFs (van Wezel et al. 2001). Par ailleurs, le rôle de cette protéine dans la suppression du mécanisme général de résistance des plantes aux virus par « *silencing* » (encore nommé VIGS pour *virus induced gene silencing*) a été démontré (Bisaro 2006; Rojas et al. 2005; Sharma and Ikegami 2010). La protéine C4, codé par l'ORF C4, semble intervenir dans l'expression et la sévérité des symptômes, dans la gamme d'hôte (Dogra et al. 2009; Latham et al. 1997; Rigden et al. 1994), et dans le mouvement de cellule à cellule et systémique du virus (Jupin et al. 1994; Teng et al. 2010). L'expression de l'ORF C4 du *Tomato leaf curl virus* dans des plantes transgéniques de *Nicotiana benthamiana* a permis d'obtenir l'expression de symptômes de virose, fournissant une preuve supplémentaire de la participation de cet ORF dans l'expression des symptômes (Selth et al. 2004). Enfin, son rôle dans la suppression de « silencing », en association ou non avec la TrAP, a également été démontré (Dogra et al. 2009).

Selon les hôtes et les virus, des ADN défectifs peuvent aussi s'accumuler durant l'infection (Behjatnia et al. 2007). Ces ADNs d'environs 1300 bases peuvent interférer avec les génomes parentaux et alors jouer un rôle dans la modulation de la sévérité des symptômes (Paximadis and Rey 2001). Dans ce cas, ils sont alors nommés *Defective Interfering DNA* (DI DNAs) pour ADN défectifs interférant (ADN DI) (Stanley et al. 1990 et pour revue voir Patil and Dasgupta 2006). L'analyse des séquences des ADN-DI suggère que ces derniers sont formés par la suppression, la duplication, l'inversion, le réarrangement et parfois l'insertion de séquences d'ADN non viral impliquant le génome viral et ses ADN satellites. Ils sont principalement constitués des ORFs complémentaires des génomes monopartites ou du génome B des bipartites et contiennent toujours l'origine de réplication (Liu et al. 1998; Patil et al. 2007). Le rôle de la plante hôte dans la formation des ADN DI semble également important, sachant qu'ils sont plus aisément détectés dans les hôtes expérimentaux plutôt que leurs hôtes naturels. La modulation de la sévérité des symptômes de la maladie par les ADN DI est considérée comme le résultat de la compétition pour les facteurs viraux de l'hôte, réduisant ainsi les niveaux d'accumulation du virus parental. Leur rôle dans l'activation du PTGS de la plante contre les transcrits viraux et le phénomène de réversion des symptômes a également été proposé (Patil and Dasgupta 2006).

c. Réplication et transcription

1) La reprogrammation cellulaire, un préalable à la réplication

La réplication de l'ADN et les enzymes associées sont confinées aux zones méristématiques et aux tissus soumis à l'endoréplication[1]. Les géminivirus sont hors des cellules méristématiques et n'ont donc pas accès à la machinerie cellulaire de la réplication de l'hôte (Peele et al. 2001).

Contrairement au TYLCV, le *Tomato golden mosaic virus* (TGMV) n'est pas restreint aux cellules vasculaires. Des hybridations *in situ* ont montré que les protéines virales Rep et REn associées à la réplication de l'ADN viral, sont présentes dans les cellules différenciées du mésophylle, de l'épiderme et les cellules vasculaires foliaires (Nagar et al. 2002). Des expériences de marquages *in vivo* avec un analogue de la thymidine, le 5-bromo-2-deoxyuridine (BrDU), ont montré que le TGMV se réplique dans le noyau des cellules contaminées (Nagar et

[1] réplication de l'ADN du noyau d'une cellule sans division

al. 2002). Des niveaux importants de BrDU ont été incorporés dans l'ADN viral et l'ADN de l'hôte, témoignant de la reprise de l'activité de la réplication de l'ADN caractéristique de la phase S cellulaire. La réplication de l'ADN viral dans des cellules infectées par le TGMV, sans division cellulaire, suggère l'endoréplication du noyau de la cellule végétale (Bass et al. 2000). Les géminivirus peuvent induire une entrée en phase active de division cellulaire ou un passage en endocycle (cycle d'endoréplication). Dans les deux cas, l'expression des gènes est reprogrammée dans les cellules différenciées pour permettre l'accumulation des facteurs cellulaires nécessaires à la réplication de l'ADN.

Les géminivirus ressemblent aux oncovirus mammifères dans leur capacité à se multiplier dans les cellules différenciées en utilisant les facteurs cellulaires de l'hôte. Le génome des polyomavirus, papillovirus et adénovirus code pour des protéines qui modifient la régulation du cycle cellulaire en se fixant aux protéines du rétinoblastome (pRb) (Herwig and Strauss 1997). Les pRb interviennent dans le blocage du cycle cellulaire en G1, en réprimant la transcription des gènes impliqués dans la phase S du cycle. Pour cela, pRb interagit avec les facteurs de transcription E2F (Sidle et al. 1996). Le complexe de liaison E2F/pRb constitue un élément clé du contrôle de la prolifération et de la différenciation de la cellule (Lavia and Jansen-Dürr 1999) et facilite le maintien à l'état quiescent de la cellule (Harbour and Dean 2000). En fin de phase G1, la phosphorylation du pRb par des kinases cyclines-dépendantes (CDKs) perturbe le complexe de liaison et permet l'expression des gènes requis pour la phase S (Kaelin 1999). Les virus ADN oncogènes contournent la voie de phosphorylation en se fixant directement aux pRb et empêchent leur fixation sur les facteurs de transcription E2F.
La voie de régulation pRb/E2F est conservée chez les plantes (Dewitte and Murray 2003). Les protéines *Retinoblasma Related* (pRbR) et E2F ont été identifiées dans diverses espèces de plantes (Durfee et al. 2000; Gutierrez et al. 2002). Les quantités de pRbR sont importantes dans les cellules différenciées (Huntley et al. 1998), en accord avec leurs rôles dans la répression des gènes nécessaires pour la prolifération cellulaire. De même, des sites de fixation aux E2F ont été identifiés sur des promoteurs de gènes (Menges et al. 2002; Ramirez-Parra et al. 2003) dont la transcription est régulée durant les phases de division et de développement cellulaire (Castellano et al. 2001; Stevens et al. 2002).
Les géminivirus, comme les virus oncogènes animaux, codent pour des protéines qui interagissent avec les protéines RbR de l'hôte. La fixation au pRbR a été montrée pour la protéine RepA de différents mastrévirus : *Maize streak virus* (MSV : Grafi et al. 1996; Horváth et al. 1998), du *Wheat dwarf virus* (WDV : Xie

et al. 1996), du *Bean yellow dwarf virus* (BeYD : Liu et al. 1999). La protéine RepA du MSV stimule la division et le développement de cal dans des cultures cellulaires de maïs quand elle est exprimée à partir d'un transgène (Gordon-Kamm et al. 2002). La RepA du MSV se fixe au pRbR via un motif LxCxE. Les protéines des virus oncogènes mammifères *SV40 large T-antigen*, *Adenovirus E1A* et *Human Papillomavirus E7* interagissent avec le Rb également grâce à ce motif LxCxE, témoignant de l'origine commune de ce mécanisme. Toutefois, ce motif LxCxE n'est pas le seul impliqué dans la fixation avec le pRbR. En effet, la protéine Rep des mastrévirus qui contient également ce motif ne peut pas se fixer au pRbR (Horváth et al. 1998). Chez les bégomovirus, des interactions entre le pRbR et les protéines virales impliquées dans la réplication virale ont été caractérisées : les protéines Rep et REn du TGMV (Ach et al. 1997; Settlage et al. 2001), la Rep du *Cabbage leaf curl virus* (CaLCuV) et la Rep du TYLCV (Arguello-astorga et al. 2004). Ces protéines ne contiennent pas non plus de motif LxCxE et interagissent donc avec le pRbR par d'autres mécanismes.

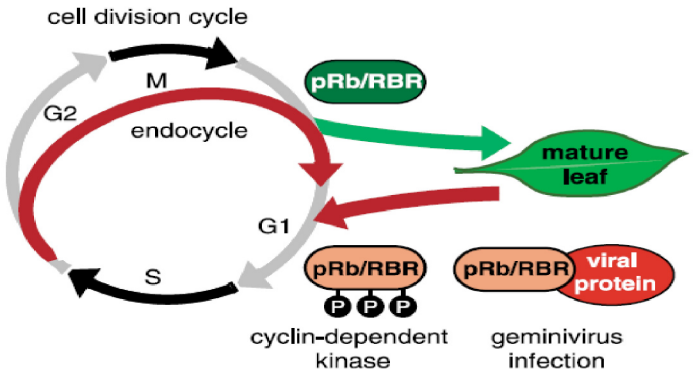


Figure 2 : Voie de régulation par le *Retinoblasma Related* (pRbR) du cycle cellulaire et de développement suite à l'infection par un géminivirus. Dans les cellules saines, la capacité de pRBR de bloquer la progression du cycle cellulaire et de favoriser la différenciation est inhibée par la phosphorylation de kinases cycline-dépendantes de la phase G1. Lors de l'infection par un géminivirus, les protéines virales interagissent avec le pRbR et l'inhibent pour établir la phase S et la capacité de réplication de l'ADN d'un cycle de division cellulaire ou d'un endocycle. D'après Hanley-Bowdoin et al (2004).

L'infection des cellules par les géminivirus conduit à la transcription de gènes en dérégulant les complexes pRBR/E2F. Des études de Nagar et al (1995) ont montré la présence de *Proliferating Cellular Nuclear Antigen* (PCNA), non

détectable dans des cellules foliaires matures saines, dans des feuilles matures de plants infectés de *N. benthamiana* par le TGMV. Cette protéine aux effets pléïotropiques est en particulier associée aux complexes de réplication de l'ADN par l'ADN polymerase δ.

La présence de l'ADN viral ou de la Rep est nécessaire dans la cellule pour exprimer le PCNA comme en témoigne son absence dans les cellules adjacentes aux cellules infectées par le TGMV. Le PCNA est aussi détecté dans des cellules différenciées transgéniques exprimant la Rep. Ces résultats prouvent que l'infection par le TGMV provoque l'accumulation de PCNA dans des cellules différenciées et que la Rep seule suffit à induire son expression.

Le promoteur du PCNA de *N. benthamiana* contient deux éléments consensus E2F nommés E2F1 et E2F2 (Egelkrout et al. 2001). Lors de l'infection par le TGMV, les protéines Rep et REn en interagissant avec le pRbR induisent la libération d'un complexe fixé au site E2F du promoteur. La transcription de PCNA est alors activée (Figure 3).

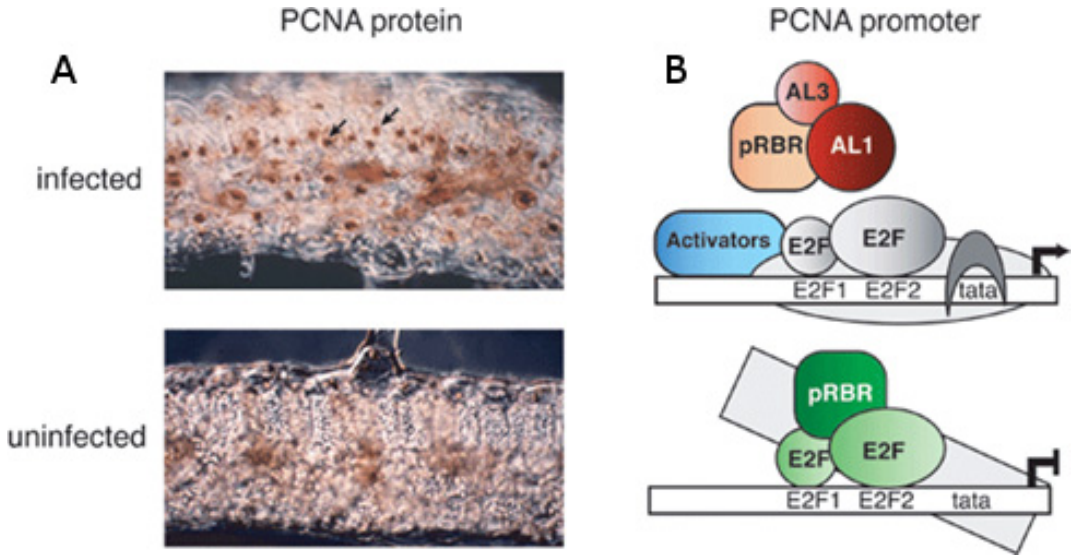


Figure 3 : Induction de l'expression de PCNA suite à l'infection par un géminivirus. (A) La protéine PCNA s'accumule dans les noyaux des cellules infectées par le *Tomato golden mosaic virus* (en haut), mais pas dans les cellules du témoin (en bas) (Nagar et al. 1995). L'antigène PCNA (en haut, flèches) a été visualisée en utilisant un anticorps anti-PCNA humaine et la détection par peroxydase (Kong et al. 2000). (B) Régulations du promoteur de PCNA dans des cellules infectées (en haut) et saines (en bas) de feuilles matures. Dans les cellules infectées par un géminivirus, AL1 et AL3 interagissent avec le pRBR pour induire la libération d'un complexe répresseur lié à des sites E2F et l'assemblage d'un promoteur actif. D'après Hanley-Bowdoin et al (2004).

Il a été démontré que les géminivirus peuvent également interagir avec d'autres facteurs cellulaires tels que les protéines de la famille des NAC comme GRAB1 et GRAB2 (Xie et al. 1999). L'interaction entre la Rep du WDV et les protéines GRAB1 et GRAB2 semble jouer un rôle dans la dérégulation du cycle cellulaire (Xie et al. 1999). De même, il a été récemment montré que l'interaction REn et Sl NAC1 (protéine de la famille des NAC, caractérisée chez la tomate *S. lycopersicum*) contribue à une augmentation de l'accumulation de l'ADN viral dans une cellule infectée (Selth et al. 2005).

2) Réplication et transcription

La réplication de l'ADN chez les géminivirus peut être divisée en plusieurs phases distinctes caractérisées par des événements spécifiques (Figure 4). Après avoir rejoint le noyau de la cellule, le génome viral ADN simple brin ou *single strand* (ADNss) est converti en une forme « super-enroulée » via un intermédiaire de réplication, l'ADN double brin ou *double strand* (ADNds). Cette première étape ne fait intervenir que des protéines de la plante hôte. La deuxième étape consiste en l'amplification d'ADNss du génome viral par un mécanisme de réplication en cercle roulant (RCR) de manière analogue aux phages ADN circulaires simple brin (Novick 1998). La protéine Rep seule est suffisante à l'initiation de la réplication en introduisant une ouverture (#) dans la séquence conservée de la tige boucle (TAATATT#AC ; Orozco and Hanley-Bowdoin 1996). Ce modèle de réplication en cercle roulant a été confirmé par microscopie électronique (Jeske et al. 2001). Ces expériences ainsi que d'autres basées sur des analyses en électrophorèse bidirectionnelle ont révélé la présence d'autres intermédiaires de réplication compatibles avec un modèle de réplication dépendante de la recombinaison (*Recombination-dependant replication*, RDR) analogues à celui du bactériophage T4 (Mosig 1998; Mosig et al. 2001).

La dernière étape permet le transport des génomes ADNss aux cellules adjacentes et l'encapsidation de génomes pour former des particules virales.
La transcription a aussi lieu dans le noyau de la cellule. Elle est bidirectionnelle depuis les séquences promotrices situées au sein de la zone intergénique pour tous les ORFs, excepté pour les ORFs C2 et C3 où la région promotrice est intégrée dans l'ORF C1. La transcription des bégomovirus est complexe, aboutissant fréquemment à des ARN messagers polycistroniques (Hanley-Bowdoin et al. 1989; Sunter and Bisaro 1989).

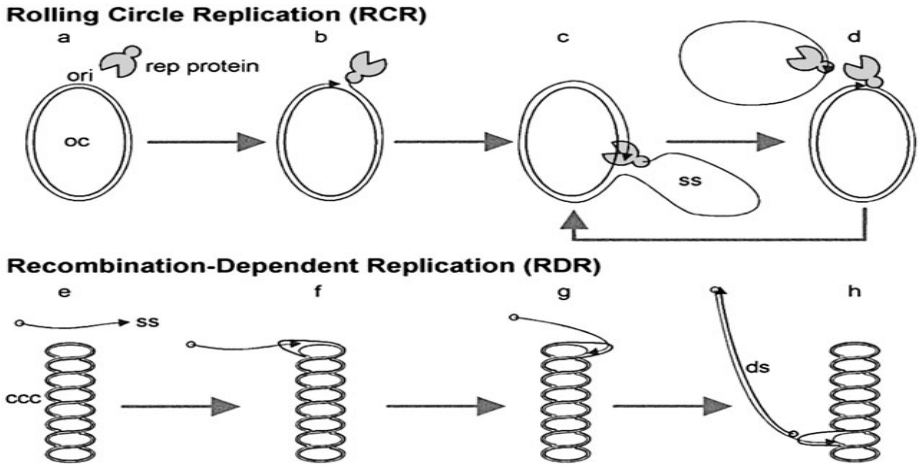


Figure 4 : Schématisation des modèles de réplication en cercle roulant (RCR) et de la réplication dépendante de la recombinaison (RDR). Etape a : accrochage de la protéine associée à la réplication (Rep) à l'origine de réplication (ori). Etape b : Ouverture de l'ADN et liaison covalente de la Rep à l'extrémité 5'. Etape c : déplacement et réplication. Etape d : Nouvelle ouverture de l'ADN, fermeture des ADNs simples brins et relargarge de la Rep. Etape e : interaction entre un ADN simple brin incomplet et la forme super-enroulée de l'ADN viral (cccDNA) à des sites homologues. Etape f : recombinaison homologue. Etape g : élongation de l'ADN simple brin. Etape h : synthèse de l'ADN complémentaire et obtention d'un ADN double brin. D'après Jeske et al (2001).

d. L'insecte vecteur, Bemisia tabaci

Les bégomovirus sont transmis aux plantes par l'intermédiaire d'un insecte vecteur, *Bemisia tabaci* (Hemiptera : Aleyrodidae), selon le mode circulant persistant. Le virus, une fois ingéré par l'insecte, qui se nourrit essentiellement de sève élaborée, va pénétrer dans le tractus intestinal par l'intermédiaire du bol alimentaire. Il va ensuite traverser une première barrière qui est la paroi de l'épithélium intestinal pour se retrouver dans l'hémolymphe de l'insecte. Les particules virales vont ensuite rejoindre, s'associer puis traverser la deuxième barrière à la transmission que représentent les glandes salivaires. Le virus circulant peut ainsi être transmis à une nouvelle plante par salivation lors de l'alimentation de l'insecte (Czosnek 2007).

B. tabaci a été décrit pour la première fois en 1889 en Grèce (Gennadius 1889). Cette "mouche blanche" a été classée dans l'ordre des *Hemiptera*, la famille *Aleyrodidae* et la sous-famille *Aleyrodinae*. *B. tabaci* est un aleurode d'environ 1 à 1,5 millimètre de long, avec un corps blanc – jaunâtre et 2 paires d'ailes blanches disposées en forme de « toit » (Figure 5).

Figure 5 : Couple d'individus adultes du biotype B de *Bemisia tabaci* en alimentation sur une feuille de chou. Photo CIRAD

B. tabaci est présent sur tous les continents excepté l'Antarctique (Perring 2001). Du fait de sa plasticité écologique, la caractérisation taxonomique de l'espèce *B. tabaci* a toujours posé et pose encore de nombreuses difficultés. Pour cette raison, les taxonomistes ont proposé d'associer cette espèce à un complexe d'espèces cryptiques, composé de nombreuses populations biologiquement et génétiquement différentes nommées « biotypes » (Brown and Bird 1995; Frohlich et al. 1999; Perring 2001). La description de ces biotypes est principalement basée sur le polymorphisme enzymatique, le polymorphisme génétique, la gamme de plantes hôtes, la fécondité, le comportement de dispersion, et la capacité à transmettre des virus de plantes (Perring 2001). A ce jour, au moins 37 biotypes ont été identifiés sur ces critères (De Barro et al. 2011). L'analyse des relations phylogénétiques entre les différents groupes génétiques de *B. tabaci*, à l'aide du gène mitochondral de la cytochrome oxydase I (COI) a permis de définir l'existence de 27 espèces cryptiques, appartenant à 11 groupes génétiques (De Barro et al. 2011; Dinsdale et al. 2010; Figure 6).

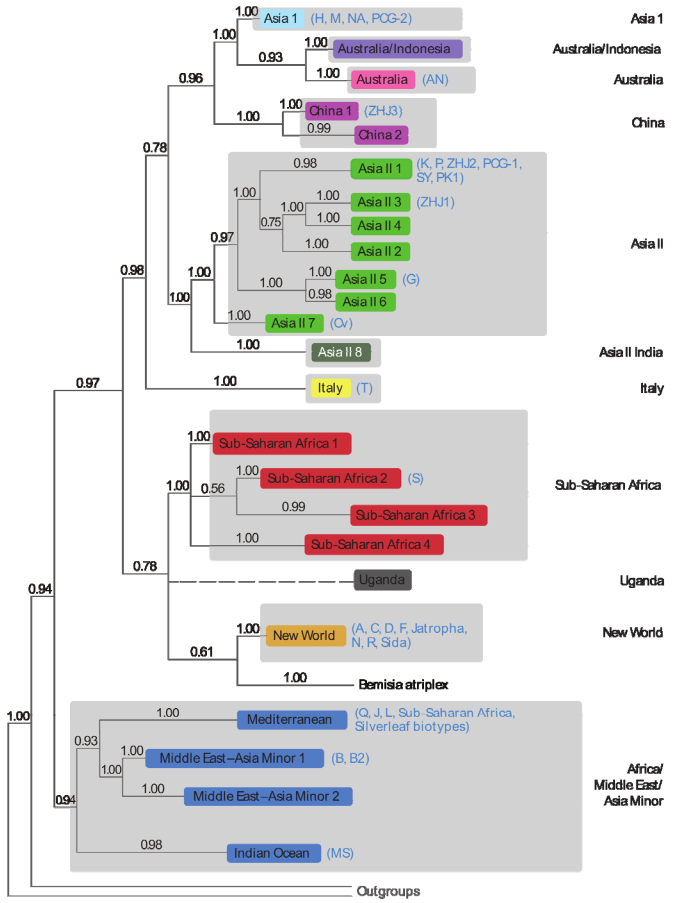


Figure 6 : Relations phylogénétiques entre les différents groupes génétiques de *Bemisia tabaci*. Arbre phylogénétique construit à partir du gène mitochondrial de 201 haplotypes, représentant les 24 espèces cryptiques (encadrement de couleur) regroupés en 11 groupes génétiques (encadrement gris). Seules les probabilités supérieures à 0.5 sont représentées. Les biotypes précédemment définis sur des caractères écologiques et biologiques sont indiqués entre parenthèses. D'après De Barro et al. (2011).

B. tabaci est reconnu comme l'une des 100 espèces les plus invasives au monde (Global Invasive Species Database http://www.issg.org/database/welcome), principalement pour ses dégâts directs et indirects. D'une part, les dégâts trophiques directs, liés à la prise de nourriture dans le phloème, sont d'autant plus préjudiciables que les densités numériques sont élevées. D'autre part, les dégâts indirects sont premièrement associés à l'excrétion de miellat produit par les aleurodes et le développement ultérieur de fumagine qui peut affecter la fonction photosynthétique de l'hôte et déprécier la valeur commerciale des produits récoltés (Byrne and Bellows 1991). Enfin, les dégâts indirects sont

principalement associés sur le plan économique aux nombreuses maladies virales transmis par *B. tabaci*. En effet, sur un total de 209 virus transmis par les aleurodes aux plantes cultivées et non cultivées, 191 espèces sont transmis par *B. tabaci* et appartiennent à quatre genres de virus : les *Begomovirus* qui sont largement majoritaire (90%) et certains *Carlavirus*, *Crinivirus*, *Ipomovirus* et *Torradovirus* (Jones 2003; Navas-Castillo et al. 2011; Figure 7).

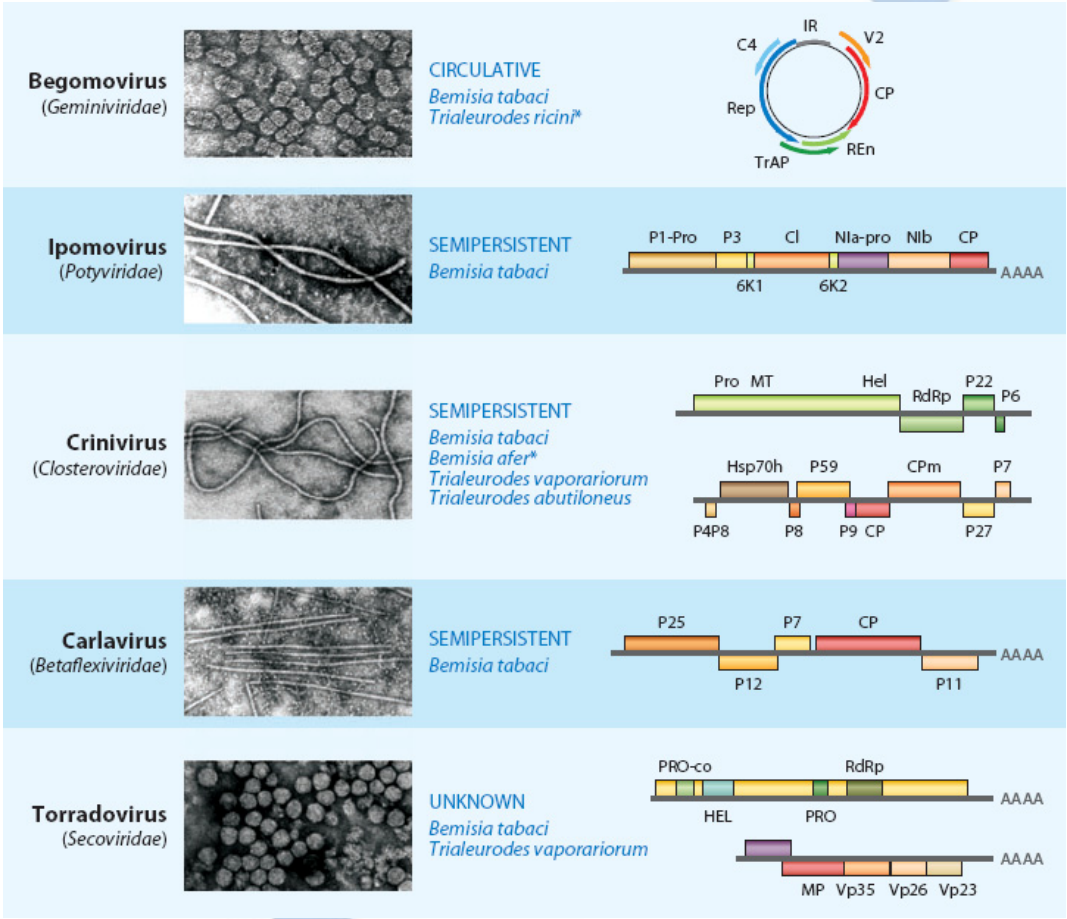


Figure 7 : Les micrographies électroniques (à gauche), le mode de transmission (au milieu), et l'organisation génomique (à droite) des principaux groupes de virus de plantes transmis par les aleurodes. Les organisations génomiques correspondent au TYLCV (begomovirus), *Cucumber vein yellowing virus* (ipomovirus), *Tomato chlorosis virus* (crinivirus), *Cowpea mild mottle virus* (carlavirus), et *Tomato torrado virus* (torradovirus). D'après Navas-Castillo et al. (2011).

Les principaux responsables de ces dégâts ont été identifiés comme les biotypes invasifs et polyphages B et Q. En effet, à partir des années 1990, le biotype B vraisemblablement originaire du Moyen-Orient se serait rapidement propagé aux dépens des autres biotypes locaux (Brown and Bird 1995). Sa propagation à

travers le monde a directement été associée à la dissémination du TYLCV responsable d'épidémies virales très dommageables sur les cultures de tomate notamment dans les Caraïbes (Polston 1994), aux Etats-Unis d'Amérique (Polston 1994), au Japon (Ueda et al. 2005) et à La Réunion (Delatte et al. 2007). Ce biotype présente des caractéristiques biologiques particulières : (1) un taux de fertilité accru (Bethke et al. 1991; Delatte et al. 2009) (2) une mobilité qui lui permet de se déplacer à longue distance (Blackmer et al. 1995) (3) une gamme de plantes hôtes très importante (Brown and Bird 1995) et (4) une résistance à certains insecticides (Brown and Bird 1995; Elbert and Nauen 2000; Horowitz et al. 2005). La pullulation d'autres biotypes de *B. tabaci* a également été associée à l'émergence de certaines maladies virales comme la pandémie sévère de la mosaïque africaine du manioc en Afrique de l'Est (Legg and Ogwal 1998; Figure 8).

Figure 8 : Pullulations d'aleurodes sur feuille de manioc (*Manihot esculenta*) directement associées à la dissémination du variant Ougandais impliqué dans l'épidémie sévère de la mosaïque du manioc en Afrique de l'Est Legg et al. (2002). Photo James Legg.

La diversité génétique ou plasticité écologique importante de *B. tabaci* avec l'existence de nombreux biotypes lui permet d'accéder à une très large gamme de plantes hôtes. Plus de 900 espèces de plantes hôtes de *B. tabaci*, appartenant à 74 familles botaniques, ont été recensées. L'ensemble de ces espèces représente 73% des plantes cultivées (Servin et al. 1999).

3. Evolution et expansion des populations virales

Malgré leurs conditions de pathogène obligatoire, les virus ont montré une grande capacité à investir de nouvelles niches écologiques. L'adaptation à ces nouvelles niches est liée à leur capacité d'évolution dont les moteurs sont un taux de mutation très élevé et une grande capacité à échanger du matériel génétique par recombinaison.

a. La mutation

La mutation génère de la variabilité génétique, base sur laquelle l'adaptation à de nouveaux environnements est rendue possible. Lors de la réplication, les mutations sont générées par des erreurs de copie du génome par les polymérases ADN ou ARN dépendantes. Les virus à ARN dont la réplication dépend des ARN polymérases ARN dépendantes ont un taux d'erreur compris entre 10^{-3} et 10^{-5} par nucléotide et par cycle de réplication. A l'inverse, les taux de mutation sont environ 1000 fois plus faibles pour les virus à ADN double brin de vertébrés qui dépendent des polymérases cellulaires pour leur réplication. Le taux de mutation de ces virus à ADN double brin est ainsi proche de celui des ADNs cellulaires estimé à 10^{-8} - 10^{-11} (Drake et al. 1998). Bien qu'utilisant également les ADN polymérases ADN dépendantes cellulaires, les virus à ADN simple brin infectant les animaux et les plantes présentent des taux de mutations bien plus élevés que ceux généralement observés avec ces enzymes (Duffy et al. 2008). Bien qu'encore incompris sur le plan mécanistique, ces résultats suggèrent que les protéines virales sont capables de détourner à leur avantage de nombreux facteurs cellulaires avec la possibilité de modifier la fidélité des polymérases cellulaires.

b. La recombinaison

La recombinaison virale est un processus permettant la genèse d'un virus en créant de nouvelles combinaisons de séquences à partir de l'ADN de deux virus parents. La recombinaison permet l'acquisition en une seule étape d'une grande variabilité génétique et donc la création de nouveaux types d'arrangements dans le génome. Conjointement, mutation et recombinaison peuvent potentiellement

fournir un accès à un polymorphisme beaucoup plus important que par mutation seule (Crameri et al. 1998).

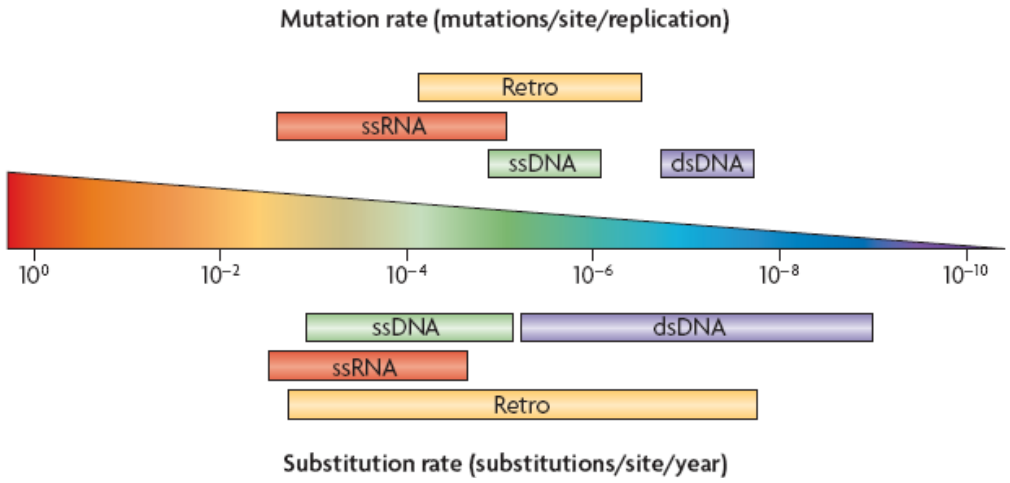


Figure 9 : Comparaison entre taux de mutation et de substitution des virus suivant la nature de leur génome. Les taux de mutations (partie haute du schéma) sont donnés en mutations par site par réplication tandis que les taux de substitutions (partie basse du schéma) sont donnés en substitution par site et par an. D'après Duffy et al. (2008).

Le phénomène de recombinaison est connu depuis longtemps chez les géminivirus avec des descriptions de recombinaison entre virus de la même espèce (Zhou et al. 1997), d'espèces différentes (Monci et al. 2002) et aussi de genres différents (Saunders and Stanley 1999). Avec l'augmentation du nombre de séquences de geminivirus disponibles dans les bases de données (Fauquet et al. 2008) et le développement de méthodes efficaces de détection des évènements de recombinaison (Martin et al. 2010), il a été montré que la recombinaison est le moteur principal de la diversification des géminivirus (Lefeuvre et al. 2007c; Padidam et al. 1999). Ces évènements de recombinaison ne sont pas distribués aléatoirement sur le génome (Lefeuvre et al. 2007c). Il a été démontré que le maintien de l'intégrité des réseaux d'interactions formés au sein d'un génome est un critère majeur de sélection des bégomovirus recombinants (Lefeuvre et al. 2007b; Figure 10).

Des virus recombinants aux propriétés biologiques et épidémiologiques supérieures aux virus parentaux ont été décrits chez les bégomovirus. Les plus importants parmi eux sont notamment (1) le *Tomato leaf curl Malaga virus*

(TYLCMalV; Monci et al., 2002), issu d'une recombinaison entre le TYLCV-Mld et le TYLCSV, qui présente une gamme de plantes hôtes plus large et (2) le variant sévère ougandais ou encore appelé souche sévère d'Ouganda de l'*East African cassava mosaic virus* (EACMV-UG) issu d'une recombinaison entre l'EACMV et l'ACMV, responsable d'une épidémie très sévère sur manioc en Afrique de l'Est, depuis la fin des années 1990, avec des conséquences humanitaires et économiques dramatiques (Zhou et al. 1997).

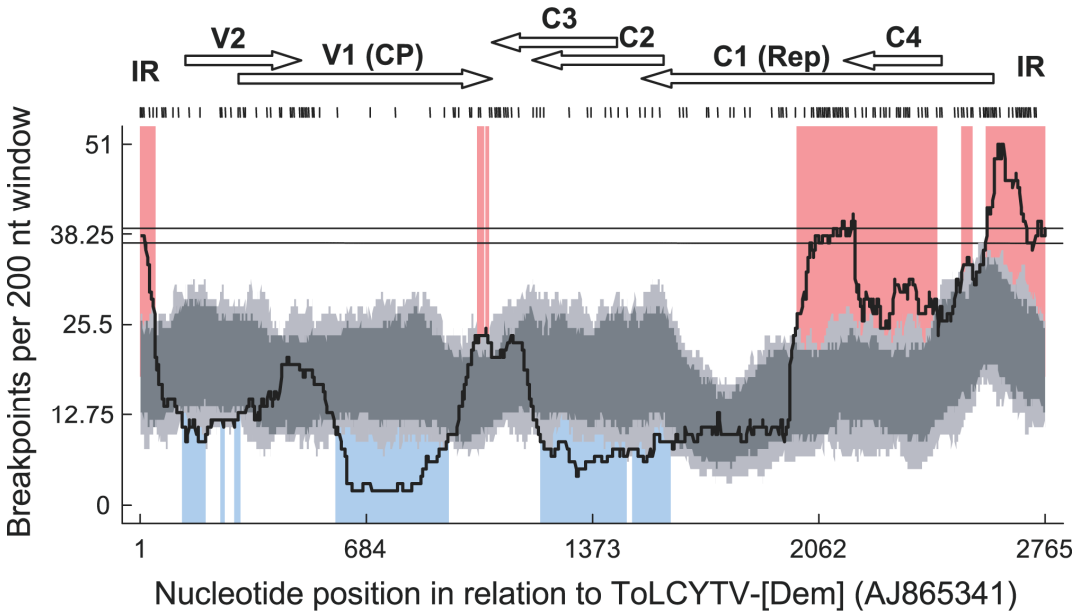


Figure 10 : Distribution des points de recombinaison détectés dans des séquences d'ADN A-*like* de bégomovirus monopartites. Les zones en rouge indiquent les « hot-spots » de recombinaisons, tandis que les zones bleues représentent les « cold-spot » de recombinaisons. Les ORF (flèches horizontales) et l'IR sont représentés sur le haut du graphique. D'après Lefeuvre et al. (2007b).

4. Diversité moléculaire des bégomovirus

Des études récentes menées notamment au Brésil (Castillo-Urquiza et al. 2008; Fernandes et al. 2008), en Asie / Inde (Kumari et al. 2011; Sharma et al. 2011) et dans les îles du Sud-Ouest de l'océan Indien (Delatte et al. 2005b; Lefeuvre et al. 2007c), ont illustré la capacité d'évolution des bégomovirus en révélant leur très grande diversité moléculaire et en particulier ceux infectant la tomate.

L'avènement du diagnostic par amplification en cercle roulant (*rolling cercle amplification*, RCA) et l'amélioration des techniques de séquençage ont permis d'accroître fortement le nombre de séquences disponibles dans les bases de données et mettre à jour une grande diversité virale certainement toujours largement sous-évaluée (Fauquet et al. 2008; Inoue-Nagata et al. 2004). Au moins 57 espèces différentes de bégomovirus capables d'infecter la tomate ont ainsi été décrites (Abhary et al. 2007). Sur la base de leur organisation génomique, de leur origine et des séquences génomiques, les bégomovirus ont été divisés en deux groupes : les bégomovirus du Nouveau Monde (New World, NW) et de l'Ancien Monde (Old World, OW) (Figure 11).

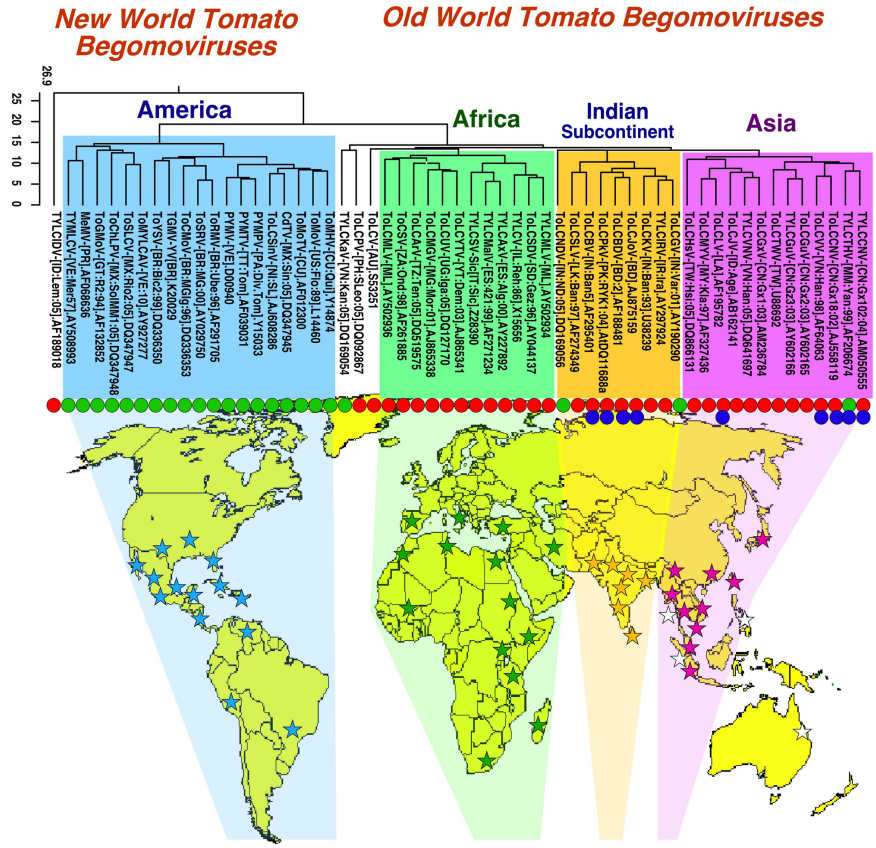


Figure 11 : Phylogéographie des 57 espèces de bégomovirus du Nouveau Monde et de l'Ancien Monde responsables de maladies sur tomate (d'après la liste de l'ICTV, 2006 ; Abhary et al. (2007).

Les bégomovirus natifs du NW sont bipartites et regroupés dans un cluster monophylétique (Abhary et al. 2007; Navas-Castillo et al. 2011). Il a été récemment montré que ces virus peuvent être accompagnés d'ADN-Alpha (Paprotka et al. 2010; Romay et al. 2010). Un grand nombre d'espèces de bégomovirus a été décrit dans le Nouveau Monde, principalement distribuées sur

les continents Américains (Nord et Sud) mais aussi dans les Iles des Caraïbes. Le Brésil illustre particulièrement la grande diversité virale des bégomovirus infectant la tomate avec au moins 14 espèces différentes décrites sur tomate (Castillo-Urquiza et al. 2008; Fernandes et al. 2008; Navas-Castillo et al. 2011).

b. Les bégomovirus de l'Ancien Monde

Les OW bégomovirus regroupent les bégomovirus originaires d'Asie en incluant l'Inde ainsi que ceux originaires d'Afrique et du bassin méditerranéen. Ces bégomovirus peuvent être mono- ou bipartites associés ou non à des ADN satellites (Abhary et al. 2007).

La maladie du tomato yellow leaf curl (TYLCD ou maladie du jaunissement et de l'enroulement foliaire de la tomate) est responsable de pertes et de dégâts considérables dans de nombreuses régions tropicales et sub-tropicales (Nakhla et al. 1994; Picó 1996; Reynaud et al. 2003). Cette maladie est causée par un complexe d'espèces de bégomovirus issues de l'Ancien Monde dont la plus connue est le TYLCV. D'un point de vue taxonomique et sur la base de leur relations phylogénétiques, tous les virus associés au TYLCD sont apparentés (TYLCVs-like) et appartiennent à six espèces nord africaines et méditerranéennes (TYLCV, TYLCSV), *Tomato yellow leaf curl Axiarqua virus* (TYLCAxV), TYLCMalV, *Tomato yellow leaf curl Mali virus* (TYLCMLV) et *Tomato leaf curl Soudan virus* (ToLCSDV) et 15 souches différentes (Figure 12; Abhary et al. 2007).

Les différentes espèces et souches de TYLCV-like forment un cluster de séquences qui diffèrent les unes des autres par des évènements de recombinaison le long du génome. C'est ainsi qu'il a été montré que la souche Israël du TYLCV (TYLCV-IL) était le fruit d'une recombinaison entre un ancêtre de la souche Mild du TYLCV (TYLCV-Mld) et un ancêtre des Tomato leaf curl virus (ToLCVs) asiatiques (Navas-Castillo et al. 2000). Des recombinaisons interspécifiques vraisemblablement plus récentes sont à l'origine du TYLCMalV et du TYLCAxV entre le TYLCV-IL et le TYLCSV, et le TYLCV-Mld et le TYLCSV, respectivement (García-Andrés et al. 2006; Monci et al. 2002; Figure 13).

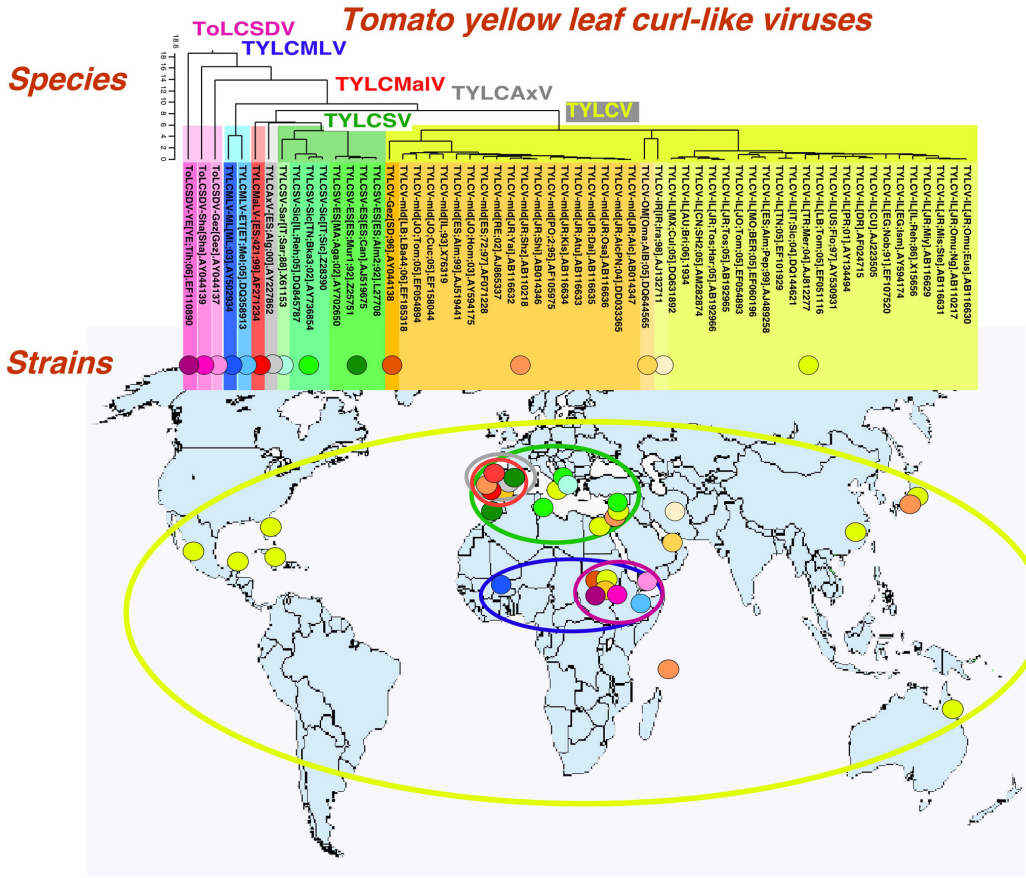


Figure 12 : Phylogéographie des six espèces nord africaines et méditerranéennes de bégomovirus communément appelées Tomato yellow leaf curl virus-like (TYLCVs-like), et de leurs 15 souches différentes (d'après Abhary et al. 2007) : *Tomato yellow leaf curl virus*-Israel (TYLCV-IL), TYLCV-Mild (TYLCV-Mld), TYLCV-Iran (TYLCV-IR), TYLCV-Oman (TYLCV-OM), TYLCV-Gezira (TYLCV-Gez), *Tomato yellow leaf curl Sardinia virus*-Spain (TYLCSV-ES), TYLCSV-Sicily (TYLCSV-Sic), TYLCSV-Italy (TYLCSV-IT), *Tomato yellow leaf curl Axiarqua virus* (TYLCAxV), *Tomato yellow leaf curl Malaga virus* (TYLCMalV), *Tomato yellow leaf curl Mali virus*-Ethiopia (TYLCMLV-ET), TYLCMLV-Mali (TYLCMLV-ML), *Tomato leaf curl Sudan virus*-Gezira (ToLCSDV-Gez), ToLCSDV-Shambat (ToLCSDV-Sha), ToLCSDV-Yemen (ToLCSDV-YE). La répartion mondiale de chaque espèce est indiquée par un cercle de couleur (d'après la liste de l'ICTV, 2006 ; Abhary et al, 2007).

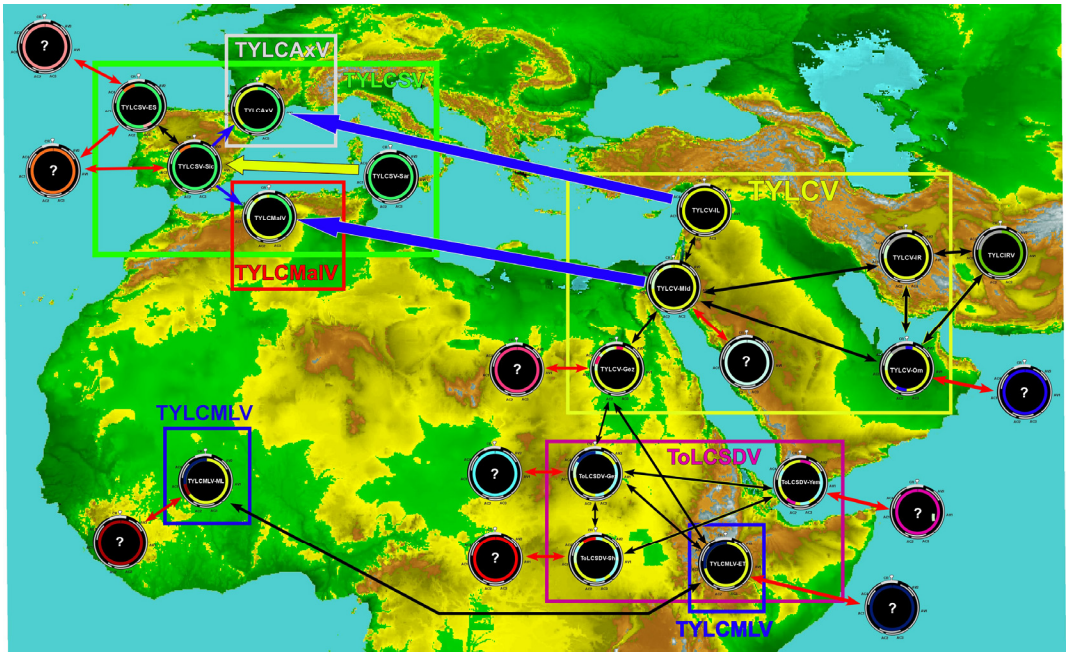


Figure 13 : Représentation schématique des relations théoriques de parenté entre les 15 souches appartenant aux six espèces du clade TYLCVs-like. Les relations de parenté sont décrites par des flèches et des cercles de couleurs représentant le génome d'ADN simple brin circulaire des bégomovirus monopartites et les régions communes issues par recombinaison. Les noms complets des souches et espèces de bégomovirus sont décrits dans la légende de la figure 10. D'après Abhary et al. (2007).

5. Emergence des bégomovirus

Depuis la fin des années 1980s, les bégomovirus sont devenus un des principaux facteurs limitant de la production dans beaucoup de régions. Dès lors, la qualification de virus émergents a souvent été associée à ces virus. La notion d'émergence couvre une large gamme de situations qu'il convient de définir :

a) émergence d'une espèce ou souche virale **connue** mais dans une **nouvelle région**
b) émergence d'une espèce ou d'une souche virale **connue** mais sur un **nouvel hôte**
c) émergence d'une espèce ou d'une souche virale **connue** mais avec des **proportions nouvelles**

d) émergence d'une espèce ou d'une souche virale **inconnue**, présentant une **valeur sélective** ou une **virulence accrue** et éventuellement capable de surmonter **une résistance**

Récemment le programme ProMED (Program for Monitoring Emerging Diseases ; www.promed-mail.org) a listé les principaux facteurs associés aux émergences virales chez les plantes : introduction de pathogènes (71% des cas), changements dans les populations de vecteurs (16%), la recombinaison (5%), le climat (5%) et enfin les pratiques culturales (5%) (Anderson et al. 2004).

L'émergence virale est généralement associée à des facteurs écologiques et moléculaires aboutissant à de nouvelles interactions virus - vecteur - plante - environnement (Fargette et al. 2006).

Le cas de l'émergence du TYLCV et des TYLCVs-like en est un parfait exemple et met en avant la contribution importante des activités humaines. Les symptômes de la maladie du TYLCD (Figure 14) ont été observés pour la première fois dans la vallée du Jourdain, dans les années 1939-1940, et décrits en 1964 sur tomate en Israël (Cohen and Harpaz 1964). Le bégomovirus associé à cette maladie a été identifié dans les années 90 comme étant le TYLCV (Antignus and Cohen 1994). En Afrique, il a été décrit la première fois au Soudan (Yassin and Nour 1965), puis s'est répandu dans tout l'Est africain. Ce complexe viral s'est depuis largement répandu à travers le monde au cours des trois dernières décennies. Le TYLCV a été décrit dans la plupart des régions tropicales et sub-tropicales, dans le bassin méditerranéen, en Asie, Moyen-Orient, en Afrique, dans les Caraïbes, aux Etats-Unis et en Amérique Latine (Bird et al. 2001; Van Brunschot et al. 2010; Delatte et al. 2005a; Gharsallah Chouchane et al. 2007; Peterschmitt et al. 1999; Polston 1994; Ueda et al. 2004; Urbino and Tassius 2003; Zambrano et al. 2007). Une dynamique d'apparition et d'expansion de variants recombinants a également été constatée tout au long des 10 dernières années (Davino et al. 2009; García-Andrés et al. 2006; Monci et al. 2002).

Dans de nombreuses régions du monde, une arrivée successive de différents variants de TYLCV a été constatée (Delatte et al. 2005a; Duffy and Holmes 2007; Idris et al. 2007; Ueda et al. 2004) et disséquée sur le plan moléculaire (Duffy and Holmes 2007) et épidémiologique (Davino et al. 2006; Sánchez-Campos et al. 1999). L'exemple des travaux menés en Espagne et en Italie à ce sujet est très significatif. Ces études ont souligné l'importance de l'ensemble des facteurs

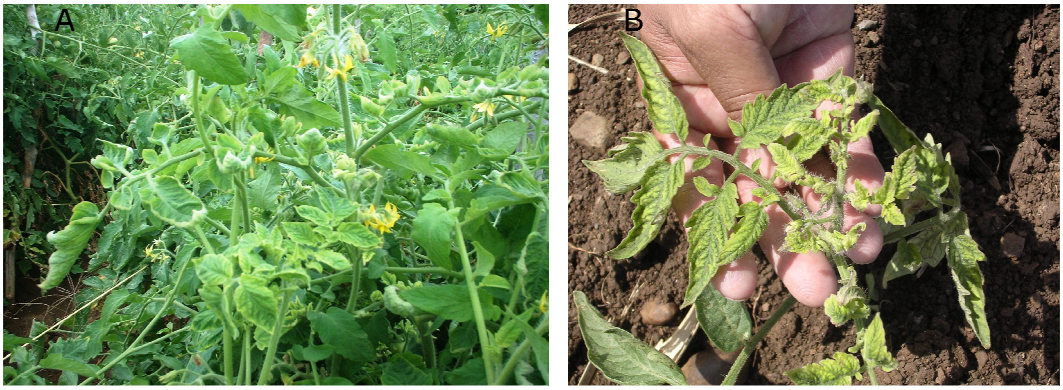


Figure 14 : Symptômes provoqués par le *Tomato yellow leaf curl virus* (TYLCV) sur plant adulte (A) et jeune plant (B) de tomate. Photo CIRAD

pouvant intervenir dans la dynamique virale, à savoir la gamme d'hôte, la *fitness*[2] des virus dans l'hôte ainsi que la capacité du virus à être transmis par les différents biotypes de *B. tabaci* (Davino et al. 2006; Monci et al. 2002; Sánchez-Campos et al. 1999).

La rapidité de l'expansion géographique du TYLCV et la description de nombreux variants ces 20 dernières années, semblent liées à la dissémination mondiale du biotype B, et plus récemment du biotype Q, de *B. tabaci* (Jones 2009). Cette expansion géographique de *B. tabaci* et des bégomovirus a directement été associée à la mondialisation et à l'intensification des échanges commerciaux de plantes ornementales et de légumes (Anderson et al. 2004; Delatte et al. 2003).

6. Le contrôle des bégomovirus

Les maladies virales sont responsables de pertes importantes à la fois sur la quantité et la qualité des productions agricoles. Une infection par le TYLCV se caractérise par des symptômes de jaunissement des feuilles avec des enroulements foliaires, un nanisme des plants et l'avortement des fleurs. Une infection à un stade précoce peut ainsi entraîner la perte totale de la production (Nakhla et al. 1994; Picó 1996).
Peu de méthodes de contrôle sont disponibles dans le cas d'une infection virale et surtout, aucun traitement curatif ne peut être appliqué. Les moyens de contrôle

[2] La *fitness* correspond au nombre de descendants viables et fertiles que produit en moyenne chaque individu d'un génotype donné. Par commodité et usage, le terme anglo-saxon fitness est préféré au terme français de « valeur sélective ».

des maladies virales peuvent se décliner en deux catégories principales : (1) les stratégies qui permettent de limiter ou de réduire au minimum l'arrivée du virus sur la parcelle et (2) les stratégies qui tendent à limiter les effets du virus sur la plante une fois qu'elle a été infectée.

a. Pratiques culturales, prophylaxie

La première des pratiques consiste à empêcher ou réduire fortement l'arrivée du virus sur la parcelle. Empêcher les introductions et les mouvements de plants contaminés vers les zones encore indemnes est une priorité dans la lutte contre les bégomovirus (Anderson et al. 2004). Les autorités sanitaires ont bénéficié du développement d'un vaste panel de tests de détection basés sur des techniques d'immunologie (Dalmon et al. 2000), d'hybridation moléculaire (Crespi et al. 1991; Czosnek et al. 1988), de PCR classique (Dalmon et al. 2000; Lefeuvre et al. 2007a) ou de PCR en temps réel (Mason et al. 2008; Papayiannis et al. 2010) pour limiter de nouvelles contaminations.

Dans les zones contaminées, l'emploi de plants sains et certifiés ainsi que la suppression des plantes adventices hôtes de bégomovirus à proximité de la parcelle (Papayiannis et al. 2011; Sánchez-Campos et al. 1999) constituent les premières mesures qui tendent à repousser l'infestation de la parcelle. De même, la rotation des cultures ainsi que l'élimination des résidus de culture sont également recommandés compte tenu du fait que les bégomovirus ont une large gamme d'hôtes dans les plantes maraîchères (Morilla et al. 2005; Navas-Castillo et al. 1999).

Les bégomovirus sont transmis exclusivement par l'aleurode *B. tabaci* qui peut être ciblé pour assurer le contrôle des maladies. Le contrôle de l'insecte vecteur peut se baser sur l'utilisation de barrières physiques (filets *insect-proof*, (Cohen and Antignus 1994), de méthodes qui perturbent le comportement de l'insecte (modification du rayonnement UV dans les serres, Antignus et al. 2001), de la lutte biologique avec des insectes auxiliaires des cultures (insectes des genres *Eretmocerus* et *Encarsia*; Picó 1996), de la résistance variétale (voir section suivante) et bien sûr de la lutte chimique. Toutefois, les emplois répétés d'insecticide favorisent le développement de résistances chez les biotypes invasifs B et Q de *B. tabaci* et n'ont montré qu'une faible efficacité pour limiter la dissémination de la maladie (Elbert and Nauen 2000; Horowitz et al. 2005; Perring et al. 1999).

b. La résistance variétale

Les pratiques culturales peuvent aider à contrôler le développement de la maladie mais sont en général insuffisantes (Antignus 2007; Polston and Lapidot 2007). Développer des cultivars résistants est la meilleure stratégie pour assurer une production acceptable en quantité et en qualité. L'emploi de cultivars résistants assure une protection efficace sans travail ni coût supplémentaire tout au long du cycle de production.

1) Les différents types de résistance aux virus

Une plante est qualifiée de résistante à un virus quand l'infection, la réplication et/ou le mouvement du virus dans la plante (Cooper and Jones 1983). On peut considérer trois classes de gènes de résistance naturelle actuellement exploitables en sélection : les gènes dominants, les gènes récessifs et les gènes quantitatifs (Maule et al. 2007). Les deux premières classes se caractérisent dans beaucoup de cas, mais pas toujours, par un déterminisme génétique simple de type qualitatif (mendélien) reposant sur un gène majeur. Les résistances quantitatives font intervenir de 2 ou 3 (oligogéniques) à de nombreux (polygéniques) facteurs de résistance (QTL) selon les cas.

Les gènes majeurs dominants aux virus appartiennent majoritairement à la classe des gènes R, qui se caractérisent par une structure moléculaire de la protéine codée de type NBS-LRR avec un domaine de type TIR (« Toll-interleukin-1 receptor ») ou CC (« coiled-coil ») à sa partie N-terminale. Ces gènes confèrent souvent une résistance totale (qualitative) au travers d'une interaction « gène pour gène » de type R/Avr, interaction qui dans la majorité des cas semble néanmoins être indirecte et complexe (McDonald and Linde 2002).

Les gènes récessifs de résistance aux virus connus résultent tous de mutations naturelles ou induites dans un facteur du complexe d'initiation de la traduction (Le Gall et al. 2011; Robaglia and Caranta 2006). Des facteurs de type eIF4E mutés ont été caractérisés comme les facteurs récessifs de résistance à plusieurs virus à ARN chez différents espèces. Bien que ce type de résistance puisse s'exprimer de façon qualitative, dans beaucoup de cas elle apparaît plutôt comme quantitative et/ou faisant partie d'une résistance de type oligo/polygénique

comme dans le cas par exemple du gène pvr2 de résistance au PVY chez le piment (Caranta et al. 1997; Maule et al. 2007).

Les gènes quantitatifs ou QTL (Quantitative Trait locus) dont les effets combinés se traduisent par un niveau final de résistance variant selon l'environnement et notamment le niveau d'infection. L'expression de cette résistance quantitative peut se mesurer par les effets sur le taux d'infection[3], l'expression des symptômes (incidence[4] et sévérité[5]) et la progression de la maladie (période de latence[6]) (McDonald and Linde 2002). Les effets d'additivité de ces facteurs quantitatifs souvent prédominent. Alors que les résistances quantitatives aux virus sont potentiellement très abondantes au sein de la biodiversité, peu d'études de cartographie de ces QTL ont été publiées et aucun QTL de résistance à un virus n'a été caractérisé au niveau moléculaire, en dehors du cas d'eIF4E, comme cité ci-dessus.

La sélection de cultivars résistants repose sur la disponibilité de caractères de résistance dans le germplasme[7], l'introgression de ces caractères dans des lignées élites aux fortes qualités agronomiques et enfin la diffusion à grandes échelles de ces cultivars pour mesurer leurs valeurs en conditions de production. Ces caractères de résistance introgressés peuvent être issus de la variabilité génétique naturelle ou induits par des traitements avec des agents mutagènes.

2) Les résistances naturelles

La recherche de résistance dans la diversité biologique naturelle peut s'effectuer dans le *pool* de gènes primaire (génotypes de la même espèce), secondaire (espèces apparentées avec de la compatibilité sexuelle) et plus rarement tertiaire (espèces ou genre avec incompatibilité sexuelle) (Harlan and Wet 1971).

---

[3] Taux d'infection : nombre de plantes contaminées par rapport au nombre de plantes évaluées.

[4] Incidence : nombre de plantes symptomatiques par rapport au nombre total de plantes de la parcelle

[5] Sévérité : gravité des symptômes.

[6] Période de latence : période entre l'inoculation et l'expression des premiers symptômes de la maladie.

[7] Germplasme : ensemble des ressources génétiques végétales à la disposition d'un sélectionneur pour la création de nouvelles variétés/lignées

Dans le cas des bégomovirus, aucune source de résistance n'a été rapportée dans l'espèce *S. lycopersicum* et les recherches se sont donc étendues aux espèces sauvages apparentées à la tomate cultivée (Pilowsky and Cohen 1974). Les recherches de sources naturelles de résistance au TYLCV ainsi qu'aux autres bégomovirus infectant la tomate, qui ont été très nombreuses depuis les années 1970, ont mis en évidence plusieurs sources de résistance dans des accessions principalement issues de *Solanum chilense*, *Solanum habrochaites*, *Solanum pimpinellifolium*, *Solanum peruvanium* (pour revue Ji et al. 2007b).

L'introgression de la résistance dans des fonds génétiques à haute valeur agronomique est un challenge majeur pour les sélectionneurs (Koornneef and Stam 2001; Strange and Scott 2005). Le développement de marqueurs moléculaires et la sélection assistée par marqueur a permis de réaliser des progrès majeurs dans la création de cultivars résistants (Foolad 2005). Les sélectionneurs ont ainsi bénéficié de la cartographie des QTL majeurs de résistance aux bégomovirus et des marqueurs liés de type PCR facilitant leur introgression dans le matériel commercial. Zamir et al (1994) ont été les premiers à cartographier un gène de résistance au TYLCV, *Ty-1* (chromosome 6 et 3 respectivement), dans l'accession *S. chilense* LA1969, qui est associé à 2 QTLs mineurs détectés sur les chromosomes 3 et 7. Ce gène majeur a été le premier à être largement utilisé par les organismes privés et publics pour la création d'hybrides commerciaux résistants, dont certains sont cultivés à grande échelle (García-Andrés et al. 2009; Picó 1996). D'autres gènes de résistance aux bégomovirus ont ensuite été cartographiés et incluent : *Ty-2* issu de l'accession *S. habrochaites* H24 (chromosome 11) (Hanson et al. 2000), *Ty-3* et *Ty-4* issus de l'accession *S. chilense* LA1932 (chromosome 6 et 3 respectivement) (Ji et al. 2007a; Ji et al. 2009) et enfin *Ty-5* issu de l'accession *S. peruvanium* TY172 (chromosome 4) (Anbinder et al. 2009).

En dehors de la résistance au virus *sensu stricto*, la résistance au vecteur *B. tabaci* est une option intéressante mais encore peu exploitée pour le contrôle des maladies causées par les bégomovirus. Des sources naturelles de résistance à *B. tabaci* ont été décrites dans des accessions originaires de *S. habrochaites*, *Solanum pennellii* et *S. pimpinellifolium* (Blauth et al. 1998; Delatte et al. 2006; Momotaz et al. 2010; Rodríguez-López et al. 2011; Simmons and Gurr 2005). Ces résistances reposent sur la sécrétion de métabolites secondaires par des trichomes glandulaires de type IV et VI présents sur les feuilles (Blauth et al. 1998; Muigai et al. 2002; Figure 14). Ces composés ont des propriétés à la fois

d'antibiose (effet négatif sur la physiologie de l'insecte) et d'antixénose (perturbation du comportement de l'insecte). L'efficacité de ces mécanismes de résistance a été démontrée pour limiter l'infection (Delatte et al. 2006) et la dissémination du virus (Rodríguez-López et al. 2011).

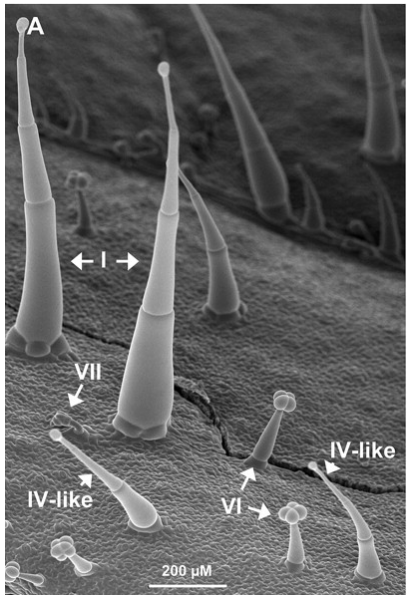


Figure 14 : Photographie en cryo-microscopie électronique de trichomes sur la surface foliaire. Les différents types de trichomes présents sont indiqués par des flèches. D'après Kang et al. (2010).

3) La résistance induite

En plus des sources naturelles de résistances aux virus, les mutations induites sont une autre source de variabilité biologique utile pour les sélectionneurs. L'induction de mutations par traitements mutagènes (radiations ou chimiques) permet la création de nouveaux allèles qui ne sont peut être pas disponibles dans le germplasme naturel. Le gène *rym3* qui confère la résistance au *Barley yellow mosaic virus* (BaYMV) a ainsi été identifié dans un mutant du cultivar « Chikurin Ibaraki 1 » (orge) traité aux rayons gamma (Ukai and Yamashita 1984). D'autre part, la mutagénèse par le méthane sulfonate d'éthyle (EMS) est un moyen simple et rentable pour saturer un génome avec des mutations (Greene et al. 2003). Le TILLING (*Targeting Induced Local Lesions IN Genomes*) utilise la mutagenèse EMS couplée à la détection des mutations d'un seul nucléotide dans un gène ciblé (Comai and Henikoff 2006). Cette technique peut être utilisée à haut-débit pour la création d'une série d'allèles d'un gène cible. Le TILLING a été utilisé pour développer des résistances aux potyvirus par mutation sur les

facteurs d'initiation à la traduction eIF4E (Nieto et al. 2007; Piron et al. 2010). Bien que prometteuses et porteuses de résultats chez les virus à ARN, ces stratégies de mutations induites n'ont pas été encore appliquées avec succès pour la résistance aux géminivirus.

4) La résistance transgénique

Bien que toujours marginales et peu acceptées par la société, les techniques de transgénèse contribuent à élargir la diversité des mécanismes de résistance utilisable par les sélectionneurs. Différentes méthodologies basées sur la résistance dérivée du pathogène (*Pathogen-derived resistance*, PDR), avec ou sans expression de protéines virales, et l'expression d'agents antiviraux non issus d'agents pathogènes ont été utilisées principalement à des fins expérimentales (pour revues voir Prins et al. 2008; Shepherd et al. 2009). En ce qui concerne la résistance aux géminivirus, notons l'efficacité des expériences de résistance dérivée du pathogène pour le MSV (Shepherd et al. 2009) et pour le TYLCV (Fuentes et al. 2006).

7. La résistance comme objectif de sélection

Les programmes d'amélioration variétale visent la diffusion de cultivars qui répondent aux attentes des producteurs à la fois au niveau des qualités agronomiques mais aussi en terme de résistance aux pathogènes. La résistance aux virus, comme pour les autres pathogènes, doit répondre à trois objectifs majeurs : le niveau (quantitatif), le spectre d'action (qualitatif) et la durabilité de la résistance.

a. Niveau de résistance

Le niveau de résistance se traduit par l'efficacité de contrôle de **la quantité de virus**. Cette quantité se mesure à plusieurs niveaux qui vont de l'organe et de la plante entière (charge virale) jusqu'au niveau de la parcelle (incidence de la maladie). Les résistances qualitatives telles que l'hypersensibilité[8] agissent à un

[8] Hypersensibilité : le virus est isolé et restreint dans de petites zones tissulaires qui se nécrosent.

stade précoce du cycle viral et empêchent ainsi la propagation virale. A l'inverse, quand la résistance repose sur des résistances quantitatives, le niveau conféré doit être suffisant pour limiter l'expansion du virus et réduire les pertes occasionnées. Ce niveau de résistance dépend de l'agressivité des populations virales à contrôler et sera d'autant plus faible si les conditions sont favorables au développement de la maladie (abondance d'inoculum primaire et secondaire, pullulations d'insectes vecteurs). Il est possible de combiner plusieurs sources de résistance qualitatives et/ou quantitatives afin d'augmenter le niveau de résistance dans un cultivar hybride. Cette stratégie de pyramidage de gènes s'avère particulièrement efficace avec la combinaison de sources de résistance agissant à différents niveaux du cycle viral : inoculation, réplication, mouvement cellule à cellule et systémique et acquisition par le vecteur (Djian-Caporalino et al. 2007).

b. Spectre d'action de la résistance

Le spectre d'action d'une résistance caractérise **son efficacité à contrôler différents isolats ou souches d'un virus**. Alors que les résistances qualitatives sont basées sur des relations spécifiques R–Avr, les résistances quantitatives sont généralement efficaces avec plusieurs souches et isolats d'un virus (McDonald and Linde 2002). L'extension du spectre d'action d'une résistance est généralement favorisée par la combinaison de gènes complémentaires. L'exploration de la diversité de la population virale à contrôler ainsi que la spécificité de la résistance des différents gènes de résistance sont autant de pré-requis à un pyramidage de gènes efficace. Chez le haricot (*Phaseolus vulgaris*), cette stratégie a été particulièrement efficace pour lutter contre le *Bean golden yellow mosaic virus* (BGYMV, famille des *Geminiviridae*) en associant un gène majeur récessif (*bgm-1*) à un gène majeur dominant (*Bgp*) et à un QTL (SW12) (Beaver and Osorno 2009; Singh et al. 2000). Ces cultivars résistants ont été diffusés en Amérique Latine et ont servi de donneurs de résistance dans de nombreux programmes de création variétale.

c. Durabilité de la résistance

Plus récemment le critère de durabilité de la résistance s'est ajouté aux critères d'évaluation de la résistance. La durabilité caractérise l'effet du déploiement de

gènes de résistance sur la dynamique évolutive des populations virales à contrôler. Les gènes de résistance exercent de fortes pressions de sélection sur les populations virales et les façonnent en favorisant l'émergence de populations adaptées, potentiellement capables de contourner ces gènes (García-Andrés et al. 2009; McDonald and Linde 2002). Prédire ces changements dans les populations et évaluer les risques de contournement constituent maintenant des étapes nécessaires dans les programmes de création variétale.

Le contournement d'une résistance est le résultat de l'apparition, la multiplication et la dispersion d'un variant virulent dans une population initialement avirulente. Chacune de ces étapes est conditionnée par les capacités évolutives de la population virale que l'on veut contrôler. En complément de la résistance, des stratégies de contrôle qui intègrent à la fois des aspects législatifs et réglementaires, et des pratiques culturales participent à préserver l'efficacité du cultivar résistant (Moury et al. 2011).

8. Problématique et objectifs

L'émergence virale représente, tout particulièrement en milieu tropical, une question d'importance fondamentale pour l'agriculture de subsistance. Notamment ces 20 dernières années, le continent Africain et sa population ont du faire face à l'émergence de nombreuses épidémies virales sur des cultures vivrières aussi essentielles que le riz avec le *Rice yellow mottle virus* (RYMV; Fargette et al. 2006), le maïs avec le MSV (Magenya et al. 2008) ou le manioc avec le complexe d'espèces de bégomovirus associé à la maladie de la mosaïque du manioc (Legg and Fauquet 2004). A partir de ces modèles d'études, l'analyse des données moléculaires et écologiques a permis d'identifier plusieurs facteurs impliqués dans leur émergence, dont : (1) l'évolution virale par recombinaison, (2) la synergie entre virus, (3) l'arrivée de nouveaux biotypes d'insectes vecteurs, (4) l'accès et l'adaptation à de nouvelles plantes hôtes (saut d'espèce), et (5) la dispersion à longue distance (mondialisation des échanges).

Le groupe des bégomovirus regroupe actuellement le plus grand nombre d'espèces virales émergentes d'importance économique. La capacité d'évolution rapide et d'adaptation des bégomovirus à de nouvelles niches écologiques (forts taux de mutation et de recombinaison), l'augmentation régulière de leur aire de répartition et la polyphagie de certains biotypes invasifs de leur insecte vecteur *B.*

tabaci font de ce complexe virus - vecteur un des plus dommageables et préoccupants.

Alors que de grands progrès ont été réalisés sur la caractérisation de la diversité moléculaire des bégomovirus et les mécanismes évolutifs associés, la caractérisation phénotypique de cette diversité demeure toujours limitée. Il convient pourtant de mieux appréhender et caractériser cette diversité pour développer des stratégies de contrôle adaptées.

Au travers du modèle des maladies à bégomovirus sur tomate, l'objectif de ce travail de thèse a été premièrement de comparer biologiquement les différentes espèces de bégomovirus présentes dans les départements d'Outre Mer et plus particulièrement les deux souches émergentes du TYLCV à l'île de La Réunion (Chapitre I). Ce volet s'est appuyé sur le développement d'un outil de détection et quantification qui a permis de mettre en évidence des différences d'accumulation virale parmi les bégomovirus monopartites et bipartites présents dans les départements français d'Outre-Mer.

Deuxièmement, nous nous sommes attachés à étudier les dynamiques épidémiologiques du TYLCV à la Réunion. L'isolement géographique de La Réunion, associé aux récentes et successives introductions de deux souches émergentes du TYLCV, a créé un environnement propice à l'étude de l'émergence virale et des facteurs épidémiologiques associés (Chapitre II). Comprendre l'interaction et les dynamiques évolutives et épidémiologiques de cette diversité virale est un préalable nécessaire pour adapter les stratégies de contrôle et plus particulièrement pour orienter la sélection et le déploiement de cultivars résistants.

Ensuite, nous nous sommes attachés à étudier la spécificité d'action des principaux QTL/gènes de résistance utilisés par les sélectionneurs (Chapitre III). Si un effet attendu du pyramidage de gènes est d'étendre le spectre d'action de la résistance, il suppose toutefois une complémentarité des mécanismes contrôlés par ces gènes face à la diversité virale à contrôler. Ainsi, ce volet s'est accompagné de la caractérisation d'une nouvelle source de résistance récessive au bégomovirus, donc contrôlant des mécanismes différents de la résistance par rapport à ceux contrôlés par les QTL/gènes majeurs jusque là utilisés.

Enfin, la dernière partie traite de l'évaluation et de la caractérisation de la résistance à *B. tabaci* exploitable dans l'accession *S. habrochaites* LA1777 aux deux souches émergentes du TYLCV. Cette partie permettra de discuter des mécanismes de résistance au vecteur pouvant être prise en compte dans une stratégie de pyramidage de gènes (Chapitre IV).

Chapitre I :
Mieux appréhender la diversité virale pour mieux la contrôler

Des études récentes ont révélé la grande diversité moléculaire des populations de bégomovirus capables d'infecter la tomate. Au moins 57 espèces différentes de bégomovirus ont ainsi été décrites (Abhary et al. 2007). Sur la base de leur organisation génomique, de leur origine et des séquences génomiques, les bégomovirus ont été divisés en deux groupes : les bégomovirus du Nouveau Monde (New World, NW) et de l'Ancien Monde (Old World, OW).

La maladie du TYLCD est responsable de pertes et de dégâts considérables dans de nombreuses régions tropicales et sub-tropicales. Cette maladie est causée par un complexe d'espèces de bégomovirus issues de l'Ancien Monde dont la plus connue est le TYLCV. Sur la base de l'identité génomique, cinq souches de TYLCV aux aires de répartition contrastées sont actuellement décrites. Alors que le TYLCV-Mld et le TYLCV-IL se sont disséminés à l'échelle mondiale, les trois autres (*i.e* TYLCV-Gez, TYLCV-IR et TYLCV-OM) sont pour le moment confinés à leur centre d'origine. En dépit de leur caractérisation moléculaire, peu d'informations sont pour le moment disponible sur d'éventuelles différences biologiques au sein de l'espèce TYLCV.

Depuis la fin des années 1980s, les bégomovirus sont devenus un des principaux facteurs limitant de la production dans beaucoup de régions. La qualification de virus émergents a souvent été associée à ces virus. Les introductions de virus et / ou des vecteurs sont les premiers facteurs associés aux émergences virales. Dans ce contexte, il est nécessaire de développer des outils de diagnostic afin d'assurer un diagnostic précoce et limiter les mouvements de plants contaminés.

Ce chapitre traite de la description d'une stratégie innovante de méthodologie pour la quantification en PCR en temps réel et son application dans la détection d'une large gamme de bégomovirus émergents. L'outil de diagnostic présenté ici est prêt à utiliser par les laboratoires impliqués dans la certification des plantes ou le diagnostic. De plus, la stratégie développée pourrait être étendu à d'autres agents pathogènes de plantes. L'application de ces PCR en temps réel a permis de mettre en évidence des données importantes et originales sur l'accumulation virale des bégomovirus bipartites et monopartites et pour la première fois, à notre connaissance, entre les deux molécules des bégomovirus bipartites.

Dans un second volet, ce chapitre présente la première description d'une différence de sévérité entre les deux principales souches émergentes du TYLCV. Cette description représente une nouveauté de première importance pour la gestion d'une des maladies de plantes les plus dévastatrices dans les régions chaudes et tempérées du monde. Ces données aideront les autorités sanitaires et les sélectionneurs à développer des stratégies de contrôle qui tiennent compte de ces phénotypes contrastés.

VIROLOGY JOURNAL

METHODOLOGY Open Access

A novel synthetic quantification standard including virus and internal report targets: application for the detection and quantification of emerging begomoviruses on tomato

Frédéric Péréfarres, Murielle Hoareau, Frédéric Chiroleu, Bernard Reynaud, Jacques Dintinger and Jean-Michel Lett*

Abstract

Background: Begomovirus is a genus of phytopathogenic single-stranded DNA viruses, transmitted by the whitefly *Bemisia tabaci*. This genus includes emerging and economically significant viruses such as those associated with Tomato Yellow Leaf Curl Disease, for which diagnostic tools are needed to prevent dispersion and new introductions. Five real-time PCRs with an internal tomato reporter gene were developed for accurate detection and quantification of monopartite begomoviruses, including two strains of the *Tomato yellow leaf curl virus* (TYLCV; Mld and IL strains), the Tomato leaf curl Comoros virus-like viruses (ToLCKMV-like viruses) and the two molecules of the bipartite *Potato yellow mosaic virus*. These diagnostic tools have a unique standard quantification, comprising the targeted viral and internal report amplicons. These duplex real-time PCRs were applied to artificially inoculated plants to monitor and compare their viral development.

Results: Real-time PCRs were optimized for accurate detection and quantification over a range of 2×10^9 to 2×10^3 copies of genomic viral DNA/μL for TYLCV-Mld, TYLCV-IL and PYMV-B and 2×10^8 to 2×10^3 copies of genomic viral DNA/μL for PYMV-A and ToLCKMV-like viruses. These real-time PCRs were applied to artificially inoculated plants and viral loads were compared at 10, 20 and 30 days post-inoculation. Different patterns of viral accumulation were observed between the bipartite and the monopartite begomoviruses. Interestingly, PYMV accumulated more viral DNA at each date for both genomic components compared to all the monopartite viruses. Also, PYMV reached its highest viral load at 10 dpi contrary to the other viruses (20 dpi). The accumulation kinetics of the two strains of emergent TYLCV differed from the ToLCKMV-like viruses in the higher quantities of viral DNA produced in the early phase of the infection and in the shorter time to reach this peak viral load.

Conclusions: To detect and quantify a wide range of begomoviruses, five duplex real-time PCRs were developed in association with a novel strategy for the quantification standard. These assays should be of a great interest for breeding programs and epidemiological surveys to monitor viral populations.

Background

The genus *Begomovirus* (family *Geminiviridae*) is a group of emerging phytopathogenic viruses transmitted by the whitefly *Bemisia tabaci* in a circulative permanent manner [1]. Begomoviruses cause severe diseases in a wide variety of plant species including many of considerable agricultural importance in tropical and subtropical areas [2]. Begomovirus genomes consist of monopartite or bipartite components of circular single strand DNA (ssDNA) [3]. The bipartite begomovirus genome is composed of two similar sized DNA molecules named DNA-A and DNA-B that share little sequence identity except for a 200nt region with at least 85% identity known as common region (CR) [4]. DNA-A component contains virus-encoded functions required for replication, transcription and encapsidation while the DNA-B component encodes proteins involved in intra- and inter-cellular viral movement [5] and symptom

* Correspondence: lett@cirad.fr
CIRAD, UMR PVBMT CIRAD-Université de la Réunion, Pôle de protection des plantes, 7 chemin de l'IRAT, 97410 Saint Pierre, Ile de la Réunion, France

development [6]. The monopartite begomovirus genome is homologous to the DNA-A component of the bipartite with an additional viral-sense ORF, the precoat or V2, implicated in viral movement and pathogenicity [7]. Whereas in monopartite begomoviruses the single DNA-A like component is sufficient for infection, for bipartite begomoviruses, both DNA components are necessary for a systemic symptomatic infection and thus must be co-transmitted into a target cell to initiate the infection [8].

Based on their genome organization, their genetic diversity, and their geographical distribution, begomoviruses have been divided into two groups: Old World (Africa, Asia, Australia and Europe) and New World (America) begomoviruses [9]. Although no native monopartite begomovirus from the New World has been described, the *Tomato yellow leaf curl virus*, (TYLCV), a monopartite begomovirus, was accidentally introduced into America [10,11], and is now widespread in North America, Central America and the Caribbean. Its global spread represents one of the most serious threats to worldwide tomato production, including temperate, subtropical and tropical areas [12]. In addition to TYLCV, a wide range of begomoviruses [13] are associated with the tomato yellow leaf curl disease and sanitation measures are essential to prevent further introductions and dispersion of these devastating viruses.

The use of real-time PCR to detect and quantify RNA and DNA viruses from plants and/or insects has become particularly appealing due to both its speed and greater accuracy compared with serological or end-point PCR [14-17].

Most notably, duplex real-time PCR, with a plant gene as internal control, allows normalisation between samples. This procedure removes any sampling, extraction or amplification bias that could hamper the analyses and permits direct comparisons between independent samples and avoids false negatives.

In this paper, we describe the development of five duplex real-time PCRs for the detection and quantification of a wide range of begomoviruses responsible for the tomato yellow leaf curl disease in French overseas departments (Martinique and Guadeloupe [18], Reunion [19,20] and Mayotte [21-23]). These diagnostic tools are coupled with an original strategy: a unique quantification standard comprising the viral and internal report targets. All five duplex PCRs were applied to artificially inoculated plants to monitor and compare the viral accumulation of a bipartite begomovirus (*Potato yellow mosaic virus*, PYMV) and monopartite begomoviruses including two strains of one of the most emergent plant viruses (TYLCV) as well as species restricted to the Comoros archipelago, collectively recorded as Tomato leaf curl Comoros virus-like viruses (ToLCKMV-like viruses).

Methods

Design of primers and probes

Alignments of complete sequences of TYLCV-IL (n = 41), TYLCV-Mld (n = 16), PYMV (n = 3, DNA-A and DNA-B) and ToLCKMV-like viruses (ToLCKMV, ToLCYTV, ToLCMohV n = 4) were performed using the Clustal-W subalignment tool [24] available in MEGA 4 [25] (the different isolates used and the sequence alignments are presented in Additional File 1 and 2). For the internal control, the sequence of the nuclear-encoded large subunit ribosomal RNA gene (*Solanum lycopersicum 25S ribosomal RNA* gene (Sl25S; GenBank: X13557) was selected. Primers and MGB-probes were designed using the Primer Express Software for real-time PCR version 3.0 (Applied Biosystems). All primers and probes were purchased commercially (Applied Biosystems, Foster City, USA). The sequences, the ORF targeted and the labels of primers and probes developed in this study are listed in Table 1.

Table 1 Primers and probes developed and used in this study

Primers/ Probes	Sequence 5'-3'	Label	Targeted ORF
F-Sl-25S	CGCCCGGTCGTACTCATAA	none	
R-Sl-25S	TCCATCGACCAGAGGCTGTT	none	NA
P-Sl-25S	CGCATCAGGTCTCCA	VIC	
PYMV-A-138-F	GCCTCTTGGCCCACTCTCTT	none	
PYMV-A-201-R	GCCATTGAACGCCATGGA	none	CP
PYMV-A-160-PMGB	ACTCAAAATGCCTAAGCG	FAM	
PYMV-B-1356-F	TGCAGACTCTCCCGGATCTAG	none	
PYMV-B-1415-R	CATCCGTATCGAGATCTGCAAA	none	MP
PYMV-B-1378-PMGB	ACGCTTGCTCCCAGC	FAM	
TYLCV-Mld-2186-F	CCTCTGACTTACTGCCTGAGTTAAGA	none	
TYLCV-Mld-2246-R	GGTCAGCAGTCAGCCAATGA	none	C4
TYLCV-Mld-2213-PMGB	CTGCGGCGTAAGC	FAM	
TYLCV-IL-2180-F	TGAGGGCCTCGGATTTATTG	none	
TYLCV-IL-2241-R	CAATCTGCCAACGACGCATA	none	C4
TYLCV-IL-2201-PMGB	CTGAATTGAGTGCTTCGG	FAM	
ToLCKMV-303-F	AGCGACCCGCCGATATAAT	none	
ToLCKMV-361-R	TTCAGTCTCCGACGCACCTT	none	CP
ToLCKMV-323-PMGB	ATTTCCACGCCCGCCT	FAM	

Construction of the quantification standard into plasmid vector

The original feature of the assay was the construction of a quantification standard comprising the five viral and the internal report targets in a single plasmid vector (Figure 1) (see Additional File 3 for a schematic representation). The amplicons targeted by the different primer/probe systems were synthesized and cloned into *Sma*I-digested pBluescript II SK using Epoch Biolabs Inc. facilities (Missouri city, TX, USA). *Escherichia coli* strain JM-109 (Promega, Paris, France) cells were transformed with this plasmid. Recombinant plasmid DNA was isolated from bacteria with the Plasmid MiniPrep Spin Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and quantified with the NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Courtaboeuf, France). The extracted plasmids were then serially diluted from 10^9 to 10^3 copies per µL in 10-fold steps, aliquoted and frozen before use as standards in each real-time PCR run. Standard curves were obtained by linear regression analysis of the threshold cycle (Ct) value of each of the two standard-dilution replicates over the log of the total amount of DNA. The Cts were automatically calculated by the StepOne Software v2.0 (Applied Biosystems, Courtaboeuf, France). The PCR efficiency (E) was calculated as follows:

$$E = e^{(\ln 10/-s)} - 1$$

where a slope s = -3.322 represents an efficiency of 100%.

Duplex real-time PCR optimizations

For the optimization of the five duplex real-time PCR assays, various primer concentrations (200-900 nM), probe concentrations (50-250 nM) and annealing-extension temperatures (60-65°C) were tested in a 15 µL reaction mix comprising 1× TaqMan universal PCR master mix (Applied Biosystems, Foster City, USA) and 2 µL DNA template. PCR reactions were carried out in the StepOnePlus real-time PCR system in fast optical 96-well reaction plates (Applied Biosystems, Courtaboeuf, France). Each sample was amplified in duplicate and a new aliquot of the standard was used in each run.

Construction of agroinfectious clones of PYMV

Full-length DNA-A and DNA-B genomes of *Potato yellow mosaic virus-Tomato* [Guadeloupe:Tomato] (PYMV-To[GP:Tom], EMBL:AY120882/AY120883, [18]) were used for the construction of infectious clones in the binary vector pCambia0380 (Cambia, Camberra, Australia). A 1166 bp *Apa*I/*Nco*I digested fragment containing the

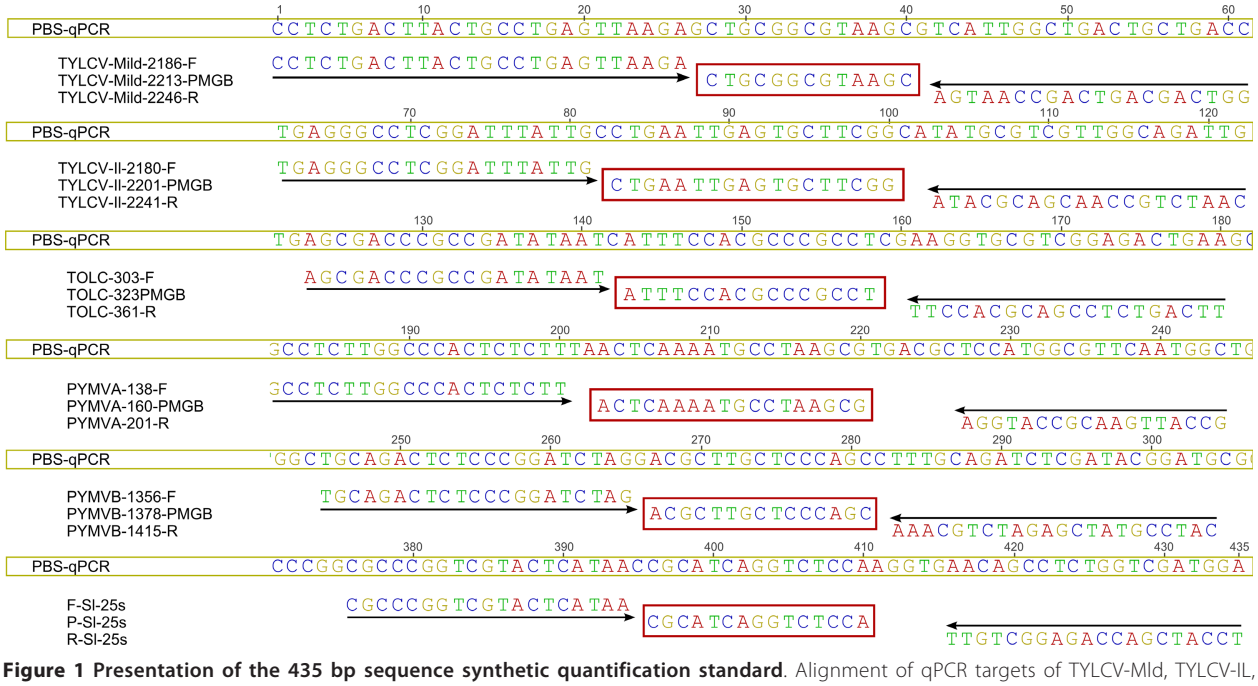


Figure 1 Presentation of the 435 bp sequence synthetic quantification standard. Alignment of qPCR targets of TYLCV-Mld, TYLCV-IL, ToLCKMV-like, PYMV (DNA-A and DNA-B), tomato internal control *Solanum lycopersicum 25S ribosomal RNA* gene (Sl25S; GenBank: X13557) with Taqman-MGB probes and forward and reverse primers.

intergenic region (IR) of the DNA-A was cloned to generate a 0.44-mer (pCambia0380-0.44). The full length monomer was cloned into *Apa*I digested pCambia0380-0.44 to generate a 1.44mer of PYMV-A. For the DNA-B, a 1367 bp *Eco*RI/*Bam*HI digested fragment containing the IR was cloned to generate a 0.54-mer (pCambia0380-0.54). The full length monomer was cloned into *Eco*RI digested pCambia0380-0.54 to generate a 1.54mer of PYMV-B. The orientation of the inserted genomes was checked by *Sac*I digestion. Recombinant plasmids were mobilized from *E. coli* strain JM-109 cells into *Agrobacterium tumefaciens* (strain C58) by triparental mating using *E. coli* HMB101 containing the plasmid helper pRK 2013 [26].

Plant inoculation and total DNA extraction

Liquid culture of *A. tumefaciens* containing the agro-infectious clones of TYLCV-IL[RE4], TYLCV-Mld[RE], ToLCKMV-[YT:Dem:03] or PYMV-To[GP:Tom] (described respectively in [20,22] and this study) were grown for 14 h and adjusted to an $OD_{600\ nm}$ of 1.0 before inoculation. For PYMV inoculation, equal amounts of *A. tumefaciens* containing PYMV-To[GP:Tom] (molecule A) and PYMV-To[GP:Tom] (molecule B) clones were mixed. Four sets of eight tomato plants *cv*. Farmer (Known-you Seed) were inoculated at the three-leaf stage by injecting 100 μL of *A. tumefaciens* culture into the stems (one set per virus).

Plants were then maintained in a complete random block design in an insect-free growth chamber at 26°C/24°C (day/night) with a 12-hour photoperiod. Virus accumulation was monitored using the duplex-real-time PCR developed in this study from the first true youngest leaf of each plant collected at 10, 20 and 30 days post-inoculation (dpi). Total DNA was extracted using the DNeasy Plant miniprep Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was finally resuspended in 100 μL (two successive elutions of 50 μL) of ultrapure water and stored at -20°C until utilization.

Quantities of virus and internal report were calculated with the corresponding standard curves and results were expressed as the log of the ratio of the quantity of virus DNA to that of plant genomic DNA [27].

Statistical analysis

The effects of the inoculated virus and the dpi were analysed on virus accumulation using an ANOVA procedure available in the R statistical software (R Development Core Team).

Results

Performance of the duplex-real-time PCR developed

Primer and TaqManMGB-probe concentrations for the duplex real-time PCR were first optimized to obtain the best efficiency in the larger linear dynamic range (data not shown). Selected conditions of the five duplex real-time PCR assays developed in this study are summarized in Table 2. All cycles begin with 2 min at 50°C then 10 min at 95°C follow by 40 two-step cycles comprising 15 s at 95°C and 1 min at the appropriate annealing-extension temperature (Table 2). In those conditions, no cross reaction was observed between the primers/probe system and non-targeted begomoviruses both in naturally field infected or artificially agro-inoculated plants (data not shown).

Typical amplification plots for the five duplex systems are shown in Figure 2. The corresponding standard curves had high correlation coefficients (R^2>0.99), and calculated PCR efficiencies ranged from 93% to 108%. The linear dynamic ranges for the virus quantification were within the range of 2×10^9 to 2×10^3 copies/μL except for PYMV-A and ToLCKMV-like viruses, with a corresponding linear dynamic range of 2×10^8 to 2×10^3 copies/μL. The quantification of the internal report was possible in the range of 2×10^9 to 2×10^3 copies/μL for the TYLCV-Mld/Sl25S and PYMV-B/Sl25S duplexes, and 2×10^7 to 2×10^3 copies/μL for the TYLCV-IL/Sl25, PYMV-A/Sl25S and ToLCKMV-like viruses/Sl25S duplexes (Table 2).

Virus accumulation in tomato plants

TYLCV-IL[RE4], TYLCV-Mld[RE], ToLCKMV-[YT:Dem:03] and PYMV-To[GP:Tom] were agro-inoculated in four sets of eight tomato plants to monitor the virus accumulation at 10, 20 and 30 dpi. All the plants inoculated with TYLCV-IL[RE4] and TYLCV-Mld[RE] produced typical yellow leaf curl and stunting symptoms between 10 and 20 dpi. All the plants inoculated with the PYMV-To[GP:Tom] developed typical symptoms of yellow mosaic on the leaves, curling and stunting between 13 and 20 dpi, confirming the pathogenicity of the partial tandem constructions. Six out of eight plants inoculated with ToLCKMV-[YT:Dem:03] became symptomatic between 15 and 25 dpi.

Effects of the virus inoculated, the dpi, and the interaction virus-dpi were highly significant on virus accumulation variations ($p < 10^{-8}$, Fisher-Snedecor's test). Significant differences were found for viral accumulation between the different begomoviruses inoculated at each date considered (Figure 3). At 10 dpi, PYMV DNA-A and DNA-B accumulated on average 88-fold and 36-fold more viral DNA respectively than the TYLCV-IL ($p < 10^{-8}$ for PYMV DNA-A and DNA-B), and in average 248-fold and 101-fold more respectively than the TYLCV-Mld ($p < 10^{-8}$ for PYMV DNA-A and DNA-B). No difference was found between the genome A and B of the PYMV ($p = 0.67$) and between the two strains of the TYLCV ($p = 0.29$). None of the inoculated plants

Table 2 Reaction conditions and assay performance of the five duplex real-time PCRs

Target	Virus		Internal report (ADN 25S)		T°m (°C)	Linear dynamic range (copies/uL)		PCR efficiency (%)	
	Primers	Probe	Primers	Probe		Virus	Internal report	Virus	Internal report
TYLCV-IL	650	150	500	100	63	2×10^9 to 2×10^3	$2 \times 10^7$to 2×10^3	96	108
TYLCV-Mld	250	200	750	150	64	2×10^9to2×10^3	2×10^9to2×10^3	103	107
PYMV-A	750	150	200	50	63	2×10^8to2×10^3	2×10^7to2×10^3	93	100
PYMV-B	900	150	200	50	63	2×10^9to2×10^3	2×10^9to2×10^3	97	100
ToLCKMV-like	750	150	300	50	62	2×10^8to2×10^3	2×10^7to2×10^3	95	104

with the ToLCKMV-[YT:Dem:03] were detected as infected at 10 dpi. At 20 dpi, PYMV DNA-A accumulated on average 7-fold and 39-fold more than TYLCV-IL and TYLCV-Mld ($p = 10^{-3}$ and $p < 10^{-8}$ respectively) without any significant differences with PYMV DNA-B ($p = 1$). TYLCV-Mld accumulated on average 5-fold less than the IL strain ($p = 8 \times 10^{-3}$) but on average 29-fold more than the ToLCKMV ($p = 4 \times 10^{-5}$). At 30 dpi, PYMV DNA-A and DNA-B accumulated on average 7-fold and 11-fold more viral DNA than the TYLCV-IL respectively ($p = 2 \times 10^{-3}$ and $p = 6 \times 10^{-5}$ for PYMV DNA-A and DNA-B respectively) without any difference between the two molecules ($p = 1$). TYLCV-Mld accumulated on average 15-fold less viral DNA than the IL strain ($p = 1 \times 10^{-5}$) and there was no difference with ToLCKMV ($p = 0.07$). The asymptomatic plants inoculated with the ToLCKMV-[YT:Dem:03] remained virus-free or undetectable by the real-time PCR at 10, 20 and 30 dpi.

Significant differences were found in the accumulation kinetics of the begomoviruses considered. PYMV DNA-A and DNA-B reached their highest viral load at 10 dpi and maintained this level at 20 and 30 dpi ($p = 1$ for DNA-A and DNA-B for 20 and 30 dpi). TYLCV-IL reached its peak viral load at 20 dpi ($p = 6 \times 10^{-4}$ with 10 dpi) and remained at this maximum viral load between 20 and 30 dpi ($p = 0.31$). TYLCV-Mld reached its peak viral load at 10 dpi and there was no difference between 10 and 20 dpi ($p = 0.046$). Between 20 and 30 dpi, the viral load decreased to the same level reached at 10 dpi ($p = 1 \times 10^{-3}$ and $p = 1$ for 20 and 30 dpi and for 30 and 10 dpi respectively). In the case of ToLCKMV, at 20 dpi only four plants showed detectable levels of virus and reached their peak viral load. At 30 dpi, six plants were quantified, their viral loads were not different as compared to 20 dpi ($p = 1$).

Discussion

During the last two decades, the spread of the highly polyphageous biotype B of *B. tabaci* has greatly contributed to the worldwide emergence of begomoviruses. These devastating viruses are one of the most important threats for tomato production in tropical and subtropical environments. Within the European Union, begomoviruses are listed on the EPPO A2 alert list and diagnostics tools are essential to prevent dispersions and new introductions. Several PCR-based methods [28,29] and real-time PCR [15,16] have been reported recently for the detection and the differentiation of strains and species of begomoviruses. We present here five real-time PCR assays including an internal report for the relative quantification of different begomoviruses in tomato plants. Those duplex real-time PCR assays are associated with a novel strategy for a unique quantification standard consisting in the cloning of both viral and internal report targets in the same plasmid. We developed and successfully applied these real-time PCR assays for the specific detection and quantification of a wide range of begomoviruses including the two emerging strains of TYLCV (IL and Mld strains), the PYMV and the ToLCKMV-like viruses.

As described previously in others studies [30,31], we used an internal report to validate and normalize the entire experiment including the processes of sampling, DNA extraction and DNA amplification. Following Mason et al. [15], we selected the *Solanum lycopersicum 25S ribosomal RNA* gene as internal report and we optimized the real-time PCR to amplify, in the same reaction, both viral and host DNA targets. The original feature of our assay was the design of a unique standard quantification comprising both the viral and the internal report targets. Recently, Lay et al [32] described a similar approach with the cloning of two Epstein-Barr virus targets in a single quantification standard. Here, we have conceived a quantification standard comprising not only the five viral targeted amplicons but also the internal report target. This approach is very useful to reduce the laborious stages of preparation of quantification standards containing known amounts of each target to a single step, and thus reduces the time and the cost of the whole assay.

The real-time PCR assays developed in this study were optimized to detect and quantify both the viral and host DNA in multiplex reactions respecting the MIQE guidelines [33]. PCR amplifications of the internal report cover six (TYLCV-IL, PYMV-A and ToLCKMV-like

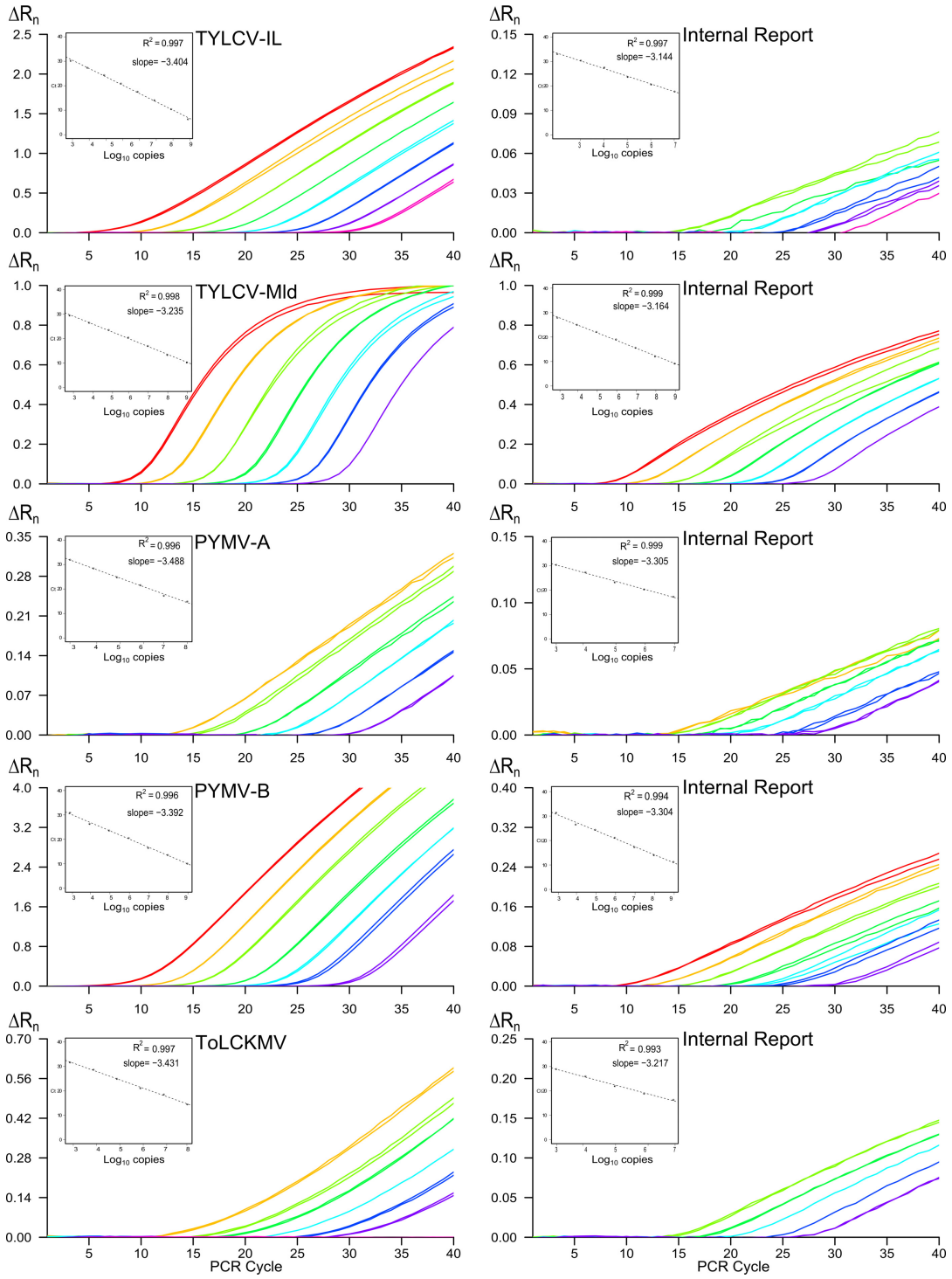


Figure 2 Amplification plots and standard curves of the five duplex real-time PCRs. Typical amplification plots for each viral target and the corresponding internal report in theirs linear dynamic ranges are represented. The corresponding standard curves are obtained by linear regression analysis of the threshold cycle (Ct) value of each of the two standard-dilution replicates over the $\log_{10}$ of the copies of DNA targets. The slope and the correlation coefficients are mentioned for each standard curve.

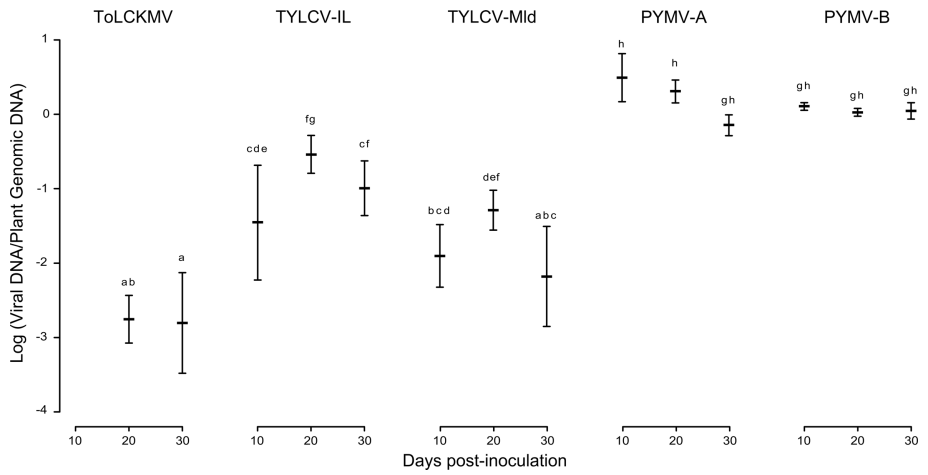


Figure 3 Average virus accumulation of TYLCV-IL, TYLCV-Mld, ToLCKMV and PYMV (DNA-A and DNA-B) in tomato plants. Data were obtained from 8 plants inoculated per virus. Vertical bars around each point represent the 95% confidence interval and the bars topped by the same letter (a to h) are not significantly different at p = 0.01 (Tuckey's HSD test). None of the inoculated plants with the ToLCKMV-[YT:Dem:03] were detected as infected at 10 dpi.

viruses) to seven (TYLCV-Mld and PYMV-B) orders of magnitude. Viral detection and quantification are possible in the range of 2×10^9 to 10^3 viral DNA copies/μL except for PYMV-A and ToLCKMV-like (2×10^8 to 10^3 viral DNA copies/μL). Those real-time PCR assays provide an accurate detection and quantification of the targeted viruses, with a higher detection limit than the ones previously described by others studies on RNA viruses [34,35] or DNA viruses [15] albeit of only a 10-fold. Advantageously, our real-time PCR assays are able to quantify both the host and viral DNA in a single run, making a direct normalisation of the quantification possible.

We successfully used the real-time PCR developed with experimentally inoculated plants to compare viral accumulation at 10, 20 and 30 dpi. These three successive viral quantifications, although unable to reflect the entire kinetics of viral accumulation, were sufficient to observe different patterns of viral accumulation between the bipartite and the monopartite begomoviruses and between the different strains and species of monopartite begomoviruses analysed.

The comparison of relative loads of viral DNA demonstrated that the bipartite PYMV accumulated more viral DNA than the two strains of TYLCV and ToLCKMV in tomato plants at each date considered. To our knowledge, it is the first demonstration of the higher viral load of a bipartite begomovirus than monopartite begomoviruses. The DNA-B component of begomoviruses encodes two viral proteins with essential functions in intra- and inter-cellular efficient movement [5] and can contribute to symptom production [6]. Although the origin of the DNA-B remains unclear [36], this component must provide selective advantages with enhanced viral fitness [36]. *A contrario* to TYLCV, the capacity of bipartite begomoviruses to escape from the phloem cells and infect the surrounding tissues could be a key element in this difference of the viral accumulation observed [7,37]. This wider tissue tropism gives the opportunity to infect more plant cells and may be the major determinant in our observed difference in viral accumulation. Our data revealed strong differences in the viral load between the bipartite and the monopartite begomoviruses at the leaf-level but the question of viral accumulation in a single infected cell remains open.

In the case of PYMV, interestingly, no difference was observed between the two genomic components accumulation at 10, 20 and 30 dpi with the higher viral loads reached at 10 dpi for both components. DNA-B of bipartite begomoviruses is necessary for viral infection, and so the two components must be co-transmitted to spread and induce systemic symptomatic infections [8] (for exception see [38]). Our data provide new insights into the replication of bipartite begomoviruses and suggest that both PYMV molecules accumulate at the same level from the early to the late phase of the infection, ensuring a further efficient transmission although we cannot exclude differential time to reach this peak viral load during the first ten days post-infection.

We also revealed differences in the patterns of viral accumulation between the two strains of TYLCV (Mld and IL strains). Experimental work using TYLCV, the monopartite ToLCV form Australia and TYLCCNV showed that the ORF C4 is implicated in viral movement [39], symptom development [40] and bypass defence mechanisms of the host [41,42]. Considering

the recombinant nature of TYLCV-IL, which shares a common origin for a portion of its genome comprising the C4 ORF with ToLCV-Asian-like ancestors [43], we can hypothesize that the C4 protein of TYLCV-IL is in part responsible for the higher fitness observed compared to the Mld strain.

Finally, we revealed strong differences between the two strains of TYLCV and the ToLCKMV. The accumulation kinetics of these two species differed both in the quantity of viral DNA produced, and in the time to reach this peak viral load. Such differences could have strong epidemiological consequences increasing the probability for an insect vector to acquire a virus from a plant with higher viral load during a longer timeframe and thus contributing to their preferential dispersions. In this study, we compared two species of monopartite begomoviruses with very different impacts and areas of distribution. While the TYLCV is considered as one of the most emergent plant viruses and has succeeded in spreading worldwide [10], the ToLCKMV-like viruses are for now confined to the Comoros archipelago with a minor impact on local production [22]. Those two contrasted epidemiological profiles coincide with the strong differences in biological properties revealed by our study, such as the higher fitness. Our study thus suggests possible reasons for the successful spread of the emergent TYLCV, as compared to the indigenous and area-restricted ToLCKMV-like viruses.

Conclusions

In this paper, we described an original real-time PCR strategy using a unique synthetic quantification standard comprising both viral and internal report targeted amplicons. The assays developed could be used to detect and quantify the four viruses studied in artificially inoculated plants. This approach is very useful in reducing the time and cost of the assays and could be extended to pathogen amplicons targeted by other real-time PCR assays. This strategy and the tools developed could be suitable and advisable for laboratories involved in plant certification or diagnosis.

Additional material

Additional file 1: Isolate, acronym and accessions numbers of the TYLCD-associated viruses used for sequences alignment and design of the primers and probes.

Additional file 2: Alignments of the targeted isolates used to design primers and probes. Taqman-MGB probes and forward and reverse primers are represented on each alignment.

Additional file 3: Schematic representation of the synthetic quantification standard.

Acknowledgements

This study was funded by the Conseil Régional de La Réunion, the European Union (FEDER, POSEIDOM) and the CIRAD. FP is a recipient of a PhD fellowship from the CIRAD. The authors wish to thank Nathalie Becker (MNHN, Saint Pierre, Ile de la Réunion, France) for reviewing the manuscript.

Authors' contributions

FP developed the assays and carried out all the DNA work. MH was involved in the agroinoculations and DNA extraction. FP and FC were involved in the statistical data analysis. FP and JML analyzed the data, prepared the manuscript and were involved in the design and conception of the study. JML, JD and BR secured funding for the project and provided ideas and comments during preparation of the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 15 March 2011 Accepted: 5 August 2011
Published: 5 August 2011

References

1. Moriones E, Navas-Castillo J: **Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide.** *Virus Res* 2000, **71**:123-134.
2. Seal S, vandenBosch F, Jeger M: **Factors Influencing Begomovirus Evolution and Their Increasing Global Significance: Implications for Sustainable Control.** *Crit Rev Plant Sci* 2006, **25**:23-46.
3. Jeske H: **Geminivirus.** *Cur Top Microbiol Immunol* 2009, **331**:185-226.
4. Hanley-Bowdoin L, Settlage S, Orozco B, Nagar S, Robertson D: **Geminiviruses: Models for Plant DNA Replication, Transcription, and Cell Cycle Regulation.** *Crit Rev Plant Sci* 1999, **18**:71-106.
5. Sanderfoot AA, Lazarowitz SG: **Getting it together in plant virus movement: cooperative interactions between bipartite geminivirus movement proteins.** *Trends Cell Biol* 1996, **6**:353-358.
6. Hussain M, Mansoor S, Iram S, Fatima AN, Zafar Y: **The Nuclear Shuttle Protein of Tomato Leaf Curl New Delhi Virus Is a Pathogenicity Determinant.** *J Virol* 2005, **79**:4434-4439.
7. Rojas MR, Jiang H, Salati R, Xoconostle-Cázares B, Sudarshana MR, Lucas WJ, Gilbertson RL: **Functional Analysis of Proteins Involved in Movement of the Monopartite Begomovirus, Tomato Yellow Leaf Curl Virus.** *Virology* 2001, **291**:110-125.
8. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL: **Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses.** *Annu Rev Phytopathol* 2005, **43**:361-394.
9. Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X: **Geminivirus strain demarcation and nomenclature.** *Arch Virol* 2008, **153**:783-821.
10. Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJA, Meredith S, Lakay F, Monjane A, Lett J, Varsani A, Heydarnejad J: **The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World.** *PLoS Pathog* 2010, **6**: e1001164-.
11. Duffy S, Holmes EC: **Multiple Introductions of the Old World Begomovirus Tomato yellow leaf curl virus into the New World.** *Appl Environ Microbiol* 2007, **73**:7114-7117.
12. Díaz-Pendón JA, Cañizares MC, Moriones E, Bejarano ER, Czosnek H, Navas-Castillo J: **Tomato yellow leaf curl viruses: ménage à trois between the virus complex, the plant and the whitefly vector.** *Mol Plant Pathol* 2010, **11**:441-450.
13. Abhary M, Patil B, Fauquet C: **Molecular Biodiversity, Taxonomy, and Nomenclature of Tomato Yellow Leaf Curl-like Viruses.** In *Tomato Yellow Leaf Curl Virus Disease.* Edited by: Henryk Czosnek. Springer Netherlands; 2007:85-118.
14. Balme-Sinibaldi V, Tribodet M, Croizat F, Lefeuvre P, Kerlan C, Jacquot E: **Improvement of Potato virus Y (PVY) detection and quantitation using PVYN- and PVYO-specific real-time RT-PCR assays.** *J Virol Methods* 2006, **134**:261-266.
15. Mason G, Caciagli P, Accotto GP, Noris E: **Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci.** *J Virol Methods* 2008, **147**:282-289.

16. Papayiannis LC, Iacovides TA, Katis NI, Brown JK: **Differentiation of Tomato yellow leaf curl virus and Tomato yellow leaf curl Sardinia virus using real-time TaqMan PCR.** *J Virol Methods* 2010, **165**:238-245.
17. Gutiérrez-Aguirre I, Mehle N, Delic D, Gruden K, Mumford R, Ravnikar M: **Real-time quantitative PCR based sensitive detection and genotype discrimination of Pepino mosaic virus.** *J Virol Methods* 2009, **162**:46-55.
18. Urbino C, Polston JE, Patte CP, Caruana M: **Characterization and genetic diversity of *Potato yellow mosaic virus* from the Caribbean.** *Arch Virol* 2004, **149**:417-424.
19. Peterschmitt M, Granier M, Mekdoud R, Dalmon A, Gambin O, Vayssieres JF, Reynaud B: **First Report of Tomato Yellow Leaf Curl Virus in Réunion Island.** *Plant Dis* 1999, **83**:303-303.
20. Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM: **The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Réunion Island.** *Plant Pathol* 2005, **54**:262-262.
21. Lett JM, Delatte H, Naze F, Reynaud B, Abdoul-Karime AL, Peterschmitt M: **A New Tomato leaf curl virus from Mayotte.** *Plant Dis* 2004, **88**:681-681.
22. Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett J: **South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group.** *J Gen Virol* 2005, **86**:1533-1542.
23. Lefeuvre P, Martin DP, Hoareau M, Naze F, Delatte H, Thierry M, Varsani A, Becker N, Reynaud B, Lett J: **Begomovirus 'melting pot' in the south-west Indian Ocean islands: molecular diversity and evolution through recombination.** *J Gen Virol* 2007, **88**:3458-3468.
24. Thompson JD, Higgins DG, Gibson TJ: **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.** *Nucleic Acids Res* 1994, **22**:4673-4680.
25. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0.** *Mol Biol Evol* 2007, **24**:1596-1599.
26. Ditta G, Stanfield S, Corbin D, Helinski DR: **Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti.** *Proc Natl Acad Sci USA* 1980, **77**:7347-7351.
27. Urbino C, Thébaud G, Granier M, Blanc S, Peterschmitt M: **A novel cloning strategy for isolating, genotyping and phenotyping genetic variants of geminiviruses.** *Virol J* 2008, **5**:135.
28. Lefeuvre P, Hoareau M, Delatte H, Reynaud B, Lett J: **A multiplex PCR method discriminating between the TYLCV and TYLCV-Mld clades of tomato yellow leaf curl virus.** *J Virol Methods* 2007, **144**:165-168.
29. Davino S, Davino M, Accotto GP: **A single-tube PCR assay for detecting viruses and their recombinants that cause tomato yellow leaf curl disease in the Mediterranean basin.** *J Virol Methods* 2008, **147**:93-98.
30. Osman F, Rowhani A: **Real-time RT-PCR (TaqMan®) assays for the detection of viruses associated with Rugose wood complex of grapevine.** *J Virol Methods* 2008, **154**:69-75.
31. López R, Asensio C, Guzman M, Boonham N: **Development of real-time and conventional RT-PCR assays for the detection of potato yellow vein virus (PYVV).** *J Virol Methods* 2006, **136**:24-29.
32. Lay M, Lucas R, Ratnamohan M, Taylor J, Ponsonby A, Dwyer D, the Ausimmune Investigator Group (AIG): **Measurement of Epstein-Barr virus DNA load using a novel quantification standard containing two EBV DNA targets and SYBR Green I dye.** *Virol J* 2010, **7**:252.
33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.** *Clin Chem* 2009, **55**:611-622.
34. Fabre F, Kervarrec C, Mieuzet L, Riault G, Vialatte A, Jacquot E: **Improvement of Barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real time RT-PCR.** *J Virol Methods* 2003, **110**:51-60.
35. Olmos A, Bertolini E, Gil M, Cambra M: **Real-time assay for quantitative detection of non-persistently transmitted Plum pox virus RNA targets in single aphids.** *J Virol Methods* 2005, **128**:151-155.
36. Briddon RW, Patil BL, Bagewadi B, Nawaz-ul-Rehman MS, Fauquet CM: **Distinct evolutionary histories of the DNA-A and DNA-B components of bipartite begomoviruses.** *BMC Evol Biol* 2010, **10**:97.
37. Morilla G, Krenz B, Jeske H, Bejarano ER, Wege C: **Tete a Tete of Tomato Yellow Leaf Curl Virus and Tomato Yellow Leaf Curl Sardinia Virus in Single Nuclei.** *J Virol* 2004, **78**:10715-10723.
38. Saunders K, Salim N, Mali VR, Malathi VG, Briddon R, Markham PG, Stanley J: **Characterisation of Sri Lankan Cassava Mosaic Virus and Indian Cassava Mosaic Virus: Evidence for Acquisition of a DNA B Component by a Monopartite Begomovirus.** *Virology* 2002, **293**:63-74.
39. Jupin I, De Kouchkovsky F, Jouanneau F, Gronenborn B: **Movement of tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4.** *Virology* 1994, **204**:82-90.
40. Rigden JE, Krake LR, Rezaian MA, Dry IB: **ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity.** *Virology* 1994, **204**:847-850.
41. Dogra SC, Eini O, Rezaian MA, Randles JW: **A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein.** *Plant Mol Biol* 2009, **71**:25-38.
42. van Wezel R, Dong X, Blake P, Stanley J, Hong Y: **Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in Nicotiana benthamiana.** *Mol Plant Pathol* 2002, **3**:461-471.
43. Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto GP, Moriones E: **Natural recombination between Tomato yellow leaf curl virus-is and Tomato leaf curl virus.** *J Gen Virol* 2000, **81**:2797-2801.

doi:10.1186/1743-422X-8-389
Cite this article as: Péréfarres *et al.*: A novel synthetic quantification standard including virus and internal report targets: application for the detection and quantification of emerging begomoviruses on tomato. *Virology Journal* 2011 **8**:389.

Differential severity between the two main emerging strains of TYLCV

Frédéric Péréfarres[1,2], Pierre Lefeuvre[1], Murielle Hoareau[1], Frédéric Chiroleu[1], Magali Thierry[1,2], Bernard Reynaud[1], Jacques Dintinger[1] and Jean-Michel Lett[1*].

(1)CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France

(2)Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France

*Corresponding author

Email addresses:

FP: frederic.perefarres@cirad.fr
PL: pierre.lefeuvre@cirad.fr
MH: murielle.hoareau@cirad.fr
FC: frederic.chiroleu@cirad.fr
MT: mag.thierry@cirad.fr
BR: bernard.reynaud@cirad.fr
JD: jacques.dintinger@cirad.fr
JML: lett@cirad.fr

Submitted to Virology Journal

Abstract

Background: During the last three decades, begomoviruses have greatly extended their geographical distribution, and become one of the most destructive groups of plant viruses in a wide variety of important plants. Tomato yellow leaf curl disease (TYLCD), one of the most devastating diseases of tomato, is caused by a complex of begomoviruses including the best-known *Tomato yellow leaf curl virus* (TYLCV).

Findings: Whereas an increasing number of TYLCV isolates are identified and molecularly characterized, little is known about differences in their biological traits, and more particularly between the two major TYLCV-Mild (-Mld) and TYLCV-Israel (-IL) strains. Here, we compared the severity of symptoms of the two strains in agro-inoculated tomato plants. Whereas the progression of severity of symptoms did not differ from one strain to the other, TYLCV-IL induced more severe symptoms than TYLCV-Mld in tomato plants.

Conclusion: To our knowledge, this is the first experimental demonstration of a differential severity of symptoms between the two main emerging TYLCV strains. These findings will help plant breeders and sanitation authorities to develop accurate control strategies that account for the contrasted phenotypes of the two strains.

Findings

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) is a group of phytopathogenic viruses causing diseases in a wide variety of plants including many of considerable economic and social importance. Tomato yellow leaf curl disease (TYLCD) is one of the most devastating plant diseases in warm and temperate regions of the world. Symptoms of the disease are yellowing and upwards curling of leaflet margins, stunting of the plants and flower abortion. Infection during early phases can lead to total loss of the crop. This disease is caused by a complex of begomovirus species including the best-known and emergent species *Tomato yellow leaf curl virus* (TYLCV). Based on sequence identities, five strains of TYLCV are currently described including Gezira (e.g. TYLCV-Gez[SD:96]), Iran (e.g. TYLCV-IR[IR:Ira:98]), Israel (e.g. TYLCV-IL[IL:Reo:86]), Mild (e.g. TYLCV-Mld[IL:93]) and Oman strains (e.g. TYLCV-OM[OM:Alb:05]) [1]. While TYLCV-IL and TYLCV-Mld are widely distributed, the three others strains are currently confined to their centres of origins (recently reviewed in [2]).

Before the introduction of TYLCV-Mld in 1997, no begomoviruses had been described in Reunion Island [3]. Severe outbreaks of TYLCD were observed in 2004 and revealed the accidental introduction of the Israel strain [4]. It is surprising that (1) despite the fact that these strains are now well characterized molecularly and (2) that their names (Israel *versus* Mild) suggest a difference in virulence, little evidence actually supports any differences in their biological traits, such as severity of symptoms.

To evaluate and compare the severity of symptoms of TYLCV-IL and TYLCV-Mld, the symptomatology of agro-inoculated tomato plants (cv. Farmer, Known You Seed) was scored in a trial repeated three times independently. Full head-to-tail DNA dimers of the cloned DNA components of Reunion isolates of TYLCV-Mld[RE:SPi:02] (EMBL: AJ865337) or TYLCV-IL[RE:SGi:RE4:04] (EMBL: AM409201) were inserted in the binary vector pCAMBIA2300 and mobilized into the C58 strain of *Agrobacterium tumefaciens* [5]. Liquid cultures of *A. tumefaciens* containing the agro-infectious clones of TYLCV-Mld[RE:SPi:02], TYLCV-IL[RE:SGi:RE4:04] or mock (native pCAMBIA2300 plasmid), were grown for 14 h and adjusted to an OD_{600nm} of 1.0 before inoculation. Each repetition consisted of 3 sets of 28 plants (one set per virus and one mock) agro-inoculated at the one-leaf growth stage with 50 µL of suspension culture injected into the stems. Plants were then maintained in a complete random block design in a growth-chamber at 26°C/24°C (day/night) with a 12-hour photoperiod. Disease severity was evaluated using symptom scoring every 3.5 days for 5 weeks post inoculation. The disease severity scale was 1, no symptom, to 10, dead plant, with numbers 1-9 corresponding to the 0-4 scale of Lapidot *et al* [6]. Disease

severity, defined as the mean score of the plant exhibiting symptoms, was calculated at each scoring date.

Nonlinear regression analyses were performed using different link functions (logistic, Cauchy, probit, loglog and cloglog) to fit the progression of disease severity. Based on likelihood and using the Akaike's Information Criterion (AIC), the loglog function appeared to be the most appropriate to fit the progression of disease severity. In this model, written as Y ~ 1 + C exp(- exp(- A (X - B) + log(- log(0.5)))), disease severity (Y) depends on three biologically relevant parameters: A is the slope of the exponential phase, (1 + C) is the disease severity at the plateau phase, and B is the time to reach 50% of the disease severity at the plateau phase. Once adjusted, the parameters of the logistic disease progression model were compared between the IL, Mld and control experiments using likelihood ratio tests (LRT). All the statistical analyses were performed using R statistical software [7].

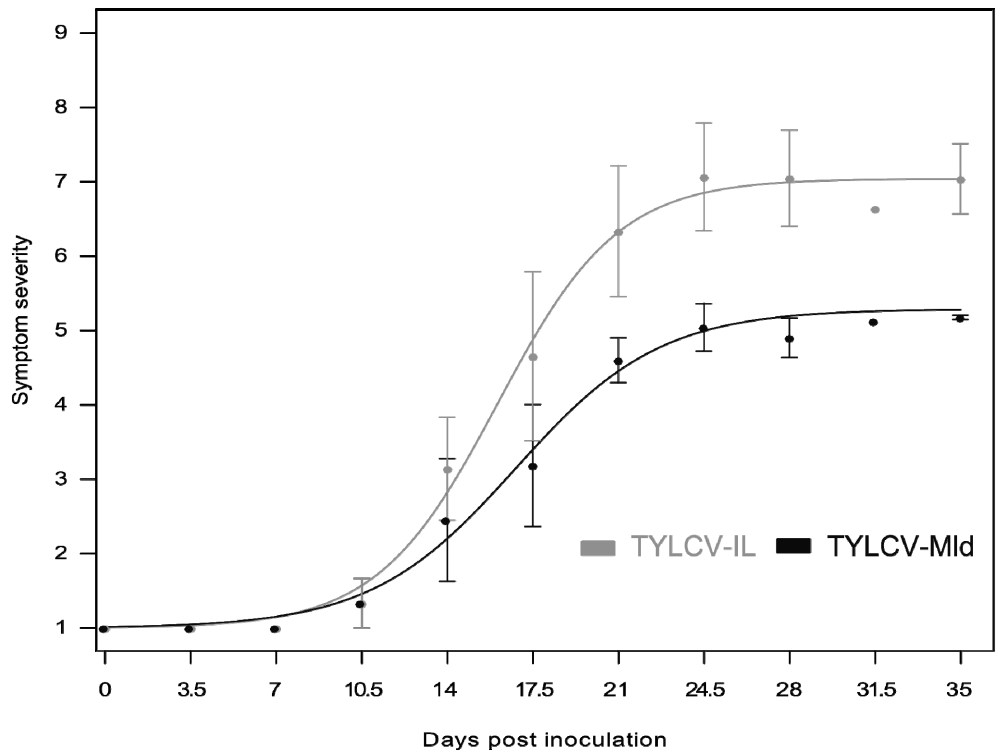


Figure 1: Estimated (solid line) and mean (dots) symptom severity of tomato yellow leaf curl disease following agro-inoculation of tomato plants with *Tomato yellow leaf curl virus* Reunion isolates of Israel or Mild strains (TYLCV-IL or TYLCV-Mld). The symptom severity scale was 1, no symptom, to 10, dead plant. Vertical bars around the dots represent the standard error. It should be noted that all the scored plants had disease severity of 1 between 0 and 7 days post inoculation (dpi) and only the first trial was scored at 31.5 dpi.

While all the mock inoculated plants remained asymptomatic throughout the experiments, tomato plants agro-inoculated by TYLCV-IL and TYLCV-Mld started

exhibiting typical yellow leaf curl and stunting symptoms between 10.5 and 14 dpi (Figure 1). Symptom severity increased before reaching a maximum value at 24.5 dpi for both strains. Whereas the parameters governing the speed of the disease progression did not significantly differ between the two strains (p=0.44 and p=0.46 for the parameters A and B respectively), at the plateau phase, the symptom severity was clearly higher for TYLCV-IL (disease severity$_{35dpi}$ of 6.9 and 5.8 for TYLCV-IL and TYLCV-Mld respectively, $p=3 \times 10^{-3}$). Together, these results demonstrate for the first time the higher symptom severity of the IL strain compared to the Mld strain in tomato plants.

Determining the molecular basis of this difference is a major challenge for plant pathologists and plant breeders to develop suitable control strategies. Interestingly, it has been proved that TYLCV-IL is a recombinant variant of the Mediterranean TYLCV-Mld with the 5' portion of its *rep* gene including the C4 ORF, which is very similar to the Asian *Tomato leaf curl Karnataka virus* (ToLCKV) [8]. Previous studies focusing on the role of C4 ORF of TYLCV and relatives revealed its implication in major pathways of the viral infection including the viral movement, symptom development and bypass defence mechanisms of the host [9–12]. Tomás *et al* recently demonstrated differential abilities of the C4 ORF of the Spain isolates of TYLCV-Mld and TYLCV-IL to overcome defence barriers of a wild relative tomato *Solanum habrochaites* including suppression of post-transcriptional gene silencing (PTGS) and/or enhancement of viral movement [13]. We thus hypothesize that the C4 protein of TYLCV-IL is also implicated in the difference in symptom severity.

In the light of the considerable difference in symptom severity between these two strains, it is necessary to accurately identify which TYLCV-strain is involved in new infection or epidemics, as the outcome will differ dramatically. In addition, since the most desirable strategy to control TYLCD is based on breeding resistant cultivars, germplasm screening should also take into account the contrasted phenotypes of these two strains for the accurate estimation of TYLCV-resistance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FP, PL and JML developed the assays. MH was involved in the agro-inoculations. FP, PL and FC were involved in analysis of statistical data. FP, PL, MT and JML analyzed the data, prepared the manuscript and were involved in the design and conception of the study. JML, JD and BR secured funding for the project's execution, and provided ideas and comments during manuscript preparation. All authors have read and approved the final manuscript.

Acknowledgements

This study was funded by the *Conseil Régional de La Réunion*, the European Union (FEDER) and CIRAD. FP is a recipient of a PhD fellowship from the CIRAD.

References

1. Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X: **Geminivirus strain demarcation and nomenclature.** *Archives of virology* 2008, **153**:783-821.

2. Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJ a., Meredith S, Lakay F, Monjane A, Lett J-M, Varsani A, Heydarnejad J: **The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World**. *PLoS Pathogens* 2010, **6**:e1001164.

3. Peterschmitt M, Granier M, Mekdoud R, Dalmon A, Gambin O, Vayssieres JF, Reynaud B: **First Report of Tomato Yellow Leaf Curl Virus in Réunion Island**. *Plant Disease* 1999, **83**:303-303.

4. Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM: **The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Reunion Island**. *Plant Pathology* 2005, **54**:262-262.

5. Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett J-M: **South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group.** *The Journal of general virology* 2005, **86**:1533-42.

6. Lapidot M, Ben-Joseph R, Cohen L, Machbash Z, Levy D: **Development of a Scale for Evaluation of Tomato yellow leaf curl virus Resistance Level in Tomato Plants.** *Phytopathology* 2006, **96**:1404-8.

7. Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RDC: **nlme: Linear and Nonlinear Mixed Effects Models**. 2009, **R package**:1-86.

8. Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto GP, Moriones E: **Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus**. *Journal of General Virology* 2000, **81**:2797-2801.

9. Jupin I, De Kouchkovsky F, Jouanneau F, Gronenborn B: **Movement of Tomato Yellow Leaf Curl Geminivirus (TYLCV): Involvement of the Protein Encoded by ORF C4**. *Virology* 1994, **204**:82-90.

10. van Wezel R, Dong X, Blake P, Stanley J, Hong Y: **Differential roles of geminivirus Rep and AC4 (C4) in the induction of necrosis in Nicotiana benthamiana.** *Molecular plant pathology* 2002, **3**:461-71.

11. Dogra SC, Eini O, Rezaian MA, Randles JW: **A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein.** *Plant molecular biology* 2009, **71**:25-38.

12. Gronenborn B: **The Tomato Yellow Leaf Curl Virus Genome and Function of its Proteins**. In *Tomato Yellow Leaf Curl Virus Disease*. edited by Czosnek H Springer Netherlands; 2007:67-84.

13. Tomás DM, Cañizares C, Abad J, Fernández-Muñoz R, Moriones E: **Resistance to tomato yellow leaf curl virus accumulation in the tomato**

wild relative Solanum habrochaites associated with the C4 viral protein.
Molecular plant-microbe interactions□: MPMI 2011, **24**:849-861.

Chapitre II :
Dynamiques épidémiologiques des populations de TYLCV-Mld et TYLCV-IL à la Réunion.

Le TYLCV est probablement une des espèces de phytovirus les mieux connues et caractérisées avec les bégomovirus infectant le manioc. Le TYLCV appartient à un complexe d'espèces de bégomovirus, les TYLCV-like, responsables d'une des maladies les plus préjudiciables sur cultures maraîchères : le TYLCD. Le TYLCV et son complexe d'espèces apparentées avec son cortège de variants ne cesse d'accroitre son aire de répartition à l'échelle mondiale. De nombreuses études ont également décrit l'apparition de variants recombinants au sein de ce complexe d'espèces.

A La Réunion, les deux souches du TYLCV actuellement décrites (TYLCV-IL et TYLCV-Mld) ont été introduites accidentellement à quelques années d'intervalle. Le TYLCV-Mld a été décrit pour la première fois en 1997 (Peterschmitt et al. 1999) et a envahi le bassin maraîcher réunionnais (Delatte et al. 2007). Le TYLCV-IL a été détecté en 2004 dans l'ouest de l'île à l'extrémité ouest du bassin maraîcher (Delatte et al. 2005).

Ces introductions successives de ces deux souches du TYLCV on fait de la Réunion un lieu privilégié pour étudier les dynamiques épidémiologiques de ce complexe viral dans un environnement tropical et insulaire. Plusieurs scénarii étaient envisageables avec soit (1) une compétition et un déplacement d'une souche par une autre, (2) un synergisme et une cohabitation des deux souches ou (3) l'apparition d'un variant recombinant plus fit et capables de déplacer les deux parents.

Comprendre l’interaction et les dynamiques évolutives et épidémiologiques de cette diversité virale est un préalable nécessaire pour adapter les stratégies de contrôle et plus particulièrement pour orienter la sélection et le déploiement de cultivars résistants.

Notre suivi épidémiologique basé sur un échantillonnage de 7 ans a permis de mettre en avant le déplacement rapide du TYLCV-Mld par le TYLCV-IL. De prédominant dans le bassin maraîcher, le TYLCV-Mld est aujourd'hui minoritaire.

Afin de déterminer les bases biologiques de cet avantage sélectif, des tests comparatifs de *fitness* et d'efficacité de transmission ont été réalisés entre ces deux souches du TYLCV. Nos essais ont mis en avant de meilleures aptitudes de la souche IL tant au niveau de la colonisation de son hôte principal que dans sa capacité de dissémination naturelle. Ces propriétés ont certainement permis au TYLCV-IL d’envahir le bassin maraîcher réunionnais et déplacer le TYLCV-Mld pourtant déjà bien implanté.

En plus de ce déplacement, un des points marquants de notre suivi épidémiologique est le maintien du TYLCV-Mld à la Réunion et la part importante de plantes co-infectées par les deux souches du TYLCV. Les infections mixtes sont

connues pour jouer un rôle majeur dans la modulation de propriétés biologiques des virus mais sont aussi un pré-requis pour permettre la recombinaison virale, principal moteur de la diversification des bégomovirus. Notre étude a mis en évidence l'assistance conférée par le TYLCV-IL dans la colonisation de l'hôte et dans la dissémination naturelle du TYLCV-Mld. De plus, il est important de noter que, pour le moment, aucun variant recombinant issu des TYLCV-Mld et TYLCV-IL n'a été détecté à la Réunion. L'ensemble de ces données épidémiologiques et expérimentales a permis la construction d'un modèle

Enfin, cette étude a également permis d'estimer pour la première fois la taille du goulet d'étranglement exercé par *B. tabaci* lors de la transmission horizontale, paramètre important dans la compréhension des dynamiques évolutives des populations virales.

Long-term viral competition monitoring: a case of epidemiological rescue

Péréfarres Frédéric[1,2], Thébaud Gaël[3], Lefeuvre Pierre[1], Hoareau Murielle[1], Chiroleu Frédéric[1], Reynaud Bernard[1] and Lett Jean-Michel[1*].

CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de la Réunion, France[1].
Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France [2].
INRA, UMR BGPI, Campus international de Baillarguet, Cirad TA A-54/K, 34398 Montpellier Cedex 5, France[3].

*Corresponding author. Mailing address: Pôle de protection des plantes - 3P, 7 chemin de l'IRAT, 97410 Saint-Pierre, Ile de la Réunion, France. Phone: +262 262499201. Fax: +262 2 62 49 92 93. E-mail: lett@cirad.fr

Abstract

Biological invasions are major threats to biodiversity and the major factors associated to emerging viral diseases. The ongoing spread of *Tomato yellow leaf curl virus* is a major concern to the sustainable tomato production throughout the world. The two main strains of TYLCV have been successively introduced in Reunion Island providing an ideal experimental field to study the invasion and competition of these two emerging strains in a new environment. In this study, a seven-year based field survey was performed following the introduction of the TYLCV-IL into a niche widely occupied by TYLCV-Mld. A displacement of the TYLCV-Mld by the newcomer TYLCV-IL was observed in this short period. To understand the factors associated with this displacement, biological traits related to fitness were measured to compare these strains. A better ecological aptitude was demonstrated providing the causes in the rapid spread and prevalence of TYLCV-IL. Moreover, because of the maintenance of TYLCV-Mld in mixed infections with TYLCV-IlL, we studied the effects of the mixed infections in these biological traits. Our study revealed complex interplay between these two strains responsible of the maintenance of TYLCV-Mld in Reunion Island.

Introduction

Biological invasions of arthropods and viruses are major factors associated with viral emerging diseases (Anderson et al. 2004). These negative effects of biological invasions are often more pronounced in small and isolated regions, such as insular habitats (Gillespie and Roderick 2002). Since the early domestication of

plants, human activity has greatly impacted viral evolution promoting virus invasions and propagations thanks to the movement of plants away from theirs centres of diversification. These processes were enhanced considerably over the last 500 – 1000 years as human activity continued to expand. Most notably, in the last 100 years the whole process is being exacerbated by global climate change and the increase in world trade in plants and plants products (Anderson et al. 2004; Jones 2009) that favour virus and vector disseminations.

During the last three decades, new begomovirus species (family *Geminiviridae*) have simultaneously emerged worldwide in a variety of plant species including many crops of considerable agricultural importance. The spread of highly polyphagous and fecund biotypes of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) has been associated to this emergence (Fargette et al. 2006; Jones 2009).
Tomato yellow leaf curl disease (TYLCD) is one of the most devastating viral diseases affecting tomato crops in warm and temperate regions of the world. Symptoms of the disease consist of upward curling of leaflet margins, reduction of leaflet area and some yellowing of young leaves. Infection also results in stunting, flower abortion and reduced plant growth. Epidemics of TYLCD can cause extensive crop losses (up to 100% if plants are infected during early growth). TYLCD was first reported in the late 1920s in the Jordan Valley (Cohen and Antignus 1994) and since then this disease has become one of the main factors limiting tomato production in many regions of Europe, Asia, Africa and more recently the Americas and Australia (for a review see Jones 2009). This disease is caused by a complex of begomovirus species referred to as Tomato yellow leaf curl viruses (TYLCVs) (Abhary et al. 2007) including the best known and emergent *Tomato yellow leaf curl virus* (TYLCV) (Jones 2009; Lefeuvre et al. 2010). These begomoviruses are transmitted in a circulative persistent manner by the whitefly *B. tabaci*. TYLCVs particles consist of two fused icosahedral capsids enclosing a unique circular single-stranded DNA (ssDNA) genome of about 2.8 kb that encodes six partially overlapping open reading frames (ORFs).

Epidemiological factors such as host range or vector transmission differences associated with TYLCD epidemics have been studied in temperate regions (Davino et al. 2006; Sánchez-Campos et al. 1999). Although local emergence and co-occurrence of multiple species were reported throughout the world (Delatte et al. 2005; Ueda et al. 2004), extensive studies in tropical environment remain scarce. Different scenarios are expected in such an epidemiological situation with competition: synergism, co-existence, displacement or emergence of new variants (Davino et al. 2006; Davino et al. 2009; Monci et al. 2002; Sánchez-Campos et al. 1999).

TYLCV was first reported in Reunion Island in 1997 as the causal agent of outbreaks of yellow leaf curl disease in tomato crops (Peterschmitt et al. 1999). Molecular characterization revealed the introduction of the Mild strain of TYLCV (TYLCV-Mld). The spread and molecular evolution of TYLCV-Mld in Reunion Island has been well documented from 1998 to 2004 (Delatte et al. 2007a; Delatte et al. 2007b). In 2004, severe symptoms of TYLCD, never seen before, were observed in the western part of the tomato cropping region. Molecular diagnosis revealed the introduction of the "Israel" strain also called "severe" strain of TYLCV (TYLCV-IL, Delatte et al. 2005). These two successive introductions into the same new ecological environment gave us the opportunity to study for the first time the invasion and competition of the two main strains of one of the most emergent virus species.

Here, we demonstrate that, over a seven-year period since its emergence in Reunion Island, the severe strain TYLCV-IL progressively displaced TYLCV-Mld which became predominantly recovered in mixed infections. We studied key biological traits that may be related to fitness differences between the two strains (within-plant virus accumulation, transmission efficiency).

Materials and Methods

Fields survey

Surveys were performed in nine locations of the main tomato-growing areas of Reunion Island (see supplemental figure 1) twice a year during the highest seasons of TYLCD epidemics (February-March and September-October, respectively at the end and the beginning of the hot-wet season). From 2004 to 2008, tomato plants (*Solanum lycopersicum*) exhibiting TYLCD symptoms from low to high severity were sampled so as to be representative of the observed severity. In 2009 and 2010, random samplings were performed every 5 m along a transect with 30 samples collected per sampling site regardless of symptomatology. All the samples were dehydrated using anhydrous calcium chloride and stored prior to DNA extraction (Bos 1977).

DNA extraction and TYLCV typing

Total DNA was extracted using the DNeasy Plant miniprep Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was resuspended in 100 µL (two successive elutions of 50 µL) of ultrapure water and stored at -20°C until utilization. To differentiate between TYLCV-IL and TYLCV-Mld, we used multiplex polymerase chain reaction (PCR) as described in Lefeuvre et al (2007).

Test for the presence of recombinant

To account for the possible emergence of a new TYLCV variant resulting from the recombination between TYLCV-IL and TYLCV-Mld, the viral replication-associated protein open reading frame (Rep ORF) of co-infected field samples was amplified and sequenced. Amplification with primers TYLCV1940F (5'-ACAACGAAATCCGTGAACAG-3') and TYLCV200R (5'-TTTTGCCTGTTCTGCTATCAC-3') for 30 PCR cycles of 94°C-30s, 55°C-30s and 72°C-60s with an initial denaturation step of 94°C-5min and a final amplification step of 72°C-5min produced 1100-bp fragments which were cloned into pGEM-T (Promega, France). *Escherichia coli* strain JM-109 (Promega, Paris, France) cells were then transformed with this plasmid. Recombinant plasmid DNA was isolated from bacteria with the Plasmid MiniPrep Spin Kit (Qiagen S.A., Courtaboeuf, France) according to manufacturer's instructions and sequenced using Macrogen Inc. sequencing service (Seoul, Korea). Seven and ten sequences were obtained from samples collected in 2008 and 2010 respectively. Additionally, sixteen full-length genomes from samples detected in 2010 as infected by TYLCV-IL (n=5), TYLCV-Mld (n=3) and both strains (n=4×2) from the different sampling sites were cloned and sequenced: circular viral DNA molecules were amplified using a TempliPhi kit (GE Healthcare) as described in Inoue-Nagata et al (2004). Full-length genomes were cloned into pGEM-3Zf or pGEM-T vectors digested with *Bam*HI or *Nco*I, respectively. *E. coli* strain JM-109 cells (Promega, Paris, France) were then transformed with this plasmid. Recombinant plasmid DNA was isolated as described above and sequenced using Macrogen Inc. sequencing service (Seoul, Korea). For both datasets (partial and full genomes), sequences were aligned along with the TYLCV-IL and TYLCV-Mld reference sequences from Reunion using the Clustal-W sub-alignment tool available in MEGA 4 before being checked for the presence of recombination (Tamura et al. 2007; Thompson et al. 1994).

Within-plant virus accumulation experiments

The within-plant virus accumulation of each TYLCV strain in tomato (cv. Farmer, Known-You Seed) was estimated by monitoring viral DNA accumulation in single and mixed infections using real-time PCR in the StepOnePlus PCR systems (Applied Biosystems, Courtaboeuf, France). Liquid cultures of *Agrobacterium tumefaciens* containing the agro-infectious clones of TYLCV-IL[RE4] and TYLCV-Mld[RE] (Delatte et al. 2005) were grown for 14 h and adjusted to an OD_{600nm} of 1.0 before inoculation. For mixed infection, equal amounts of *A. tumefaciens* containing TYLCV-IL[RE4] and TYLCV-Mld[RE] clones were mixed. Three sets of tomato plantlets at the three-leaf growth stage were agroinoculated with TYLCV-IL[RE4] and/or TYLCV-Mld[RE] by injecting 50 µL of *A. tumefaciens* culture into the stems. Plants were then maintained in an insect-proof greenhouse in a complete randomized block design.

The apex including the last three leaves of plants inoculated respectively by TYLCV-IL[RE4] (n=10 to 15), TYLCV-Mld[RE] (n=12 to 17) and both clones (n=20) were randomly sampled between 2 and 35 days post inoculation (see supplemental material Table 1 in annexes). Samples were dehydrated and ground with Tissue Lyser (Qiagen, Courtaboeuf, France); 20 mg were then collected for total DNA extraction as described above. Viral and genomic DNA were quantified using the real-time duplex PCR described elsewhere (Perefarres et al. 2011). Quantities of virus and of the internal reporter were calculated with the corresponding standard curves and the normalized viral load was defined as the ratio of the log of virus DNA quantity to the log of plant genomic DNA quantity (Mason et al. 2008).

Insect transmission assays

Non-viruliferous *B. tabaci* colonies (biotype B) were reared on cabbage plants (*Brassica oleracea*) in growth chamber at 25°C day and 20°C night, 70% relative humidity and a 16-h photoperiod. Synchronous female adults were caged onto tomato plants (cv. Farmer, Known-You Seed) inoculated by TYLCV-IL, TYLCV-Mld or both four weeks beforehand and were given a 72-h acquisition access period (AAP). To avoid bias on transmission efficiency estimates from mixed infections, viral load was estimated using real-time PCR as described above and only plants with ratio of the two TYLCV strains ranging from 0.95 to 1.05 were used. After the AAP, adults were collected individually and deposited on tomato plantlets (cv. Farmer, Known-You Seed) at the one-leaf growth stage for a 72-h inoculation access period (IAP). Only plantlets with living *B. tabaci* after the IAP were considered for the estimation of transmission efficiencies. Plantlets were then sprayed with insecticide and maintained in an insect-proof greenhouse. After five weeks, symptoms were scored and plants were tested for TYLCV-IL and/or TYLCV-Mld presence using multiplex PCR as described above (Lefeuvre et al. 2007). Two independent transmission experiments were performed for single infections, and three for mixed infections.

Within-insect viral quantification

Non-viruliferous *B. tabaci* colonies (biotype B) were reared on healthy cabbage plants (*Brassica oleracea*) in growth chamber at 25°C day and 20°C night, 70% relative humidity and a 16-h photoperiod. Ten synchronous female adults were caged onto tomato plants (cv. Farmer, Known-You Seed) inoculated by TYLCV-IL or TYLCV-Mld four weeks beforehand and were given a 72-h AAP. The within-insect viral load of each TYLCV strain was estimated by real-time PCR using the StepOnePlus PCR systems (Applied Biosystems, Courtaboeuf, France). Total DNA was extracted (Delatte et al. 2007c) and stored at -20°C until utilization. To standardize the viral load in insects, we quantified the amount of insect DNA by targeting a reporter gene,

the *Bemisia tabaci 18S ribosomal RNA* gene. We added 2µL of total DNA extraction to a 25 µL reaction mix comprising 1x TaqMan universal PCR master mix (Applied Biosystems, Foster City, USA), 44.4 nM F-IL/Mld-1445 (5'-GCCTGAGGAGCAGTGATGAGT-3'), 600 nM R-IL/Mld-1556 (5'-ACCAATAAGGCGTAAGCGTGTAG-3'), 150 nM MGB-IL/Mld-1471 (5'-TGTGCGTGAATCCA-3'), 266 nM F-Bt-18S (5'-CGGAGAGGGAGCCTGAGAA-3'), 266.6 nM R-Bt-18S (5'-CCGGGAGTGGGTAATTTGC-3') and 100 nM MGB-Bt-18S (5'-CGGCTACCACATCC-3'). Quantities of virus and of the internal reporter were calculated with the corresponding standard curves and the normalized viral load was defined as the ratio of the log of viral DNA quantity to the log of insect genomic DNA quantity.

Statistical analyses

All statistical analyses were performed using the R statistical software (R Development Core Team). Normalized viral load was monitored from 2 to 35 days post inoculation, and nonlinear regression analyses were performed for different models (logistic, Cauchy, probit, loglog and cloglog). Each of the models was used to fit the data and, on the basis of the likelihood, the best model was chosen. Using Akaike's Information Criterion (AIC), the loglog function appeared to be the most appropriate to model TYLCV replication *in planta*. In this model, written as Y ~ C exp(- exp(- A (X - B) + log(- log(0.5)))), the normalized TYLCV quantity (Y) depends on three biologically relevant parameters: A is the slope of the exponential phase, C is the viral load at the final plateau, and B is the time to reach 50% of this viral load. Once adjusted, the parameters of the logistic growth model were compared using likelihood ratio tests (LRT).

Transmission efficiencies were compared using chi-square tests under a generalized linear model (GLM) with the logit link function and a binomial underlying distribution. Tests plants of transmission experiments from mixed infected plants were divided into four categories: infected by both strains, infected by TYLCV-Mld (alone or mixed), infected by TYLCV-IL (alone or mixed), not infected. These proportions were then compared under a GLM with the logit link function and a Poisson underlying distribution. Viral accumulation within-insect were compared between strains using an ANOVA procedure.

The number of effectively transmitted viral genomes V was inferred using a maximum likelihood approach. For transmission tests from single-infected hosts, assuming that V has a Poisson distribution with mean λ; then, the maximum likelihood estimate for λ can be obtained from the null class of the Poisson

distribution: P(V=0)=exp(-λ), hence λ=-ln[P(V=0)], where the empirical estimate of P(V=0) is the proportion of non-infected test plants. For transmission tests from mixed-infected hosts, assuming that V_M for the Mild strain and V_I for the IL strain have independent Poisson distributions with means λ_M and λ_I, respectively. Among the n test plants, let d be the number of mixed infections, m the number of single infections by the Mild strain and i the number of single infections by the IL strain. The likelihood function for the experiment is thus proportional to:

$P(V_M \geq 1, V_I \geq 1)^d \times P(V_M = 0, V_I \geq 1)^i \times P(V_M \geq 1, V_I = 0)^m \times P(V_M = 0, V_I = 0)^{n-d-i-m}$.

The resulting log-likelihood function is:

$$L \propto (d+i)\ln(1-e^{-\lambda_I}) + (d+m)\ln(1-e^{-\lambda_M}) - \lambda_M(n-d-m) - \lambda_I(n-d-i).$$

Maximizing this function requires $\partial L/\partial\lambda_M=0$ and $\partial L/\partial\lambda_I=0$; hence the maximum likelihood estimates λ_M=-ln(1-(d+m)/n) and λ_I=-ln(1-(d+i)/n). Again, these estimates are related to the proportion of test plants not infected by each strain. Confidence intervals (CI) were derived using the profile-likelihood method.

Results

Rapid displacement of TYLCV-Mld by the newcomer TYLCV-IL

Table 1: Results of the survey conducted between 2004 and 2010 in Reunion Island and analysis of the samples for infection by *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and / or strain Israel (TYLCV-IL).

Season	Sampled sites	Sampling	Collected samples	Positive Samples (%)			Infected samples
				TYLCV-IL	TYLCV-Mld	Co-infected	
2003-2004	-	symptomatic	39	0	39 (100)	0	39
2004-2005	18	symptomatic	63	1 (3.8)	12 (46.2)	13 (50)	26
2005-2006	16	symptomatic	79	27 (43.5)	9 (14.5)	26 (41.9)	62
2006-2007	15	symptomatic	58	32 (62.7)	6 (11.8)	13 (25.5)	51
2007-2008	9	symptomatic	90	45 (71.4)	0 (0)	18 (28.6)	63
2008-2009	10	random	225	20 (57.1)	6 (17.1)	9 (25.7)	35
2009-2010	12	random	421	50 (72.5)	11 (15.9)	8 (11.6)	69

The repartition of both strains of TYLCV in Reunion Island was analysed from 975 samples collected between 2004 and 2010 in the main tomato-producing areas (Table 1). Whereas sampling in the first four years was based on symptomatology (i.e. only symptomatic plants were collected), random samplings were performed in 2009 and 2010, providing an estimate of disease prevalence. From these latter, 15%

(2009) and 16.4% (2010) of the samples were infected with at least one strain of TYLCV.

Since the introduction of the TYLCV-IL strain, the evolution of the proportion of both strains in field samples clearly indicates a displacement of TYLCV-Mld by TYLCV-IL (Figure 1). Whereas TYLCV-Mld was dominant in 2004, it progressively decreased in prevalence, with an increasing incidence of TYLCV-IL. If the symptom-based sampling strategy could have somehow biased the analysis with a preferential collection of one strain compared to the other, the extensive random samplings performed from 2009 to 2010 confirmed the displacement of the Mld strain, with 72.5% of the TYLCV-positive samples infected with the IL strain alone, 15.9% by the Mld strain alone, and 11.6% co-infected.

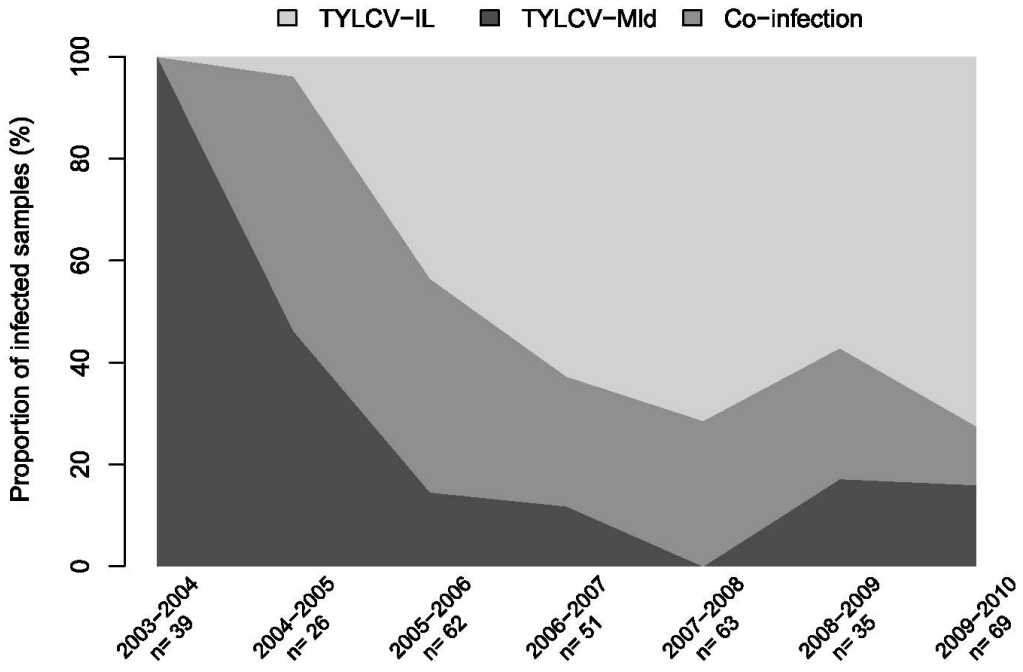


Figure 1: Dynamics of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL) in Reunion Island from 2004 to 2010

Whereas TYLCV-Mld was rapidly displaced by TYLCV-IL, it is interesting to notice that it persists and predominantly in mixed infections (Figure 1, Table 1). Because mixed infections were frequent and begomoviruses are known to be highly recombinogenic, it was important to determine whether a new recombinant between TYLCV-Mld and TYLCV-IL had appeared and reached a significant prevalence. Put it another way, we determined if the viruses recovered from the field were still the TYLCV-IL and TYLCV-Mld strains introduced in Reunion or the progeny of their recombination. As the Rep

ORF is the only portion of the genome allowing for a clear differentiation between these strains (Navas-Castillo et al. 2000), we cloned and sequenced this ORF from seven and ten samples collected in 2008 and 2010 respectively. None of the sequenced samples presented any recombination pattern and all the sequences were of TYLCV type. These analyses were repeated in 2010 with full-length genomes and confirmed the previous results with all sequences being fully of TYLCV-IL type (n=9) or TYLCV-Mld type (n=7). This indicates that a new recombinant viral strain is not likely to be prevalent in Reunion Island on tomato crops and that the Mild strain of TYLCV was displaced by the IL strain and not by a recombinant strain.

TYLCV-IL is better at replicating within the plant alone but not in competition

Within-plant accumulation of the two TYLCV strains was evaluated in agro-inoculated tomato plants using real time PCR assays. We used a completely randomized block design to avoid any risk of block effects due to differential environmental factors that might interfere with plant physiology and thus viral accumulation. Viral accumulation kinetics were measured in the three youngest (apical) leaves as a closely related begomovirus accumulates evenly among these leaves (Mason et al. 2008). Preliminary studies revealed that the kinetics of viral accumulation fitted a logistic growth-model, characterized by an exponential increase in viral load before reaching a plateau.

To accurately infer the model parameters, we decided to maximize the number of samples collected during the first days post inoculation. GNLS models were then fitted to the experimental data and high correlation coefficients of 0.991 and 0.985 were obtained for TYLCV-Mld and TYLCV-IL models respectively. The parameters of the logistic growth model (Table 2) were compared using likelihoods ratio tests (LRT), and it was clearly apparent that TYLCV-IL accumulates faster than TYLCV-Mld (50% of the viral load at the plateau reached 0.8 days before TYLCV-Mld, $p<10^{-8}$) and to a higher level (5.9 fold, $p=10^{-7}$).

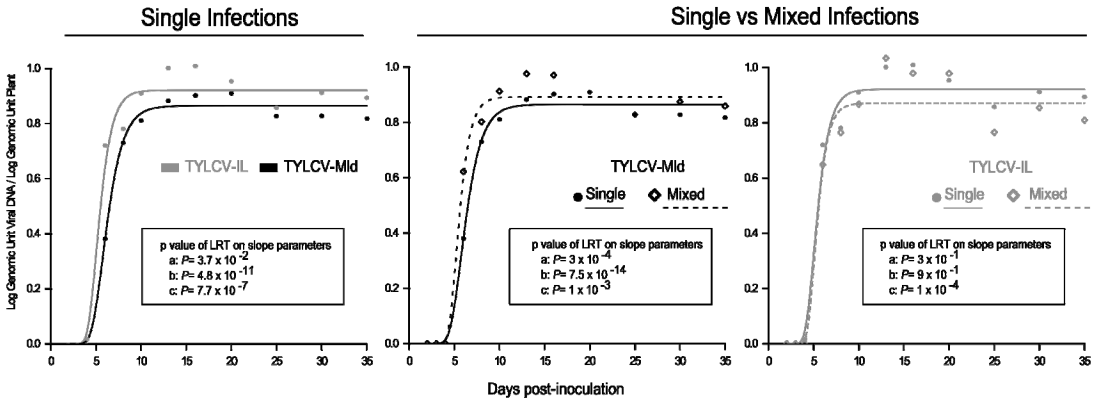


Figure 2: Estimated (solid) and mean (dot) normalized viral load after agro-inoculations of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and / or Israel (TYLCV-IL) in tomato plants.

When it came to within-plant accumulation in mixed infections it appeared that TYLCV-Mld accumulates to a higher viral load (2 fold, p=0.001) and faster than in single infection, reaching 50% of the maximum viral load 0.7 days earlier ($p<10^{-8}$). In contrast, TYLCV-IL accumulates less in mixed infection (3.5 fold, $p=1x10^{-4}$) than in single infection (no difference was found in the rate of viral accumulation). Moreover, in mixed infections none of the three parameters was different between TYLCV-IL and TYLCV-Mld, reflecting similar within-plant accumulations.

Table 2: Estimated parameters (confident intervals at 95%) of the logistic growth models of within-plant accumulation of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL) in single and mixed infections.

Infection	Virus	*Loglog* model parameters		
		A	B	C
Single	TYLCV-IL	1,06 (0,18)	5,38 (0,2)	0,92 (0,02)
	TYLCV-Mld	0,81 (0,11)	6,21 (0,12)	0,86 (0,01)
Mixed	TYLCV-IL	1,25 (0,29)	5,4 (0,19)	0,87 (0,02)
	TYLCV-Mld	1,19 (0,21)	5,52 (0,14)	0,89 (0,01)

TYLCV-IL is better transmitted than TYLCV-Mld in single but not in mixed infections

The rapid spread of TYLCV on tomato crops in Reunion Island was clearly associated with the proliferation of the invasive biotype B of *B. tabaci* (Delatte et al. 2006). The transmission experiments with individual synchronous female adults were performed twice independently from plants in single infections. Significant differences were found between the two TYLCV strains with transmission efficiencies of 82.9% and 91.0% for TYLCV-IL and 59.0% and 65.8% for TYLCV-Mld in the two experiments

($p=6x10^{-4}$, Table 3). In line with these results, the mean numbers of efficiently transmitted genomes were also significantly different: 1.0 (CI: 0.71–1.4) for TYLCV-Mld and 2.0 (CI: 1.4–2.8) for TYLCV-IL. As we revealed that TYLCV-IL accumulates more in tomato plants, it could be argued that the difference observed in the transmission efficiency is mainly due to a difference in *B. tabaci* ability to acquire the two TYLCV strains. However, after an AAP of 72 h, no significant differences were found in the viral load between the two strains in *B. tabaci* biotype B ($p=0.87$, Figure 3), indicating that the higher transmission rate of the IL strain results from a more efficient inoculation or initiation of infection.

Table 3: Results of transmission experiments conducted from tomato to tomato with *Bemisia tabaci* adult females from colonies of biotypes B.

IAP (days)	Single infections		Mixed infections		
	TYLCV-Mld	TYLCV-IL	TYLCV-Mld	TYLCV-IL	Both
<3	23/38	11/13	15/108	17/108	73/108
3	38/60	45/52	9/95	11/95	68/95

Transmission experiments were performed three times independently with individual synchronous female adults of biotype B of *B. tabaci* fed on co-infected plants with the same amounts of TYLCV-IL and TYLCV-Mld. This experiment resulted in significantly ($p<10^{-4}$ in each case) more plants co-infected (75.8%) than infected by either TYLCV-IL alone (12.3%) or TYLCV-Mld alone (11.9%). Moreover no difference ($p=0.996$) was found between the proportion of plants infected by TYLCV-IL alone or TYLCV-Mld alone (Table 3). Congruently, the mean numbers of efficiently transmitted genomes were very similar in these experiments: 1.7 (CI: 1.3–2.1) for TYLCV-Mld and 1.7 (CI: 1.3–2.2) for TYLCV-IL.

Regarding the transmission efficiencies, it appeared that TYLCV-Mld is significantly better transmitted from mixed infected plants than in single infection with in average transmission efficiencies of 61% and 89% respectively ($p=6.2 \times 10^{-6}$). In contrast, the transmission efficiencies of TYLCV-IL from single or mixed infected plants were similar (86% and 88% respectively, $p=0.628$).

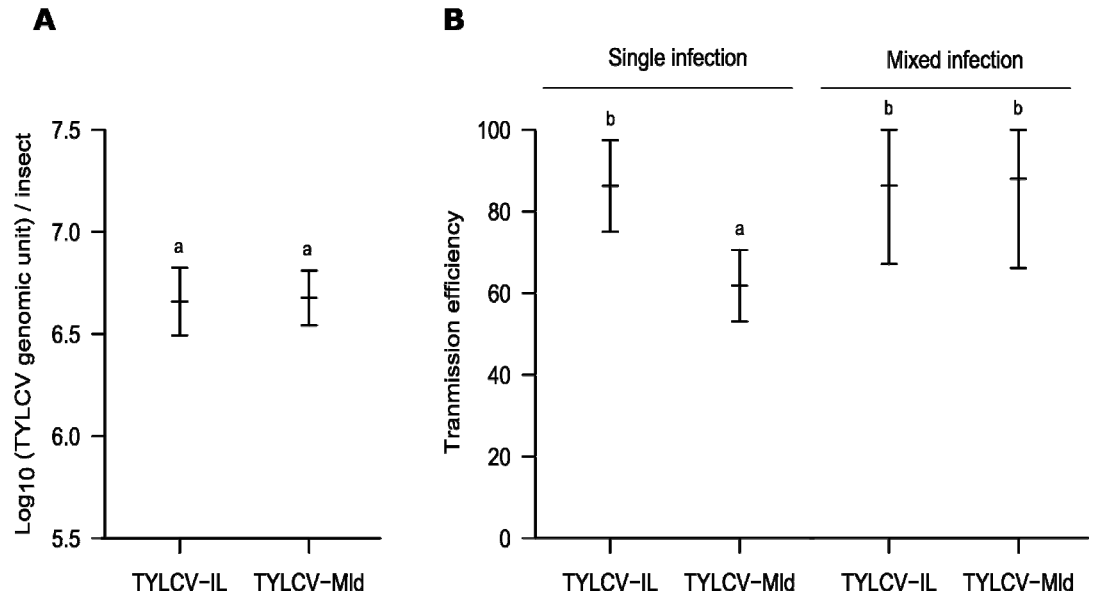


Figure 3. **A**: Logarithm of the number of viral genomes of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL) per individual *Bemisia tabaci* estimated by quantitative polymerase chain reaction after a 72h acquisition access period. Vertical bars around each point represent the 95% confidence interval and the bars topped by the same letter are not significantly different at p = 0.01 (Fisher-Snedecor's test). **B**: Results of transmission experiments conducted from tomato infected by TYLCV-Mld and / or TYLCV-IL with *B. tabaci* adult females from colonies of biotypes B. Vertical bars around each point represent the 95% confidence interval and the bars topped by the same letter are not significantly different at p = 0.01 (Tukey's all-pair comparison test).

Discussion

Identifying the factors involved in pathogen emergence and evaluating their relative roles is a prerequisite to understand why some pathogens are most likely to become successful invaders and deploy efficient disease management strategies.

The ongoing spread of TYLCV is one of the main threats to tomato production in all temperate parts of the world and TYLCV (IL and Mld strain) is still expanding its distribution area (Lett et al. 2011; Stonor et al. 2003; Zambrano et al. 2007). After the introduction in 1998 of TYLCV-Mld in the tropical and insular environment of Reunion Island, the recent introduction of the TYLCV-IL gave us the unique opportunity to follow and study the competition between the two main strains of one of the most emergent plant virus. Whereas their prevalence remains lower than those revealed in other similar epidemics (Sánchez-Campos et al. 1999), these two viral strains nevertheless became the main factor limiting tomato production, with up to 80% of losses in cultures (Reynaud et al. 2003, Rimbaud et al. 2012), leading to the progressive abandonment of the open field culture system. In Reunion Island,

these two emergent strains of TYLCV were introduced successively, and the inter-strain competition led to the rapid displacement of TYLCV-Mld by the newcomer TYLCV-IL

In order to understand the ecological and evolutionary factors providing the IL strain with a selective advantage over the Mld strain, we examined two factors that might explain the observed shift of viral populations: i) within-plant accumulation in their main host, ii) transmission efficiency by *B. tabaci*. These factors were studied both in single-infections and in mixed infections.

Our study revealed that when in single infection in its principal host, TYLCV-IL perform better in both transmission and replication resulting in the preferential natural spread that we observed during our epidemiological surveys. Surprisingly, Ohnishi *et al* did not report any differences in experimental transmission efficiencies between Japanese isolates of Mld and IL strains of TYLCV (72 and 75% respectively) that could be explained by differences in experimental design and/or *B. tabaci* populations (Ohnishi et al. 2010).

The correlation between transmission efficiency and within-plant accumulation is generally accepted although studies on circulative and propagative viruses remain scarce (Froissart et al. 2010). Here, as the difference in within-plant accumulation between TYLCV-Mld and TYLCV-IL did not affect their acquisitions and accumulations by *B. tabaci*, the variation in the ability to be inoculated or to initiate an infection seemed therefore primordial. Whereas each vector bears around seven millions viral genomes, our estimations of the number of efficiently transmitted viral genomes upheld this better ability of TYLCV-IL compared to TYLCV-Mld (mean estimates of 2 and 1 effectively transmitted viral particle respectively). Our estimations are surprisingly low and similar to those calculated for viruses transmitted in a non-persistent manner (Betancourt et al. 2008; Moury et al. 2007). These original data bolster the implication of random genetic drift in the evolution of viral population and will be of primary interest in the design of viral evolution models of viruses transmitted in a circulative permanent manner.

Another striking characteristic of the field survey was the persistence of TYLCV-Mld and predominantly in mixed infections promoting virus-virus interactions (Figure 1, Table 1). Mixed infections of plant viruses are common in nature, and a number of important virus diseases of plants are the outcomes of interactions between causative agents (Fabre et al. 2010; Gómez et al. 2009; Pita et al. 2001). Multiple infections can lead to a variety of intra host virus–virus interactions with dramatic sanitary consequences and possibly promoting changes in the genetic structure of the viral population.

Since recombination is a major factor driving begomoviruses evolution (Padidam et al. 1999; Seal et al. 2006), it was intriguing to investigate the presence of a new variant resulting from the recombination between the two strains of TYLCV. Our results indicate that the predominant viruses circulating in Reunion Island are still of parental type. If genetic exchanges had occurred new recombinant variants may have been outcompeted by the parental and thus been eliminated from the population after purifying selection (Escriu et al. 2007).

In addition to recombination, interactions between the two co-occurring viruses can have strong epidemiological consequences on the modulation of biological traits, with phenomena of synergism or interference (Alves-Júnior et al. 2009; Fondong et al. 2000; Martin and Elena 2009). It was therefore relevant to determine if the mixed infections could hamper and/or improve the biological traits related to viral fitness of the two strains. Within-plant accumulation of the two strains was hence compared in single and mixed infections. Importantly, both within-plant accumulations kinetics were affected by the presence of a competitor. On the one hand, TYLCV-Mld could benefit from the presence of TYLCV-IL to replicate better (i.e faster and more) than alone; on the other hand TYLCV-IL was less efficient in replicating, resulting in a drop in viral load compared to the single infection. In these experiments, recombination events were also considered to avoid false interpretations. Several observations strongly support that its impact in our experimental design is negligible. First, as recombination is a stochastic event, it is not supposed to affect systematically all the replicates and thus affect our experimental design. Secondly, Garcia-Andrés *et al* estimated the recombinant frequencies in a begomovirus population during a mixed infection between TYLCSV and TYLCV and never observed recombinants at 30 and even 60 dpi (García-Andrés et al. 2007).

The molecular basis of the assistance conferred by TYLCV-IL to TYLCV-Mld remains nevertheless to be explored. Considering the plant as an ecological niche, the reduction of the maximum viral load for TYLCV-IL in mixed infection with TYLCV-Mld could be due to the depletion of a limited resource implying therefore the limitation of the population growth (Polechova and Storch 2008). Morilla *et al* (2004) demonstrate with two related begomoviruses that the number of phloem nuclei cells attainable and/or susceptible to the virus was similar (1.5%) in both single and mixed infections (Morilla et al. 2004). In mixed infection, we can hypothesize that the cells infected by TYLCV-Mld were thus not available for TYLCV-IL which could not accumulate as much as in single infection.

In complement to within-plant accumulation, effects of the mixed infection were also evaluated for the transmission by *B. tabaci*. These experiments confirmed the ability of *B. tabaci* to acquire and transmit successfully these two strains of TYLCV (Ohnishi

et al. 2010) and predominantly both strains simultaneously after an acquisition from plants with the same amount of TYLCV-IL and TYLCV-Mld. Considering the transmission efficiencies, whereas TYLCV-Mld appeared to be better transmitted from co-infected plants than from single-infected plants, the presence of TYLCV-Mld did not significantly hinder the transmission of TYLCV-IL in comparison to single-infected plants. Taken together, these observations highlight the benefits of the mixed infections for the natural spread of TYLCV-Mld. Although out-competed and displaced in a short period, TYLCV-Mld could take advantage of TYLCV-IL to persist in Reunion Island while the expectation was its rapid extinction. In the mean time, this assistance to TYLCV-Mld could have limited the spread of TYLCV-IL, but interestingly, despite a drop in viral load in mixed infected plants, its transmission efficiency by *B. tabaci* is not significantly impaired. Overall, this should result in an increased prevalence for the TYLCD in the landscape, and a longer time before the complete extinction of the Mild strain.

A shift in viral populations could also involve other epidemiological factors. An extended host range could have contributed to this displacement like reported in others studies (Gómez et al. 2009; Sánchez-Campos et al. 1999). However, in Reunion Island, since tomato is cultivated all year long, TYLCV does not need alternative hosts throughout the year. In addition, a field survey in Florida also indicated the absence of alternative hosts (Adkins et al. 2011). It would be of particular interest to build epidemiological models derived from our experimental and field data to assess the relevance of our compared parameters (Jeger et al. 2011).

In conclusion, while most attention in virology research has traditionally been given to properties of individual virus species (Syller 2011), our study highlights complex interplays between two strains of one of the most emergent plant virus following their successive introductions in the insular and tropical environment of Reunion Island. These interactions led to the maintenance of residual TYLCV-Mld infections which should be taken into account for virus control and resistant-cultivar deployment.

Acknowledgments

The authors thank M. Grondin, D. Fontaine and J. Vuillemot for their excellent technical assistance. This study was funded by the CIRAD and the FEDER. F. Péréfarres is a recipient of a Phd fellowship from the CIRAD.

References

Abhary M, Patil B, Fauquet C (2007) Molecular Biodiversity, Taxonomy, and Nomenclature of Tomato Yellow Leaf Curl-like Viruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 85-118

Adkins S, Webster CG, Kousik CS, Webb SE, Roberts PD, Stansly P a, Turechek WW (2011) Ecology and management of whitefly-transmitted viruses of vegetable crops in Florida. Virus Research 159:110-114.

Alves-Júnior M, Alfenas-Zerbini P, Andrade EC, Esposito D a, Silva FN, F da Cruz AC, Ventrella MC, Otoni WC, Zerbini FM (2009) Synergism and negative interference during co-infection of tomato and Nicotiana benthamiana with two bipartite begomoviruses. Virology 387:257-66.

Anderson PK, Cunningham A a, Patel NG, Morales FJ, Epstein PR, Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends in Ecology & Evolution 19:535-44.

Betancourt M, Fereres A, Fraile A, García-Arenal F (2008) Estimation of the effective number of founders that initiate an infection after aphid transmission of a multipartite plant virus. Journal of Virology 82:12416-12421.

Bos L (1977) Persistence of infectivity of three viruses in plant material dried over CaCl2 and stored under different conditions. Netherlands Journal of Plant Pathology 83:217-220.

Cohen S, Antignus Y (1994) Tomato yellow leaf curl virus, a whitefly-borne geminivirus of tomatoes. Advances in Diseases Vector Research 10:259-288.

Davino S, Napoli C, Davino M, Accotto GP (2006) Spread of Tomato yellow leaf curl virus in Sicily: partial displacement of another geminivirus originally present. European Journal of Plant Pathology 114:293-299.

Davino S, Napoli C, Dellacroce C, Miozzi L, Noris E, Davino M, Accotto GP (2009) Two new natural begomovirus recombinants associated with the tomato yellow leaf curl disease co-exist with parental viruses in tomato epidemics in Italy. Virus Research 143:15-23.

Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM (2005) The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Reunion Island. Plant Pathology 54:262-262.

Delatte H, David P, Granier M, Lett J, Goldbach R, Peterschmitt M, Reynaud B (2006) Microsatellites reveal extensive geographical, ecological and genetic contacts between invasive and indigenous whitefly biotypes in an insular environment. Genetical Research 87:109–124.

Delatte H, Holota H, Moury B, Reynaud B, Lett J-M, Peterschmitt M (2007a) Evidence for a founder effect after introduction of Tomato yellow leaf curl virus-mild in an insular environment. Journal of molecular evolution 65:112-8.

Delatte H, Lett J, Lefeuvre P, Reynaud B, Peterschmitt M (2007b) An insular environment before and after TYLCV introduction. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 13-23

Delatte H, Reynaud B, Granier M, Thornary L, Lett JM, Goldbach R, Peterschmitt M (2007c) A new silverleaf-inducing biotype Ms of Bemisia tabaci (Hemiptera: Aleyrodidae) indigenous to the islands of the south-west Indian Ocean. Bulletin of Entomological Research.

Escriu F, Fraile A, Garcı F (2007) Constraints to Genetic Exchange Support Gene Coadaptation in a Tripartite RNA Virus. PLoS Pathogens 3(1): e8.

Fabre F, Chadoeuf J, Costa C, Lecoq H, Desbiez C (2010) Asymmetrical over-infection as a process of plant virus emergence. Journal of Theoretical Biology 265:377-88.

Fargette D, Konaté G, Fauquet C, Muller E, Peterschmitt M, Thresh JM (2006) Molecular ecology and emergence of tropical plant viruses. Annual Review of Phytopathology 44:235-60.

Fondong VN, Pita JS, Rey ME, de Kochko a, Beachy RN, Fauquet CM (2000) Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. The Journal of General Virology 81:287-97.

Froissart R, Doumayrou J, Vuillaume F, Alizon S, Michalakis Y (2010) The virulence–transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. Philosophical Transactions of the Royal Society B: Biological Sciences 365:1907-1918.

García-Andrés S, Tomás DM, Sánchez-Campos S, Navas-Castillo J, Moriones E (2007) Frequent occurrence of recombinants in mixed infections of tomato yellow leaf curl disease-associated begomoviruses. Virology 365:210-9.

Gillespie RG, Roderick GK (2002) Arthropods in islands: Colonization, Speciation, and Conservation. Annual Review of Entomology 47:595-632.

Gómez P, Sempere RN, Elena SF, Aranda M a (2009) Mixed infections of Pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. Journal of Virology 83:12378-87.

Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage [phi]29 DNA polymerase. Journal of Virological Methods 116:209-211.

Jeger MJ, van den Bosch F, Madden LV (2011) Modelling virus- and host-limitation in vectored plant disease epidemics. Virus Research 159:215-222.

Jones RAC (2009) Plant virus emergence and evolution: origins, new encounter scenarios, factors driving emergence, effects of changing world conditions, and prospects for control. Virus Research 141:113-30.

Lefeuvre P, Hoareau M, Delatte H, Reynaud B, Lett J-M (2007) A multiplex PCR method discriminating between the TYLCV and TYLCV-Mld clades of tomato yellow leaf curl virus. Journal of Virological Methods 144:165-8.

Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJ a., Meredith S, Lakay F, Monjane A, Lett J-M, Varsani A, Heydarnejad J (2010) The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World. PLoS Pathogens 6:e1001164.

Lett J-M, Péréfarres F, Hoareau M, Lefeuvre P, De Bruyn A, Dottin M, Prior P, Wicker E, Umaharan P (2011) Tomatoes showing yellow leaf curl symptoms in the island of Grenada exhibit an infection with Tomato yellow leaf curl virus either alone or in combination with Potato yellow mosaic virus. New Disease Reports 24:19.

Martin S, Elena SF (2009) Application of game theory to the interaction between plant viruses during mixed infections. Journal of General Virology 90:2815-2820

Mason G, Caciagli P, Accotto GP, Noris E (2008) Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci. Journal of Virological Methods 147:282-289.

Monci F, Sanchez-Campos S, Navas-Castillo J, Moriones E (2002) A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. Virology 303:317-326.

Morilla G, Jeske H, Bejarano ER, Wege C (2004) Tête à Tête of Tomato Yellow Leaf Curl Virus and Tomato Yellow Leaf Curl Sardinia Virus in Single Nuclei. Journal of Virology 78:10715-10723.

Moury B, Fabre F, Senoussi R (2007) Estimation of the number of virus particles transmitted by an insect vector. Proceedings of the National Academy of Sciences of the United States of America 104:17891-6.

Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto GP, Moriones E (2000) Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. Journal of General Virology 81:2797-2801.

Ohnishi J, Kitamura T, Terami F, Honda K-ichiro (2010) Co-transmission of Tomato yellow leaf curl virus (TYLCV)-Mld and TYLCV-IL by the whitefly Bemisia tabaci. Journal of General Plant Pathology 77:54-59.

Padidam M, Sawyer S, Fauquet CM (1999) Possible emergence of new geminiviruses by frequent recombination. Virology 265:218-25.

Perefarres F, Hoareau M, Chiroleu F, Reynaud B, Dintinger J, Lett J-M (2011) A novel synthetic quantification standard including virus and internal report targets: application for the detection and quantification of emerging begomoviruses on tomato. Virology journal 8:389.

Peterschmitt M, Granier M, Mekdoud R, Dalmon A, Gambin O, Vayssieres JF, Reynaud B (1999) First Report of Tomato Yellow Leaf Curl Virus in Réunion Island. Plant Disease 83:303-303.

Pita JS, Fondong VN, Sangaré A, Otim-Nape GW, Ogwal S, Fauquet CM (2001) Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. The Journal of General Virology 82:655-65.

Polechova J, Storch D (2008) Ecological Niche. In: Ecology BFBT-E of (ed)Academic Press, Oxford, pp 1088-1097

Reynaud B, Wuster G, Delatte H, Soustrade I, Jean-Michel L, Gambin O, Peterschmitt M (2003) Les maladies à bégomovirus chez la tomate dans les départements français d'Outre-Mer - Le Tomato yellow leaf curl virus (TYLCV) à la Réunion. Phytoma, la défense des végétaux 13-17.

Rimbaud L, Cottineau JS, Avril JP, Sorres V, Festin C, Minatchy J, Suzanne W, Leroux K, Tilma P, Maillary L, Dupuis S, Hostachy B, Lett JM (2012) Le Tomato yellow leaf curl virus à La Réunion 10 ans après les premières épidémies : Impact sur la filière tomate, état des lieux et principales méthodes de lutte. Phytoma, la défense des végétaux (sous presse).

SYLLER J (2011) Facilitative and antagonistic interactions between plant viruses in mixed infections. Molecular Plant Pathology (sous presse).

Seal SE, Jeger MJ, Van den Bosch F (2006) Begomovirus Evolution and Disease Management. In: Karl Maramorosch AJS and JMTBT-A in VR (ed) Advances in Virus Research. Academic Press, pp 297-316

Stonor J, Hart P, Gunther M, DeBarro P, Rezaian M a. (2003) Tomato leaf curl geminivirus in Australia: occurrence, detection, sequence diversity and host range. Plant Pathology 52:379-388.

Sánchez-Campos S, Navas-Castillo J, Camero R, Soria C, Díaz J a, Moriones E (1999) Displacement of Tomato Yellow Leaf Curl Virus (TYLCV)-Sr by TYLCV-Is in Tomato Epidemics in Spain. Phytopathology 89:1038-43.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24:1596-1599.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-80.

Ueda S, Kimura T, Onuki M, Hanada K, Iwanami T (2004) Three distinct groups of isolates of Tomato yellow leaf curl virus in Japan and construction of an infectious clone. Journal of General Plant Pathology 70:232-238-238.

Zambrano K, Carballo O, Geraud F, Chirinos D, Fernández C, Marys E (2007) First Report of Tomato yellow leaf curl virus in Venezuela. Plant Disease 91:768-768.

Chapitre III :
Spectre d'action des principaux gènes de résistance aux bégomovirus et caractérisation d'une résistance récessive.

Dans le cas d'une infection virale, l'éventail des méthodes de lutte demeure restreint et notamment aucune méthode curative applicable en condition de culture n'est actuellement disponible. En complément de la prophylaxie, l'utilisation de cultivars résistant est le meilleur moyen pour assurer un contrôle efficace des bégomovirus tout au long du cycle de production.

Dans le cas des bégomovirus, aucune source de résistance n'a été reportée dans l'espèce *S. lycopersicum* et les recherches se sont étendues aux espèces sauvages apparentées à la tomate cultivée (Pilowsky & Cohen 1974). Les recherches de sources naturelles de résistance au TYLCV ainsi qu'à d'autres bégomovirus infectant la tomate ont été très nombreuses depuis les années 1970 et ont pu mettre en évidence des accessions principalement issues de *Solanum chilense*, *Solanum habrochaites*, *Solanum pimpinellifolium*, *Solanum peruvianum* (pour revue Ji et al. 2007).

Une des principales difficultés dans le développement de cultivars résistants aux bégomovirus réside dans la grande diversité virale à contrôler. Dans un schéma de sélection, le pyramidage de gènes est une stratégie efficace pour augmenter le niveau et étendre le spectre d'action de la résistance. Cette stratégie repose toutefois sur la complémentarité des gènes de résistance combinés face à la diversité virale à contrôler.

Ce chapitre présente l'exploration de la spécificité d'action des principaux gènes de résistance utilisés dans les programmes de création variétale. Pour cela, notre étude a confronté différentes lignées portant les gènes de résistance *Ty-1*, *Ty-2* ou *Ty-3* à un vaste panel représentant la diversité naturelle des bégomovirus incluant des bipartites (PYMV) et monopartites (ToLCKMV et TYLCV). Ce chapitre présente également la caractérisation d'une nouvelle source de résistance originaire de *S. pimpinellifolium*. Notre étude s'est attachée à décrire non seulement les bases génétiques de cette résistance mais également son spectre d'action vis-à-vis de notre vaste panel de bégomovirus.

Ce volet de la thèse a permis de dessiner les contours de la spécificité de la résistance conférée par ces différents gènes de résistance et émettre des hypothèses sur les bases moléculaires sous jacentes. De plus, compte tenu des différences d'expression de la résistance de ces différentes accessions, notre étude servira aux sélectionneurs à orienter les programmes de création en combinant efficacement les gènes issus de *S. chilense, S. habrochaites et S. pimpinellifolium*.

Resistance to begomoviruses: phenotype and spectrum of action of a novel recessive resistance and the main resistance genes.

Péréfarres Frédéric[1,2], Hoareau Murielle[1], Moretti André[3], Chiroleu Frédéric[1] , Lett Jean-Michel[1], Reynaud Bernard[1] and Dintinger Jacques[1].

1 CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France

2 Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France

3 INRA, UR1052, Génétique et Amélioration des Fruits et Légumes, Génétique et amélioration des plantes, Centre de Recherche PACA, 84143 Montfavet Cedex, France.

Abstract

Begomovirus is a genus of phytopathogenic single-stranded DNA viruses, transmitted by the whitefly *Bemisia tabaci*. This genus includes emerging and economically significant viruses and notably the *Tomato yellow leaf curl virus* which have reached a worldwide distribution. In addition to TYLCV, a wide range of begomoviruses are associated with the tomato yellow leaf curl disease. Control measures are often limited in viral infections and mainly rely on the use of resistant cultivars. Because of the great viral diversity to control plant breeders face a tremendous challenge to develop broad spectrum resistance. This study reports the evaluation of the spectrum of action of the main resistance genes used by seed companies confronted to the TYLCV (Mld and IL strains), *Tomato leaf curl Comoros virus* (ToLCKMV) and the *Potato yellow mosaic virus* (PYMV). Besides, we characterized a novel source of resistance originating from *Solanum pimpinellifolium* to assess its value in the diversification of resistance sources. Whereas the line carrying *Ty-2* exhibited a specific resistance to TYLCV-IL, lines carrying *Ty-1*, *Ty-3* and *Ty-3a* expressed a broad tolerance to the wide panel of begomoviruses. Similarly, LA2187-5 exhibited a broad resistance to this panel of viruses, although the conferred level was not sufficient under high inoculation pressure with TYLCV-IL. Our study also revealed the recessive monogenic basis of the resistance conferred to PYMV by LA2187-5. This resistance appears therefore to be a good candidate for further use in breeding programs for the diversification of resistance sources.

Keywords

Begomovirus, resistance, tomato yellow leaf curl disease, spectrum of action, recessive resistance

Introduction

Begomoviruses (genus *Begomovirus*, Family *Geminiviridae*) is a group of phytopathogenic viruses transmitted by the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). Since the early 1990s, begomoviruses have emerged worldwide causing diseases in a wide variety of plants of considerable agricultural importance and become one of the most destructive groups of plant viruses. The spread and proliferation of highly polyphagous and fecund biotypes of *B. tabaci* have greatly promoted this emergence (Fargette et al. 2006).

Tomato yellow leaf curl disease (TYLCD) is among the most devastating diseases of tomato (*Solanum lycopersicum* L.) crops in sub-tropical and tropical areas worldwide. Its ongoing spread represents a major threat to the sustainable tomato production. Symptoms of the disease are upward curling of leaflet margins, reduction of leaflet area and some yellowing of young leaves. Infection results also in stunting, flower abortion, decrease in plant growth. Epidemics of TYLCD can cause extensive crop losses with up to 100% when plants are infected at early stage (Picó 1996).

Begomovirus genomes consist of monopartite or bipartite components of circular single strand DNA (ssDNA) (for a review see Jeske 2009). Based on their genome organization, their genetic diversity, and their geographical distribution, begomoviruses have been divided into two groups: Old World (Africa, Asia, Australia and Europe) and New World (North and South America) begomoviruses (Abhary et al. 2007). Although no native monopartite begomovirus from the New World has been described, the *Tomato yellow leaf curl virus* (TYLCV), a monopartite begomovirus, was accidentally introduced into America, and is now widespread in North America, Central America and the Caribbean (Duffy and Holmes 2007). Additionally, TYLCV has greatly extended its distribution area in the Old World, which now stretches from Spain (west) to Japan (east) and to Reunion Island and Australia in the south (recently reviewed in Lefeuvre et al. 2010).

The control of tomato-infecting begomoviruses based on the control of whitefly populations including cropping practices and insecticide treatments is often ineffective in limiting viral spread and has raised environmental concerns (Elbert and Nauen 2000). Breeding for resistant cultivars has become the most desirable strategy to control the tomato-infecting begomoviruses. Unfortunately, no resistance to begomoviruses within the germplasm of *S. lycopersicum* has been reported so far (Pilowsky and Cohen 1974). The screenings were subsequently extended to wild species related to the cultivated tomato and lead to the discovery of resistance sources in accessions mainly originating from *S. chilense, S. habrochaites, S. pimpinellifolium and S. peruvianum* (for a review see Ji et al. 2007b).

The most widely used resistance gene *Ty-1* is derived from *S. chilense* accession LA1969 and is already deployed in large cropping areas (García-Andrés et al. 2009). Additional begomovirus resistance genes were then mapped in wild species and include *Ty-2* from *S. habrochaites* accession H24 (Hanson et al. 2000), *Ty-3* (accession LA2779)*, Ty-3a* and *Ty-4* (accession LA1932) from *S. chilense* (Ji et al. 2007a; Ji et al. 2009) and finally *Ty-5* from *S. peruvanium* accession TY172 (Anbinder et al. 2009). Recently, additional unmapped recessive genes have also been reported from accessions originating from *S. pimpinellifolium* (Boissot et al. 2008; de Castro et al. 2008) and derived from the commercial hybrid 'Tyking' (Bian et al. 2007; García-Cano et al. 2008; Giordano et al. 2005).

Because of the great viral diversity encountered, even at the field level where co-occurrence of multiple begomoviruses is common (Castillo-Urquiza et al. 2008; Lefeuvre et al. 2007b), plant breeders face a tremendous challenge to develop broad spectrum resistance. The combining of resistance genes (also called genes pyramiding) is a useful strategy to extend the spectrum of action of hybrid cultivars (Mejía et al. 2005; Singh et al. 2000; Vidavski et al. 2008). To achieve this aim, it is important to challenge the resistance genes in multi-site screenings or to different tomato-infecting begomoviruses. Despite studies that report some limits of the *Ty-1* resistance gene against diverse tomato-infecting begomoviruses (Boiteux et al. 2007; Mejía et al. 2005), such studies with inbred lines carrying other resistance genes remain scarce.

This study reports the analysis of the spectrum of action of elite cultivars carrying the main resistance genes used by seed companies in their breeding programs (i.e *Ty-1*, *Ty-2*, *Ty-3* and *Ty-3a*). The resistance evaluation was carried out with agro-inoculations of a wide panel of begomoviruses so as to be representative of the high viral diversity requiring control. We selected bipartite and monopartite begomoviruses with contrasted biological properties and areas of distribution. While the two strains of TYLCV (Mld and IL) are considered some of the most emergent plant virus, the other monopartite *Tomato leaf curl Comoros virus* (ToLCKMV) is currently restricted to Comoros Archipelago (Delatte et al. 2005b). In addition to these monopartite begomoviruses, resistant accessions were also screened with a bipartite begomovirus, the *Potato yellow mosaic virus* (PYMV) originating from the New World and present in Central America and the Caribbean (Urbino et al. 2004). Moreover, *B. tabaci*-mediated inoculations with the two emerging strains of TYLCV (Mld and IL) were performed to evaluate the stability of the conferred resistance under high inoculation pressure. Finally, the recently characterized resistance from the accession LA2187-5 originating from *S. pimpinellifolium* was also evaluated by exploring its spectrum of action and its genetic basis to assess its potential value in the diversification of resistance sources.

Materials and methods

Plant and virus materials

The commercial tomato hybrid cv Farmer (Known You Seed Co.) was used as the susceptible control in all experiments. Four inbred tomato lines carrying the homozygous *Ty-1*, *Ty-2*, *Ty-3* or *Ty-3a* resistance genes (kindly provided by G. Bonnet, Syngenta Seeds, Aramon, France) were used in this study. *Ty-1* was originally mapped from *S. chilense* accession LA1969 (Zamir et al. 1994), *Ty-2* from *S. habrochaites* accession H24 (Hanson et al. 2000), *Ty-3* from *S. chilense* accession LA2779 (Ji et al. 2007a), and *Ty-3a* from *S. chilense* accession LA1932 (Ji et al. 2007a). Accession LA2187-5 originating from *S. pimpinellifolium* was first reported in Guadeloupe during a screening for resistance to PYMV (Boissot et al. 2008).

Virus sources

Full head-to-tail DNA dimers of the cloned DNA components of TYLCV-Mld[RE:SPi:02] (EMBL: AJ865337), TYLCV-IL[RE:SGi:RE4:04] (EMBL: AM409201) and ToLCKMV-[YT:Dem:03] (EMBL: AJ865341) were inserted in the binary vector pCAMBIA2300 and mobilized into the C58 strain of *Agrobacterium tumefaciens* (Delatte et al. 2005a; Delatte et al. 2005b). Full-length DNA-A and DNA-B genomes of *Potato yellow mosaic virus*-Tomato [Guadeloupe:Tomato] (PYMV-To[GP:Tom], EMBL:AY120882/AY120883) were used for the construction of infectious clones in the binary vector pCambia0380 as described elsewhere (Perefarres et al. 2011).

Agro-inoculation experiments

Liquid culture of *A. tumefaciens* containing the agro-infectious clones of TYLCV-IL[RE4], TYLCV-Mld[RE], ToLCKMV-[YT:Dem:03], PYMV-To[GP:Tom] or mock (native pCAMBIA2300 plasmid) were adjusted to an OD_{600nm} of 1.0 before inoculation. For the bipartite PYMV inoculation, equal amounts of *A. tumefaciens* containing PYMV-To[GP:Tom] (molecule A) and PYMV-To[GP:Tom] (molecule B) clones were mixed. Tomato plantlets at the three-leaf growth stage (n=10 to 20 for each accession) were inoculated by injecting 50 µL of *A. tumefaciens* culture into the stems with a syringe. Two independent experiments were performed for each virus.

Whitefly-mediated inoculation experiments

Non-viruliferous *B. tabaci* colonies of the biotype B were reared on cabbage plants (*Brassica oleracea*) in a growth chamber at 25°C day and 20°C night, 70% relative humidity and a 16h photoperiod. Viruliferous whiteflies were obtained

after a 72h-acquisition access period (AAP) on tomato plants cv Farmer agro-inoculated five weeks before following the method described in the agro-inoculation experiments. After the AAP, 50 adults were collected and deposited on each tomato seedlings at the one-leaf growth stage (n=10 to 20 for each accession) individually placed under microcages for a 72h-inoculation access period (IAP). At the end of the IAP, insects were killed when the tomato seedlings were sprayed with insecticide (Confidor®). Negative controls consisted in mock inoculated plants (non viruliferous whiteflies). Three independent experiments were performed with TYLCV-Mld and TYLCV-IL.

Experimental design and symptom rating

Plants were maintained in a completely randomized block design in an insect-proof growth chamber at 26°C/24°C (day/night) with a 12h photoperiod (agro-inoculation experiments) or in an insect-proof glasshouse (*B. tabaci*-mediated inoculations). Symptoms were scored twice a week during 35 days post inoculation (dpi). The rating scale was 1, no symptoms, to 10, dead plant, with numbers 1-9 corresponding to the scale of Lapidot et al (2006). At 35dpi, the apical part of the plant including the three youngest leaves was collected before being dehydrated and ground with Tissue Lyser (Qiagen, Courtaboeuf, France). Total DNA extraction was performed from 20 mg of ground plant material using the DNeasy Plant miniprep Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was then resuspended in 100 µL of ultrapure water (two successive elutions of 50 µL) and stored at -20°C before use.

Virus detection

PCR was carried out to detect viral presence in samples collected at 35 dpi in both agro-inoculation and *B. tabaci*-mediated inoculation experiments. For TYLCV-Mld and TYLCV-IL, PCR was performed as described in Lefeuvre et al (2007a). For PYMV and ToLCKMV, viral detection was performed in a 25 µL reaction mixture containing 1 mM MgCl2, 1x PCR reaction buffer, 0.3 mM of each dNTP, 250 nM of each primer (PYMV primer sequences, PYMVA-F: 5'- CTATTGTCAGGCGGTTCTGG-3'; PYMVA-R: 5'- ATGTGGGCAAGGGTCTTAGG-3'; ToLCKMV primer sequences, ToLCKMV-F: 5'- AGGCTTTCAGGGGTGCA-3'; ToLCKMV-R: 5'-GTCGTTTCAGCATCAAAGC-3'), 2.5U of Red Gold Star DNA polymerase (Eurogentec, Belgium) and 2 µL of DNA template. Reactions consisted of 35 PCR cycles of 94°C-30s, 58°C-30s and 72°C-60s with an initial denaturation step at 94°C-5min and a final amplification step of 72°C-10min in a GeneAmp PCR System 9700 thermocycler.

Inheritance study

The inheritance of resistance conferred by LA2187-5 was studied with the analysis of the generations derived from the cross between the resistant LA2187-5 and the susceptible CRA66 lines. The family generations consisted of the two parental lines (LA2187-5 n=41; CRA66 n=47), the interspecific F_1 hybrid (n=50), the backcross with the resistant parent (F_1 x LA2187-5, referred as BCPR; n=108), the backcross with the susceptible parent (F_1 x CRA66, referred as BCPS; n=96) and the F_2 (n=236). Study of segregation for resistance in a Mendelian approach was done by grouping plants into resistant and susceptible classes. Segregation ratios were tested for goodness-of-fit to theoretical ratios for the hypothesis that a single locus and two loci control the resistance.

Real-time PCR detection of PYMV

The quantification of PYMV was performed as described in Péréfarres et al (2011). Each sample was amplified in duplicate and a new aliquot of the standard was used in each run. Quantities of virus and internal report were calculated with the corresponding standard curves and results were expressed as the log of the ratio of the quantity of virus DNA to that of plant genomic DNA.

Variables description and statistical analysis

Disease severity, defined as the mean symptom severity of plants exhibiting symptoms, and, disease incidence, defined as the number of symptomatic plants were calculated at each scoring date. Viral incidence, defined as the number of plants detected as infected at 35 dpi was also calculated.

All statistical analyses were performed using the R statistical software (Team 2008). Nonlinear regression analyses were performed using different link functions (logistic, Cauchy, probit, loglog and cloglog) to fit the progression of disease severity (Pinheiro et al. 2009). Based on the likelihood and using the Akaike's Information Criterion (AIC), the loglog function appeared to be the most appropriate to fit the disease severity progression. In this model, written as $Y \sim 1 + C \exp(- \exp(- A (X - B) + \log(- \log(0.5))))$, the disease severity (Y) is dependent on three biologically relevant parameters: A is the slope of the exponential phase, (1 + C) is the disease severity at the plateau phase, and B is the time to reach 50% of the disease severity at the plateau phase. Once adjusted, the parameters of the logistic disease progression model were compared between the different accessions after TYLCV-IL, TYLCV-Mld, PYMV and ToLCKMV inoculations using likelihood ratio tests (LRT). In the cases where symptom progression did not fit a logistic distribution (*i.e* cases of symptom regression), effects of the dpi and the accession were analysed on symptom severity for each inoculated virus using an ANOVA procedure. Effects of the

accession, the dpi and the virus were analysed on disease incidence using chi-square tests under a generalized linear model (GLM) (with the logit link function and a binomial underlying distribution). Effects of the accession and the virus were analysed on viral incidence using chi-square tests under a generalized linear model (GLM) (with the logit link function and a binomial underlying distribution).

Results

Inheritance of resistance from LA2187-5

The most contrast in phenotypes between the susceptible parent and LA2187-5 could be identified by viral incidence or viral load at 20 dpi after PYMV agro-inoculation (data not shown). The genetic analysis using a Mendelian approach was hence attempted using these two criteria. Nine plants out 41 were detected as infected for LA2187-5 while 41 plants out 47 were for CRA66 (Table 1). Since 48 F_1 plants out of 50 inoculated were infected at 20 dpi, the recessive inheritance of the resistance was assumed. Whereas the segregations of the BCPR and BCPS did not support single or two recessive gene models, segregation of the F_2 generation (168 infected and 68 healthy) supported the model of one recessive gene controlling the resistance to PYMV at P=0.18. Similarly, regarding the viral load at 20 dpi, in the light of the F_1 segregation and the deviation from normality observed in the F_2, the implication of one major recessive locus associated with the resistance to PYMV could be assumed (Figure 1).

Table 1: Goodness of fit (Chi^2 and P) for genetic models of resistance to *Potato yellow mosaic virus* (PYMV) in a family of generations of a cross between the resistant 'LA2187-5' and the susceptible 'CRA66' tomato lines.

Generation	Number of plants			One recessive gene			Two recessive genes		
	Total	Healthy	Infected	Expected (R:S)	Goodness of fit		Expected (R:S)	Goodness of fit	
					Chi^2	P		Chi^2	P
LA2187-5	41	32	9	1-0			1-0		
CRA66	47	6	41	0-1			0-1		
F1	50	2	48	0-1			0-1		
F2	236	68	168	1-3	1.83	0.1761	1-15		<0.0001
BCPR	108	38	70	1-1	9.48	0.002	1-3	5.98	0.01
BCPS	96	24	72	0-1	Inf	<0.0001	0-1	Inf	<0.0001

Spectrum of action of the resistance genes

Different phenotypes were observed between the accessions following the agro-inoculations with ToLCKMV, TYLCV-IL, TYLCV-Mld and PYMV (Figure 2, Table 2). As the effects of the accessions, the virus and the interaction of accession x virus were highly significant on viral incidence, we performed separate GLMs for each accession and for each virus (Table 2).

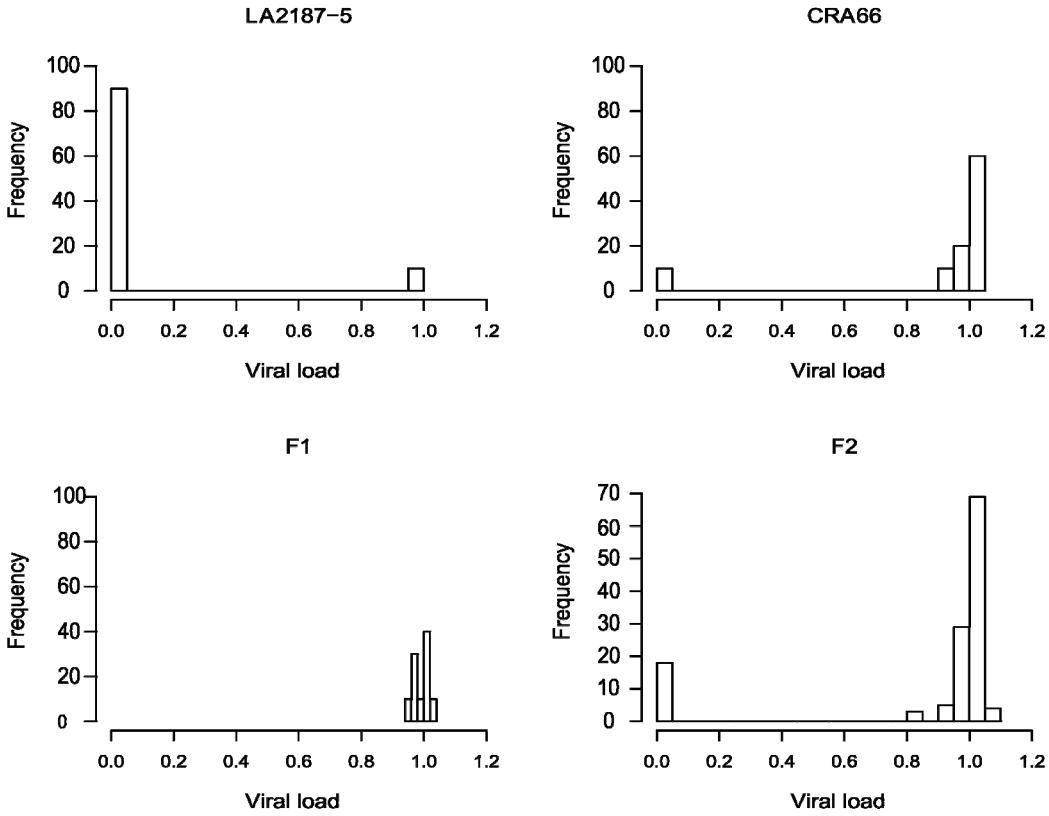


Figure 1: Frequency distributions of *Potato yellow mosaic virus* (PYMV) viral load in a family of generations of a cross between the resistant 'LA2187-5' and the susceptible 'CRA66' tomato genotypes.

Table 2: Results of the agro-inoculation experiments of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL), *Tomato leaf curl Comoros virus* (ToLCKMV) and *Potato yellow mosaic virus* (PYMV).

Accession	TYLCV-IL	TYLCV-Mld	ToLCKMV	PYMV
Farmer	34/35 (α) (b)	30/34 (α) (bc)	12/20 (na)	24/24 (α) (b)
Ty-1	23/33 (α) (b)	18/27(α) (abc)	0/20 (na)	15/20(α) (b)
Ty-2	7/35 (α) (a)	26/29 (β) (c)	0/22 (na)	17/20 (β) (b)
Ty-3	25/28 (β) (b)	9/22 (α) (ab)	0/22 (na)	8/20 (α) (ab)
Ty-3a	20/32 (α) (b)	10/24 (α) (ab)	0/21 (na)	8/15 (α) (ab)
LA2187-5	18/31 (β) (b)	10/33 (αβ) (a)	0/27 (na)	2/23 (α) (a)

Number of infected plants on number of inoculated plants. Values followed by the same letter (a to c) (vertical sense) and (α to β) are not significantly different at $P = 0.05$ (Tukey's all-pair comparison test)

After ToLCKMV agro-inoculation, only the susceptible control Farmer was found infected (viral incidence = 60%), exhibiting mild symptoms from 10.5 dpi,

reaching a disease severity of 2.2 at 35 dpi. All the resistant accessions remained asymptomatic and no virus was detected by our PCR assay at 35 dpi.

Similarly, when agro-inoculated with TYLCV-IL, only the susceptible control Farmer exhibited TYLCD symptoms which started at 7 dpi and reached their maximum at 35 dpi (disease severity$_{35dpi}$ = 7.4). Regarding viral incidence at 35 dpi, the Ty-2 inbred line was significantly less infected (viral incidence = 20%) than all the others accessions whose viral incidence ranged from 58% to 97%.

When agro-inoculated with TYLCV-Mld, while the accessions Ty-1, Ty-3, Ty-3a and LA2187-5 remained asymptomatic throughout the experiments, accession Ty-2 and the susceptible control first exhibited symptoms at 7 dpi reaching a maximum value at 35 dpi. Whereas the parameters governing the speed of the progression of disease severity were not significantly different between these two accessions, the disease severity at the plateau phase was clearly higher for Farmer (disease severity$_{35dpi}$ of 6.5 and 3.8 for Farmer and Ty2, respectively, $p<10^{-8}$). Regarding the viral incidence, accession Ty-2 (89%) and Farmer (88%) were significantly more infected than the remaining accessions whose viral incidence ranged from 30% to 66%.

When agro-inoculated with PYMV, only accession Ty-2 and Farmer exhibited symptoms that started at 14 dpi and reached a maximum value at 24.5 dpi. None of the three parameters governing the progression of the disease severity was significantly different between Farmer and Ty-2 (disease severity$_{35dpi}$ = 4.1 and 3.7, for Farmer and Ty-2, respectively). Concerning the viral incidence, accession LA2187-5 (viral incidence = 20%) was less infected than the other accessions whose viral incidence ranged from 40% to 100%.

A separate analysis with GLM model was performed on accessions to compare viral incidence between the four inoculated viruses (Table 2). Accessions Ty-1 and Ty-3a did not have significant differences between the different agro-inoculated viruses except with ToLCKMV that was only detected on Farmer. In contrast, the inbred with Ty-2 was significantly more infected by TYLCV-Mld and PYMV than TYLCV-IL ($p<10^{-4}$ and $p=10^{-4}$ respectively). Similarly, accession Ty-3 was found to be significantly more infected by TYLCV-IL than TYLCV-Mld (p=0.006) and PYMV (p=0.005). Finally, LA2187-5 was significantly less infected by TYLCV-IL than PYMV (p=0.002), but without any differences with TYLCV-Mld (p=0.05). Also, no significant difference was found between PYMV and TYLCV-Mld (p=0.19) for LA2187-5.

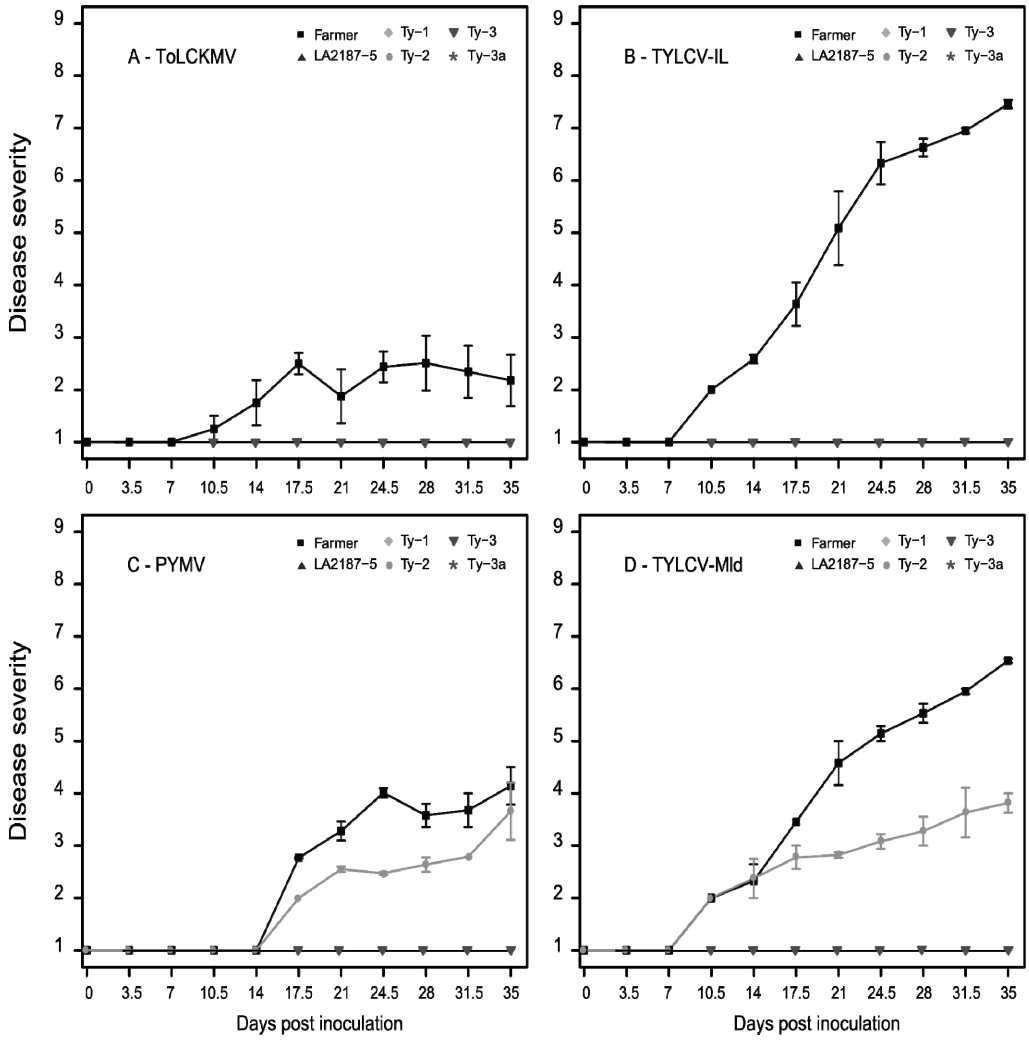


Figure 2: Time course analysis of symptom severity following agro-inoculation of tomato plants with *Tomato leaf curl Comoros virus* (ToLCKMV) (A), *Tomato yellow leaf curl virus* Reunion isolates of Israel strains (B) (TYLCV-IL), *Potato yellow mosaic virus* (PYMV) (C) and TYLCV Reunion isolates of Mild strains (TYLCV-Mld) (D). The symptom severity scale was 1, no symptom, to 10, dead plant. Vertical bars around the dots represent the standard error.

Expression of resistance to TYLCV (Mld and IL strains) under high inoculum pressure *B. tabaci*-mediated inoculations

Similar to the agro-inoculation experiment, only accession Ty-2 and the susceptible control Farmer exhibited TYLCD symptoms after TYLCV-Mld *B. tabaci*-mediated inoculations. Symptoms were first observed at 7 dpi and 10.5 dpi (for Farmer and Ty-2, respectively) and then increased before reaching a maximum value at 31.5 dpi. Models of disease severity progressions of Ty-2 and Farmer only differed from each other by the disease severity at the plateau phase (disease severity$_{35dpi}$ of 5.9 and 2.8 for Farmer and Ty2 respectively, $p<10^{-8}$).

When it came to TYLCV-IL *B. tabaci*-mediated inoculations, contrasted phenotypes were observed between the accessions and also between the trials. Hence, accessions Ty-1, Ty-2, Ty-3 and Ty-3a became symptomatic in the first two experiments conducted at the beginning of the hot/wet season (October) contrary to the third experiment conducted at the end of the hot/wet season (May). In the cases of accessions LA2187-5 and Farmer, similar patterns were observed irrespective the experiments. Moreover, while symptoms remained on Farmer and LA2187-5, remissions occurred in accessions Ty-1, Ty-2, Ty-3 and Ty-3a (Figure 3). Because of these strong differences, we conducted a two-way analysis to accurately compare these contrasting phenotypes i) models were fitted to compare the disease severity progression on LA2187-5 and Farmer and ii) an ANOVA procedure was performed to compare the effects of accessions and dpi on disease incidence and symptom severity excluding the last trial.

The first analysis focused on LA2187-5 and Farmer which exhibited similar phenotypes after TYLCV-IL *B. tabaci*-mediated inoculations irrespective the experiments. The first symptoms were observed at 7 and 10.5 dpi for Farmer and LA2187-5, respectively and reached a maximum value at 31.5 dpi and 14 dpi for Farmer and LA2187-5 respectively (Figure 3 and 4). Models of disease severity progressions of LA2187-5 and Farmer only differed from each other at the plateau phase (disease $severity_{35dpi}$ = 3.6 and 7 for LA2187-5 and Farmer respectively) (Figure 4).

The second analysis was performed to compare the disease severity and the disease incidence between the different accessions after TYLCV-IL *B. tabaci*-mediated inoculations (Figure 3). Effects of the dpi, the accessions and the interaction of accessions x dpi were highly significant on disease severity ($p<10^{-8}$) and on disease incidence. At 17.5 dpi and 31.5 dpi, accessions Ty-1 and Ty-3a first exhibited mild symptoms (disease $severity_{max}$ = 2.5 and 3 respectively) which were less than those of Farmer and LA2187-5. Moreover, disease incidence of Ty-1 and Ty-3a were the lowest among the accessions and reached maximums of 5% and 3% respectively. Accessions Ty-2 and Ty-3 differed from LA2187-5 and Farmer by their patterns of disease progression. While disease incidence reached 80% (Ty-2) and 63% (Ty-3) at 14 dpi and 17.5 dpi, remission occurred and only 5% (Ty-2) and 3% (Ty-3) were finally symptomatic at 35 dpi. Moreover, symptoms were less on Ty-2 and Ty-3 (disease $severity_{max}$ = 2.9 and 3, respectively) than those of Farmer (disease $severity_{35dpi}$ = 7).

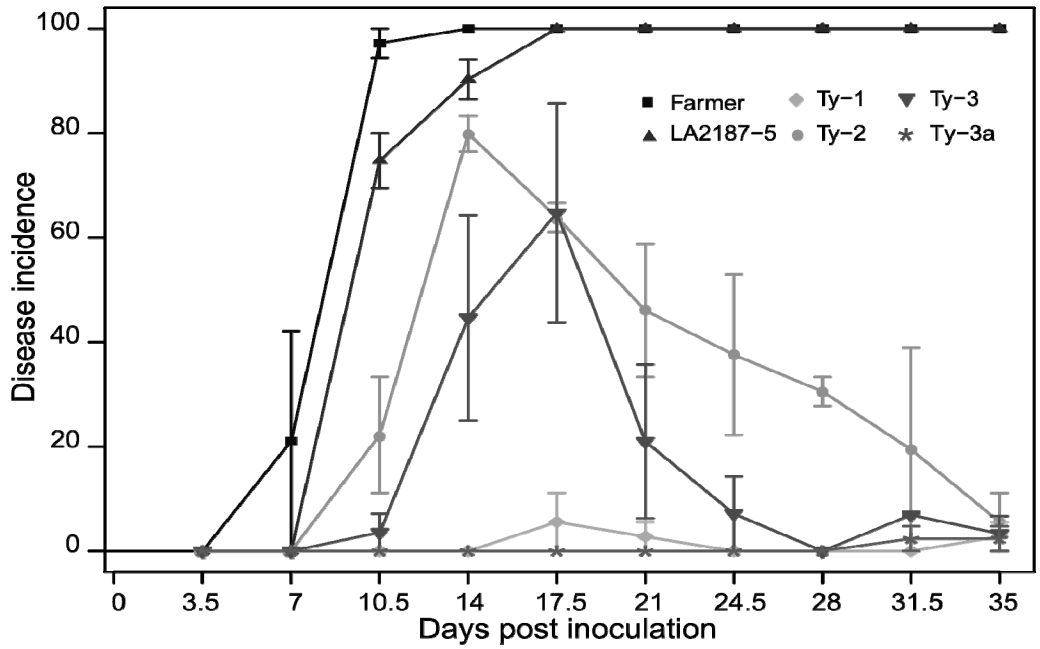


Figure 3: Mean disease incidence following *Bemisia tabaci*-mediated inoculations with *Tomato yellow leaf curl virus* Reunion isolates of Israel strain (TYLCV-IL). Vertical bars around the dots represent the standard error. It should be noted that only the first two experiments are represented.

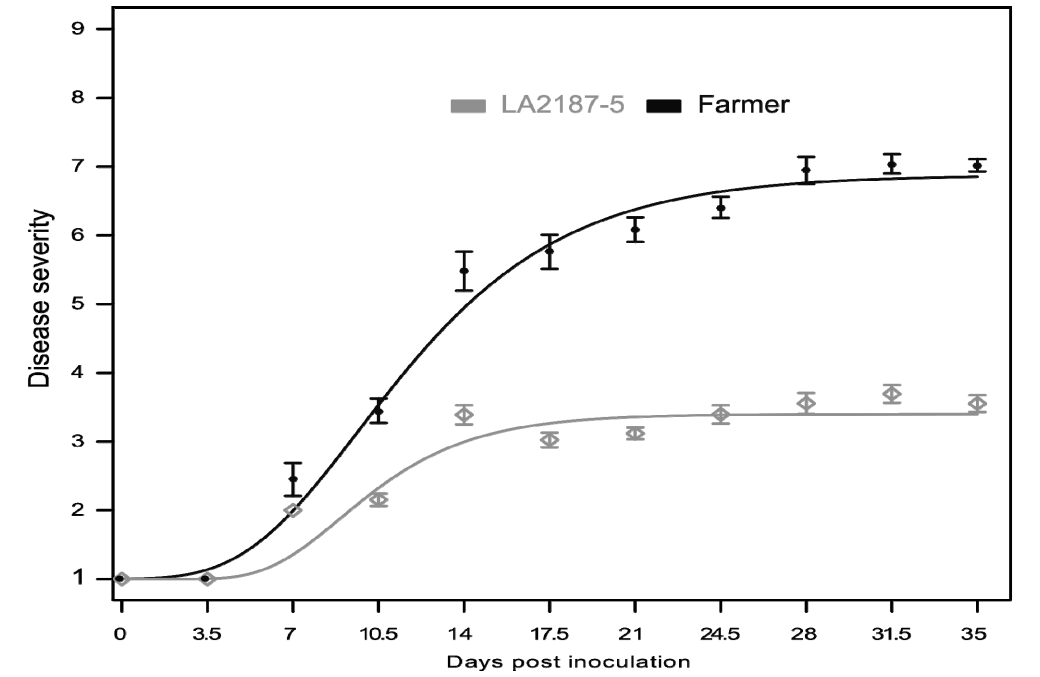


Figure 4: Estimated (solid line) and mean (dots) symptom severity of tomato yellow leaf curl disease following *Bemisia tabaci*-mediated inoculations with *Tomato yellow leaf curl virus* Reunion isolates of Israel strain (TYLCV-IL). The symptom severity scale was 1, no symptom, to 10, dead plant. Vertical bars around the dots represent the standard error.

Discussion

The diversification of resistance sources and their pyramiding is an attractive strategy to improve the level and the spectrum of action of resistance to TYLCD-associated viruses. Investigation of resistance sources to assess their complementarities is a prerequisite for the efficient combination of resistant genes. In this study, we explored the genetic basis of the resistance originating from *S. pimpinellifolium* accession LA2187-5. Besides, we faced the major resistance genes used today in breeding program and LA2187-5 to a wide range of begomoviruses implicated in the TYLCD throughout the world to evaluate the spectrum of action of these resistance sources.

Among the inoculated begomoviruses, ToLCKMV was the only virus unable to infect the resistant accessions. We previously reported that ToLCKMV was less efficient to successfully infect a susceptible tomato resulting in a delay in the viral accumulation kinetic compared to other monopartite (TYLCV-IL and TYLCV-Mld) and bipartite begomoviruses (PYMV) (Perefarres et al. 2011). Here, we uphold that ToLCKMV does not represent a major threat to tomato production. Nevertheless, as begomoviruses are known to be highly recombinogenic, ToLCKMV could still have important epidemiological issues with the emergence of new pathotypes from its progeny (Martin et al. 2011; Vuillaume et al. 2011).

Following agro-inoculation with TYLCV-Mld, TYLCV-IL and PYMV, accessions carrying the resistance genes *Ty-1*, *Ty-3* or *Ty-3a* expressed a broad tolerance to this panel of begomoviruses. In contrast, the accession carrying the *Ty-2* resistance gene exhibited a specific resistance to TYLCV-IL but was susceptible to TYLCV-Mld and PYMV. It could be argued that differences in genetic background in our accessions may also contribute to the responses following inoculation. Nevertheless, as already reported for *Ty-1* if such contribution could exist, it would have been minor compared to the introgressed resistance gene (García-Andrés et al. 2009). Because of the great viral diversity to control, the knowledge of the spectrum of action of these different resistance genes is a prerequisite not only for their appropriate deployment, but also for the pyramiding of complementary resistance genes. This is the first study that confronted these different sources of resistance to such a wide viral diversity under similar experimental controlled conditions. Our data bolstered the wide tolerance of the *Ty-1* gene to monopartite and bipartite begomoviruses implicated in the TYLCD as suggested in a previous report (Boiteux et al. 2007). The two other genes *Ty-3* and *Ty-3a,* originating from *S. chilense* accessions LA2779 and LA1932 respectively, were initially screened for TYLCV and *Tomato mottle virus* (ToMoV) resistance (Ji et al. 2007a). Our data suggest that these resistance genes also may induce a wide tolerance to monopartite and bipartite begomoviruses. Interestingly, the three *Ty-1*, *Ty-3* and *Ty-3a* genes were similarly located on the chromosome 6 which may suggest similar origins and mechanisms associated

with these resistance genes. The first characterization of the resistance gene *Ty-2* was carried out with two different viruses and lead to the description of a specific tolerance to TYLCV/ToLCV strains in Taiwan, but susceptibility to TYLCV strains from northern India (Hanson et al. 2000). These results were, however, somewhat ambiguous as screenings were not performed under similar environmental conditions and the differences in resistance expression could therefore result from differences in inoculated begomoviruses but also in environmental factors or whitefly populations (Hanson et al. 2000). Similarly, our study suggests that the resistance conferred by *Ty-2* is actually specific to the IL strain of TYLCV and is not efficient against others tomato-infecting begomoviruses such as TYLCV-Mld or PYMV. Finally, our data emphasized the high level of resistance conferred by LA2187-5 to PYMV reported from whiteflies and grafting tests in Guadeloupe (Boissot et al. 2008) with the lowest viral incidence among all the evaluated accessions. Moreover, we highlighted its efficiency with the widespread TYLCV-Mld and TYLCV-IL. These two emerging strains have reached a worldwide distribution and co-occurrence of these two strains have also been reported throughout the world (Delatte et al. 2005a; Ueda et al. 2004). The resistance conferred by LA2187-5 could therefore be used as a supplementary source of a broad spectrum resistance in breeding programs.

The accessions were also exposed to massive infestations with viruliferous *B. tabaci* to evaluate the stability of these resistances. Even under high inoculum pressures with TYLCV-Mld, no difference was observed compared to agro-inoculations experiments and confirmed the efficiencies of accessions carrying the resistance genes *Ty-1*, *Ty-3* and *Ty-3a*. Similarly, LA2187-5 did not exhibit any TYLCD symptoms in those conditions and strengthened its suitability for the efficient control of TYLCV-Mld.
In these conditions of TYLCV-IL high inoculation pressure, resistance conferred by LA2187-5 was not sufficient although the symptoms were attenuated compared to the susceptible control. The other inbred lines could also produce some TYLCD symptoms but with a lower incidence and severity compared to LA2187-5. Besides, a recovery from the disease was observed in those lines while symptoms remained observable in LA2187-5. These differential phenotypes suggest different antiviral defences associated with these resistance genes. Recovery from viral disease, including decrease in symptom incidence and severity, has been associated with gene silencing mechanisms (Covey et al. 1997). Begomoviruses are known to be able both to trigger and suppress gene silencing pathways possibly leading to the recovery from infection (Bisaro 2006; Rodríguez-Negrete et al. 2009). The balance between the suppression and the triggering of these two mechanisms can affect therefore the viral infection outcome. As this balance can be influenced by environmental conditions, it may explain the contrasted results between our trials and the recovery after a massive infestation observed in the accessions Ty-1, Ty-2, Ty-3 and Ty-3a.

Evidence was provided here that the accession Ty-2 exhibited a narrow spectrum of action limited to TYLCV-IL. Interestingly, considering the recombinant nature of TYLCV-IL which shares a common origin for a portion of the genome comprising the C4 ORF with ToLCV-Asian-like ancestors (Navas-Castillo et al. 2000), the two strains of TYLCV (Mld and IL) mainly differ by the C4 ORF. Experimental work has associated the C4 ORF in different pathways involved in viral infection and bypass defence mechanisms of the host (Gronenborn 2007). Similarly, Tomás et al recently reported a similar pattern in differential abilities of Spain isolates of TYLCV-Mld and TYLCV-IL to counter host defences conferred by a wild resistant tomato accession also originating from *S. habrochaites* (Tomás et al. 2011). In this later case, authors demonstrated that the C4 protein was involved in the successful systemic infection of TYLCV-Mld. Allelism analyses would certainly give new insights in the relationships between these resistance gene both originating from *S. habrochaites*.

The study of the inheritance of the resistance conferred by LA2187-5 to PYMV suggested that a major recessive gene is implicated. While dominant resistance is generally based on a "gene-for-gene" model (Flor 1971), recessive resistance usually results in passive mechanism in which the loss of an interaction between viral protein(s) and host factor(s) prevents a complete replication cycle of the virus. Natural recessive resistances to RNA viruses have been particularly documented and revealed the role of eukaryotic translation initiation factors 4E (Robaglia and Caranta 2006). The absence of this plant factor or its presence in a mutated version prevents from its physical interaction with the potyviral genome-linked protein (VPg) resulting in the host resistance (for a review see Le Gall et al. 2011). In the case of begomoviruses, natural recessive resistance have been reported for *Tomato chlorotic mottle virus* (Giordano et al. 2005), TYLCV (García-Cano et al. 2008) and *Tomato leaf curl virus* (ToLCV) (Bian et al. 2007). For the moment, the molecular basis of these recessive resistances remains to be explored. In order to investigate this recessive resistance to begomoviruses, a recombinant inbred lines (RILs) population deriving from a cross between LA2187-5 and a susceptible line is currently underway in our laboratory and will be used to identify the locus of resistance. This mapping study will be coupled with a candidate genes approach by searching cellular factors that could colocalise in the same genomic region than genes/QTL of resistance. Similarly to what was recently performed for the *Ty-5*-based recessive resistance from S. peruvianum (Anbinder et al., 2009), this approach could constitute the first step towards the comprehension of recessive resistance to begomoviruses.

An efficient and durable resistance strategy relies on the availability and the pyramiding of resistance genes acting at different steps of the virus cycle (inoculation process, within-cell multiplication, cell-to-cell and systemic movement and finally acquisition by vectors) and/or recognizing different targets in the virus genome. Although the molecular basis remains to be explored, the

contrasted phenotypes of known resistance genes suggest different mechanisms operating in accessions from *S. chilense*, *S. habrochaites* and *S*. *pimpinellifolium* and their combination could contribute to build an efficient and durable resistance to the TYLCD-associated viruses. In complement to the virus resistance that we evaluated here, the strategy of gene pyramiding could also include resistance genes operating against *B. tabaci*. Since the vector resistance is supposed to be non-virus specific, its combination with virus resistance would be a particularly appealing strategy for the efficient control of the wide viral diversity implicated in the TYLCD. The exploration within the germplasm *S. lycopersicum* and wild tomato relatives has revealed resistance sources *to B. tabaci* (Delatte et al. 2006; Rodríguez-López et al. 2011) which could contribute to a durable control of TYLCD.

Acknowledgments

The authors thank M. Grondin and D. Fontaine for their excellent technical assistance, J.W Scott and S. Nibouche for very helpful comments and discussions during manuscript preparation. This study was funded by the CIRAD and the FEDER. F. Péréfarres is a recipient of a Phd fellowship from the CIRAD.

References

Abhary M, Patil B, Fauquet CM (2007) Molecular Biodiversity, Taxonomy, and Nomenclature of Tomato Yellow Leaf Curl-like Viruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 85-118

Anbinder I, Reuveni M, Azari R, Paran I, Nahon S, Shlomo H, Chen L, Lapidot M, Levin I (2009) Molecular dissection of Tomato leaf curl virus resistance in tomato line TY172 derived from Solanum peruvianum. TAG Theoretical and applied genetics 119:519-30.

Bian X-Y, Thomas MR, Rasheed MS, Saeed M, Hanson P, De Barro PJ, Rezaian MA (2007) A Recessive Allele (tgr-1) conditioning tomato resistance to geminivirus infection is associated with impaired viral movement. Phytopathology 97:930-937.

Bisaro DM (2006) Silencing suppression by geminivirus proteins. Virology 344:158-68.

Boissot N, Urbino C, Dintinger J, Pavis C (2008) Vector and graft inoculations of Potato yellow mosaic virus reveal recessive resistance in Solanum pimpinellifolium. Annals of Applied Biology 152:263-269.

Boiteux LS, Oliveira VR, Silva CH, Makishima N, Inoue-Nagata AK, Fonseca MEDN, Giordano LDB (2007) Reaction of tomato hybrids carrying the Ty-1 locus to Brazilian bipartite Begomovirus species. Horticultura Brasileira 25:020-023.

Castillo-Urquiza GP, Beserra JEA, Bruckner FP, Lima ATM, Varsani A, Alfenas-Zerbini P, Murilo Zerbini F (2008) Six novel begomoviruses infecting tomato and associated weeds in Southeastern Brazil. Archives of virology 153:1985-1989.

de Castro AP, Díez MJ, Nuez F (2008) Exploiting Partial Resistance to Tomato yellow leaf curl virus Derived from Solanum pimpinellifolium UPV16991. Plant Disease 92:1083-1090.

Covey SN, Al-Kaff NS, Langara A, Turner DS (1997) Plants combat infection by gene silencing. Nature 385:781-782.

Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM (2005a) The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Reunion Island. Plant Pathology 54:262-262.

Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett JM (2005b) South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. The Journal of general virology 86:1533-1542.

Delatte H, Holota H, Reynaud B, Dintinger J (2006) Characterisation of a quantitative resistance to vector transmission of Tomato yellow leaf curl virus in Lycopersicon pimpinellifolium. European Journal of Plant Pathology 114:245-253.

Duffy S, Holmes EC (2007) Multiple introductions of the Old World begomovirus Tomato yellow leaf curl virus into the New World. Applied and Environmental Microbiology 73:7114-7117.

Elbert A, Nauen R (2000) Resistance of Bemisia tabaci (Homoptera: Aleyrodidae) to insecticides in southern Spain with special reference to neonicotinoids. Pest Management Science 56:60-64.

Fargette D, Konaté G, Fauquet C, Muller E, Peterschmitt M, Thresh JM (2006) Molecular ecology and emergence of tropical plant viruses. Annual Review of Phytopathology 44:235-260.

Flor HH (1971) Current Status of the Gene-For-Gene Concept. Annual Review of Phytopathology 9:275-296.

Le Gall O, Aranda MA, Caranta C (2011) Plant resistance to viruses mediated by translation initiation factors. In: Caranta C, Aranda MA, Tepfer M, Lopez-Moya J. (eds) Recent advances in plant virology. Caister Academic Press, pp 177-194

García-Andrés S, Tomás DM, Navas-Castillo J, Moriones E (2009) Resistance-driven selection of begomoviruses associated with the tomato yellow leaf curl disease. Virus Research 146:66-72.

García-Cano E, Resende RO, Boiteux LS, Giordano LB, Fernández-Muñoz R, Moriones E (2008) Phenotypic expression, stability, and inheritance of a recessive resistance to monopartite begomoviruses associated with tomato yellow leaf curl disease in tomato. Phytopathology 98:618-627.

Giordano LB, Silva-Lobo VL, Santana FM, Fonseca MEN, Boiteux LS (2005) Inheritance of resistance to the bipartite Tomato chlorotic mottle begomovirus derived from Lycopersicon esculentum cv. "Tyking."Euphytica 143:27-33.

Gronenborn B (2007) The Tomato Yellow Leaf Curl Virus Genome and Function of its Proteins. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 67-84

Hanson PM, Breeding P, Hall E, Green S, Tanksley SD, Chen H-mei, Kuo G, Fang D, Chen J-tzu (2000) Mapping a wild tomato introgression associated with Tomato yellow leaf curl virus resistance in a cultivated tomato line. Journal of the American Society for Horticultural Science 125:15-20.

Jeske H (2009) Geminiviruses. Current Topics in Microbiology and Immunology 331:185-226.

Ji Y, Schuster DJ, Scott JW (2007a) Ty-3, a begomovirus resistance locus near the Tomato yellow leaf curl virus resistance locus Ty-1 on chromosome 6 of tomato. Molecular Breeding 20:271-284.

Ji Y, Scott JW, Hanson P, Graham E, Maxwell D (2007b) Sources of Resistance, Inheritance, and Location of Genetic Loci Conferring Resistance to Members of the Tomato-Infecting Begomoviruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 343-362

Ji YF, Scott JW, Schuster DJ, Maxwell DP (2009) Molecular mapping of Ty-4, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. Journal of the American Society for Horticultural Science 134:281-288.

Lapidot M, Ben-Joseph R, Cohen L, Machbash Z, Levy D (2006) Development of a Scale for Evaluation of Tomato yellow leaf curl virus Resistance Level in Tomato Plants. Phytopathology 96:1404-8.

Lefeuvre P, Hoareau M, Delatte H, Reynaud B, Lett J-M (2007a) A multiplex PCR method discriminating between the TYLCV and TYLCV-Mld clades of tomato yellow leaf curl virus. Journal of Virological Methods 144:165-168.

Lefeuvre P, Martin DP, Hoareau M, Naze F, Delatte H, Thierry M, Varsani a, Becker N, Reynaud B, Lett J-M (2007b) Begomovirus "melting pot" in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. The Journal of General Virology 88:3458-3468.

Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJA, Meredith S, Lakay F, Monjane A, Lett J-M, Varsani A, Heydarnejad J (2010) The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World. PLoS Pathogens 6:e1001164.

Martin DP, Lefeuvre P, Varsani A, Hoareau M, Semegni J-Y, Dijoux B, Vincent C, Reynaud B, Lett J-M (2011) Complex Recombination Patterns Arising during Geminivirus Coinfections Preserve and Demarcate Biologically Important Intra-Genome Interaction Networks. PLoS Pathog 7:e1002203.

Mejía L, Teni R, Vidavski F, Czosnek H, Lapidot M, Nakhla MK, Maxwell DP (2005) Evaluation of tomato germplasm and selection of breeding lines for resistance to begomoviruses in Guatemala. Acta horticulturae 695:251-255.

Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto GP, Moriones E (2000) Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. Journal of General Virology 81:2797-2801.

Perefarres F, Hoareau M, Chiroleu F, Reynaud B, Dintinger J, Lett J-M (2011) A novel synthetic quantification standard including virus and internal report targets: application for the detection and quantification of emerging begomoviruses on tomato. Virology Journal 8:389.

Picó B (1996) Viral diseases causing the greatest economic losses to the tomato crop. II. The Tomato yellow leaf curl virus — a review. Scientia Horticulturae 67:151-196.

Pilowsky M, Cohen S (1974) Inheritance of Resistance to Tomato Yellow Leaf Curl Virus in Tomatoes. Phytopathology 64:632-635.

Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RDC (2009) nlme: Linear and Nonlinear Mixed Effects Models. R package:1-86.

Robaglia C, Caranta C (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends in plant science 11:40-45.

Rodríguez-López MJ, Garzo E, Bonani JP, Fereres a, Fernández-Muñoz R, Moriones E (2011) Whitefly Resistance Traits Derived from the Wild Tomato Solanum pimpinellifolium Affect the Preference and Feeding Behavior of Bemisia tabaci and Reduce the Spread of Tomato yellow leaf curl virus. Phytopathology 101:1191-201.

Rodríguez-Negrete E a, Carrillo-Tripp J, Rivera-Bustamante RF (2009) RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. Journal of Virology 83:1332-1340.

Singh SP, Morales FJ, Miklas PN, Terán H (2000) Selection for Bean Golden Mosaic Resistance in Intra- and Interracial Bean Populations. Crop Science 40:1565-1572.

Team RDC (2008) A language and environment for statistical computing. R Foundation for Statistical Computing.

Tomás DM, Cañizares C, Abad J, Fernández-Muñoz R, Moriones E (2011) Resistance to tomato yellow leaf curl virus accumulation in the tomato wild relative Solanum habrochaites associated with the C4 viral protein. Molecular Plant-Microbe Interactions 24:849-861.

Ueda S, Kimura T, Onuki M, Hanada K, Iwanami T (2004) Three distinct groups of isolates of Tomato yellow leaf curl virus in Japan and construction of an infectious clone. Journal of General Plant Pathology 70:232-238.

Urbino C, Polston JE, Patte CP, Caruana M-L (2004) Characterization and genetic diversity of potato yellow mosaic virus from the Caribbean. Archives of Virology 149:417-424.

Vidavski F, Czosnek H, Gazit S, Levy D, Lapidot M (2008) Pyramiding of genes conferring resistance to Tomato yellow leaf curl virus from different wild tomato species. Plant Breeding 127:625-631.

Vuillaume F, Thébaud G, Urbino C, Forfert N, Granier M, Froissart R, Blanc S, Peterschmitt M (2011) Distribution of the phenotypic effects of random homologous recombination between two virus species. PLoS pathogens 7:e1002028.

Zamir D, Ekstein-Michelson I, Zakay Y, Navot N, Zeidan M, Sarfatti M, Eshed Y, Harel E, Pleban T, van-Oss H, Kedar N, Rabinowitch HD, Czosnek H (1994) Mapping and introgression of a tomato yellow leaf curl virus tolerance gene, TY-1. Theoretical and Applied Genetics 88:141-146.

Chapitre IV :
Exploration de la résistance quantitative conférée par LA1777 aux deux souches émergentes du TYLCV.

Les programmes de création variétale doivent aboutir à la diffusion de cultivars qui répondent aux attentes des producteurs à la fois au niveau des qualités agronomiques mais aussi en terme de résistance aux pathogènes. La résistance aux virus, comme pour les autres pathogènes, doit répondre à trois objectifs majeurs : le niveau, le spectre d'action et la durabilité de la résistance.

Cette stratégie repose sur la disponibilité de gènes de résistance complémentaires, en d'autres termes de gènes de résistance qui reconnaissent différentes cibles dans le génome du virus. Le contournement de la résistance implique alors plusieurs substitutions nucléotidiques augmentant alors la probabilité d'induire un coût adaptatif pour le virus.

Dans la stratégie de pyramidage de gènes, une stratégie alternative repose sur la combinaison de gènes de résistance agissant à différents niveaux du cycle viral de l'hôte: processus d'inoculation, multiplication intracellulaire, mouvements de cellule à cellule et mouvement systémique et enfin l'acquisition par des vecteurs. La résistance aux vecteurs est censée être non spécifique du virus transmis et sa combinaison avec la résistance du virus est une stratégie particulièrement intéressante pour le contrôle efficace de la grande diversité virale impliquée dans le TYLCD et les maladies causées par bégomovirus en général.

Ce chapitre présente la caractérisation de la résistance conférée par l'accession LA1777 aux deux souches émergentes du TYLCV (TYLCV-Mld et TYLCV-IL). Cette accession est connue pour sa résistance à *B. tabaci* et a déjà été utilisée comme matériel de base dans des programmes de création variétale. Dans ce contexte, des tests d'inoculations avec des doses croissantes d'insectes virulifères ont été réalisés pour évaluer le niveau et la stabilité de cette résistance. De plus, grâce à l'utilisation de lignées quasi-isogéniques dérivées du croisement de LA1777 et du parent sensible E6203, notre étude a également pu explorer les bases génétiques associées à cette résistance.

Characterization of a quantitative resistance from *Solanum habrochaites* LA1777 to two emerging strains of TYLCV (TYLCV-Mld and TYLCV-IL).

F. Péréfarres[1,2], V. Lepitre[1], C. Jacquard[1,2] , B. Reynaud[1] and J. Dintinger[1*]

1.CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de la Réunion, France.

2.Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France .

*Corresponding author. Mailing address: Pôle de protection des plantes - 3P, 7 chemin de l'IRAT, 97410 Saint-Pierre, Ile de la Réunion, France. Phone: +262 262499201. Fax: +262 2 62 49 92 93. E-mail: jacques.dintinger@cirad.fr

Keywords
TYLCV, virus resistance, vector resistance, quantitative resistance, begomovirus

Abstract

Begomoviruses (family *Geminiviridae*) are transmitted by the whitefly *Bemisia tabaci* and contain monopartite or bipartite circular single-stranded (ss)DNA genomes. They have emerged as severe problems in the production of agricultural and horticultural crops worldwide. Most notably *Tomato yellow leaf curl virus* has reached a worldwide distribution and became one of the most devastating viruses of tomato crops. Control measures are scarce and require the development of resistant cultivars. This study reports the characterization and evaluation of the resistance conferred by LA1777 to the two main strains of TYLCV: TYLCV-Mld and TYLCV-IL. To evaluate the level of resistance conferred, increasing inoculative pressures were performed with *B. tabaci*. In addition, through the use of near-isogenic lines derived from the crossing of LA1777 and the susceptible parent E6203, our study also explored the genetic basis associated with this resistance. The accession LA1777 provided an efficient protection against both strains characterised by attenuated symptoms and lower disease incidence. However, this resistance was partially overcome with high inoculation pressures reflecting a quantitative resistance. Grafting inoculations and the screenings of the NILs could highlight two NILs ILTA-1554-11 and ILTA1304-7 with specific resistance to TYLCV-IL and TYLCV-Mld respectively. Although the resistance to *B. tabaci* conferred by LA1777 wil be difficult to exploit in breeding programs, our study could reveal virus resistance that should be of primer interest in the diversification of the resistance sources to TYLCV.

Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating tomato (*Solanum lycopersicum*) diseases and has become the main limiting production factor in tropical and warm regions worldwide. TYLCD infections in tomato plants result into some yellowing and upwards curling of leaflet margins, severe stunting and flower abortion possibly leading to a total loss of production. This disease is caused by a complex of begomoviruses species transmitted in a circulative permanent manner by the whitefly *Bemisia tabaci*. During the last three decades, the proliferation of highly polyphageous and fecund biotypes of *B. tabaci* have greatly contributed to the spread and the emergence of begomoviruses (Jones 2009).

A wide range of begomoviruses including at least six species and 15 related strains are associated with the TYLCD (Abhary et al. 2007). Among the tomato-infecting begomoviruses, the *Tomato yellow leaf curl virus* (TYLCV) is the most widespread and notably its two major strains TYLCV-Mld and TYLCV-IL, which have reached a worldwide distribution (recently reviewed in Lefeuvre et al. 2010). Control of TYLCD based on controlling the vector population through the use of physical barriers and/or intensive chemical sprays is often ineffective at reducing viral spread and has raised environmental concerns (Elbert and Nauen 2000; Perring et al. 1999). Breeding for resistance is therefore the most suitable strategy for the efficient control of TYLCD. Unfortunately, all the cultivated tomato (*S lycopersicum*) accessions screened for TYLCV resistance were found to be susceptible (Pilowsky and Cohen 1974). However, sources of resistance to TYLCV and others begomoviruses were found in wild tomato accessions mainly originating from *S. chilense*, *S. habrochaites, S. pimpinellifolium* and *S. peruvianum* (for a review see Ji et al. 2007). Plant breeders started to introgress the most promising identified resistance genes into elite commercial cultivars, thanks to the development of closely linked molecular markers (Ji et al. 2007). Although some of these resistance genes may exhibit a broad spectrum of action, resistance conferred in commercial cultivars may not be sufficient because of the great viral diversity requiring control, especially at the field level where occurrence of multiple begomoviruses is common (Fernandes et al. 2008; Lefeuvre et al. 2007).

The combination of different resistance genes in the same cultivar (i.e the pyramiding of resistance genes) is a useful strategy to create broad-spectrum and eventually more durable resistance (Beaver and Osorno 2009; Palloix and Ordon 2011; Singh et al. 2000). This strategy relies on the availability of complementary resistance genes, in other words in resistance genes which recognize different targets in the virus genome. The evolution towards virulence (i.e resistance breakdown) will therefore require more mutations increasing the chances to confer fitness penalties to the virus. In the framework of gene pyramiding, an alternative strategy relies on the combination of resistance genes acting at

different levels of the viral cycle in the host: inoculation process, within-cell multiplication, cell-to-cell and systemic movement and finally acquisition by vectors. Since the vector resistance is supposed to be non-specific for the transmitted virus, its combination with virus resistance would be a particularly appealing strategy for the efficient control of the wide viral diversity implicated in the TYLCD.

Natural resistances to *B. tabaci* have been reported in wild tomato relatives including accessions originating from *S. habrochaites*, *S. pennellii* and *S. pimpinellifolium* (Blauth et al. 1998; Momotaz et al. 2010; Rodríguez-López et al. 2011; and for a review see Simmons and Gurr 2005). These resistances result from secretions of allelochemicals by glandular trichomes (trichomes type IV and VI) present on stems and leaves. The *S. habrochaites* accession LA1777 is notably known as a source of resistance due to volatile compounds from trichomes of type IV with repellent and fumigant activities against *B. tabaci* (Muigai et al. 2002). The accession LA1777 was also previously reported during TYLCV and tomato-infecting begomoviruses resistance screenings (Maruthi et al. 2003; Vidavsky and Czosnek 1998). This resistance was then supposed to be the result of mechanisms operating at the withefly-plant interface.

The aim of this study was to evaluate the level of resistance conferred by the accession LA1777 to the two emerging strains of TYLCV (i.e TYLCV-Mld, TYLCV-IL) with increasing *B. tabaci*-mediated inoculation pressures. In addition, we explored the mechanisms associated to this resistance using graft-inoculation and the screening of near isogenic lines (NILs) derived from a cross between the susceptible accession E6203 and LA1777.

Material and methods

Plant material

The accession LA1777 and 52 near isogenic lines (NILs) from the ninety-nine NILs developed from the cross LA1777 x E6203 (Monforte and Tanksley 2000) were assayed for TYLCV resistance. Accessions WVA106 and M82 and the commercial hybrid cv Farmer (Known You Seed Co.) were used as susceptible controls during *B. tabaci*-mediated and grafting inoculations.

Virus and insect sources

The virus isolates used in this study were the TYLCV-Mld[Re] (GenBank: AJ865337) and the TYLCV-IL[Re4] (GenBank: AM409201) originating from Reunion Island (Delatte et al. 2005; Peterschmitt et al. 1999). Tomato plantlets cv Farmer were agro-inoculated as described elsewhere (Perefarres et al. 2011)

with TYLCV-IL or TYLCV-Mld and then used as the sources of the viruses in two separate viruliferous whitefly mass-rearing.
Adults of *B. tabaci* used in this study were obtained from a population isolated in Reunion Island. This population started from nymphs collected and subsequently reared on cabbage plants (*Brassica oleracea*) in a growth chamber at 25°C day and 20°C night, 70% relative humidity and a 16h photoperiod. The population biotype was identified using cytochrome oxydase (COI) markers (Delatte et al. 2007). *B. tabaci* nymphs at the L4 stage were transferred from cabbage plants to TYLCV-infected tomato plants in 1.0 x 1.0 x 1.5m insect-proof cages in a greenhouse. Whiteflies were maintained on TYLCV-infected tomato plants until their use in transmission tests.

Bemisia-tabaci **mediated inoculations under increasing pressures**

For each trial, a split-plot design was used with three blocks. The three main plots per block corresponded to three inoculation access periods (i.e IAP: 6, 24, and 96 h), and three subplots per main plot corresponded to the three tested accessions; LA1777, M82 and WVA106. Within each trial, the blocks corresponded to the insect-proof cages placed in an insect-proof greenhouse. Inside each cage, each subplot consisted of 16 two-leaf stage plantlets for each accession and a large amount of insects that previously had fed on infected tomato plants. The three IAPs were carried out in the same cage removing the plantlets from whitefly exposure after 6, 24 or 96 h. The mean number of feeding insects was estimated for each IAP to determine the efficiency of inoculation (see *cniwh* description later). The transmission efficiency (proportion of viruliferous insects) was determined on a sample of 100 whiteflies collected from the insect-proof cages and representing the whole population used in the trial. Whiteflies from this sample were individually deposited under microcages containing a tomato plantlet cv Farmer at the two-leaf stage. After a 72 h IAP, plantlets were sprayed with an insecticide (Confidor®, Bayer, Basel, Switzerland), and maintained in an insect-proof greenhouse. Symptoms presence was recorded 21 days later and samples were collected for viral detection. Three independent trials were performed for TYLCV-Mld and TYLCV-IL *B. tabaci*-mediated inoculations under increasing pressures.

Grafting trials

Thirty day-old seedlings of each accession were side graft-inoculated with a stem from TYLCV-infected source plants of tomato cv. Farmer as a scion. Each trial consisted in 30-50 tomato plantlets per accession.

Screening NILs against TYLCV-Mld and TYLCV-IL resistance

52 NILs derived from the cross LA1777 x E6203 were screened for TYLCV-IL and TYLCV-Mld resistance (Monforte and Tanksley 2000). Each trial consisted in ten plants for each accession at the one-leaf growth stage individually placed under microcages for a 24h- IAP with 15 viruliferous whiteflies. Accessions LA1777 and E6203 and the commercial cv Farmer were used as controls in each trial. Negative controls consisted in mock inoculated plants (non viruliferous whiteflies) and healthy plants. Four independent trials were performed: two for TYLCV-Mld and two for TYLCV-IL.

Experimental design, symptom assessment and viral detection

After *B. tabaci*-mediated inoculations, insects were removed from the tomato plantlets which were sprayed with insecticide (Confidor®, Bayer, Basel, Switzerland). Inoculated plants were maintained in an insect-proof greenhouse for symptom rating twice a week during 31.5 days post-inoculation (dpi). The disease severity scale was 1, no symptoms to 10, dead plant, with numbers 1-9 corresponding to the scale of Lapidot et al (2006). The apical part of the plant including the three youngest leaves was collected at 31.5 dpi and viral presence was confirmed using TAS-ELISA (Agden) following the manufacturer's instructions.

Variable descriptions

The variables were calculated on a subplot basis. Disease severity, defined as the mean symptom severity of the plant exhibiting symptoms and disease incidence defined as the number of plants becoming symptomatic or not were calculated at each rating date.
The inoculation pressure was estimated using the cumulative number of inoculative whiteflies according to the formula: $cniwh_{ijk} = r_i \sum^{k}_{n=1} x_{ijn} \, . \, t_n$ where $cniwh_{ijk}$ is the cumulative number of inoculative whiteflies x hours at the *k*th IAP (*k*=1 for 6 h, 2 for 24h and 3 for 96h) in the *j*th cage of the *i*th trial; x_{ijn}, the mean number of whiteflies per plant in the *j*th cage of the *i*th trial during t_n hours; t_n, the number of hours corresponding to 1st IAP (6 h, n = 1), the difference between 2nd and 1st IAP (18 h, n = 2), or the difference between 3rd and 2nd IAP (72 h, n = 3); and r_i, the proportion of inoculative whiteflies overall estimated on the *i*th trial (Delatte et al. 2006).

Trichome counting

The second pairs of proximal leaflets were collected on the youngest first true leaf on each NIL and both parents of at least three 50 to 55 day-old plants. Density of type IV and type VI trichomes were evaluated on the abaxial surface of the leaflet

under a binocular microscope at 100x magnification. Trichomes were counted in the interior middle section in a 2 mm^2 surface.

Statistical analysis

All statistical analyses were performed using the R statistical software (Team 2008). Effects of the IAP, the trial, the blocks on *cniwh* values were analysed using an ANOVA procedure. Effects of the inoculation pressure, the dpi, the accession and the interaction accession x inoculation pressure on disease incidence were analysed using a generalized linear model (GLM) (with the logit link function and a binomial underlying distribution) and likelihood ratio tests based on chi-square distribution. Effects of the inoculation pressure and the accession on symptom severity were analysed at 31.5 dpi using the ANOVA procedure.

Results

Quantitative resistance to TYLCV-IL and TYLCV-Mld conferred by LA1777

Effects of the trials, the blocks and the IAP were highly significant on *cniwh* values following TYLCV-Mld and TYLCV-IL inoculations. Inoculation pressures were therefore divided into three classes based on *cniwh* values: low (4.5 to 112.2 and 3.22 to 228.78 for TYLCV-Mld and TYLCV-IL respectively), medium (114 to 490 and 252.21 to 868.62 for TYLCV-Mld and TYLCV-IL respectively), and high (576 to 2080 and 890.35 to 3279.68 for TYLCV-Mld and TYLCV-IL respectively) (Table 1).

Table 1: Estimation of the cumulative number of inoculative whiteflies x hours (*cniwh* values) (means and standard error) in experiments of increasing *Bemisia tabaci*-mediated inoculation of the Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL).

Accession	Trial	TYLCV-Mld			TYLCV-IL		
		6h[a]	24h[a]	96h[a]	6h[a]	24h[a]	96h[a]
LA1777	1	6 (0.1)	33 (0.2)	171(0.5)	34 (49)	187 (7)	751 (24)
	2	40 (0.1)	186 (2)	695 (0.8)	40 (8)	182 (1)	1067 (21)
	3	104 (0.5)	506 (3)	1765 (15)	29 (46)	187 (8)	921 (32)
WVA106	1	6 (0.1)	33 (0.2)	171 (0.5)	58 (28)	321 (29)	1551 (122)
	2	40 (0.1)	185 (2)	695 (0.8)	61 (49)	280 (32)	1152 (71)
	3	105 (0.4)	499 (2)	1792 (14)	67 (77)	439 (62)	1112 (107)
M82	1	6 (0.1)	33 (0.2)	171 (0.5)	48 (2)	296 (4)	987 (5)
	2	40 (0.1)	183 (2)	693 (0.9)	39 (4)	222 (3)	859 (4)
	3	105 (0.4)	499 (2.4	1777 (15)	49 (2)	202 (4)	888 (7)

a :Inoculation access period (total time in hours during which the seedlings were in the cages with viruliferous insects).

As the effects of the inoculation pressure, the dpi, the accession and the interaction accession x inoculation pressure were highly significant on disease incidence, we performed separate GLMs for each inoculation pressure and

accession. Disease incidence at 31.5 dpi of LA1777 was significantly lower than those of M82 and WVA106 whatever the inoculation pressures with TYLCV-Mld and TYLCV-IL (Figure 1). In the case of TYLCV-Mld, while the effect of the inoculation pressure was not significant for M82 and WVA106 with ~100% of infected plants in all cases, disease incidence at 31.5 dpi of LA1777 following high inoculation pressure (88%) was significantly higher than those following low (44%) and medium (56%) inoculation pressures. Similar phenotypes were observed after TYLCV-IL inoculations with disease incidences of ~100% at 31.5 dpi for WVA106 at all three inoculation pressures. For LA1777, disease incidence at 31.5 dpi was significantly higher under the high inoculation pressure (53%) compared to those at low (16%) and medium (43%) inoculation pressures.

The disease severity for TYLCV-Mld and TYLCV-IL inoculations was also compared between the different accessions. For TYLCV-Mld, symptoms were more severe on WVA106 ($sev_{31.5dpi}$ = 4.7) compared to M82 ($sev_{31.5dpi}$ = 3.8) and LA1777 ($sev_{31.5dpi}$ = 3.3). For TYLCV-IL the inoculation pressure was significant on symptom severity for accessions M82 and WVA106, but not for LA1777. Overall inoculation pressure symptoms on LA1777 were attenuated ($sev_{31.5dpi}$ = 4.7) compared to the accessions M82 ($sev_{31.5dpi}$ = 7.3) and WVA106 ($sev_{31.5dpi}$ = 7.4).

Grafting trials

Disease incidences were compared between the accessions LA1777, M82 and WVA106 after grafting-inoculations of TYLCV-Mld and TYLCV-IL (Table 2). All accessions had high disease incidences ranging from 89% to 100% after TYLCV-IL-grafting inoculation. For TYLCV-Mld grafting-inoculation, accessions M82 and WVA106 were highly infested (85% and 100% respectively) compared to LA1777 (23%). Moreover, symptoms on LA1777 (sev_{35dpi}=3) were attenuated compared to those on M82 and WVA106 (sev_{35dpi} = 5.3 and 4.9 respectively).

Table 2: Disease incidence following grafting-inoculations of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL). Accessions LA1777, M82 and WVA106 were tested.

Virus	Accession	Effectif	$Incidence_{35dpi}$ (%)
TYLCV-IL	LA1777	36	89
	M82	50	94
	WVA106	47	100
TYLCV-Mld	LA1777	21	23
	M82	21	85
	WVA106	19	100

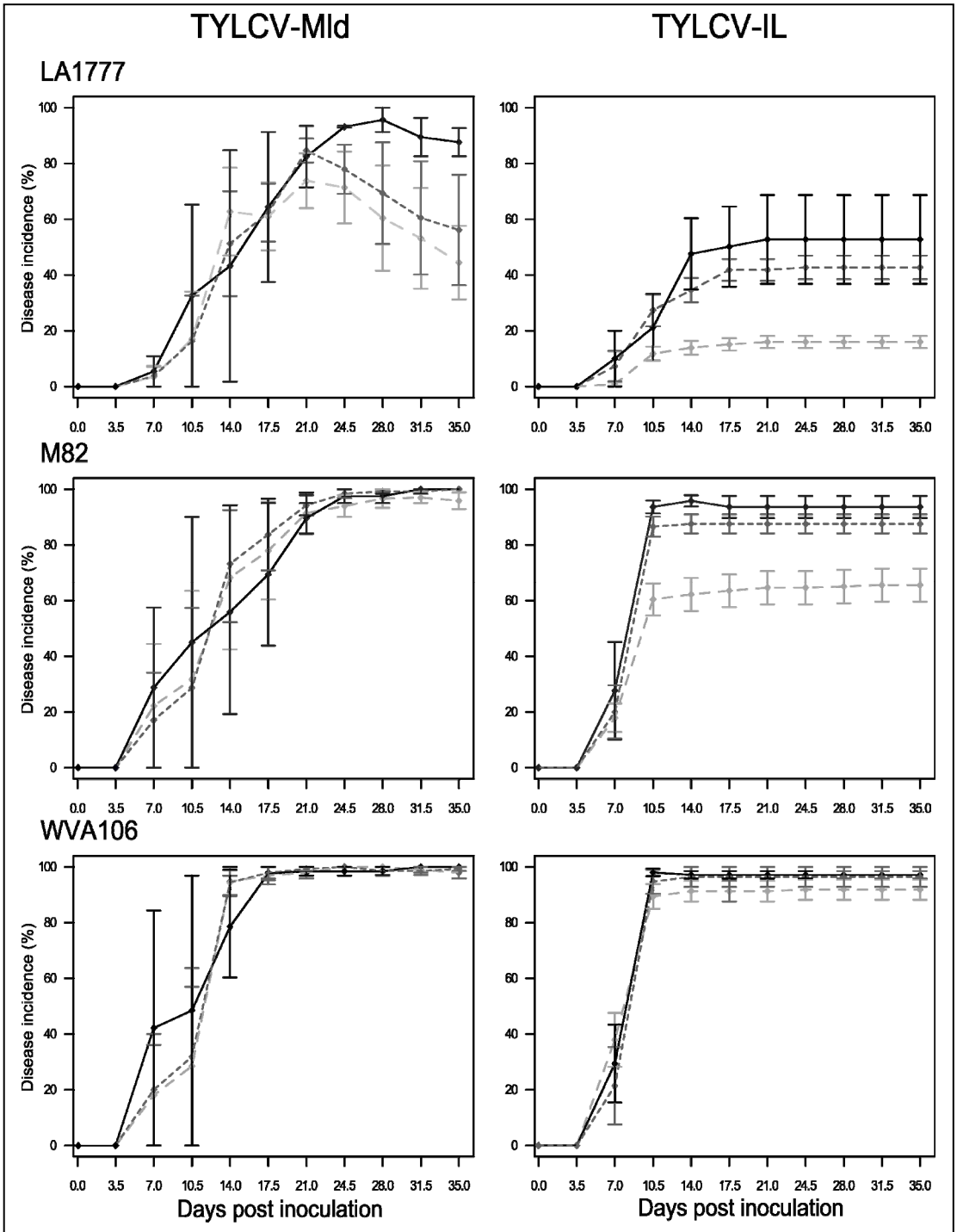


Figure 1: Time course analysis of mean disease incidence following *Bemisia tabaci*-mediated inoculations of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and Israel (TYLCV-IL) under low (- - -), medium (- - -) and high (**- - -**) inoculation pressures. Accessions LA1777, M82 and WVA106 were tested. Verticals bars represent the standard error.

NILs screening for TYLCV-Mld and TYLCV-IL resistance

Contrasted phenotypes were observed between the different NILs derived from the cross LA1777 x E6203 during the screening experiments for TYLCV (Mld and IL strains) resistance (Figure 2a and 2b). After TYLCV-Mld inoculation, NIL IL-TA1304-7 had similar disease incidence (5%) to the resistant parent LA1777 (0%) while all the other NILs ranged from 67% to 100% being closer to the susceptible parent E6203 (100%). In addition to disease incidence, NIL IL-TA1304-7 ($sev_{31.5dpi}$ = 2.2) had attenuated symptoms compared to the susceptible parent E6203 ($sev_{31.5dpi}$ = 5.2) and the remaining NILs that ranged from $sev_{31.5dpi}$ = 3.6 to $sev_{31.5dpi}$ = 6.1.

For TYLCV-IL, only NIL IL-TA1554-11 had a phenotype similar to LA1777 characterized by a lower disease incidence (30%) and attenuated symptom severity ($sev_{31.5dpi}$ = 5.7). The remaining NILs had disease incidence ranging from 67% to 100% and symptom severity close to the susceptible parent E6203, this included ILTA1304-7 (95%). Density of type IV and type VI trichomes was evaluated on both parent LA1777 and E6203 and on each NIL derived from their cross (Table 3). Accession LA1777 had a high density of type IV trichome while no trichome was observed on E6203. Interestingly, none of the NIL screened for TYLCV resistance presented a phenotype similar to the resistant parent LA1777 and their density of trichomes type IV ranged from 0 to 2.

Discussion

Since adequate control of TYLCD is mainly based on the use of resistant cultivars, the screenings and the evaluation of resistance sources are prerequisites in breeding programs. As reported in previous studies, accession LA1777 has been used in different breeding programs to develop TYLCV-resistant cultivars (Maruthi et al. 2003; Vidavsky and Czosnek 1998). When compared to the susceptible controls M82 and WVA106, LA1777 exhibited an efficient protection against TYLCV-Mld and TYLCV-IL. It is important to notice that this resistance is characterized both by a lower disease incidence and attenuated symptom severity. Our study confirms the resistance conferred by LA1777 to TYLCV-IL (Maruthi et al. 2003) and highlights also its potential for an efficient control of TYLCV-Mld, the other emerging strain of TYLCV. TYLCV-IL and TYLCV-Mld have reached a worldwide distribution and co-occurrence of these two strains have also been reported throughout the world (Delatte et al. 2005; Ueda et al. 2004). Accession LA1777 could therefore be used as a source of a broad spectrum resistance to these two strains in breeding programs.

In complement to the spectrum of action conferred, the resistance evaluation also relied on the study of its stability through high inoculum pressure inoculations.

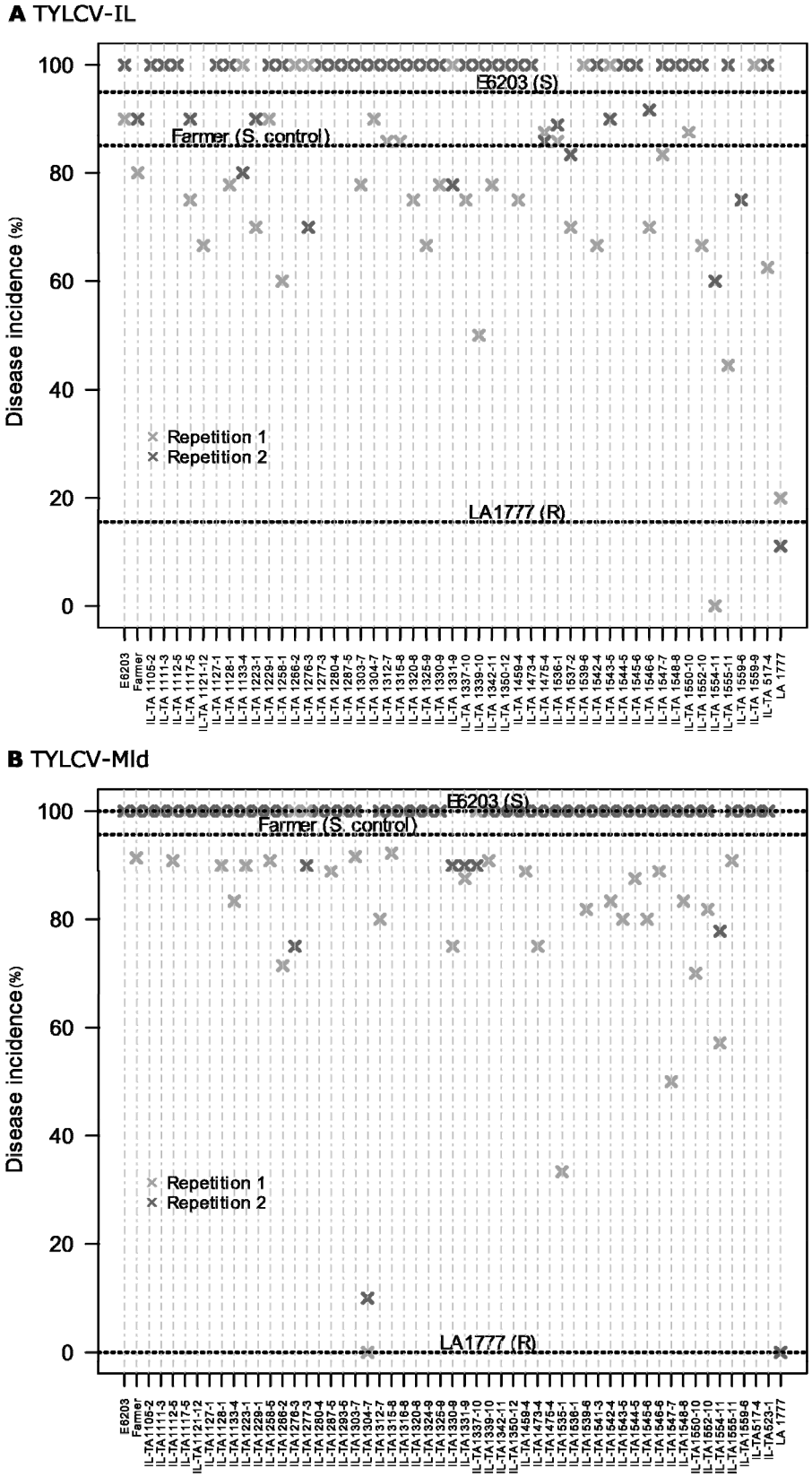


Figure 2: Disease incidence of the 52 near isogenic lines (NILs) after *Bemisia tabaci*-mediated inoculations with the Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-IL) (A) and Israël (TYLCV-Mld) (B). The two repetitions are represented.

We confronted LA1777 to increasing inoculation pressure with viruliferous *B. tabaci*. The level of the inoculation pressure was estimated through the estimation of cumulative number of inoculative whiteflies per hour in order to represent not only the duration of the exposure to viruliferous whiteflies but also the effect of the cumulative number of inoculative whiteflies. Importantly, the so-called low pressure inoculation pressure with TYLCV-Mld and TYLCV-IL of our experimental design was sufficient for a 100% disease incidence of the susceptible control WVA106. Albeit always less than the susceptible controls, disease incidence of accession LA1777 increased with the *B. tabaci*-mediated inoculation pressure. These results indicate that the resistance conferred by LA1777 is quantitative and can be overcome, at least partially, by an intense exposure to viruliferous whiteflies. A similar pattern was observed with the accession INRA-Hirsute following increasing inoculation pressures with TYLCV-Mld (Delatte et al. 2006). The resistance conferred by INRA Hirsute was associated with a lower disease incidence and was supposed to be a consequence of a disrupted feeding behaviour of *B. tabaci.*

In the light of the similar patterns between these two accessions, it was intriguing to explore the mechanism associated with the resistance of LA1777 to TYLCV-Mld and TYLCV-IL and in particular to assess the part of the resistance due to the resistance to the vector and to the virus. Indeed, the accession LA1777 is known as source of resistance to *B. tabaci* due to naturally occurring allelo-chemicals secreted by single-lobed glandular trichomes (type IV) (Muigai et al. 2002). These compounds have demonstrated high levels of repellent and fumigant activity against *B. tabaci*. To bypass these resistance mechanisms associated to vector resistance, grafting-inoculations with TYLCV-Mld and TYLCV-Il were performed. Interestingly, whereas the resistance conferred by LA1777 was not sufficient against TYLCV-IL, LA1777 exhibited resistance to TYLCV-Mld with both a lower disease incidence and attenuated symptom severity. Similar results were obtained with grafting inoculation of TYLCV-IL (Vidavsky and Czosnek 1998) but our results suggest that accession LA1777, in complement to the characterized vector resistance, also confers an effective resistance to TYLCV-Mld.

With the aim of exploring the resistance to TYLCV (Mld and IL strains), we could benefit of a set of NILs derived from the cross between LA1777 and the susceptible accession E6203 (Monforte and Tanksley 2000). NILs are a powerful tool to rapidly map new genes and are of particular interest for the study and exploration of quantitative traits including important agronomical traits such as flowering time, fruit characters and in our case, resistance to pathogens. The screenings performed with TYLCV-IL and TYLCV-Mld *B. tabaci*-mediated inoculations highlighted two NILs with specific resistance to TYLCV-Mld or TYLCV-IL. Whereas the line IL-TA1554-11 exhibited a specific resistance to TYLCV-IL with a similar phenotype to the resistant parent LA1777, the line IL-TA1304-7 expressed a specific resistance to TYLCV-Mld. It should be noted that the

resistance to TYLCV-IL is associated with a portion of the chromosome 11 of LA1777 while the resistance to TYLCV-Mld relies on a portion of the chromosome 7 from LA1777. However, none of the screened NILs, including ILTA1554-11 and ILTA1304-7, presented a density of trichomes type IV similar to the resistant parent LA1777 which suggest that the resistance conferred by these NILs is against the virus and not the whitefly. Taken together, these observations clearly indicate that the effective resistance of the accession LA1777 relies on complementary mechanisms involving resistance to the vector *B. tabaci* based on type IV trichomes and also to the virus TYLCV (Mld and IL strains). The genetic and molecular bases of these resistances remain nonetheless to be explored.

The development of TYLCV-resistant cultivars combining vector and virus resistance is an attractive strategy for the efficient and durable control of TYLCD. Mapping studies of vector resistance in wild tomato relatives, including *S. penneli*, *S. habrochaites*, and *S. pimpinnelifolium*, have revealed the genetic complexity of this character (Blauth et al. 1998; Fernández-Muñoz et al. 2000; Momotaz et al. 2010). This is in accordance with the absence of type IV-trichomes in the NILs derived from the cross between LA1777 and the cultivated tomato E6203. Therefore, introgression of this character in cultivated tomato genetic background will be difficult to manage and may appears as an unrealistic option. Nevertheless, the recently characterized accession *S. pimpinnelifolium* TO-937 exhibits a relatively simple mode of inheritance of the resistance to the two-spotted spider mite involving type IV glandular trichomes (Fernández-Muñoz et al. 2003). This accession could be used as a source of resistance to *B. tabaci* and gave promising results to reduce the spread of TYLCD (Rodríguez-López et al. 2011).

While LA1777 was known as a source of resistance to *B. tabaci*, our study could reveal loci involved in virus resistance in this accession. A portion of the chromosome 11 from LA1777 spanning marker TG36 is hence associated with a specific resistance to TYLCV-IL (Monforte and Tanksley 2000). Interestingly, Hanson et al (2000) mapped the TYLCV-resistance gene Ty-2 in this region of the chromosome 11 from the accession *S. habrochaites* H24. Similarly, Tomás et al (2011) revealed a specific resistance to the Spain isolate of TYLCV-IL in an accession belonging to the wild tomato relative *S. habrochaites*. In this later case, there was however no information on the mapping of this resistance gene. It would be therefore of particular interest to study eventual allelism relationships between these resistance genes originating from *S. habrochaites*.

As our study suggests that genomic regions of chromosome 7 and 11 from LA1777 are associated with quantitative resistance to TYLCV-IL and TYLCV-Mld, it could be interesting to cross NILs ILTA1304-7 and ILTA1554-11 in order to screen lines recombining for these two regions and resistant to both TYLCV strains. Otherwise, segregating populations of new NILs derived from crosses between

these two NILs and the susceptible parent E6203 could permit to map accurately the genes/QTL associated with this partial resistance and develop closely linked markers.

Plant resistance breakdown relies on the appearance, the multiplication and finally the dissemination of a virulent variant from an initially avirulent population. The combination of resistance genes from different origins and controlling complementary mechanisms makes this scenario unlikely. In the absence of qualitative resistance to TYLCV and tomato-infecting begomoviruses, the accurate exploitation and combination of quantitative resistance will be useful for a broad spectrum and efficient resistance. In this framework, the quantitative resistance conferred by LA1777 to TYLCV (Mld and IL strains) may be valuable and could be integrated in breeding programs in complement to the major resistance genes already widely used by seed companies.

References

Abhary M, Patil B, Fauquet CM (2007) Molecular Biodiversity, Taxonomy, and Nomenclature of Tomato Yellow Leaf Curl-like Viruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 85-118

Beaver JS, Osorno JM (2009) Achievements and limitations of contemporary common bean breeding using conventional and molecular approaches. Euphytica 168:145-175.

Blauth SL, Churchill GA, Mutschler MA (1998) Identification of quantitative trait loci associated with acylsugar accumulation using intraspecific populations of the wild tomato, Lycopersicon pennellii. TAG Theoretical and Applied Genetics 96:458-467.

Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM (2005) The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Reunion Island. Plant Pathology 54:262-262.

Delatte H, Holota H, Reynaud B, Dintinger J (2006) Characterisation of a Quantitative resistance to vector transmission of Tomato yellow leaf curl virus in Lycopersicon pimpinellifolium. European Journal of Plant Pathology 114:245-253.

Delatte H, Reynaud B, Granier M, Thornary L, Lett JM, Goldbach R, Peterschmitt M (2007) A new silverleaf-inducing biotype Ms of Bemisia tabaci (Hemiptera: Aleyrodidae) indigenous to the islands of the south-west Indian Ocean. Bulletin of Entomological Research 95: 29-35.

Elbert A, Nauen R (2000) Resistance of Bemisia tabaci (Homoptera: Aleyrodidae) to insecticides in southern Spain with special reference to neonicotinoids. Pest Management Science 56:60-64.

Fernandes FR, de Albuquerque LC, de Britto Giordano L, Boiteux LS, de Avila AC, Inoue-Nagata AK (2008) Diversity and prevalence of Brazilian bipartite begomovirus species associated to tomatoes. Virus Genes 36:251-258.

Fernández-Muñoz R, Domínguez E, Cuartero J (2000) A novel source of resistance to the two-spotted spider mite in Lycopersicon pimpinellifolium (Jusl .) Mill .: its genetics as affected by interplot interference. Journal of Economic Entomology 111:169-173.

Fernández-Muñoz R, Salinas M, Álvarez M, Cuartero J (2003) Inheritance of resistance to two-spotted spider mite and glandular leaf trichomes in wild tomato Lycopersicon pimpinellifolium (Jusl.) Mill. Journal of the American Society for Horticultural Science 128:188-195.

Hanson PM, Breeding P, Hall E, Green S, Tanksley SD, Chen H-mei, Kuo G, Fang D, Chen J-tzu (2000) Mapping a wild tomato introgression associated with Tomato yellow leaf curl virus resistance in a cultivated tomato line. Journal of the American Society for Horticultural Science 125:15-20.

Ji Y, Scott J, Hanson P, Graham E, Maxwell D (2007) Sources of Resistance, Inheritance, and Location of Genetic Loci Conferring Resistance to Members of the Tomato-Infecting Begomoviruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 343-362

Jones RAC (2009) Plant virus emergence and evolution: origins, new encounter scenarios, factors driving emergence, effects of changing world conditions, and prospects for control. Virus Research 141:113-30.

Lapidot M, Ben-Joseph R, Cohen L, Machbash Z, Levy D (2006) Development of a scale for evaluation of Tomato yellow leaf curl virus resistance level in tomato plants. Phytopathology 96:1404-1408.

Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJA., Meredith S, Lakay F, Monjane A, Lett JMM, Varsani A, Heydarnejad J (2010) The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World. PLoS Pathogens 6:e1001164.

Lefeuvre P, Martin DP, Hoareau M, Naze F, Delatte H, Thierry M, Varsani A, Becker N, Reynaud B, Lett JM (2007) Begomovirus "melting pot" in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. The Journal of General Virology 88:3458-3468.

Maruthi MN, Czosnek H, Vidavski F, Tarba S-yedidia (2003) Comparison of resistance to Tomato leaf curl virus (India) and Tomato yellow leaf curl virus (Israel) among Lycopersicon wild species , breeding lines and hybrids. European Journal of Plant Pathology 1-11.

Momotaz A, Scott JW, Schuster DJ (2010) Identification of Quantitative Trait Loci Conferring Resistance to Bemisia tabaci in an F 2 Population of Solanum lycopersicum · Solanum habrochaites Accession LA1777. Journal of the American Society for Horticultural Science 135:134-142.

Monforte AJ, Tanksley SD (2000) Development of a set of near isogenic and backcross recombinant inbred lines containing most of the Lycopersicon hirsutum genome in a L. esculentum genetic background: A tool for gene mapping and gene discovery. Genome 43:803-813.

Muigai S, Schuster D, Snyder J, Scott J, Bassett M, McAuslane H (2002) Mechanisms of resistance *Lycopersicon* germplasm to the whitefly *Bemisia argentifolii.* Phytoparasitica 30:347-360.

Palloix A, Ordon F (2011) Advanced Breeding for Virus Resistance in Plants. In: Caranta C, Aranda MA, Tepfer M, LopezMoya JJ (eds) Recent advances in plant virology. Caister Academic Press, Wymondham, pp 195-218

Perefarres F, Hoareau M, Chiroleu F, Reynaud B, Dintinger J, Lett JM (2011) A novel synthetic quantification standard including virus and internal report targets: application for the detection and quantification of emerging begomoviruses on tomato. Virology Journal 8:389.

Perring TM, Gruenhagen NM, Farrar CA (1999) Management of plant viral diseases through chemical control of insect vectors. Annual Review of Entomology 44:457-481.

Peterschmitt M, Granier M, Mekdoud R, Dalmon A, Gambin O, Vayssieres JF, Reynaud B (1999) First Report of Tomato Yellow Leaf Curl Virus in Réunion Island. Plant Disease 83:303-303.

Pilowsky M, Cohen S (1974) Inheritance of resistance to Tomato yellow leaf curl virus in tomatoes. Phytopathology 64:632-635.

Rodríguez-López MJ, Garzo E, Bonani JP, Fereres a, Fernández-Muñoz R, Moriones E (2011) Whitefly resistance traits derived from the wild tomato Solanum pimpinellifolium affect the preference and feeding behavior of Bemisia tabaci and reduce the spread of Tomato yellow leaf curl virus. Phytopathology 101:1191-1201.

Simmons AT, Gurr GM (2005) Trichomes of Lycopersicon species and their hybrids: effects on pests and natural enemies. Agricultural and Forest Entomology 7:265-276.

Singh SP, Morales FJ, Miklas PN, Terán H (2000) Selection for Bean golden mosaic resistance in intra- and interracial bean populations. Crop Science 40:1565-1572.

Team RDC (2008) A language and environment for statistical computing. R Foundation for Statistical Computing.

Tomás DM, Cañizares C, Abad J, Fernández-Muñoz R, Moriones E (2011) Resistance to tomato yellow leaf curl virus accumulation in the tomato wild relative Solanum habrochaites associated with the C4 viral protein. Molecular Plant-Microbe Interactions 24:849-861.

Ueda S, Kimura T, Onuki M, Hanada K, Iwanami T (2004) Three distinct groups of isolates of Tomato yellow leaf curl virus in Japan and construction of an infectious clone. Journal of General Plant Pathology 70:232-238.

Vidavsky F, Czosnek H (1998) Tomato breeding lines resistant and tolerant to Tomato yellow leaf curl virus issued from Lycopersicon hirsutum. Phytopathology 88:910-914

Discussion

Discussion générale

Les virus émergents sont définis comme ceux qui sont récemment apparus ou ceux dont les populations ont récemment augmenté en prévalence, en pathogénicité et/ou en répartition géographique. C'est le cas notamment des bégomovirus infectant la tomate, qui sont devenus le principal facteur limitant de la production dans beaucoup de régions tropicales, subtropicales et tempérées à travers le monde. L'émergence virale est généralement associée à des facteurs écologiques et moléculaires aboutissant à de nouvelles interactions virus - vecteur - plante - environnement (Fargette et al. 2006). Comprendre ces interactions et intégrer les différentes composantes de ce complexe sont des préalables nécessaires au développement de stratégies de contrôle et notamment à l'orientation des programmes de création variétale. Dans un premier volet, ce travail de thèse a eu comme objectifs de caractériser biologiquement la diversité des populations virales impliquées dans des maladies causées par des bégomovirus sur la tomate, d'étudier les interactions entre ces populations et leurs conséquences dans les dynamiques épidémiologiques (Chapitres I et II). Dans un second volet portant sur la recherche et la caractérisation de sources de résistance nous nous sommes attachés (1) à considérer la diversité virale à contrôler, et (2) à intégrer des sources de résistance complémentaires de celles déjà utilisées (Chapitres III et IV).

L'émergence et la dissémination des bégomovirus à travers le Monde

La globalisation et l'intensification des échanges commerciaux ont favorisé la dissémination de virus et de biotypes polyphages et invasifs de *B. tabaci*. Ces disséminations ont contribué à l'émergence des bégomovirus qui sont devenus le principal facteur limitant de la production agricole dans de nombreuses régions tropicales, subtropicales et tempérées du monde (Jones 2009; Moriones et al. 2011; Navas-Castillo et al. 2011). Le cas le plus emblématique est celui du TYLCV, qui est aujourd'hui présent en Afrique, Asie, Amérique du Nord et Amérique du Sud (pour revue Lefeuvre et al. 2010) et qui continue d'étendre son aire de répartition dans les Antilles (Lett et al. 2011) et en Océanie (Péréfarres et al. sous presse ; en annexe).

Ces introductions de virus et/ou de vecteurs sont les premiers facteurs associés aux phénomènes d'émergence (Anderson et al. 2004). Le développement d'un outil de diagnostic pour les bégomovirus présents dans les départements de l'Outre-Mer français permettra d'assurer un diagnostic précoce, de limiter les mouvements de plants contaminés et de réaliser des veilles épidémiologiques (Chapitre I). La stratégie innovante (PCR quantitative associée à un plasmide synthétique) décrite dans ce travail pourra aisément s'étendre à d'autres pathosystèmes et modèles d'études. Ce test sera prochainement transféré aux

services de l'agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) afin d'être intégré et comparé aux méthodes officielles déjà utilisées.

Les introductions successives à la Réunion des deux souches émergentes du TYLCV accompagnées du biotype B de *B. tabaci* ont profondément modifié les pratiques agricoles et progressivement conduit à l'abandon de la culture de la tomate en plein champ (Rimbaud et al. 2012). Aujourd'hui, le TYLCV et *Ralstonia solanacearum*, agent responsable du flétrissement bactérien, sont les principaux facteurs limitants de la production de la tomate à la Réunion et responsables de pertes importantes (Reynaud et al. 2003 ; Rimbaud et al. 2012).

Les introductions successives du TYLCV-Mld et du TYLCV-IL (Peterschmitt et al. 1999; Delatte et al. 2005a) ont fait de la Réunion un lieu privilégié pour étudier les dynamiques épidémiologiques dans un environnement insulaire et tropical entre deux souches d'une des espèces de phytovirus les plus préjudiciables.

Disséquer les dynamiques épidémiologiques pour orienter les stratégies de contrôle

Autour du bassin méditerranéen, zone d'origine des TYLCV-*like*, en Espagne et en Italie précisément, l'étude des dynamiques d'évolution et de la compétition au sein du complexe des TYLCV-*like* a démontré l'apparition de nombreux variants recombinants, mettant en exergue des phénomènes de compétition entre ceux-ci (Sánchez-Campos et al. 1999; Monci et al. 2002; Davino et al. 2006; García-Andrés et al. 2007; Davino et al. 2009).

De par son isolement géographique et sa petite taille, l'île de la Réunion est un lieu privilégié pour étudier les phénomènes d'invasions biologiques et de compétitions, qu'ils concernent les arthropodes (Duyck et al. 2006; Delatte et al. 2009), les plantes (Baret et al. 2005) ou les virus (Delatte et al. 2007). Avant les introductions successives des TYLCV-Mld et TYLCV-IL, aucun bégomovirus n'avait été décrit à la Réunion. Sur la base d'une veille épidémiologique de 7 ans sur l'ensemble du bassin maraîcher réunionnais, notre étude de la dynamique temporelle de l'épidémie du TYLCV a permis de mettre en évidence un **déplacement rapide du TYLCV-Mld par le TYLCV-IL**. En dépit d'aptitudes inférieures à la colonisation de l'hôte et à la dissémination naturelle, nos suivis épidémiologiques ont révélé la persistance du TYLCV-Mld à la Réunion. Nos travaux ont mis en avant l'importance des interactions et du synergisme entre ces deux souches du TYLCV, tant au niveau de la colonisation de l'hôte que de leur dissémination naturelle. Ces interactions ont conduit au **sauvetage épidémiologique du TYLCV-Mld à la Réunion** et assuré le maintien de cette souche dans un environnement où elle était pourtant désavantagée (Chapitre II).

Les infections mixtes de phytovirus sont communes dans la nature et peuvent être à l'origine d'interactions avec des conséquences sanitaires et épidémiologiques majeures (Fabre et al. 2010; Gómez et al. 2009; Lecoq et al. 2011; Martin and Elena 2009). Dans le cas des bégomovirus notamment, la co-infection est un pré-requis nécessaire à la recombinaison, principal moteur de la diversification des bégomovirus et également responsable d'émergences majeures (Fondong et al. 2000; García-Andrés et al. 2007; Idris and Brown 2005; Zhou et al. 1997). Notre étude a révélé l'importance de ces interactions virus-virus non pas dans la création de diversité virale par recombinaison mais dans le maintien de cette diversité virale à la Réunion. Il conviendra de poursuivre ces suivis épidémiologiques afin de suivre à plus long terme l'évolution de ce complexe viral et plus particulièrement le sauvetage épidémiologique du TYLCV-Mld par le TYLCV-IL et/ou l'apparition d'un recombinant entre le TYLCV-Mld et le TYLCV-IL.

Il est important de noter que Thierry *et al* (soumis) ont récemment rapporté l'introduction du biotype Q de *B. tabaci* à la Réunion. Les caractéristiques biologiques du biotype Q introduit n'ont pas été encore étudiées mais ce biotype est surtout redouté pour sa capacité de résistance à de nombreux insecticides (Horowitz et al. 2005), ainsi que pour son avantage écologique sur certaines plantes hôtes par rapport au biotype B (Muñiz 2000; Muñiz and Nombela 2001). Ces deux biotypes invasifs diffèrent également dans leur efficacité de transmission des bégomovirus et en particulier en ce qui concerne le TYLCV (Sánchez-Campos et al. 1999). Les interactions entre le biotype Q nouvellement établi et les deux autres biotypes B et Ms déjà implantés sont encore inconnues à la Réunion et mériteront toute notre attention. De même, les conséquences de cette introduction sur la dynamique épidémiologique du TYLCV à la Réunion devront être évaluées et intégrées par la suite dans notre modèle épidémiologique.

Notre étude a permis de mettre en évidence le maintien de la souche Mld à la Réunion. Cette population de TYLCV-Mld, bien que minoritaire aujourd'hui, devra être prise en considération dans le développement de stratégies de contrôle et particulièrement lors du déploiement de cultivars résistants qui devront assurer un contrôle efficace des deux souches du TYLCV à la Réunion.

B. tabaci, acteur majeur dans la dynamique évolutive des bégomovirus

Le transfert horizontal du virus d'un hôte à un autre, étape clé dans le cycle viral, est assuré par des vecteurs biologiques pour beaucoup de virus animaux et végétaux. Les vecteurs de phytovirus ont un rôle majeur dans leur dissémination mais jouent également un rôle prépondérant dans les dynamiques évolutives des populations virales.

La polyphagie des biotypes invasifs de *B. tabaci* a permis de multiplier les contacts (1) entre les virus et (2) avec de nouvelles plantes hôtes. Ces infections mixtes ont permis de façonner la diversité virale grâce notamment à la recombinaison et à la pseudo-recombinaison (échanges de composant(s) génomique(s) dans le cas des bipartites), événements communs chez les bégomovirus et principaux moteurs de leur diversification (Lefeuvre et al. 2007c; Padidam et al. 1999; Seal et al. 2006). L'introduction du biotype B de *B. tabaci* dans de nouveaux écosystèmes a permis à de nombreux bégomovirus de s'extirper de la flore naturelle et contaminer les plantes cultivées comme la tomate. Ce phénomène est notamment illustré par la récente émergence et la grande diversité virale des bégomovirus impliquée dans les épidémies sur tomate au Brésil depuis le milieu des années 1990. Autrefois mineurs, les bégomovirus sont aujourd'hui le principal facteur limitant de la production des cultures maraîchères au Brésil (Castillo-Urquiza et al. 2008; Fernandes et al. 2008; Navas-Castillo et al. 2011).
Dans le cas des transferts horizontaux des phytovirus, les vecteurs orientent également les dynamiques évolutives des populations virales à la fois en exerçant une pression de sélection et aussi par dérive génétique. Les effets de la sélection et de la dérive génétique ont été décrits et quantifiés principalement sur le modèle des *Potyvirus* (Legavre et al. 1996; Moury et al. 2007; Ng and Falk 2006). Ainsi, la pression de sélection exercée par le puceron vecteur sur le phytovirus a pu être démontrée en supprimant la contrainte de la transmission grâce à des séries répétées d'inoculations mécaniques. Des variants issus de ces expériences étaient alors peu ou pas transmissibles par puceron (Legavre et al. 1996; Ng and Falk 2006). En outre, Moury et al (2007) ont pu déterminer la taille du goulet d'étranglement lors de la transmission horizontale en estimant qu'entre 0.5 et 3.2 particules virales effectives (i.e. nombre de particules virales à l'origine d'une infection) de *Potato virus Y* (PVY, genre *Potyvirus*) sont transmises par un puceron. Des données similaires ont été ensuite rapportées chez le *Cucumber mosaic virus* (CMV, Bromoviridae) (Betancourt et al. 2008), virus à ARN également transmis selon le mode non circulant et non persistant par puceron.

Ce travail de thèse a pu pour la première fois estimer la taille du goulet d'étranglement exercé par *B. tabaci* lors de la transmission d'un virus à ADN selon le mode circulant et persistant. Jusqu'alors, l'effet de *B. tabaci* sur les populations virales avait été mis en évidence en observant le changement de compositions de populations virales recombinantes suite à une transmission horizontale (García-Andrés et al. 2007). Cette étude n'avait pu toutefois dissocier l'effet de la pression de sélection de la dérive génétique exercée par *B. tabaci* sur cette population virale. Notre travail a permis de quantifier ce goulet d'étranglement en estimant entre **1 à 2 particules virales effectives de TYLCV transmises par *B. tabaci*.** Ces données mettent en avant l'importance du phénomène de dérive génétique qui opère lors de la transmission d'un hôte à un autre hôte et la contribution majeure de cet événement dans les dynamiques

évolutives des populations virales. Ces événements répétés de sévères goulets d'étranglements lors de la transmission horizontale du virus pourraient faire de la dérive génétique aléatoire un processus aussi sinon plus important que la sélection dans les dynamiques évolutives des populations virales (García-Arenal et al. 2001; Sala and Wain-Hobson 2000). Avec un faible nombre de particules virales fondatrices, l'importance relative de la sélection et de la dérive génétique dépendra alors du nombre d'événements d'inoculations qui est lié à la densité de populations des insectes vecteurs. Ces données sont d'un intérêt majeur dans la conception de modèles d'évolution virale et notamment les modèles d'évolution de la virulence (Escriu et al. 2003; Moury et al. 2007) ou de la durabilité de gènes de résistance (Fabre et al. 2009).

Quelles sont les bases moléculaires des différences phénotypes ?

L'avènement de la stratégie d'amplification en cercle roulant (*rolling circle amplification*, RCA) de l'ADN circulaire des bégomovirus basée sur l'utilisation de la polymérase Phi29, associé à la baisse du coût de séquençage, a fortement contribué aux progrès récents dans la caractérisation moléculaire des bégomovirus. Malgré ces avancées, la caractérisation phénotypique de cette diversité virale n'a pas suivi la même trajectoire.

Ce travail a permis de mettre en évidence des profils contrastés en terme d'accumulation virale (Chapitre I), de sévérité des symptômes (Chapitre I), et d'efficacité de transmission (Chapitre II), avec des conséquences épidémiologiques (Chapitre II) et dans le contrôle de la maladie (Chapitre I, III). Déterminer les bases moléculaires de ces profils phénotypiques demeure un vaste défi pour les pathologistes. De nombreuses analyses théoriques des facteurs qui façonnent l'évolution des deux composantes du pouvoir pathogène des virus (aspect qualitatif : virulence ou gamme d'hôte ; aspect quantitatif : agressivité) ont été rapportées au cours des trois dernières décennies, avec des prédictions sur l'évolution de ces composantes suivant différents scénarios (Sacristán and García-Arenal 2008). Le volet expérimental n'a pour l'heure pas suivi. De plus, les phytopathologistes ont montré un plus grand intérêt dans la composante qualitative de la pathogénicité que dans sa composante quantitative. Ce déséquilibre est probablement lié à l'utilisation extensive de la résistance qualitative pour contrôler les maladies infectieuses des cultures (García-Arenal and McDonald 2003). Dans les dix dernières années, des progrès majeurs ont été réalisés dans la compréhension des bases moléculaires de la virulence (i.e. contournement de gènes de résistance, changement d'hôtes) (Woolhouse et al. 2005). A l'inverse, on en sait encore peu sur les bases moléculaires des composantes quantitatives du pouvoir pathogène d'un virus (*fitness*, efficacité de transmission, agressivité et incidence). Dans le cas des phytovirus, les travaux se sont principalement bornés à étudier l'effet de mutations ponctuelles et, plus rarement, l'effet de la distribution de ces mutations sur la *fitness* ou la sévérité

des symptômes (Carrasco et al. 2007; Desbiez et al. 2010; Sanjuán et al. 2004). Pour le modèle des bégomovirus, ces études sont plus rares et se sont limitées à l'étude de délétions ou mutations ponctuelles sur l'efficacité de transmission par le vecteur (Caciagli et al. 2009; Höhnle et al. 2001), la réplication ou la sévérité des symptômes (Bull et al. 2007; Stanley et al. 1992; Sung and Coutts 1995). Récemment Yaakov et al (2011) ont pu mettre en évidence l'effet d'une mutation ponctuelle de la protéine CP du TYLCV-IL et notamment son incapacité à induire des symptômes. Lors de nos veilles épidémiologiques à la Réunion, nous avons pu isoler et caractériser un isolat « faible » du TYLCV-IL. Cet isolat diffère de 14 mutations sur le génome dont cinq provoquent un changement d'acides aminés. Des données préliminaires semblent indiquer que cet isolat est incapable de provoquer des symptômes à la fois sur tomate et *N. benthamiana*, tout en se multipliant. Des travaux de mutagénèse dirigée sur le génome du TYLCV-IL sont actuellement en cours afin de révéler les bases moléculaires de ce changement de phénotype.

Malgré son rôle prépondérant dans les dynamiques évolutives, les effets de la recombinaison sur les variations phénotypiques des virus, et des bégomovirus notamment, restent largement à évaluer. Pour l'heure, des avancées ont été faites surtout dans la compréhension des facteurs qui contraignent et façonnent la distribution des recombinaisons au sein d'un génome (Lefeuvre et al. 2007b). Il a été ainsi montré que les réseaux d'interactions entre les acides aminés (repliement correct des protéines) et les nucléotides (structure secondaire des génomes) sont préservés par recombinaison (Lefeuvre et al. 2007b; Martin et al. 2011). Un des futurs challenges sera d'étudier dans quelle mesure la recombinaison peut influer sur l'agressivité ou la *fitness* d'un virus. Des travaux récents ont concerné pour la première fois cet aspect et ont permis d'étudier l'effet de la distribution de la recombinaison sur les propriétés phénotypiques (taux d'infection et accumulation virale) d'une banque de recombinants synthétiques issus du TYLCV-Mld et du ToLCKMV (Vuillaume et al. 2011). Ces auteurs n'ont toutefois pas pu associer clairement des variations phénotypiques à des variations génomiques. Cette expérience pourrait être répétée en utilisant comme base dans la banque de recombinants des virus parents aux profils biologiques plus contrastés (TYLCV-IL et ToLCKMV par exemple). Il pourrait être alors envisagé d'étudier non seulement les *loci* associés à ces traits biologiques mais également leurs relations épistatiques par approche de génétique quantitative.

Un des facteurs majeurs contraignant la recombinaison est le maintien de l'architecture des réseaux d'interactions protéiques et génomiques. La viabilité de l'ensemble des clones synthétiques développés par Vuillaume et al. (2011) témoigne des niveaux importants de plasticité des bégomovirus. Des progrès récents réalisés sur les techniques de double hybride à grande échelle ont permis d'établir les cartes d'interactions entre protéines virales et également entre

protéines virales et celles de l'hôte (Friedel and Haas 2011; Lee et al. 2011; Rual et al. 2005). Les banques de recombinants synthétiques ou obtenus *in vivo* pourraient alors être de précieux outils pour des recherches sur le potentiel d'évolution des complexes d'interactions entre protéines virales et également entre protéines virales et protéines de l'hôte, ainsi que sur les conséquences de ces modifications / perturbations d'interactions sur les propriétés biologiques des virus.

Résistances actuelles et à venir pour le contrôle durable des bégomovirus

Dans le cas d'une infection virale, l'éventail des méthodes de lutte demeure restreint et notamment aucune méthode curative applicable en conditions de culture n'est actuellement disponible. En complément de la prophylaxie, l'utilisation de cultivars résistants est le meilleur moyen pour assurer un contrôle efficace des bégomovirus tout au long du cycle de production (Antignus 2007; Picó 1996; Polston and Lapidot 2007). De nombreux *screening* ont été menés depuis le milieu des années 1970s afin de repérer des sources de résistance au TYLCV et aux autres bégomovirus associés au TYLCD (Agrama and Scott 2006; Banerjee and Kalloo 1987a; Banerjee and Kalloo 1987b; Griffiths and Scott 2001; Hassan et al. 1984; Ji et al. 2007; Kasrawi 1989; Mejía et al. 2005; Picó et al. 2000; Pilowsky and Cohen 1974). La principale difficulté dans la création de cultivars résistants aux bégomovirus réside dans la grande diversité des virus à contrôler (Abhary et al. 2007; Fauquet et al. 2008).

Dans un schéma de sélection, le pyramidage de gènes est en théorie une stratégie efficace pour augmenter le niveau de la résistance et étendre le spectre d'action de celle-ci en combinant des gènes contrôlant des mécanismes de résistance complémentaires. Ce travail de thèse a permis de dessiner les contours de la spécificité de la résistance conférée par les principaux gènes de résistance utilisés dans les programmes d'amélioration variétale des sociétés semencières (Chapitre III). Nos résultats suggèrent que la combinaison des gènes de résistance provenant de *S. chilense* et *S. habrochaites* pourraient contrôler efficacement le TYLCV et d'autres bégomovirus impliqués dans le TYLCD. Les bases moléculaires sous-jacentes restent néanmoins à explorer et seront de précieux indicateurs de la pertinence de la combinaison de ces gènes. Pour l'heure, les profils phénotypiques obtenus (gamme de spécificité, expression de symptômes, sévérité des symptômes) semblent indiquer que les gènes *Ty-1* et *Ty-3* originaires de *S. chilense* contrôleraient plutôt des mécanismes de résistance à spectre large caractérisés par des infections asymptomatiques des plants, par opposition avec le gène *Ty-2* originaire de *S. habrochaites* qui contrôlerait lui une résistance spécifique de la souche IL du TYLCV. L'efficacité de ces résistances peut s'avérer toutefois limitée sous de fortes pressions d'inoculation (Picó 1996 et chapitre III) et souligne l'importance de la caractérisation de nouvelles sources de résistance et de leur pyramidage.

La diversification des cibles pour la lutte génétique a également pour objectif l'identification de mécanismes de résistance distincts de ceux contrôlés par les facteurs dominants ou partiellement dominants. Il s'agit ici d'exploiter la capacité qu'ont les plantes d'échapper à l'infection par un virus grâce à l'absence ou la non-fonctionnalité d'un facteur cellulaire nécessaire au virus pour accomplir son cycle d'infection. Le cas le mieux documenté est celui du facteur d'initiation de la traduction eIF4E, pour lequel il a été démontré que des mutations naturelles étaient à l'origine de résistances récessives aux potyvirus et à d'autres virus à ARN (Albar et al. 2006; Marandel et al. 2009; Nieto et al. 2006; Ruffel et al. 2002 et pour revues Le Gall et al. 2011; Robaglia and Caranta 2006). Les mécanismes, qui caractérisent ce type de résistance dite constitutive, sont associés à des gènes récessifs dont on a démontré qu'ils pouvaient être de type qualitatif, mais également dans d'autres cas, être l'un des composants d'une résistance quantitative oligogénique. C'est le cas notamment chez le piment, avec le locus récessif *pvr2*, dont certains allèles contrôlent une résistance partielle au PVY, et ont été initialement cartographiés comme locus quantitatif (Caranta et al. 1997; Maule et al. 2007).

Les géminivirus, comme tous les virus, dépendent totalement de facteurs de la plante hôte pour se répliquer et envahir les tissus. Une mutation d'un de ces facteurs peut aboutir à un blocage du cycle viral et ainsi à la résistance de la plante. Certains gènes de l'hôte codant pour des protéines impliquées dans la réplication de l'ADN ou la régulation du cycle cellulaire ont été identifiés comme des candidats potentiels pour explorer les mécanismes moléculaires de la résistance récessive aux géminivirus (Castillo 2003; Castillo et al. 2004; Selth et al. 2005; Settlage et al. 2001; Xie et al. 1999). D'autres candidats peuvent être impliqués comme par exemple des gènes de la plante codant pour des protéines permettant le mouvement du virus (Lee et al. 1994; Mcgarry et al. 2003; Selth et al. 2006; Zhou et al. 2011). Dans ce contexte, la résistance récessive au PYMV identifiée dans LA2187-5 (Chapitre III) est particulièrement intéressante en vue de l'étude des bases génétiques et moléculaires de ce type de résistance aux bégomovirus. Ce travail a permis de mettre en évidence le large spectre ainsi que le caractère monogénique récessif de cette résistance conférée par LA2187-5. Une descendance en lignées recombinantes (RIL) a pu être développée et utilisée pour constituer une carte génétique basée sur des marqueurs de type *Single nucleotide polymorphism* (SNP) (556 marqueurs polymorphes pour 817 monomorphes entre LA2187-5 et CRA66). La cartographie d'un facteur majeur récessif de résistance permettra de voir une éventuelle co-localisation avec un gène candidat pour lequel un marqueur moléculaire polymorphe a pu être obtenu. Une co-localisation éventuelle avec un tel facteur cellulaire de la plante constituerait une piste intéressante pour explorer plus avant les bases génétiques et moléculaires de cette résistance récessive naturelle. Le séquençage comparé et l'analyse exon-intron, chez le parent résistant (LA2187-5) et le parent sensible

(CRA66), pourraient permettre de comprendre le déterminisme moléculaire de cette résistance récessive.
Anbinder et al. (2009) ont récemment cartographié le QTL majeur *Ty-5* associé à une résistance récessive au TYLCV dans l'accession *S. peruvianum* 'TY172'. De manière intéressante, il a été montré que ce locus co-localisait sur la carte génétique avec un marqueur de *SlNAC1*, un gène de la famille des NAC dont l'implication dans la réplication du TYLCV via son interaction avec la REn est connue (Selth et al. 2005). Dans cette même étude, les auteurs ont également détecté un QTL mineur de résistance au TYLCV co-localisant avec un gène de la famille *SUMO*. Ces gènes codent pour des protéines qui sont connues pour leur rôle dans le contrôle du cycle cellulaire et il a pu être montré que l'interaction entre la protéine Rep du TYLCSV et du TGMV avec des protéines de la famille *SUMO* est nécessaire dans l'accomplissement du cycle viral (Castillo et al. 2004). Si les bases génétiques et moléculaires de résistances reposant sur des mutations dans des facteurs cellulaires de la plante restent à découvrir, les deux gènes identifiés constituent des candidats prioritaires dans l'étude de la résistance récessive aux bégomovirus.

Les progrès réalisés dans la technique de double hybride à grande échelle ont permis d'établir les cartes d'interactions entre protéines virales, mais également, de dévoiler des interactions encore inconnues entre les protéines virales et celles de l'hôte (Calderwood et al. 2007 et pour revue voir Friedel and Haas 2011). L'application de ces techniques aux bégomovirus devrait notamment permettre de cibler chez l'hôte des interacteurs des protéines virales. La stratégie d'induction de résistance par *TILLING* s'est avérée particulièrement prometteuse pour la création d'allèles de résistance à des virus à ARN (Nieto et al. 2007). Cette stratégie pourrait de façon similaire se révéler utile dans la diversification des sources de résistance utilisables par les sélectionneurs pour le contrôle du TYLCV et des autres bégomovirus infectant la tomate, en orientant la création de mutants dans les gènes impliqués dans l'accomplissement du cycle viral.

Dans la stratégie de pyramidage de gènes visant à contrôler les bégomovirus, les sélectionneurs ont également cherché à intégrer des facteurs de la résistance au vecteur *B. tabaci* basés notamment sur la présence de trichomes (Blauth et al. 1998; Muigai et al. 2002 et pour revue Simmons and Gurr 2005). En complément des résistances aux virus, la résistance au vecteur constitue une option complémentaire et avantageuse pour augmenter le niveau et le spectre de la résistance tout en ciblant une autre étape du cycle viral. Nous avons confirmé l'intérêt de l'accession résistante à *B. tabaci* 'LA1777' (Momotaz et al. 2010; Muigai et al. 2002) pour le contrôle des deux souches émergentes du TYLCV (Chapitre IV). En dépit de son efficacité à limiter l'infection par le TYLCV-IL et le TYLCV-Mld comme nous l'avons démontré, la résistance conférée par LA1777 n'est pas directement exploitable dans des programmes d'amélioration compte tenu de la complexité de ce caractère de résistance (Chapitre IV et Momotaz et

al. 2010). Néanmoins, le screening de lignées quasi-isogéniques de *S. lycopersicum* issues du croisement de LA1777 et E6203 a mis en évidence dans cette accession des facteurs d'une résistance partielle au TYLCV-IL et TYLCV-Mld qui sont exploitables en sélection. Plus intéressant encore, la résistance conférée au TYLCV-IL correspond à une portion du génome déjà associée à la résistance dans une autre accession de *S. habrochaites* résistante spécifiquement au TYLCV-IL (Chapitre III, Hanson et al. 2000; Monforte and Tanksley 2000). Ces résultats suggèrent l'existence de mécanismes conservés au sein de l'espèce *S. habrochaites*. Il conviendrait toutefois de caractériser plus finement ces *loci* afin d'établir d'éventuelles relations alléliques.
Rodríguez-López et al. (2011) ont récemment décrit l'utilisation de la résistance à *B. tabaci* conférée par l'accession *S. pimpinellifolium* 'TO-937' afin de limiter l'infection par le TYLCV et la dissémination de la maladie. Cette lignée portant une résistance à l'insecte apparemment contrôlée par un facteur majeur constitue une source potentielle de diversification de la résistance aux bégomovirus justifiant une étude de cartographie génétique et la recherche de marqueurs moléculaires liés au gène/QTL majeur responsable.
D'une façon générale, la caractérisation de nouvelles sources de résistances originaires de *S. pimpinellifolium*, espèce apparentée la plus proche de la tomate cultivée et présentant le moins d'effets négatifs en tant que source de caractères d'intérêt, intéresse en priorité les sélectionneurs dans leur stratégie de diversification des sources de résistance pour un contrôle efficace et durable des bégomovirus.

Quel devenir des populations virales ?

L'activité humaine a été un des facteurs les plus importants responsables de l'émergence des bégomovirus, principalement à travers la dissémination des biotypes invasifs de *B. tabaci* et des virus qu'ils transmettent. De même, depuis la domestication des plantes, l'intensification de l'agriculture associée à la monoculture à grande échelle a également contribué à l'émergence des maladies virales en favorisant leurs propagations (Morales and Anderson 2001). Les résistances génétiques ont permis de réduire l'impact des maladies virales chez de nombreuses plantes cultivées. Cependant, comme pour tout couple hôte-parasite, les populations virales s'adaptent à ces résistances qui exercent de fortes pressions de sélection et jouent un rôle dans les dynamiques évolutives des virus. Les bégomovirus, comme la plupart des virus de plantes, infectent plusieurs espèces et génotypes d'hôtes cultivés et sauvages. Pour l'heure, on ne sait pas grand chose de l'effet de la plante hôte sur les dynamiques évolutives des populations virales et dans quelle mesure elle contribue à la structuration des populations virales. Le projet VirPop initié durant cette thèse a pour but de contribuer à la compréhension des mécanismes d'émergences en étudiant les dynamiques d'évolution (taux de mutation et de recombinaison) et le potentiel adaptatif des populations virales de bégomovirus à l'échelle de l'hôte. Afin

d'estimer les contraintes adaptatives liées aux changements d'hôte, ces mesures seront réalisées pour des populations simples (mono-infection) et complexes (co-infection) sur des plantes sauvages, cultivées sensibles et cultivées résistantes. Ces données serviront à estimer les pressions de sélection exercées par ces différents hôtes rencontrés par les populations virales à l'échelle d'un paysage agricole.

La pression de sélection exercée par les cultivars résistants déployés à grande échelle peut aboutir à l'émergence de populations virales adaptées à ces cultivars et aux contournements des gènes de résistance (i.e. lorsque l'adaptation des populations virales provoque des pertes de rendement supérieures au seuil économique acceptable ; Johnson 1984). Alors que certains gènes de résistance s'avèrent efficaces pendant de nombreuses années (cas du gène *N* de résistance au *Tobacco mosaic virus* (TMV) chez le tabac ; García-Arenal and McDonald 2003), d'autres ont été contournés en quelques années, voire quelques mois (cas du gène *Tm-1* de résistance au *Tomato mosaic virus* (ToMV) ; Meshi et al. 1988). Alors qu'aucune relation claire n'a pour l'instant pu être établie entre la structure, la séquence ou la fonction des gènes de résistance et leur durabilité, l'analyse de la dynamique et de la génétique des populations virales a permis des avancées dans la compréhension de la durabilité des résistances. Le contournement d'une résistance peut être découpé en trois étapes distinctes (Moury et al. 2011). La première étape consiste en l'apparition de variants viraux virulents, capables de contourner la résistance, à partir des populations virales avirulentes (i.e. non compatibles). Ces variants virulents sont ensuite en compétition dans la cellule et dans la plante avec le reste de la population virale pour leur multiplication et l'infection de la plante. Enfin, la dernière étape du contournement consiste en la dissémination de ce variant entre les plantes de la parcelle puis au delà au niveau de l'agroécosystème. Ces trois étapes du contournement de la résistance sont à mettre en relation avec les cinq forces évolutives qui façonnent le devenir des populations virales : alors que la mutation et la recombinaison génèrent de la diversité dans les populations, la sélection, la dérive génétique et la migration jouent un rôle dans l'évolution de la fréquence des variants dans la population.

Compte tenu de la multiplicité des paramètres intervenant lors du contournement d'une résistance, l'analyse expérimentale de l'effet de chacun de ces facteurs est rendue difficile. Le recours à la modélisation a permis de faire des progrès dans la compréhension des dynamiques épidémiologiques (Jeger et al. 2011; Matthews and Haydon 2007) et des mécanismes d'adaptation au déploiement de cultivars résistants ou de contournement des résistances qualitatives (Van Den Bosch et al. 2006; Fabre et al. 2009). En intégrant les facteurs opérant de l'échelle de la cellule végétale jusqu'à l'échelle de la population de l'hôte, les travaux de Fabre *et al* (2009) ont permis de réaliser des avancées significatives dans l'estimation de la contribution relative des facteurs épidémiologiques, génétiques et évolutifs, et de leurs interactions associés à l'émergence de variants virulents. Le premier

facteur associé au contournement d'une résistance est d'origine génétique et correspond au nombre de mutations nécessaires pour acquérir la virulence, en accord avec ce qui avait déjà été suggéré par Harrison (2002).

Il reste encore de nombreux progrès à réaliser avant d'envisager l'application de tels modèles d'évolution de la virulence ou de la durabilité des gènes de résistance chez les bégomovirus. Ce travail de thèse a permis d'apporter les premières composantes de ces modèles notamment dans la caractérisation de facteurs épidémiologiques associés à l'émergence virale, comme l'estimation de la taille du goulet d'étranglement exercé par *B. tabaci* lors de la transmission d'une plante à l'autre. Pour l'heure, une des lacunes majeures dans le modèle des bégomovirus réside dans le manque de connaissances sur les bases moléculaires des gènes de résistance aux bégomovirus et plus généralement aux virus à ADN. Les améliorations techniques dans le génotypage et la cartographie fine devraient permettre dans un futur proche de percer les mécanismes de ces résistances ainsi que les bases moléculaires du contournement de ces gènes. L'intégration d'estimateurs de la durabilité de la résistance dans des modèles reliant les dynamiques évolutives et épidémiologiques des populations virales permettra le développement d'outils pertinents d'aide à la décision pour la création et le déploiement de cultivars résistants à l'échelle de l'agroécosystème.

Les techniques de génotypage haut débit permettent d'envisager maintenant la cartographie très fine de ces gènes/QTL de résistance aux bégomovirus chez la tomate, ce qui est un avantage incontestable pour les sélectionneurs en leur permettant d'introgresser la résistance avec le minimum d'effets agronomiques défavorables. Cependant, on ne connaît rien de la nature moléculaire de ces gènes/QTL, des mécanismes de résistance mis en œuvre dans la plante et des risques de contournement par le virus. Actuellement, les seuls modèles d'évaluation de la durabilité de gènes de résistance concernent les virus à ARN (Fabre et al. 2009; Janzac et al. 2009). Concernant les bégomovirus, compte tenu des manques de connaissances sur les gènes de résistance et des événements nucléotidiques associées à leur contournement (mutation, recombinaison, pseudo-recombinaison), il est encore trop tôt pour concevoir des modèles reliant les facteurs génétiques de la plante, les dynamiques évolutives et épidémiologiques des populations virales. En conclusion, il est difficile de prévoir la durabilité des résistances qui sont pour l'instant très efficaces, qu'ont tendance à utiliser certains sélectionneurs et qui reposent sur des combinaisons alléliques de *Ty-1* et *Ty-2* par exemple. Dans l'immédiat, il s'agit donc de poursuivre l'exploration des autres sources de résistance (récessive et insecte vecteur) et/ou de tolérance, de façon à pouvoir diversifier à l'avenir les facteurs génétiques contrôlant les bégomovirus dans les cultivars de tomate.

Références bibliographiques

Références bibliographiques

Abhary M, Patil B, Fauquet CM (2007) Molecular Biodiversity, Taxonomy, and Nomenclature of Tomato Yellow Leaf Curl-like Viruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 85-118.

Ach RA, Durfee T, Miller AB, Taranto P, Hanley-Bowdoin L, Zambryski PC, Gruissem W (1997) RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Molecular and Cellular Biology 17:5077-5086.

Agrama HA, Scott JW (2006) Quantitative Trait Loci for Tomato Yellow Leaf Curl Virus and Tomato Mottle Virus Resistance in Tomato. Journal of the American Society for Horticultural Science 131:267-272.

Albar L, Bangratz-Reyser M, Hébrard E, Ndjiondjop M-N, Jones M, Ghesquière A (2006) Mutations in the eIF(iso)4G translation initiation factor confer high resistance of rice to Rice yellow mottle virus. The Plant Journal: for cell and molecular biology 47:417-26.

Anbinder I, Reuveni M, Azari R, Paran I, Nahon S, Shlomo H, Chen L, Lapidot M, Levin I (2009) Molecular dissection of Tomato leaf curl virus resistance in tomato line TY172 derived from Solanum peruvianum. Theoretical and Applied Genetics 119:519-30.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends in Ecology & Evolution 19:535-44.

Antignus Y (2007) The management of tomato yellow leaf curl virus in greenhouses and the open field, a strategy of manipulation. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 263-278.

Antignus Y, Cohen S (1994) Cloning of tomato yellow leaf curl virus (TYLCV) and the complete nucleotide sequence of a mild infectious clone. Phytopathology 84:707-712.

Antignus Y, Nestel D, Cohen S, Lapidot M (2001) Ultraviolet-Deficient Greenhouse Environment Affects Whitefly Attraction and Flight-Behavior. Environmental Entomology 30:394-399.

Arguello-astorga G, Lopez-ochoa L, Kong L-jie, Orozco BM, Settlage SB, Hanley-bowdoin L (2004) A Novel Motif in Geminivirus Replication Proteins Interacts with the Plant Retinoblastoma-Related Protein. Journal of Virology 78:4817-4826.

Banerjee M, Kalloo MK (1987a) Inheritance of tomato leaf curl virus resistance in Lycopersicon hirsutum f. glabratum. Euphytica 36:581–584.

Banerjee MK, Kalloo MK (1987b) Sources and inheritance of resistance to leaf curl virus in Lycopersicon. Theoretical and Applied Genetics 73:707-710.

Baret S, Le Bourgeois T, Strasberg D (2005) How would Rubus alceifolius, an intrusive exotic species, progressively colonize the entirety of a humid tropical forest. Canadian Journal of Botany 83:219-226.

Bass HW, Nagar S, Hanley-Bowdoin L, Robertson D (2000) Chromosome condensation induced by geminivirus infection of mature plant cells. Journal of Cell Science 113:1149-1160.

Beaver JS, Osorno JM (2009) Achievements and limitations of contemporary common bean breeding using conventional and molecular approaches. Euphytica 168:145-175.

Behjatnia SAA, Dry IB, Rezaian MA (2007) Characterization and transient replication of tomato leaf curl virus defective DNAs. Archives of Virology 152:1127-38.

Betancourt M, Fereres A, Fraile A, García-Arenal F (2008) Estimation of the effective number of founders that initiate an infection after aphid transmission of a multipartite plant Virus. Journal of Virology 82:12416-12421.

Bethke J, Paine TD, Nuessly GS (1991) Comparative Biology, Morphometrics, and Development of Two Populations of Bemisia tabaci (Homoptera: Aleyrodidae) on Cotton and Poinsettia. Annals of the Entomological Society of America 84:407-411.

Bird J, Idris AM, Rogan D, Brown JK (2001) Introduction of the Exotic Tomato yellow leaf curl virus-Israel in Tomato to Puerto Rico. Plant Disease 85:1028.

Bisaro DM (2006) Silencing suppression by geminivirus proteins. Virology 344:158-68.

Blackmer JL, Lindley VA, Byrne DN (1995) Histological examination of flight muscle development and breakdown in *Bemisia tabaci* (Homoptera: Aleyrodidae): Relationship to age and flight behvior. Journal of Morphology 226:213-221.

Blauth SL, Churchill GA, Mutschler MA (1998) Identification of quantitative trait loci associated with acylsugar accumulation using intraspecific populations of the wild tomato, Lycopersicon pennellii. Theoretical and Applied Genetics 96:458-467.

Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X, Fauquet CM (2008) Recommendations for the classification and nomenclature of the DNA-β satellites of begomoviruses. Archives of Virology 153:763-781.

Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, Rishi N, Siwatch SS, Zafar Y, Abdel-Salam AM, Markham PG (2004) Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus–DNA β complexes. Virology 324:462-474.

Briddon RW, Patil BL, Bagewadi B, Nawaz-ul-Rehman MS, Fauquet CM (2010) Distinct evolutionary histories of the DNA-A and DNA-B components of bipartite begomoviruses. BMC Evolutionary Biology 10:97.

Briddon RW, Pinner MS, Stanley J, Markham PG (1990) Geminivirus coat protein gene replacement alters insect specificity. Virology 177:85-94.

Briddon RW, Stanley J (2006) Subviral agents associated with plant single-stranded DNA viruses. Virology 344:198-210.

Brown JK, Bird J (1995) Variability within the *Bemisia tabaci* species complex and its relation to new epidemics caused by geminiviruses. Memoria, IV Taller Latinoamericano sobre moscas blancas y geminivirus 16:18.

Bull SE, Briddon RW, Sserubombwe WS, Ngugi K, Markham PG, Stanley J (2007) Infectivity, pseudorecombination and mutagenesis of Kenyan cassava mosaic begomoviruses. Journal of General Virology 88:1624-1633.

Byrne DN, Bellows TS (1991) Whitefly Biology. Annual Review of Entomology 36:431-457.

Caciagli P, Medina Piles V, Marian D, Vecchiati M, Masenga V, Mason G, Falcioni T, Noris E (2009) Virion stability is important for the circulative transmission of tomato yellow leaf curl sardinia virus by *Bemisia tabaci*, but virion access to salivary glands does not guarantee transmissibility. Journal of Virology 83:5784-95.

Calderwood M a, Venkatesan K, Xing L, Chase MR, Vazquez A, Holthaus AM, Ewence AE, Li N, Hirozane-Kishikawa T, Hill DE, Vidal M, Kieff E, Johannsen E (2007) Epstein-Barr virus and virus human protein interaction maps. Proceedings of the National Academy of Sciences of the United States of America 104:7606-11.

Caranta C, Lefebvre V, Palloix A (1997) Polygenic Resistance of Pepper to Potyviruses Consists of a Combination of Isolate-Specific and Broad-Spectrum Quantitative Trait Loci. Molecular Plant-Microbe Interactions 10:872-878.

Carrasco P, de la Iglesia F, Elena SF (2007) Distribution of fitness and virulence effects caused by single-nucleotide substitutions in Tobacco Etch virus. Journal of Virology 81:12979-84.

Castellano MM, del Pozo JC, Ramirez-Parra E, Brown S, Gutierrez C (2001) Expression and Stability of Arabidopsis CDC6 Are Associated with Endoreplication. The Plant Cell Online 13:2671-2686.

Castillo AG, Kong LJ, Bejarano ER (2004) Interaction between a Geminivirus Replication Protein and the Plant Sumoylation System. Journal of Virology 78:2758-2769.

Castillo-Urquiza GP, Beserra JEA, Bruckner FP, Lima ATM, Varsani A, Alfenas-Zerbini P, Murilo Zerbini F (2008) Six novel begomoviruses infecting tomato and associated weeds in Southeastern Brazil. Archives of Virology 153:1985-9.

Cohen S, Antignus Y (1994) Tomato yellow leaf curl virus, a whitefly-borne geminivirus of tomatoes. Advances in Diseases Vector Research 10:259-288.

Cohen S, Harpaz I (1964) Periodic, rather than continual acquisition of a new tomato virus by its vector, the tobacco whitefly *Bemisia tabaci*. Entomologia Experimentalis et Applicata 7:155-166.

Comai L, Henikoff S (2006) TILLING: practical single-nucleotide mutation discovery. The Plant Journal 45:684-694.

Cooper JI, Jones AT (1983) Responses of plants to viruses: proposals for the use of terms. Phytopathology 73:127-128.

Crameri A, Raillard SA, Bermudez E, Stemmer WPC (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 391:288-291.

Crespi S, Accotto GP, Caciagli P, Gronenborn B (1991) Use of digoxigenin-labelled probes for detection and host-range studies of tomato yellow leaf curl geminivirus. Research in Virology 142:283-288.

Cui X, Li G, Wang D, Hu D, Zhou X (2005) A Begomovirus DNAβ-Encoded Protein Binds DNA, Functions as a Suppressor of RNA Silencing, and Targets the Cell Nucleus. Journal of Virology 79:10764-10775.

Czosnek H (2007) Interactions of Tomato Yellow Leaf Curl Virus with its Whitefly Vector . In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 157-170

Czosnek H, Ber R, Navot N, Zamir D (1988) Detection of Tomato Yellow Leaf Curl Virus in Lysates of Plants and Insects by Hybridization with a Viral DNA Probe. Plant Disease 72:949-951.

Dalmon A, Cailly M, David C (2000) Comparison of serological and molecular techniques for detection of Tomato yellow leaf curl begomovirus*. EPPO Bulletin 30:457-462.

Davino S, Napoli C, Davino M, Accotto GP (2006) Spread of Tomato yellow leaf curl virus in Sicily: Partial displacement of another geminivirus originally present. European Journal of Plant Pathology 114:293-299.

Davino S, Napoli C, Dellacroce C, Miozzi L, Noris E, Davino M, Accotto GP (2009) Two new natural begomovirus recombinants associated with the tomato yellow leaf curl disease co-exist with parental viruses in tomato epidemics in Italy. Virus Research 143:15-23.

De Barro PJ, Liu S-S, Boykin LM, Dinsdale AB (2011) Bemisia tabaci: a statement of species status. Annual Review of Entomology 56:1-19.

Delatte H, Dalmon A, Rist D, Soustrade I, Wuster G, Lett JM, Goldbach RW, Peterschmitt M, Reynaud B (2003) *Tomato yellow leaf curl virus* can be acquired and transmitted by *Bemisia tabaci* (Gennadius) from tomato fruit. Plant Disease 87:1297-1300.

Delatte H, Holota H, Naze F, Peterschmitt M, Reynaud B, Lett JM (2005a) The presence of both recombinant and nonrecombinant strains of Tomato yellow leaf curl virus on tomato in Reunion Island. Plant Pathology 54:262-262.

Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett J-M (2005b) South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. The Journal of General Virology 86:1533-42.

Delatte H, Holota H, Reynaud B, Dintinger J (2006) Characterisation of a quantitative resistance to vector transmission of Tomato yellow leaf curl virus in Lycopersicon pimpinellifolium. European Journal of Plant Pathology 114:245-253.

Delatte H, Lett JM, Lefeuvre P, Reynaud B, Peterschmitt M (2007) An insular environment before and after TYLCV introduction. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 13-23

Delatte H, Duyck PF, Triboire A, David P, Becker N, Bonato O, Reynaud B (2009) Differential invasion success among biotypes: case of *Bemisia tabaci*. Biological Invasions 11:1059-1070.

Desbiez C, Girard M, Lecoq H (2010) A novel natural mutation in HC-Pro responsible for mild symptomatology of Zucchini yellow mosaic virus (ZYMV, Potyvirus) in cucurbits. Archives of virology 155:397-401.

Dewitte W, Murray JAH (2003) The plant cell cycle. Annual Review of Plant Biology 54:235-264.

Dinsdale A, Cook L, Riginos C, Buckley YM, Barro PD (2010) Refined global analysis of *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) mitochondrial cytochrome oxidase 1 to identify species level genetic boundaries. Annals of the Entomological Society of America 103:196-208.

Djian-Caporalino C, Lefebvre V, Sage-Daubèze AM, Palloix A (2007) Capsicum. In: Singh (ed) Genetic Resources, Chromosome Engineering, and Crop Improvement series, Volume 3, Vegetable crops, R.J., ed. CRC Press, Boca Raton, FL, pp 185-243

Dogra SC, Eini O, Rezaian MA, Randles JW (2009) A novel shaggy-like kinase interacts with the Tomato leaf curl virus pathogenicity determinant C4 protein. Plant Molecular Biology 71:25-38.

Drake JW, Charlesworth B, Charlesworth D, Crow JF (1998) Rates of Spontaneous Mutation. Genetics 148:1667-1686.

Dry IB, Krake LR, Rigden JE, Rezaian MA (1997) A novel subviral agent associated with a geminivirus: The first report of a DNA satellite. Proceedings of the National Academy of Sciences 94:7088-7093.

Duffy S, Holmes EC (2007) Multiple introductions of the Old World begomovirus Tomato yellow leaf curl virus into the New World. Applied and Environmental Microbiology 73:7114-7.

Duffy S, Shackelton LA, Holmes EC (2008) Rates of evolutionary change in viruses: patterns and determinants. Nature reviews Genetics 9:267-76.

Durfee T, Feiler HS, Gruissem W (2000) Retinoblastoma-related proteins in plants: homologues or orthologues of their metazoan counterparts? Plant Molecular Biology 43:635-642.

Duyck P-F, David P, Junod G, Brunel C, Dupont R, Quilici S (2006) Importance of competition mechanisms in successive invasions by polyphagous tephritids in La Reunion. Ecology 87:1770-1780.

Egelkrout EM, Robertson D, Hanley-Bowdoin L (2001) Proliferating Cell Nuclear Antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves. The Plant Cell Online 13:1437-1452.

Elbert A, Nauen R (2000) Resistance of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides in southern Spain with special reference to neonicotinoids. Pest Management Science 56:60-64.

Escriu F, Fraile A, García-Arenal F (2003) The evolution of virulence in a plant virus. Evolution 57:755-765.

Fabre F, Bruchou C, Palloix A, Moury B (2009) Key determinants of resistance durability to plant viruses: insights from a model linking within- and between-host dynamics. Virus Research 141:140-9.

Fabre F, Chadoeuf J, Costa C, Lecoq H, Desbiez C (2010) Asymmetrical over-infection as a process of plant virus emergence. Journal of Theoretical Biology 265:377-88.

Fargette D, Konaté G, Fauquet CM, Muller E, Peterschmitt M, Thresh JM (2006) Molecular ecology and emergence of tropical plant viruses. Annual Review of Phytopathology 44:235-60.

Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X (2008) Geminivirus strain demarcation and nomenclature. Archives of Virology 153:783-821.

Fernandes FR, de Albuquerque LC, de Britto Giordano L, Boiteux LS, de Avila AC, Inoue-Nagata AK (2008) Diversity and prevalence of Brazilian bipartite begomovirus species associated to tomatoes. Virus Genes 36:251-8.

Fondong VN, Pita JS, Rey ME, de Kochko a, Beachy RN, Fauquet CM (2000) Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. The Journal of General Virology 81:287-97.

Foolad MR (2005) Deployment of Molecular Markers and Marker-assisted Selection in Tomato Breeding. HortScience 40:1114.

Friedel CC, Haas J (2011) Virus–host interactomes and global models of virus-infected cells. Trends in Microbiology 19:501-508.

Frohlich DR, Torres-Jerez I, Bedford ID, Markham PG, Brown JK (1999) A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. Molecular Ecology 8:1683-1691.

Fuentes A, Ramos P, Fiallo E, Callard D, Sánchez Y, Peral R, Rodríguez R, Pujol M (2006) Intron–hairpin RNA derived from replication associated protein *C1* gene confers immunity to Tomato yellow leaf curl virus infection in transgenic tomato plants. Transgenic Research 15:291-304.

García-Andrés S, Monci F, Navas-Castillo J, Moriones E (2006) Begomovirus genetic diversity in the native plant reservoir Solanum nigrum: Evidence for the presence of a new virus species of recombinant nature. Virology 350:433-42.

García-Andrés S, Tomás DM, Sánchez-Campos S, Navas-Castillo J, Moriones E (2007) Frequent occurrence of recombinants in mixed infections of tomato yellow leaf curl disease-associated begomoviruses. Virology 365:210-9.

García-Andrés S, Tomás DM, Navas-Castillo J, Moriones E (2009) Resistance-driven selection of begomoviruses associated with the tomato yellow leaf curl disease. Virus Research 146:66-72.

García-Arenal F, Fraile A, Malpica JM (2001) Variability and genetic structure of plant virus populations. Annual Review of Phytopathology 39:157-186.

García-Arenal F, McDonald BA (2003) An analysis of the durability of resistance to plant viruses. Phytopathology 93:941-952.

Gennadius P (1889) Disease of tobacco plantations in the Trikonia. The aleurodid of tobacco. Ellenike Georgia 5:1-13.

Gharsallah Chouchane S, Gorsane F, Nakhla MK, Maxwell DP, Marrakchi M, Fakhfakh H (2007) First report of Tomato yellow leaf curl virus-Israel species infecting tomato, pepper and bean in Tunisia. Journal of Phytopathology 155:236-240.

Gómez P, Sempere RN, Elena SF, Aranda MA (2009) Mixed infections of Pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. Journal of Virology 83:12378-87.

Gordon-Kamm W, Dilkes BP, Lowe K, Hoerster G, Sun X, Ross M, Church L, Bunde C, Farrell J, Hill P, Maddock S, Snyder J, Sykes L, Li Z, Woo Y-min, Bidney D, Larkins BA (2002) Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. Proceedings of the National Academy of Sciences 99:11975-11980.

Grafi G, Burnett RJ, Helentjaris T, Larkins BA, De Caprio JA, Sellers WR, Kaelin WG (1996) A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. Proceedings of the National Academy of Sciences 93:8962-8967.

Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics 164:731-740.

Griffiths PD, Scott JW (2001) Inheritance and Linkage of Tomato Mottle Virus Resistance Genes Derived from Lycopersicon chilense Accession LA 1932. Journal of the American Society for Horticultural Science 126:462-467.

Grubman MJ, Baxt B (2004) Foot-and-Mouth Disease Foot-and-Mouth Disease. Clinical Microbiology Reviews 17:465-493.

Gutierrez C (2002) Strategies for geminivirus DNA replication and cell cycle interference. Physiological and Molecular Plant Pathology 60:219-230.

Gutierrez C, Ramirez-Parra E, Castellano MM, del Pozo JC (2002) G1 to S transition: more than a cell cycle engine switch. Current Opinion in Plant Biology 5:480-486.

Hanley-Bowdoin L, Elmer JS, Rogers SG (1989) Functional expression of the leftward open reading frames of the A component of Tomato golden mosaic virus in transgenic tobacco plants. The Plant Cell Online 1:1057-1067.

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (2000) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Critical reviews in biochemistry and molecular biology 35:105-140.

Hanley-Bowdoin L, Settlage S, Robertson D (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. Molecular plant 5:149-156.

Hanson PM, Breeding P, Hall E, Green S, Tanksley SD, Chen H-mei, Kuo G, Fang D, Chen J-tzu (2000) Mapping a wild tomato introgression associated with Tomato yellow leaf curl virus resistance in a cultivated tomato line. Journal of the American Society for Horticultural Science 125:15-20.

Harbour JW, Dean DC (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. Genes & Development 14:2393-2409.

Harlan JR, Wet JMJ de (1971) Toward a rational classification of cultivated plants. Taxon 20:509-517.

Harrison BD (2002) Virus variation in relation to resistance-breaking in plants. Crop Research 181-192.

Hassan AA, Mazayd HM, Moustafa SE, Nassar SH, Nakhla MK, Sims WL (1984) Inheritance of resistance to tomato yellow leaf curl virus derived from Lycopersicon cheesmanii and Lycopersicon hirsutum. HortScience 19:574-575.

Herwig S, Strauss M (1997) The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. European Journal of Biochemistry 246:581-601.

Höhnle M, Höfer P, Bedford ID, Briddon RW, Markham PG, Frischmuth T (2001) Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible Abutilon mosaic virus isolate. Virology 290:164-71.

Holmes EC, Rambaut A (2004) Viral evolution and the emergence of SARS coronavirus. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 359:1059-1065.

Horowitz AR, Kontsedalov S, Khasdan V, Ishaaya I (2005) Biotypes B and Q of Bemisia tabaci and their relevance to neonicotinoid and pyriproxyfen resistance. Archives of Insect Biochemistry and Physiology 58:216-225.

Horváth GV, Pettkó-Szandtner A, Nikovics K, Bilgin M, Boulton M, Davies JW, Gutiérrez C, Dudits D (1998) Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis. Plant Molecular Biology 38:699-712.

Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J, Makker J, Walker E, Jackman M, Xie Q, Bannister AJ, Kouzarides T, Gutiérrez C, Doonan JH, Murray JAH (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. Plant Molecular Biology 37:155-169.

Idris AM, Guerrero JC, Brown JK (2007) Two Distinct Isolates of Tomato yellow leaf curl virus threaten tomato production in Arizona and Sonora, Mexico. Plant Disease 91:910.

Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage [phi]29 DNA polymerase. Journal of Virological Methods 116:209-211.

Janzac B, Fabre F, Palloix A, Moury B (2009) Constraints on evolution of virus avirulence factors predict the durability of corresponding plant resistances. Molecular Plant Pathology 10:599-610.

Jeger MJ, van den Bosch F, Madden LV (2011) Modelling virus- and host-limitation in vectored plant disease epidemics. Virus Research 159:215-222.

Jeske H, Lütgemeier M, Preiss W (2001) DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. The EMBO Journal 20:6158-67.

Ji Y, Schuster DJ, Scott JW (2007a) Ty-3, a begomovirus resistance locus near the Tomato yellow leaf curl virus resistance locus Ty-1 on chromosome 6 of tomato. Molecular Breeding 20:271-284.

Ji Y, Scott JW, Hanson PM, Graham E, Maxwell D (2007b) Sources of resistance, inheritance, and location of genetic loci conferring resistance to members of the tomato-infecting begomoviruses. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 343-362

Ji YF, Scott JW, Schuster DJ, Maxwell DP (2009) Molecular mapping of Ty-4, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. Journal of the American Society for Horticultural Science 134:281-288.

Johnson R (1984) A Critical Analysis of Durable Resistance. Annual Review of Phytopathology 22:309-330.

Jones DR (2003) Plant viruses transmitted by whiteflies. European Journal of Plant Pathology 109:195-219.

Jones RAC (2009) Plant virus emergence and evolution: origins, new encounter scenarios, factors driving emergence, effects of changing world conditions, and prospects for control. Virus Research 141:113-30.

Jupin I, De Kouchkovsky F, Jouanneau F, Gronenborn B (1994) Movement of Tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4. Virology 204:82-90.

Kaelin WG (1999) Functions of the retinoblastoma protein. BioEssays 21:950-958.

Kang J-H, Shi F, Jones AD, Marks MD, Howe GA (2010) Distortion of trichome morphology by the hairless mutation of tomato affects leaf surface chemistry. Journal of Experimental Botany 61:1053-1064.

Kasrawi MA (1989) Inheritance of Resistance to Tomato Yellow Leaf Curl Virus (TYLCV) in Lycopersicon pimpinellifolium. Plant Disease 73:435.

Kong LJ, Orozco BM, Roe JL, Nagar S, Ou S, Feiler HS, Durfee T, Miller a B, Gruissem W, Robertson D, Hanley-Bowdoin L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. The EMBO Journal 19:3485-95.

Koornneef M, Stam P (2001) Changing paradigms in plant breeding. Plant Physiology 125:156-9.

Kumari P, Singh A, Sharma V, Chattopadhyay B, Chakraborty S (2011) A novel recombinant tomato-infecting begomovirus capable of transcomplementing heterologous DNA-B components. Archives of Virology 156:769-783.

Latham JR, Saunders K, Pinner MS, Stanley J (1997) Induction of plant cell division by beet curly top virus gene C4. The Plant Journal 11:1273-1283.

Lavia P, Jansen-Dürr P (1999) E2F target genes and cell-cycle checkpoint control. BioEssays 21:221-230.

Lecoq H, Fabre F, Joannon B, Wipf-Scheibel C, Chandeysson C, Schoeny a, Desbiez C (2011) Search for factors involved in the rapid shift in Watermelon mosaic virus (WMV) populations in South-eastern France. Virus Research 1-9.

Lee S, Stenger DC, Bisaro DM, Davies KR (1994) Identification of loci in Arabidopsis that confer resistance to geminivirus infection. The Plant Journal 6:525-535.

Lee S, Salwinski L, Zhang C, Chu D, Sampankanpanich C, Reyes NA, Vangeloff A, Xing F, Li X, Wu T-T, Sahasrabudhe S, Deng H, LaCount DJ, Sun R (2011) An integrated approach to elucidate the intra-viral and viral-cellular protein interaction networks of a gamma-herpesvirus. PLoS Pathog 7:e1002297.

Lefeuvre P, Hoareau M, Delatte H, Reynaud B, Lett J-M (2007a) A multiplex PCR method discriminating between the TYLCV and TYLCV-Mld clades of tomato yellow leaf curl virus. Journal of Virological Methods 144:165-168.

Lefeuvre P, Lett J-M, Reynaud B, Martin DP (2007b) Avoidance of Protein Fold Disruption in Natural Virus Recombinants. PLoS Pathogens 3:e181.

Lefeuvre P, Martin DP, Hoareau M, Naze F, Delatte H, Thierry M, Varsani A, Becker N, Reynaud B, Lett J-M (2007c) Begomovirus "melting pot" in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. The Journal of General Virology 88:3458-3468.

Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJ a., Meredith S, Lakay F, Monjane A, Lett J-M, Varsani A, Heydarnejad J (2010) The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World. PLoS Pathogens 6:e1001164.

Le Gall O, Aranda M a, Caranta C (2011) Plant resistance to viruses mediated by translation initiation factors. In: Caranta C, Aranda M a, Tepfer M, Lopez-Moya J. (eds) Recent advances in plant virology. Caister Academic Press, pp 177-194

Legavre T, Maia IG, Casse-Delbart F, Bernardi F, Robaglia C (1996) Switches in the mode of transmission select for or against a poorly aphid-transmissible strain of potato virus Y with reduced helper component and virus accumulation. Journal of General Virology 77:1343-1347.

Legg JP, Ogwal S (1998) Changes in the incidence of African cassava mosaic virus disease and the abundance of its whitefly vector along south–north transects in Uganda. Journal of Applied Entomology 122:169-178.

Legg JP, French R, Rogan D, Okao-Okuja G, Brown JK (2002) A distinct *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) genotype cluster is associated with the epidemic of severe cassava mosaic virus disease in Uganda. Molecular Ecology 11:1219-1229.

Legg JP, Fauquet CM (2004) Cassava mosaic geminiviruses in Africa. Plant Molecular Biology 56:585-599.

Lett J-M, Péréfarres F, Hoareau M, Lefeuvre P, De Bruyn A, Dottin M, Prior P, Wicker E, Umaharan P (2011) Tomatoes showing yellow leaf curl symptoms in the island of Grenada exhibit an infection with Tomato yellow leaf curl virus either alone or in combination with Potato yellow mosaic virus. New Disease Reports 24:19.

Liu L, Saunders K, Thomas CL, Davies JW, Stanley J (1999) Bean yellow dwarf virus RepA, but not Rep, binds to maize retinoblastoma protein, and the virus tolerates mutations in the consensus binding motif. Virology 256:270-279.

Liu Y, Robinson DJ, Harrison BD (1998) Defective forms of cotton leaf curl virus DNA-A that have different combinations of sequence deletion, duplication, inversion and rearrangement. Journal of General Virology 1501-1508.

Magenya OEV, Mueke J, Omwega C (2008) Significance and transmission of maize streak virus disease in Africa and options for management: A review. African Journal of Biotechnology 7:4897-4910.

Marandel G, Salava J, Abbott A, Candresse T, Decroocq V (2009) Quantitative trait loci meta-analysis of Plum pox virus resistance in apricot (Prunus armeniaca L.): new insights on the organization and the identification of genomic resistance factors. Molecular Plant Pathology 10:347-360.

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P (2010) RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26:2462-2463.

Martin DP, Lefeuvre P, Varsani A, Hoareau M, Semegni J-Y, Dijoux B, Vincent C, Reynaud B, Lett J-M (2011) Complex recombination patterns arising during geminivirus coinfections preserve and demarcate biologically important intra-genome interaction networks. PLoS Pathog 7:e1002203.

Martin S, Elena SF (2009) Application of game theory to the interaction between plant viruses during mixed infections. Journal of General Virology 90:2815-2820.

Mason G, Caciagli P, Accotto GP, Noris E (2008) Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in *Bemisia tabaci*. Journal of Virological Methods 147:282-289.

Matthews L, Haydon D (2007) Introduction. Cross-scale influences on epidemiological dynamics: from genes to ecosystems. Journal of The Royal Society Interface 4:763-765.

Maule AJ, Caranta C, Boulton MI (2007) Sources of natural resistance to plant viruses: status and prospects. Molecular Plant Pathology 8:223-231.

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential and durable resistance. Annual Review of Phytopathology 40:349-379.

Mcgarry RC, Barron YD, Carvalho MF, Hill JE, Gold D, Cheung E, Kraus WL, Lazarowitz SG (2003) A Novel Arabidopsis Acetyltransferase Interacts with the Geminivirus Movement Protein NSP. Journal of Virology 15:1605-1618.

Mejía L, Teni R, Vidavski F, Czosnek H, Lapidot M, Nakhla MK, Maxwell DP (2005) Evaluation of tomato germplasm and selection of breeding lines for resistance to begomoviruses in Guatemala. Acta horticulturae 695:251-255.

Menges M, Hennig L, Gruissem W, Murray JAH (2002) Cell Cycle-regulated Gene Expression in Arabidopsis. Journal of Biological Chemistry 277:41987-42002.

Meshi T, Motoyoshi F, Adachi A, Watanabe Y, Takamatsu N, Okada Y (1988) Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, Tm-1. The EMBO journal 7:1575-1581.

Momotaz A, Scott JW, Schuster DJ (2010) Identification of quantitative trait loci conferring resistance to *Bemisia tabaci* in an F2 population of Solanum lycopersicum · Solanum habrochaites Accession LA1777. Journal of the American Society for Horticultural Science 135:134-142.

Monci F, Sanchez-Campos S, Navas-Castillo J, Moriones E (2002) A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. Virology 303:317-326.

Monforte AJ, Tanksley SD (2000) Development of a set of near isogenic and backcross recombinant inbred lines containing most of the Lycopersicon hirsutum genome in a L. esculentum genetic background: A tool for gene mapping and gene discovery. Genome 43:803-813.

Morales FJ, Anderson PK (2001) The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America. Archives of Virology 146:415-441.

Morilla G, Janssen D, García-Andrés S, Moriones E, Cuadrado IM, Bejarano ER (2005) Pepper (Capsicum annuum) is a dead-end host for Tomato yellow leaf curl virus. Phytopathology 95:1089-97.

Moriones E, Navas-Castillo J, Díaz-Pendón JA (2011) Emergence of Begomoviruses Diseases. In: Caranta C, Aranda M a, Tepfer M, Lopez-Moya J. (eds) Recent advances in plant virology. Caister Academic Press, pp 301-320

Mosig G (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. Annual Review of Genetics 32:379-413.

Mosig G, Gewin J, Luder A, Colowick N, Vo D (2001) Two recombination-dependent DNA replication pathways of bacteriophage T4, and their roles in mutagenesis and horizontal gene transfer. Proceedings of the National Academy of Sciences 98:8306-8311.

Moury B, Fabre F, Senoussi R (2007) Estimation of the number of virus particles transmitted by an insect vector. Proceedings of the National Academy of Sciences of the United States of America 104:17891-6.

Moury B, Fereres A, Garcia-Arenal F, Lecoq H (2011) Sustainable management of plant resistance to viruses. In: Caranta C, Aranda MA, Tepfer M, LopezMoya JJ (eds) Recent advances in plant virology. Caister Academic Press, Wymondham, pp 219-236

Muigai S, Schuster D, Snyder J, Scott JW, Bassett M, McAuslane H (2002) Mechanisms of resistance Lycopersicon germplasm to the whitefly *Bemisia argentifolii* Phytoparasitica 30:347-360.

Muñiz M (2000) Host suitability of two biotypes of Bemisia tabaci on some common weeds. Entomologia Experimentalis et Applicata 95:63-70.

Muñiz M, Nombela G (2001) Differential Variation in Development of the B- and Q-Biotypes of Bemisia tabaci (Homoptera: Aleyrodidae) on Sweet Pepper at Constant Temperatures. Environmental Entomology 30:720-727.

Nagar S, Pedersen TJ, Carrick KM, Hanley-Bowdoin L, Robertson D (1995) A Geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. The Plant Cell Online 7:705-719.

Nagar S, Hanley-Bowdoin L, Robertson D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. The Plant Cell Online 14:2995-3007.

Nakhla MK, Maxwell DP, Martinez R, Carvalho M, Gilbertson RL (1994) Widespread occurrence of the eastern mediterranean strain of Tomato yellow leaf curl geminivirus in tomatoes in the Dominican Republic. Plant Disease 78:926-926.

Navas-Castillo J, Sanchez-Campos S, Diaz JA, Saez-Alonso E, Moriones E (1999) Tomato yellow leaf curl virus-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. Plant Disease 83:29-32.

Navas-Castillo J, Sánchez-Campos S, Noris E, Louro D, Accotto GP, Moriones E (2000) Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. Journal of General Virology 81:2797-2801.

Navas-Castillo J, Fiallo-Olivé E, Sánchez-Campos S (2011) Emerging virus diseases transmitted by whiteflies. Annual Review of Phytopathology 49:219-48.

Nawaz-Ul-Rehman MS, Nahid N, Mansoor S, Briddon RW, Fauquet CM (2010) Post-transcriptional gene silencing suppressor activity of two non-pathogenic alphasatellites associated with a begomovirus. Virology 405:300-8.

Nawaz-Ul-Rehman MS, Fauquet CM (2009) Evolution of geminiviruses and their satellites. FEBS letters 583:1825-32.

Ng JCK, Falk BW (2006) Virus-Vector Interactions Mediating Nonpersistent and Semipersistent Transmission of Plant Viruses. Annual Review of Phytopathology 44:183-212.

Nieto C, Piron F, Dalmais M, Marco CF, Moriones E, Gómez-Guillamón ML, Truniger V, Gómez P, Garcia-Mas J, Aranda MA, Bendahmane A (2007) EcoTILLING for the identification of allelic variants of melon eIF4E, a factor that controls virus susceptibility. BMC Plant Biology 7:34.

Novick RP (1998) Contrasting lifestyles of rolling-circle phages and plasmids. Trends in Biochemical Sciences 23:434-8.

Orozco BM, Hanley-Bowdoin L (1996) A DNA structure is required for geminivirus replication origin function. Journal of Virology 70:148-158.

Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M (2008) Strategies for antiviral resistance in transgenic plants. Molecular Plant Pathology 9:73-83.

Padidam M, Sawyer S, Fauquet CM (1999) Possible emergence of new geminiviruses by frequent recombination. Virology 265:218-25.

Papayiannis LC, Iacovides TA., Katis NI, Brown JK (2010) Differentiation of Tomato yellow leaf curl virus and Tomato yellow leaf curl Sardinia virus using real-time TaqMan® PCR. Journal of Virological Methods 165:238-245.

Papayiannis LC, Katis NI, Idris AM, Brown JK (2011) Identification of weed hosts of Tomato yellow leaf curl virus in Cyprus. Plant Disease 95:120-125.

Paprotka T, Metzler V, Jeske H (2010) The first DNA 1-like α satellites in association with New World begomoviruses in natural infections. Virology 404:148-157.

Patil B, Dasgupta I (2006) Defective interfering DNAs of plant viruses. Critical Reviews in Plant Sciences 25:47-64.

Patil BL, Dutt N, Briddon RW, Bull SE, Rothenstein D, Borah BK, Dasgupta I, Stanley J, Jeske H (2007) Deletion and recombination events between the DNA-A and DNA-B components of Indian cassava-infecting geminiviruses generate defective molecules in Nicotiana benthamiana. Virus Research 124:59-67.

Patil BL, Fauquet CM (2009) Cassava mosaic geminiviruses: actual knowledge and perspectives. Molecular Plant Pathology 10:685-701.

Paximadis M, Rey MEC (2001) Genome organization of Tobacco leaf curl Zimbabwe virus, a new distinct monopartite begomovirus associated with subgenomic defective DNA molecules. Journal of General Virology 715:3091-3097.

Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, Eagle P, Hanley-Bowdoin L, Robertson D (2001) Silencing of a meristematic gene using geminivirus-derived vectors. The Plant Journal 27:357-366.

Perring TM (2001) The *Bemisia tabaci* species complex. Crop Protection 20:725-737.

Perring TM, Gruenhagen NM, Farrar CA (1999) Management of plant viral diseases through chemical control of insect vectors. Annual Review of Entomology 44:457-481.

Peterschmitt M, Granier M, Mekdoud R, Dalmon A, Gambin O, Vayssieres JF, Reynaud B (1999) First report of Tomato yellow leaf curl virus in Réunion Island. Plant Disease 83:303-303.

Picó B (1996) Viral diseases causing the greatest economic losses to the tomato crop. II. The Tomato yellow leaf curl virus — a review. Scientia Horticulturae 67:151-196.

Picó B, Sifres A, Elía M, Díez M, Nuez F (2000) Searching for new resistance sources to tomato yellow leaf curl virus within a highly variable wild *Lycopersicon* genetic pool. Acta Physiologiae Plantarum 22:344-350.

Pilowsky M, Cohen S (1974) Inheritance of resistance to Tomato yellow leaf curl virus in tomatoes. Phytopathology 64:632-635.

Piron F, Nicolaï M, Minoïa S, Piednoir E, Moretti A, Salgues A, Zamir D, Caranta C, Bendahmane A (2010) An induced mutation in tomato eIF4E leads to immunity to two potyviruses. PloS one 5:e11313.

Polston JE (1994) First report of a Tomato yellow leaf curl-like geminivirus in the western hemisphere. Plant Disease 78:831B-831B.

Polston JE, Lapidot M (2007) Management of tomato yellow leaf curl virus: US and Israel perspectives. In: Czosnek H (ed) Tomato Yellow Leaf Curl Virus Disease. Springer Netherlands, pp 251-262.

Ramirez-Parra E, Fründt C, Gutierrez C (2003) A genome-wide identification of E2F-regulated genes in Arabidopsis. The Plant Journal 33:801-811.

Reynaud B, Wuster G, Delatte H, Soustrade I, Lett JM, Gambin O, Peterschmitt M (2003) Les maladies à bégomovirus chez la tomate dans les départements français d'Outre-Mer - Le Tomato yellow leaf curl virus (TYLCV) à la Réunion. Phytoma, la défense des végétaux 13-17.

Rigden JE, Krake LR, Rezaian MA, Dry IB (1994) ORF C4 of Tomato leaf curl geminivirus is a determinant of symptom severity. Virology 204:847-850.

Robaglia C, Caranta C (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends in Plant Science 11:40-45.

Rodríguez-López MJ, Garzo E, Bonani JP, Fereres A, Fernández-Muñoz R, Moriones E (2011) Whitefly resistance traits derived from the wild tomato Solanum pimpinellifolium affect the preference and feeding behavior of *Bemisia tabaci* and reduce the spread of Tomato yellow leaf curl virus. Phytopathology 101:1191-1201.

Rojas MR, Jiang H, Salati R, Xoconostle-Cázares B, Sudarshana MR, Lucas WJ, Gilbertson RL (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. Virology 291:110-125.

Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annual Review of Phytopathology 43:361-394.

Romay G, Chirinos D, Geraud-Pouey F, Desbiez C (2010) Association of an atypical alphasatellite with a bipartite New World begomovirus. Archives of Virology 155:1843-1847.

Rual J-F, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, Li N, Berriz GF, Gibbons FD, Dreze M, Ayivi-Guedehoussou N, Klitgord N, Simon C, Boxem M, Milstein S, Rosenberg J, Goldberg DS, Zhang LV, Wong SL, Franklin G, Li S, Albala JS, Lim J, Fraughton C, Llamosas E, Cevik S, Bex C, Lamesch P, Sikorski RS, Vandenhaute J, Zoghbi HY, Smolyar A, Bosak S, Sequerra R, Doucette-Stamm L, Cusick ME, Hill DE, Roth FP, Vidal M (2005) Towards a proteome-scale map of the human protein-protein interaction network. Nature 437:1173-1178.

Ruffel S, Dussault M-H, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). The Plant journal: for cell and molecular biology 32:1067-75.

Sacristán S, García-Arenal F (2008) The evolution of virulence and pathogenicity in plant pathogen populations. Molecular Plant Pathology 9:369–384.

Sala M, Wain-Hobson S (2000) Are RNA Viruses Adapting or Merely Changing? Journal of Molecular Evolution 51:12-20.

Sánchez-Campos S, Navas-Castillo J, Camero R, Soria C, Díaz J a, Moriones E (1999) Displacement of Tomato yellow leaf curl virus (TYLCV)-Sr by TYLCV-Is in tomato epidemics in Spain. Phytopathology 89:1038-1043.

Sanjuán R, Moya A, Elena SF (2004) The distribution of fitness effects caused by single-nucleotide substitutions in an RNA virus. Proceedings of the National Academy of Sciences of the United States of America 101:8396-8401.

Saunders K, Norman A, Gucciardo S, Stanley J (2004) The DNA β satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (βC1). Virology 324:37-47.

Saunders K, Stanley J (1999) A nanovirus-like DNA component associated with yellow vein disease of Ageratum conyzoides: evidence for interfamilial recombination between plant DNA viruses. Virology 264:142-152.

Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, Lavenir R, Pardigon N, Reynes JM, Pettinelli F, Biscornet L, Diancourt L, Michel S, Duquerroy S, Guigon G, Frenkiel MP, Bréhin AC, Cubito N, Desprès P, Kunst F, Rey FA, Zeller H, Brisse S (2006) Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS medicine 3:e263.

Seal SE, vandenBosch F, Jeger MJ (2006) Factors influencing begomovirus evolution and their increasing global significance: implications for sustainable control. Critical Reviews in Plant Sciences 25:23-46.

Selth LA, Randles JW, Rezaian MA (2004) Host responses to transient expression of individual genes encoded by Tomato leaf curl virus. Molecular Plant-Microbe Interactions 17:27-33.

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA (2005) A NAC domain protein interacts with Tomato leaf curl virus replication accessory protein and enhances viral replication. Journal of Virology 17:311-325.

Servin R, Martínez JL, Hirales L (1999) Weeds and cultivated hosts of the silverleaf whitefly *Bemisia argentifolii* bellows and perring in Baja California Sur, Mexico. Southwestern Entomologist 24:31-36.

Settlage SB, Miller AB, Gruissem W, Hanley-Bowdoin L (2001) Dual interaction of a geminivirus replication accessory factor with a viral replication protein and a plant cell cycle regulator. Virology 279:570-576.

Sharma P, Ikegami M (2010) Tomato leaf curl Java virus V2 protein is a determinant of virulence, hypersensitive response and suppression of posttranscriptional gene silencing. Virology 396:85-93.

Sharma P, Matsuda N, Bajet N, Ikegami M (2011) Molecular analysis of new isolates of *Tomato leaf curl Philippines virus* and an associated betasatellite occurring in the Philippines. Archives of Virology 156:305-312.

Shepherd D, Martin DP, Thomson J (2009) Transgenic strategies for developing crops resistant to geminiviruses. Plant Science 176:1-11.

Sidle A, Palaty C, Dirks P, Wiggan O, Kiess M, Gill RM, Wong AK, Hamel PA (1996) Activity of the retinoblastoma family proteins, pRB, p107, and p130, during cellular proliferation and differentiation. Critical Reviews in Biochemistry and Molecular Biology 31:237-271.

Simmons AT, Gurr GM (2005) Trichomes of Lycopersicon species and their hybrids: effects on pests and natural enemies. Agricultural and Forest Entomology 7:265-276.

Singh SP, Morales FJ, Miklas PN, Terán H (2000) Selection for Bean golden mosaic resistance in intra- and interracial bean populations. Crop Science 40:1565-1572.

Smith GJD, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JSM, Guan Y, Rambaut A (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125.

Stanley J, Frischmuth T, Ellwood S (1990) Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. Proceedings of the National Academy of Sciences of the United States of America 87:6291-5.

Stanley J, Latham JR, Pinner MS, Bedford I, Markham PG (1992) Mutational analysis of the monopartite geminivirus beet curly top virus. Virology 191:396-405.

Stevens R, Mariconti L, Rossignol P, Perennes C, Cella R, Bergounioux C (2002) Two E2F sites in the Arabidopsis MCM3 promoter have different roles in cell cycle activation and meristematic expression. Journal of Biological Chemistry 277:32978-32984.

Strange RN, Scott PR (2005) Plant Disease: A Threat to Global Food Security. Annual Review of Phytopathology 43:83-116.

Sung YK, Coutts RHA (1995) Mutational analysis of potato yellow mosaic geminivirus. Journal of General Virology 76:1773-1780.

Sunter G, Bisaro DM (1989) Transcription map of the B genome component of tomato golden mosaic virus and comparison with A component transcripts. Virology 173:647-655.

Teng K, Chen H, Lai J, Zhang Z, Fang Y, Xia R, Zhou X, Guo H, Xie Q (2010) Involvement of C4 protein of beet severe curly top virus (family Geminiviridae) in virus movement. PloS one 5:e11280.

Ueda S, Kimura T, Onuki M, Hanada K, Iwanami T (2004) Three distinct groups of isolates of Tomato yellow leaf curl virus in Japan and construction of an infectious clone. Journal of General Plant Pathology 70:232-238.

Ueda S, Takeuchi S, Okabayashi M, Hanada K, Tomimura K, Iwanami T (2005) Evidence of a new *Tomato yellow leaf curl virus* in Japan and its detection using PCR. Journal of General Plant Pathology 71:319-325.

Ukai Y, Yamashita A (1984) Induced mutation for resistance to barley yellow mosaic virus. Japan Agricultural Research Quarterly 17:255–259.

Urbino C, Tassius K (2003) First Report of Tomato yellow leaf curl virus in Tomato in Guadeloupe. Plant Disease 87:1397.

Van Brunschot SL, Persley DM, Geering ADW, Campbell PR, Thomas JE (2010) Tomato yellow leaf curl virus in Australia: distribution, detection and discovery of naturally occurring defective DNA molecules. Australasian Plant Pathology 39:412-423.

Van Den Bosch F, Akudibilah G, Seal S, Jeger M (2006) Host resistance and the evolutionary response of plant viruses. Journal of Applied Ecology 43:506-516.

Vanitharani R, Chellappan P, Fauquet CM (2005) Geminiviruses and RNA silencing. Trends in Plant Science 10:144-51.

Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens E, Estes MK, Lemon S, Maniloff J, Mayo MA, McGeoch D, Pringle CR, Wickner RB (2000) Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press London, pp1044. San Diego: Academic Press London.

van Wezel R, Liu H, Tien P, Stanley J, Hong Y (2001) Gene C2 of the monopartite geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. Molecular Plant-Microbe Interactions 14:1125-1128.

Vuillaume F, Thébaud G, Urbino C, Forfert N, Granier M, Froissart R, Blanc S, Peterschmitt M (2011) Distribution of the phenotypic effects of random homologous recombination between two virus species. PLoS pathogens 7:e1002028.

Woolhouse MEJ, Taylor LH, Haydon DT (2001) Population Biology of Multihost Pathogens. Science 292:1109-1112.

Woolhouse MEJ, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. Trends in Ecology & Evolution Evolution 20:238-244.

Xie Q, Sanz-Burgos AP, Hannon GJ, Gutiérrez C (1996) Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. The European Molecular Biology Organization Journal 15:4900-4908.

Xie Q, Sanz-Burgos A, Guo H, García J, Gutiérrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Molecular Biology 39:647-656.

Yaakov N, Levy Y, Belausov E, Gaba V, Lapidot M, Gafni Y (2011) Effect of a single amino acid substitution in the NLS domain of Tomato yellow leaf curl virus-Israel (TYLCV-IL) capsid protein (CP) on its activity and on the virus life cycle. Virus Research 158:8-11.

Yassin AM, Nour MA (1965) Tomato leaf curl diseases in the Sudan and their relation to tobacco leaf curl. Annals of Applied Biology 56:207-217.

Zambrano K, Carballo O, Geraud F, Chirinos D, Fernández C, Marys E (2007) First Report of Tomato yellow leaf curl virus in Venezuela. Plant Disease 91:768-768.

Zamir D, Ekstein-Michelson I, Zakay Y, Navot N, Zeidan M, Sarfatti M, Eshed Y, Harel E, Pleban T, van Oss H, Kedar N, Rabinowitch HD, Czosnek H (1994) Mapping and introgression of a tomato yellow leaf curl virus tolerance gene, TY-1. Theoretical and Applied Genetics 88:141-146.

Zhou X, Liu Y, Calvert L, Munoz C, Otim-Nape GW, Robinson DJ, Harrison BD (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. Journal of General Virology 78:2101-2111.

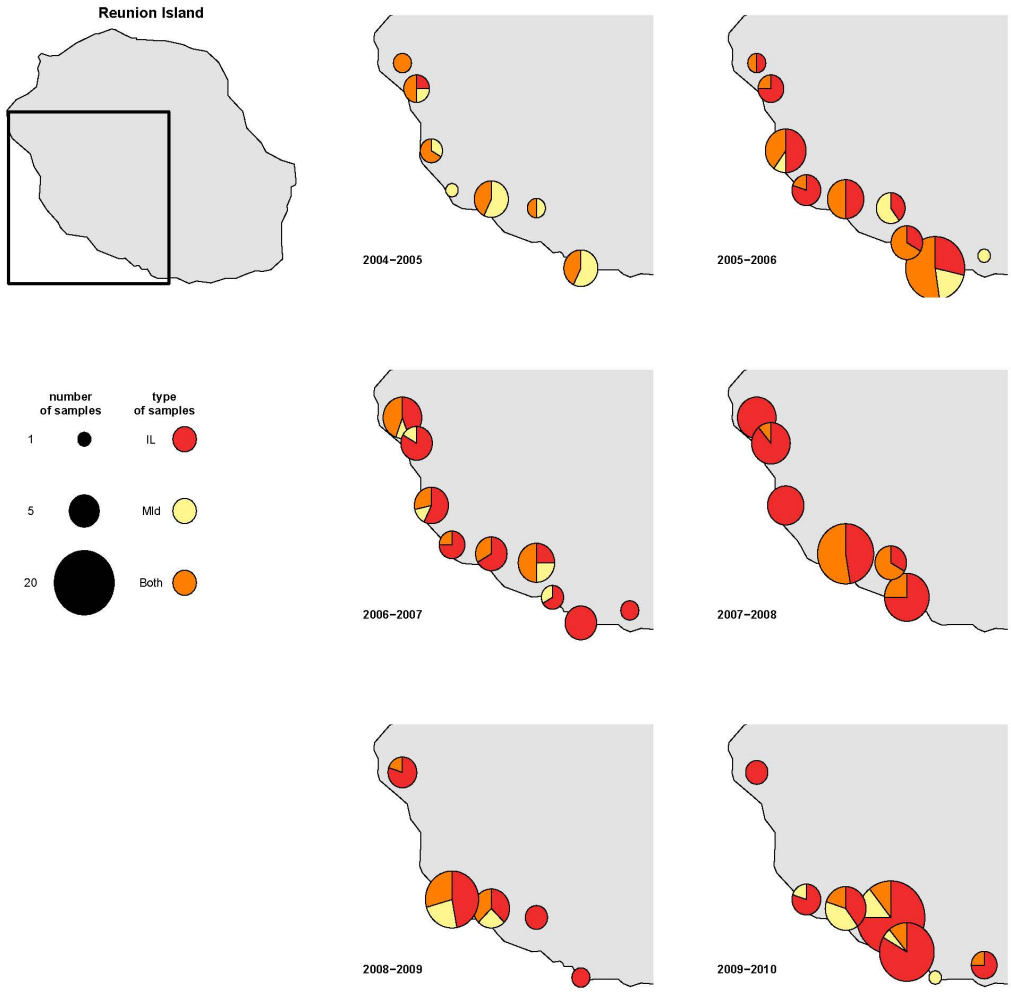
Zhou Y, Rojas MR, Park M-R, Seo Y-S, Lucas WJ, Gilbertson RL (2011) Histone H3 Interacts and Colocalizes with the Nuclear Shuttle Protein and the Movement Protein of a Geminivirus . Journal of Virology 85 :11821-11832.

Annexes

Supplementary material Chapter II

Table 1: Number of analysed samples by real-time PCR during the within-plant virus accumulation experiments following agro-inoculations of Reunion isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and / or Israel (TYLCV-IL).

Days post-inoculation	TYLCV-IL	TYLCV-Mld	TYLCV-IL+TYLCV-Mld
2	12	12	10
3	12	12	10
4	14	17	19
6	14	15	14
8	13	14	16
10	14	14	19
13	6	10	8
16	9	12	10
20	10	12	10
25	9	12	10
30	7	12	14
35	8	7	14



Supplementary Figure 1: Dynamics of Tomato yellow leaf curl virus strain Mild (TYLCV-Mld) and Israel (TYLCV-IL) in Reunion Island from 2004 to 2010. Pie graph size depends of the number of tested samples. Pie graph portions represent the proportion of samples of each type with yellow for TYLCV-Mld, red for TYLCV-IL and orange for co-infection.

Arch Virol (2011) 156:483–487
DOI 10.1007/s00705-010-0854-2

BRIEF REPORT

Molecular and biological characterization of *Pepper yellow vein Mali virus* (PepYVMV) isolates associated with pepper yellow vein disease in Burkina Faso

Fidèle Tiendrébéogo · Pierre Lefeuvre · Murielle Hoareau · Valentin S. Edgar Traoré · Nicolas Barro · Frédéric Péréfarres · Bernard Reynaud · Alfred S. Traoré · Gnissa Konaté · Jean-Michel Lett · Oumar Traoré

Received: 3 May 2010 / Accepted: 25 October 2010 / Published online: 16 November 2010

Abstract Yellow vein disease (YVD) is a major problem in pepper in West Africa. Despite the recent implication of a begomovirus in YVD in Mali and in Burkina Faso, the aetiology of the disease remains unclear. Using symptomatic samples from the main vegetable cultivation regions in Burkina Faso, 10 full-length DNA-A-like begomovirus sequences were obtained, each showing 98% nucleotide identity to pepper yellow vein Mali virus (PepYVMV). The host range was determined after construction of a viral clone for agroinfection. Severe symptoms developed in tomato and *Nicotiana benthamiana*. By contrast, no symptoms developed in either commercial or local pepper cultivars, demonstrating that the aetiology of YVD is not only associated with the presence of PepYVMV.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-010-0854-2) contains supplementary material, which is available to authorized users.

F. Tiendrébéogo · N. Barro · A. S. Traoré
Laboratoire de Biochimie & Biologie Moléculaire, CRSBAN/UFR/SVT, Université de Ouagadougou, 03 BP 7021 Ouagadougou 03, Burkina Faso
e-mail: fidelet@gmail.com

N. Barro
e-mail: nicolas_barro@univ-ouaga.bf

A. S. Traoré
e-mail: astraore@univ-ouaga.bf

F. Tiendrébéogo · P. Lefeuvre · M. Hoareau · F. Péréfarres · B. Reynaud · J.-M. Lett
CIRAD, UMR 53 PVBMT CIRAD-Université de la Réunion, Pôle de Protection des Plantes, 7 Chemin de l'IRAT, 97410 Saint Pierre, La Réunion, France

P. Lefeuvre
e-mail: pierre.lefeuvre@cirad.fr

M. Hoareau
e-mail: murielle.hoareau@cirad.fr

Text

Begomoviruses (family *Geminiviridae)* cause serious diseases in several economically important crops [8]. Begomoviruses have monopartite or bipartite genomes and infect dicotyledonous plants. With the exception of tomato yellow leaf curl virus (TYLCV), which was introduced inadvertently into the New World, all of the monopartite begomoviruses have been found in Africa, Asia and the Australian subcontinent, occasionally associated with ssDNA satellites [2]. Common symptoms induced by begomoviruses include leaf-curling, mosaic, and vein yellowing, often accompanied by stunting and severe yield losses.

In Burkina Faso, peppers are widely used as condiments and are a major source of income for many small-scale farmers, but they are susceptible to yellow vein disease (YVD), which occur in West Africa [16, 17]. The symptoms

F. Péréfarres
e-mail: frederic.perefarres@cirad.fr

B. Reynaud
e-mail: bernard.reynaud@cirad.fr

J.-M. Lett
e-mail: lett@cirad.fr

V. S. E. Traoré · G. Konaté · O. Traoré (✉)
Laboratoire de Virologie et de Biotechnologies Végétales, Institut de l'Environnement et de Recherches Agricoles (INERA), 01 BP 476 Ouagadougou 01, Burkina Faso
e-mail: ohmar@hotmail.com

V. S. E. Traoré
e-mail: traorevalentin@yahoo.fr

G. Konaté
e-mail: gnataramen@yahoo.fr

of YVD resemble those induced by begomovirus infections and are clearly distinct from mosaic symptoms already reported to be caused in this country by pepper veinal mottle virus (PVMV) or tobacco mosaic virus (TMV) [1, 10]. Recently, a new monopartite begomovirus, pepper yellow vein Mali virus (PepYVMV) has been shown to be associated to YVD in pepper in Mali and Burkina Faso, even if its role in the disease is still unclear [16, 17]. Nevertheless, despite the importance of yellow vein disease in Burkina Faso and several West African countries [16, 17], very few studies have so far characterized the complete genomes of PepYVMV isolates and clearly unravelled the aetiology of the YVD.

During the years 2005 to 2009, pepper fields were surveyed around Ouagadougou, Bazéga, Pô and Banfora, a few of the main areas of Burkina Faso where vegetables are cultivated. A total of 25 leaf samples presenting YVD symptoms were randomly collected from sweet pepper (*Capsicum annuum*) and hot pepper (*Capsicum frutescens*) and preserved by dehydration. None of the leaf samples showed any obvious symptoms attributable to other pepper-infecting viruses. Serological tests using polyclonal antibodies for detection of PVMV and TMV, both of which occur in this country, were negative (data not shown). Total DNA was extracted using a DNeasy Plant Minikit (Qiagen). Samples were then tested for the presence of begomoviruses using a polymerase chain reaction (PCR)-based assay employing a degenerate primer set (VD360-CD1266) [4]. PCR products of the expected size were obtained with 14 samples. No PCR products were obtained with primers designed to amplify DNA betasatellite sequences [3]. Full-length viral genomes were amplified from the PCR-positive samples by rolling-circle amplification (RCA) using the kit TempliPhi^{TM}, GE Healthcare [9]. After digestion with the restriction enzyme *Bam*HI, monomeric genomes of about 2.8 kb were ligated into pGEM-3Zf+ (Promega Biotech), and one clone per sample was sequenced by primer walking (Macrogen, South Korea). Ten full-length DNA-A sequences were successfully obtained. More than 98% nucleotide identity was found among the 10 isolates. The highest nucleotide identity (98%; BLASTn, NCBI) was found with pepper yellow vein Mali virus (PepYVMV, AY502935).

Four of the ten full-length DNA-A components were 2779 nt long, four others were 2786 nt long, and the last two were 2787 and 2778 nt long, respectively. Nucleotide sequence analysis showed that the genome organization of the 10 DNA-A components was typical of that of Old World monopartite begomoviruses: six open reading frames (ORFs) were distinguished, corresponding to the V1 and V2 genes on the viral-sense strand and the C1, C2, C3 and C4 genes on the complementary strand [13]. The intergenic region (IR) sequences extending from nt 304 to 312 contained an inverted repeat (underlined) capable of forming a stem-loop structure (5'- GCGGCCATCCDTAT AATATTACCGGATCCGC -3') and included a conserved nonanucleotide (5'TAATATT↓AC3') that is present in almost all geminivirus virion-strand replication origins (arrow). Five sequences (accession numbers FM876848, FM876850, FM876851, FM876852 and FN555171) showed the same deletion of seven nucleotides in the IR between the nonanucleotide and the start codon of the replication-associated protein gene (C1).

For phylogenetic reconstruction, the 10 complete genome sequences reported here were aligned with the only publicly available complete genome sequence of PepYVMV from Africa, a Chinese isolate of PepYVMV and several African monopartite and bipartite begomoviruses, using the ClustalW alignment tool [15] implemented in MEGA4 [14]. Similarities between sequences were calculated in MEGA 4 with pairwise deletion of gaps. The optimal model of sequence evolution ($GTR+I+G_4$), determined using ModelTest [12], was used for maximum-likelihood (ML) phylogenetic reconstruction with PHYML_v2.4.4 [7]. The degree of support for individual branches within the resulting phylogenetic tree was assessed with 1000 full ML bootstrap iterations (Fig. 1). The tree was visualized using FigTree v1.1.1 software.

Pairwise sequence analysis indicated that the 10 new PepYVMV genomes from Burkina Faso had 96 to 99.9% identity amongst themselves and shared between 95.9 and 98.6% similarity with *Pepper yellow vein Mali virus*-[Mali:2003] (PepYVMV-[ML:03]; AY502935), and 92.3 to 96% with *Pepper yellow vein Mali virus*-[China: Fujian:2006] (PepYVMV-[CN:Fuj:06]; AM691547) (Supplementary Table). The ML consensus tree also confirmed the clustering with PepYVMV isolates reported elsewhere and showed the close relatedness with tobacco leaf curl Zimbabwe virus from Zimbabwe (TbLCZV-[ZW:01]) and tomato curly stunt virus from South Africa (ToCSV-[ZA:Ond:02]) (Fig. 1). In accordance with standard begomovirus classification criteria [6], we classified this group of isolates from Burkina Faso as belonging to PepYVMV ("in accordance with the ICTV proposition" [Claude M. Fauquet, Personal communication]). Accordingly, we named the 10 isolates PepYVMV-[BF:Baz: SPe:07], PepYVMV-[BF:Baz:HPe1:07], PepYVMV-[BF: Oua:SPe1:08], PepYVMV-[BF:Oua:HPe:08], PepYVMV-[BF:Oua:SPe2:08], PepYVMV-[BF:Baz:HPe2:08], PepYVMV-[Ban:Hpe1:09], PepYVMV-[Ban:Hpe2:09], PepYVMV-[Ban:Hpe3:09] and PepYVMV-[Pô:Hpe:08] (Fig. 1). The genetic diversity observed among some isolates of Burkina Faso and China (<93% nucleotide identity) suggests the occurrence of several PepYVMV strains (Supplementary Table).

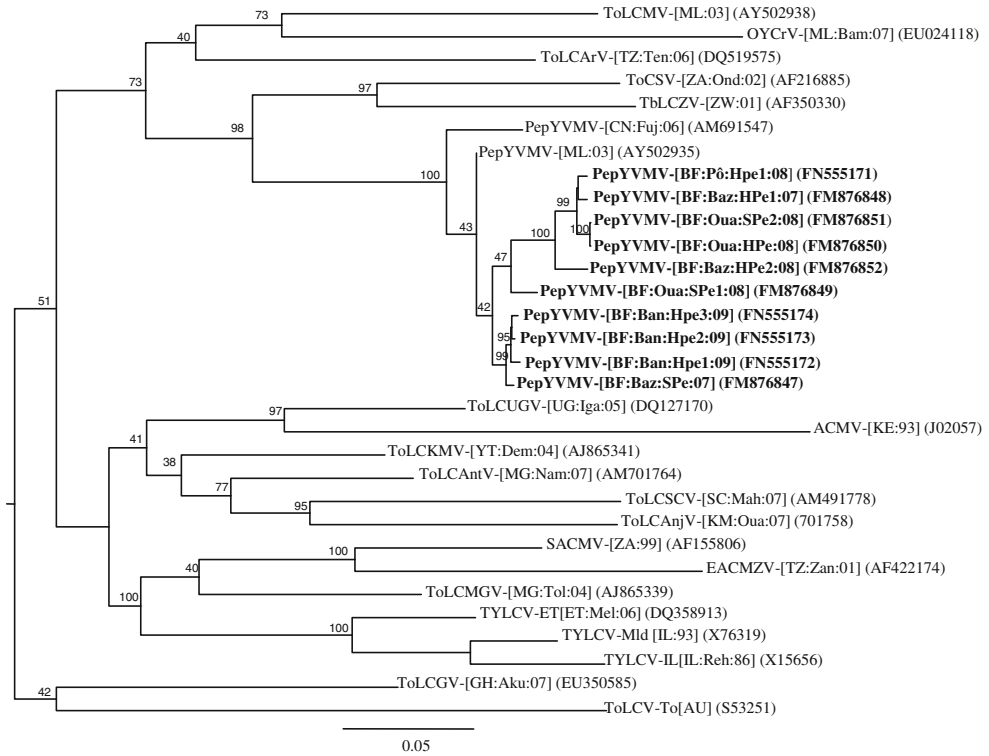


Fig. 1 Maximum-likelihood tree showing the relationships between the complete genomes of Burkina Faso isolates of *Pepper yellow vein Mali virus* (PepYVMV) and other representative begomoviruses sequences. Sequences reported in this study (in bold) are the following: *Pepper yellow vein Mali virus*-[Burkina Faso:Bazega: sweet pepper:2007] (PepYVMV-[BF:Baz:SPe:07]; FM876847), *Pepper yellow vein Mali virus*-[Burkina Faso:Bazega:hot pepper1:2007] (PepYVMV-[BF:Baz:HPe1:07]; FM876848), *Pepper yellow vein Mali virus*-[Burkina Faso:Ouagadougou:sweet pepper1:2008] (PepYVMV-[BF:Oua:SPe1:08]; FM876849), *Pepper yellow vein Mali virus*-[Burkina Faso:Ouagadougou:hot pepper:2008] (PepYV MV-[BF:Oua:HPe:08]; FM876850), *Pepper yellow vein Mali virus*-[Burkina Faso:Ouagadougou:sweet pepper2:2008] (PepYVMV-[BF: Oua:SPe2:08]; FM876851), *Pepper yellow vein Mali virus*-[Burkina Faso:Bazega:hot pepper2:2008] (PepYVMV-[BF:Baz:HPe2:08]; FM876852), *Pepper yellow vein Mali virus*-[Burkina Faso :Banfora:hot pepper1:2009] (PepYVMV-[BF:Ban:Hpe1:09]; FN555172), *Pepper yellow vein Mali virus*-[Burkina Faso:Banfora:hot pepper2:2009] (PepYVMV-[BF:Ban:Hpe2:09]; FN555173), *Pepper yellow vein Mali virus*-[Burkina Faso:Banfora:hot pepper3:2009] (PepYVMV-[BF:Ban:Hpe3:09]; FN555174) and *Pepper yellow vein Mali virus*-[Burkina Faso:Pô:hot pepper:2008] (PepYVMV-[BF:Pô:Hpe:08]; FN555171). Representative begomovirus sequences used for comparison are: *Tomato leaf curl virus Ghana virus*-[Ghana:Akumadan:2007] (ToLCGV-[GH:Aku:07]; EU350585), *Tomato leaf curl Mali virus*-[Mali:2003] (ToLCMV-[ML:03]; AY502938), *Okra yellow crinkle virus*-[Mali:Bamako:2007] (OY CrV-[ML:Bam:07]; EU024118), *Tomato leaf curl Arusha virus*-[Tanzania:Tengeru:2006] (ToLCArV-[TZ:Ten:06]; DQ519575), *Tomato curly stunt virus*-[South Africa:Ondenberg:2002] (ToCSV-[ZA:Ond:02]; AF261885), *Tobacco leaf curl Zimbabwe virus*-[Zimbabwe:2001] (TbLCZV-[ZW:01]; AF350330), *Pepper yellow vein Mali virus*-[China:Fujian:2006] (PepYVMV-[CN:Fuj:06]; AM691547), *Pepper yellow vein Mali virus*-[Mali:2003] (PepYVMV-[ML:03]; AY502935), *Tomato leaf curl Madagascar virus*-[Madagascar:Toliary:2004] (ToLCMGV-[MG:Tol:04]; AJ865339), *South African cassava mosaic virus*-[South Africa:1999] (SACMV-[ZA:99]; AF155806), *East African cassava mosaic Zanzibar virus*-[Tanzania:Zanzibar:2001] (EACMZV-[TZ:Zan:01]; AF422174), *Tomato leaf curl Uganda virus*-[Uganda:Iganga:2005] (ToLCUGV-[UG:Iga:05]; DQ127170), *African cassava mosaic virus*-[Kenya:1993] (ACMV-[KE:93]; J02057), *Tomato leaf curl Comoros virus*-[Mayotte:Dembeni:2004], (ToLCKMV-[YT:Dem:04]; AJ865341), *Tomato leaf curl Antsiranana virus*-[Madagascar:Namakely:2007] (ToLCAntV-[MG:Nam:07]; AM701764), *Tomato leaf curl Seychelles virus*-[Seychelles:Mahe:2007] (ToLCSCV-[SC:Mah:07]; AM491778), *Tomato leaf curl Anjouan virus*-[Comoros:Ouani:2007] (ToLCAnjV-[KM:Oua:07]; AM701758), *Tomato yellow leaf curl virus*-Ethiopia [Ethiopia:Melkassa:2006] (TYLCV-ET[ET:Mel:06]; DQ358913), *Tomato yellow leaf curl virus*-Mild [Israel:1993] (TYLCV-Mld[IL:93]; X76319), *Tomato yellow leaf curl virus*-Israel [Israel:Rehovot:1986] (TYLCV-IL[IL:Reh:86]; X15656) and *Tomato leaf curl virus*-Tomato [Australia] (ToLCV-To[AU]; S53251)

To determine the infectivity of the clones, one isolate (accession number FM876848) was generated in the binary vector pCAMBIA0380 (Cambia). A 969-bp *Bam*HI-*Nco*I-digested fragment containing the intergenic region (IR) was cloned to generate a 0.34-mer (pCAMBIA0380-0.34). The full-length monomer was cloned into *Bam*HI-digested pCAMBIA0380-0.34 to generate a 1.34-mer. Recombinant plasmids were mobilized from *Escherichia coli* JM-109 cells (Promega) into *Agrobacterium tumefaciens* (strain C58) by triparental mating using *E. coli* HMB101 containing the plasmid helper pRK 2013 [5]. Host-range determination was performed using the agroinoculation

Table 1 Infectivity and partial host range of a Burkina Faso isolate of *Pepper yellow vein Mali virus* (PepYVMV) associated to yellow vein disease and determined by agroinoculation

Plants	cultivars	Infectivity (% of infected plants)	
		Symptoms [a]	Viral DNA [b]
Tomato	Farmer	9/34 (26%)***	9/34 (26%)
Pepper	Yollo Wonder	0/34 (0%)	2/34 (6%)
	JBF†	0/21 (0%)	5/21 (24%)
	RBF†	0/21 (0%)	5/34 (15%)
	Cayenne Long	0/21 (0%)	5/34 (15%)
Nicotiana benthamiana		34/34 (100%)***	34/34 (100%)

Infectivity: Number of infected plants/number of inoculated plants, [a] Verified by visual observation of symptoms, [b] confirmed by PCR amplification of viral genomic fragment

†: Local pepper cultivar

***: Severe symptoms

system with commercial tomato (cv. Farmer), local pepper (cvs. Jaune Burkina Faso [JBF], Rouge Burkina Faso [RBF]), commercial pepper (Cayenne Long, Yollo Wonder) and *Nicotiana benthamiana*. Ten-day-old seedlings were agro-inoculated with *Agrobacterium* cells ($OD_{600} \sim 1.0$) using a needle [11], and symptoms of infection were evaluated 30 days post-inoculation. *Nicotiana benthamiana* seedlings were severely affected and developed up-curling, swilling and yellowing in leaves. Inoculated tomato seedlings also developed severe symptoms of leaf curling, yellowing, and severe stunting. Unlike whitefly transmission of PepYVMV to pepper [16], agro-inoculated pepper plants did not develop symptoms. All of the agro-inoculated plants were tested for the presence of viral DNA. PepYVMV DNA was detected in top leaves of 100% of *N. benthamiana*, 26% of tomato, 6% of pepper cv. Yollo Wonder, 24% of pepper cv. JBF, 15% of pepper cv. RBF and 15% of pepper cv. Cayenne Long (Table 1). These results are consistent with the findings of Zhou et al. [17]. The lack of symptoms on local peppers (cvs. JBF, RBF and Yollo Wonder) demonstrated that the yellow vein disease did not relate to the nature of peppers widely grown in Burkina Faso.

PepYVMV and related viruses (Fig. 1) naturally infect solanaceous plant species domesticated in the New World. Consequently, these viruses are possibly African indigenous begomoviruses that evolved to infect exotic plants such as pepper, tomato and tobacco. Thus, African indigenous host plant species should be identified in order to better understand the genetic diversity of African monopartite begomoviruses and to devise adequate control measures against PepYVMV and its related viruses.

In conclusion, our results suggest that YVD in BF is neither associated with a complex of begomoviruses nor with DNA satellites but instead with the Old World monopartite PepYVMV. Typical yellow vein symptoms were not induced on peppers under artificial conditions using agroinoculation. Moreover, both mechanical and seed transmission tests failed to reproduce the disease in peppers [16]. These results suggest the need for further investigation on the role of both the vector and environmental factors in the development of pepper yellow vein disease.

Acknowledgments This study was supported by the following institutions: International Foundation for Science (IFS) fellowship no. C/4472-1 to F. Tiendrébéogo, AIRES-Sud: a programme from the French Ministry of Foreign and European Affairs implemented by the Institut de Recherche pour le Développement (IRD-DSF), CRSBAN/UFR-SVT (University of Ouagadougou), CIRAD, Conseil Régional de La Réunion, European Union (FEDER) and GIS Centre de recherche et de veille sanitaire sur les maladies émergentes dans l'Océan Indien (N°PRAO/AIRD/CRVOI/08/03). The technical assistance of Mr. Martial Grondin is gratefully appreciated. FT completed this research as part of his PhD Degree.

References

1. Barro N, Tiendrébéogo F, Traoré AS, Konaté G (2007) Symptoms and some ecological aspects of important vegetable crops viral diseases in Burkina Faso. Ann Bot Afr Ouest 4:1-12
2. Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X, Fauquet CM (2008) Recommendations for the classification and nomenclature of DNA-β satellites of begomoviruses. Arch Virol 153:763-781
3. Briddon RW, Bull SE, Mansoor S, Amin I, Markham PG (2002) Universal primers for the PCR-mediated amplification of DNA β; a molecule associated with some monopartite begomoviruses. Mol Biotechnol 20:315-318
4. Delatte H, Martin DP, Naze F, Golbach RW, Reynaud B, Peterschmitt M, Lett JM (2005) South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. J Gen Virol 86:1533-1542
5. Ditta G, Stanfield S, Corbin D, Helsinki DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc Natl Acad Sci USA 77:7347-7351

6. Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X (2008) Geminivirus strain demarcation and nomenclature. Arch Virol 153:783-821
7. Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online-a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 33:557-559
8. Harrison BD, Robinson DJ (1999) Natural genomic and antigenic variation in whitefly-transmitted Geminiviruses (Begomoviruses). Annu Rev Phytopathol 37:369-398
9. Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage φ29 DNA polymerase. J Virol Methods 116:209-211
10. Konaté G, Traoré O (1999) Characterization and distribution of Pepper veinal mottle virus in West Africa. Cahiers Agric 8:129-131
11. Paximadis M, Rey MEC (2001) Genome organization of Tobacco leaf curl Zimbabwe virus, a new distinct monopartite begomovirus associated with subgenomic defective DNA molecules. J Gen Virol 82:3091-3097
12. Posada D (2006) ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Res 34:W700-3
13. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu Rev Phytopathol 43:361-394
14. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599
15. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progessive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680
16. Tiendrébéogo F, Traoré VSE, Barro N, Traoré AS, Konaté G, Traoré O (2008) Characterization of Pepper *yellow vein Mali virus* in *Capsicum* sp. in Burkina Faso. Plant Pathol J 7:155-161
17. Zhou YC, Noussourou M, Kon T, Rojas MR, Jiang H, Chen LF, Gamby K, Foster R, Gilbertson RL (2008) Evidence of local evolution of tomato-infecting begomovirus species in West Africa: characterization of tomato leaf curl Mali virus and tomato yellow leaf crumple virus from Mali. Arch Virol 153:693-706

New Disease Reports

Tomatoes showing yellow leaf curl symptoms in the island of Grenada exhibit an infection with *Tomato yellow leaf curl virus* either alone or in combination with *Potato yellow mosaic virus*

J.-M. Lett [1]*, F. Péréfarres [1,2], M. Hoareau [1], P. Lefeuvre [1], A. De Bruyn [1,2], M. Dottin [3], P. Prior [1], E. Wicker [1,4] and P. Umaharan [5]

[1] *CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France*; [2] *Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France*; [3] *Ministry of Agriculture, Saint Georges, Grenada, West Indies*; [4] *CIRAD, UPR Horticulture, Pôle de Recherche Agro-environnementale, 97285 Le Lamentin, Martinique, France*; [5] *Department of Plant Science, The University of the West Indies, St. Augustine, Republic of Trinidad and Tobago*

*E-mail: lett@cirad.fr

Received: *11 May 2011.* ***Published:*** *02 Nov 2011.* ***Keywords:*** *begomovirus, TYLCD*

In March 2007, severe symptoms of leaf curling and yellowing resembling tomato yellow leaf curl disease were observed on tomato (*Solanum lycopersicum*) plants with a very high incidence in six sites on Grenada Island (Fig. 1; Table 1). Eleven leaf samples from tomato presenting the strongest symptoms were collected. Samples were tested for the presence of begomoviruses using polymerase chain reaction (PCR) assay with sets of degenerate primers designed to amplify parts of the DNA-A and DNA-B components (Table 1; Delatte *et a*l., 2005; Rojas *et al*., 1993). PCR products of the expected sizes, obtained with all DNA-A and DNA-B sets of primers for nine and three tomato samples, respectively, suggested the presence of Old World monopartite and New World bipartite begomoviruses. The nine partial DNA-A PCR products obtained with primers FD382-RD1038 were cloned and sequenced (EMBL-GenBank-DDBJ Accession Nos. FM163453 to FM163459, FM163462, FM163463). The highest nucleotide identity of 99% (BLASTn, NCBI) was obtained with the Old World monopartite *Tomato yellow leaf curl virus*-Israel (TYLCV-IL) isolates from Caribbean Islands (EF490995, AF024715). Similarly, the three partial DNA-B sequences obtained with primers PBL1V2040-PCRC1 (FM163460, FM163461 and FM163464) shared the highest nucleotide identity of 96% with the New World bipartite *Potato yellow mosaic virus*-Trinidad [Trinidad & Tobago] (PYMV-TT[TT], AF039032) DNA-B.

To confirm the molecular characterisation of the begomoviruses, full-length viral genomes were amplified from two PCR-positive samples (Table 1) by rolling-circle amplification, cloned using a set of restriction enzymes and sequenced (Inoue-Nagata *et al*., 2004). The complete DNA-A genome sequences obtained with *Nco*I (FR851297, FR851298), with 100% nucleotide identity, showed the highest sequence identity of 99% with isolates of TYLCV-IL ([Texas], AF039032; [Puerto Rico], AF039032). The complete DNA-A and DNA-B genome sequences obtained with *Sal*I, *Bam*HI and *Eco*RI (FR851299 to FR851302) showed the highest sequence identity of 96% with the Trinidad & Tobago strain of PYMV DNA-A and DNA-B (AF039031 and AF039032, respectively; Umaharan *et al*., 1998). The phylogenetic reconstruction with publicly available complete genome sequences confirmed the relationship of Grenada isolates of TYLCV-IL with the isolates from the United States, the Caribbean Islands and Central America, and of PYMV with the unique isolate of TT strain described in Trinidad & Tobago (Fig. 2).

To our knowledge, this is the first report of the Old World TYLCV and the New World PYMV implicated in yellow leaf curl disease on tomato in Grenada. This description confirms the invasion of the Lesser Antilles in the Caribbean from north to south by the Israel strain, also called the "severe" strain of TYLCV. The proximity between the island of Grenada and South America, where the "severe" strain of TYLCV has never been described, to our knowledge represents a new occurrence of first importance for the regional management of emerging crop diseases and regulatory institutions.

Acknowledgements

This study was conducted under the Project Grenada-Vitropic Moko Survey and the molecular analysis was funded by the Conseil Régional de La Réunion, the European Union (FEDER) and the CIRAD.

References

Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett JM, 2005. South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. *Journal of General Virology* **86**, 1533-1542. [doi:10.1099/vir.0.80805-0]

Guindon S, Gascuel O, (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52** , 696-704. [doi:10.1080/10635150390235520]

Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T, 2004. A simple method for cloning the complete begomovirus genome using the bacteriophage Phi29 DNA polymerase. *Journal of Virological Methods* , 209-211. [doi:10.1016/j.jviromet.2003.11.015]

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P, 2010. RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**, 2462-2463. [doi:10.1093/bioinformatics/btq467]

Rojas MR, Gilbertson RL, Russell DR, Maxwell DP, 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Disease* **77**, 340-347.

Umaharan P, Padidam M, Phelps RH, Beachy RN, Fauquet CM, 1998. Distribution and diversity of geminiviruses in Trinidad and Tobago. *Phytopathology* **88**, 1262-1268.

Figure 1

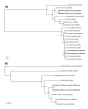


Figure 2

To cite this report: Lett J-, Péréfarres F, Hoareau M, Lefeuvre P, De Bruyn A, Dottin M, Prior P, Wicker E, Umaharan P, 2011. Tomatoes showing yellow leaf curl symptoms in the island of Grenada exhibit an infection with *Tomato yellow leaf curl virus* either alone or in combination with *Potato yellow mosaic virus*. *New Disease Reports* **24**, 19. [doi:10.5197/j.2044-0588.2011.024.019]

 This report was published on-line at www.ndrs.org.uk where high quality versions of the figures can be found.

Arch Virol
DOI 10.1007/s00705-011-1199-1

BRIEF REPORT

Differential disease phenotype of begomoviruses associated with tobacco leaf curl disease in Comoros

M. Thierry · P. Lefeuvre · M. Hoareau · F. Péréfarres · H. Delatte · B. Reynaud · D. P. Martin · J.-M. Lett

Received: 12 September 2011 / Accepted: 17 November 2011

Abstract In the 2000s, tobacco plantations on the Comoros Islands were afflicted with a previously unobserved tobacco leaf curl disease characterised by symptoms of severe leaf curling and deformation. Previous molecular characterization of potential viral pathogens revealed a complex of African monopartite tobacco leaf curl begomovirus (TbLCVs). Our molecular investigation allowed the characterization of a new monopartite virus involved in the disease: tomato leaf curl Namakely virus (ToLCNamV). Agroinoculation experiments indicated that TbLCVs and tomato leaf curl viruses (ToLCVs) can infect both tomato and tobacco but that infectivity and symptom expression fluctuate depending on the virus and the plant cultivar combination.

M. Thierry · P. Lefeuvre · M. Hoareau · F. Péréfarres · H. Delatte · B. Reynaud · J.-M. Lett (✉)
CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France
e-mail: jean-michel.lett@cirad.fr

M. Thierry
e-mail: mag.thierry@cirad.fr

P. Lefeuvre
e-mail: pierre.lefeuvre@cirad.fr

M. Hoareau
e-mail: murielle.hoareau@cirad.fr

F. Péréfarres
e-mail: frederic.perefarres@cirad.fr

H. Delatte
e-mail: helene.delatte@cirad.fr

B. Reynaud
e-mail: bernard.reynaud@cirad.fr

M. Thierry · F. Péréfarres
Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France

D. P. Martin
Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, Cape Town 7925, South Africa
e-mail: Darrin.Martin@uct.ac.za

The family *Geminiviridae* comprises plant-infecting viruses with single-strand DNA genomes encapsidated within unique twinned icosahedral particles. Most of the economically important diseases caused by geminiviruses are attributable to members of the genus *Begomovirus*, a group of whitefly (*Bemisia tabaci* Gennadius)-transmitted viruses that infect dicotyledonous plants [1]. Begomoviruses have monopartite or bipartite genomes consisting of one (DNA-A or DNA-A-like) or two (DNA-A and DNA-B) ssDNA components, respectively [2].

Tobacco leaf curl disease (TbLCD) has been reported in both the New and Old Worlds and has been associated with a complex of tobacco leaf curl begomoviruses [3–7]. In the 2000s, tobacco (*Nicotiana tabacum*) plants from the Island of Grande Comore showing severe leaf curl symptoms indicative of TbLCD were sampled, and molecular analysis indicated the presence of two begomoviruses: tobacco leaf curl Zimbabwe virus (TbLCZV) and tobacco leaf curl Comoros virus (TbLCKMV) [8]. We now report the molecular characterisation of a third monopartite begomovirus, tomato leaf curl Namakely virus (ToLCNamV), which is probably an additional member of the virus species complex responsible for TbLCD on these islands. We also report the phylogenetic relationships between the three distinct monopartite begomoviruses responsible for the disease on these islands and assess their partial host ranges, infectivities and symptom phenotypes.

Published online: 21 December 2011

Tobacco samples showing viral disease symptoms such as leaf curling, deformation and yellowing were collected from individual plants in 2004 and 2005 on the island of Grande Comore (Comoros Archipelago). Total plant DNA was extracted using a DNeasy Plant MiniKit (QIAGEN). Begomovirus DNA-A genome fragments were amplified by PCR using three degenerate primer sets: AV494-AC1048 [9], VD360-CD1266 [10] and FD382-RD1038 [11]. PCR reactions were carried out as described previously [11]. The presence/absence of a DNA-B genome, a DNA betasatellite and potyviruses were assessed using universal PCR primers for these different DNA species [12–14].

Circular viral DNA molecules were amplified using the TempliPhi Kit (GE Healthcare) as described previously [15]. Full genome-length *Bam*HI restriction fragments (~2.8 kb) were ligated into the pBC-KS plasmid. A complete DNA-A-like component was sequenced by primer walking (Macrogen, South Korea). The likely identity of this full DNA-A-like sequence was determined using NCBI-BLAST (http://www.ncbi.nlm.nih.gov). Potential mixed infections involving these three tobacco-associated begomoviruses were tested for by PCR using four different combinations of specific primers (Table 1).

Full genome sequences of tobacco-associated begomoviruses characterised in this study (ToLCNamV-[Comoros:Foubouni:Tobacco:2005], [KM:Fou:Tb:05]; FN600540) and previously in Grande Comore (TbLCZV-[Comoros:Foumboudziouni:2005], [KM:Fbz:05]; AM701756 and TbLCKMV-[Comoros:Foubouni:05], [KM:Fou:05]; AM701762) [8], as well as fourteen other full DNA-A and DNA-A-like sequences of related viruses obtained from public sequences databases (http://www.ncbi.nlm.nih.gov/), were aligned. Multiple sequence alignments were constructed using the ClustalW alignment tool [16] available in MEGA4 [17] and by manual editing. A maximum-likelihood (ML) tree was constructed with PHYML 3.0 [18] under GTR+I+G, the best-fitting nucleotide substitution model determined by ModelTest [19]. Statistical support for individual branches was estimated with 1000 bootstrap replicates. Detection of recombination events was carried out using methods implemented in RDP3 [20].

To test the infectivity of the three tobacco-associated begomoviruses (TbLCKMV, TbLCZV and the isolate KM:Fou:Tb:05), agroinfectious clones (partial DNA head-to-tail dimers) were used to infect both tomato and tobacco seedlings. To assess the infectivity of ToLCNamV-[KM:Fou:Tb:05], we decided to use an agroinfectious clone of a closely related virus already available in our laboratory (tomato leaf curl Comoros virus-[Mayotte:Dembeni:2003], ToLCKMV-[YT:Dem:03]; AJ865341). Agroinfectious clones for TbLCKMV and TbLCZV viral genomes were constructed as partial tandem repeats in the binary vector, pCAMBIA2300. The *Pst*I (1452)-*Bam*HI (149) intergenic region fragment of the TbLCKMV genome was first cloned into pCAMBIA2300, and the full-length *Bam*HI fragment was then inserted to produce a 1.52mer tandem repeat named pCambia2300-KM:Fou:05-1.52. For the TbLCZV genome, the *Sal*I (2256) - *Bam*HI (144) intergenic region fragment was ligated to pCambia2300, and the full-length DNA-A-like *Bam*HI fragment was then inserted to produce a 1.23mer tandem repeat named pCambia2300-KM:Fbz:05-1.23. Insert orientation within partial tandem repeats was confirmed by estriction digestion with the appropriate enzymes. The resulting recombinant plasmids were mobilized from JM109 *E. coli* cells (Promega) into *Agrobacterium tumefaciens* (strain C58) by triparental mating using *E. coli* HMB101 containing the plasmid helper pRK2013 [21].

Agroinoculations were performed on tomato (*Solanum lycopersicum cv*. Farmer) and tobacco (*Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum* (*cvs* Samsun and Xanthi)) seedlings at the third-leaf stage by stem puncture [22]. Plants were kept in a high-confinement growth chamber maintained at 25°C with a 16-h photoperiod for 30 days. Total DNA was extracted from the leaves at the apical meristem for each plant tested and used for virus detection by PCR amplification using degenerate primers FD382/RD1082 [11].

Besides the Grand Comorian tobacco viruses identified previously by Lefeuvre *et al*. [8], our investigation revealed the presence of a member of an additional begomovirus species infecting tobacco on this island. According to ICTV guidelines, KM:Fou:Tb:05 was classified as an isolate of tomato leaf curl Namakely virus (ToLCNamV-[KM:Fou:

Table 1 Nucleotide sequence primers used to detect mixed infection in tobacco plants sampled in Grande Comore

Begomovirus detected	Primer name	Primer set sequences (5′→3′)	Amplicon length (bp)
TbLCKMV	Tb-F901	AGTCAGAGGCTCTTAGAG	1275
	TbTo-R2175	GATGCAAGGAGGCTGCTA	
TbLCZV	Tb-F901	AGTCAGAGGCTCTTAGAG	1275
	Tb-R2175	CTGAAGCCGCTATGGCTA	
ToLCNamV	To-F828	GTGAAGAATGATTTGAGA	1348
	TbTo-R2175	GATGCAAGGAGGCTGCTA	

Tb:05]; FN600540), since the complete genome sequence was most similar to ToLCNamV-[Comoros:Dimadjou:2001] ([KM:Dim:01]; AM701761; 96.2% DNA-A-wide identity) and ToLCNamV-[Madagascar:Namakely:2001] ([MG:Nam:01]; AM701764; 90.7% identity). No begomovirus DNA-B, betasatellite DNA or potyvirus RNA were detected using diagnostic universal primers. Also, no mixed infections of TbLCKMV, TbLCZV or ToLCNamV were detected in the tobacco samples. Besides confirming evidence of the recombination events detected previously by Lefeuvre *et al*. [8], we did not reveal any additional evidence of recombination within the different ToLCNamV genomes.

A maximum-likelihood tree was constructed with 17 full-length genome sequences under the GTR+I+G nucleotide substitution model (Fig. 1). The phylogenetic analysis revealed two distinct groups: G1 and G2. The G1 group included monopartite begomovirus sequences that have previously only been found in cultivated solanaceous plants (tomato, pepper, tobacco). The ToLCNamV-[KM:Fou:Tb:05] isolate belonged to the G2 group, which included both monopartite and bipartite begomovirus sequences previously found on solanaceous and cassava plants.

Following agroinoculation of various *Nicotiana* species and tomato with isolates of TbLCKMV, TbLCZV and ToLCKMV, symptoms generally first appeared after 15 days. Infections were tested by PCR amplification of the V1 ORF (using degenerate primers FD382-RD1038 [11]) of the three viruses from total DNA extracted from symptomatic plants 30 days after agroinfection. Tomato, *N. benthamiana*, *N. glutinosa*, *N. tabacum* cv. Xanthi and *N. tabacum* cv. Samsun seedlings were all susceptible to infection with all three isolates, with systemic viral DNA being detectable within the apical meristems of both symptomatic and asymptomatic plants (Table 2). All three

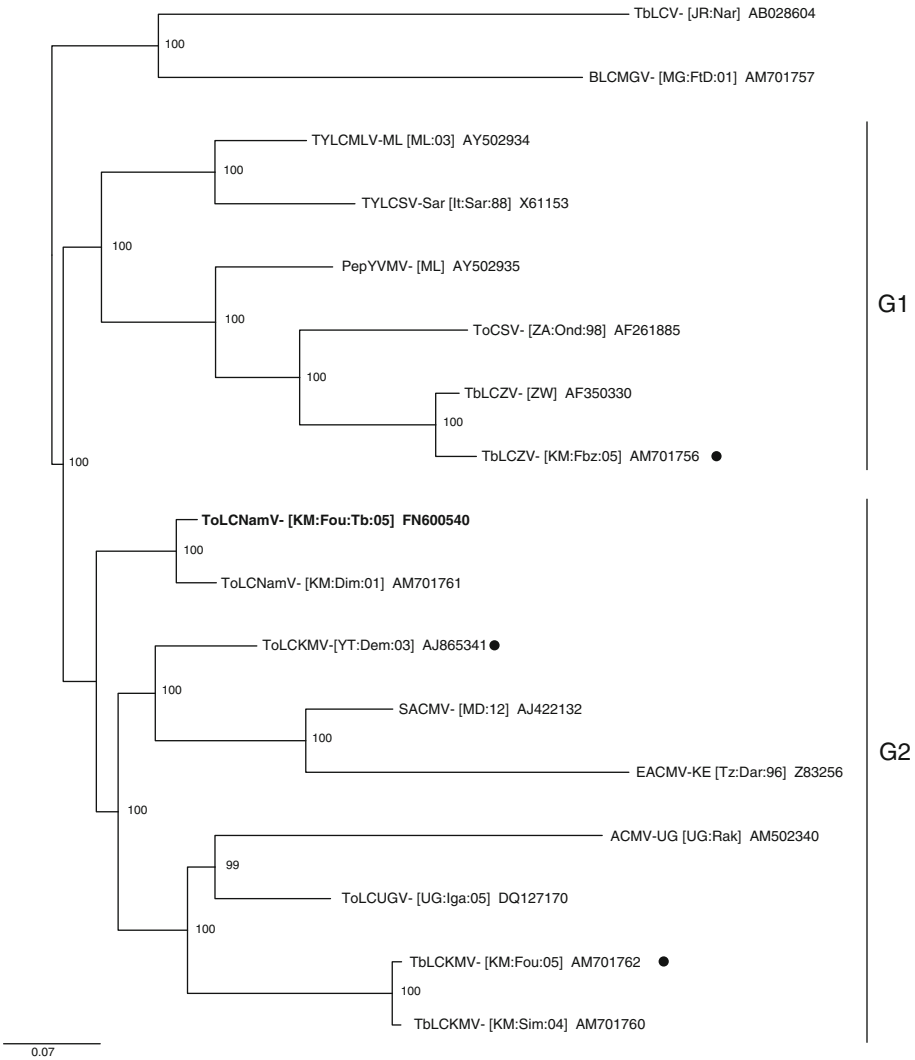


Fig. 1 Maximum-likelihood phylogenetic tree based on complete nucleotide sequences of 17 representative begomoviruses isolates. The tree was constructed using PhyML 3.0 [18] and rooted with BLCMGV-[MG:FtD:01] as an outgroup. Numbers associated with branches indicate their bootstrap support values. Only values greater than 80% are shown. Two phylogenetic groups, G1 and G2, are denoted by vertical lines. Genetic distances represented by horizontal lines are indicated by the scale bar. Dots show the three isolates used in experiments for agroinoculation of tobacco and tomato seedlings. The isolate represented in bold (ToLCNamV-[Fou:KM:Tb:05]) correspond to the new full-length genome obtained in this study

Table 2 Infectivity, symptoms and partial host range of tobacco-associated begomoviruses from Grande Comore 30 days after agroinoculation

Inoculum/host	Symptoms (%)*	Type of symptoms**	Detection of viral DNA by PCR***
ToLCKMV-[YT:Dem:03]			
S. lycopersicum	13/23 (56)	LC	13/23 (56)
N. benthamiana	14/15 (93)	LR	15/15 (100)
N. glutinosa	0/10 (0)	No	6/10 (60)
N. tabacum cv. Samsun	0/10 (0)	No	1/10 (10)
N. tabacum cv. Xanthi	0/10 (0)	No	9/10 (90)
TbLCZV-[KM:Fbz:05]			
S. lycopersicum	9/27 (33)	LC	9/27 (33)
N. benthamiana	16/16 (100)	LC	16/16 (100)
N. glutinosa	3/10 (30)	LC, MY, SL, Mt, St	3/10 (30)
N. tabacum cv. Samsun	0/10 (0)	No	7/10 (70)
N. tabacum cv. Xanthi	8/10 (80)	LD, Bls	10/10 (100)
TbLCKMV-[KM:Fou:05]			
S. lycopersicum	11/23 (48)	LC	12/23 (52)
N. benthamiana	14/16 (88)	SL	15/16 (94)
N. glutinosa	0/10 (0)	No	10/10 (100)
N. tabacum cv. Samsun	0/10 (0)	No	10/10 (100)
N. tabacum cv. Xanthi	10/10 (100)	Bls, LD, SL, LR	10/10 (100)

* Number of plants showing symptoms/number of plants agroinoculated (percentage)

** LC, leaf curling; MY, mild yellowing; St, stunting; LD, leaf distortion; LR, leaf rolling; Bls, blistering; SL, small leaves; Mt, mottling; No, no symptoms detected on any inoculated plants

*** Number of plants where viral DNA was confirmed by polymerase chain reaction in systemic leaves/number of plants agroinoculated (percentage)

of the isolates (ToLCKMV-[YT:Dem:03], TbLCZV-[KM:Fbz:05] and TbLCKMV-[KM:Fou:05]) were able to infect tomato and *N. benthamiana*. Whereas *N. tabacum* cv. Samsun plants displayed no signs of infection by any of the tested isolates, infection symptoms on *N. glutinosa* and *N. tabacum* cv. Xanthi plants varied from one isolate to the other. ToLCKMV-[YT:Dem:03] did not produce any symptoms in *N. glutinosa* and the *N. tabacum* cvs. despite viral DNA being detectable in 60% of *N. glutinosa* plants, 90% of *N. tabacum* cv. Xanthi plants and 10% of *N. tabacum* cv. Samsun plants. Unlike ToLCKMV-[YT:Dem:03], TbLCZV-[KM:Fbz:05] produced symptoms such as leaf curling, mild yellowing, mottling and stunting on both *N. tabacum* cv. Xanthi (80% of plants) and *N. glutinosa* (30% of plants; Fig. 2). TbLCKMV-[KM:Fou:05] produced no symptoms in inoculated *N. tabacum* cv. Samsun plants, but it produced blistering, leaf distortion, stunting and leaf rolling symptoms in 100% of inoculated *N. tabacum* cv. Xanthi plants (Fig. 2). Therefore, although the three viruses were all able to infect both tomato and tobacco seedlings, they displayed qualitative differences in their infectivity and the symptoms they induced in different species and cultivars.

We have cloned and sequenced a new isolate of ToLCNamV from a Grande Comorian tobacco sample showing tobacco leaf curl disease symptoms. Whereas this virus has been described previously on Grande Comore and Madagascar, it has only been detected in tomatoes. Although TbLCZV has been described previously in tobacco, it has never before been found outside Zimbabwe in this host [23].

Agroinoculation experiments with TbLCKMV-[KM:Fou:05], TbLCZV-[KM:Fbz:05] and ToLCKMV-[YT:Dem:03] confirmed that both tomato and tobacco are hosts of these three viruses. Although they all cause leaf curling or leaf rolling on tomato plants, their replication in tobacco is not always associated with the presence of symptoms. Additionally, all three viruses consistently induced symptoms in the tobacco cv. Xanthi, but symptoms varied from species to species.

It is important to point out that none of the three isolates tested produced as severe TbLCD symptoms as those described in the field by Lett *et al.* [24] and Paximadis *et al.* [25]. There could be a number of possible reasons for this discrepancy. First, plants in our experimental evaluations were subject to optimal temperature, lighting and soil

Fig. 2 Symptoms induced on *Nicotiana benthamiana* inoculated with **A**) ToLCKMV-[YT:Dem:03], **B**) TbLCKMV-[KM:Fou:05], and **C**) TbLCZV-[KM:Fbz:05] and on tomato inoculated with **D**) TbLCKMV-[KM:Fou:05]

conditions, whereas TbLCD-afflicted plants under field conditions were not. Any of these environmental factors may have increased the resistance of plants under our controlled experimental conditions [26]. Second, we performed our evaluation with commercial cultivars, whereas plants displaying TbLCD symptoms in Zimbabwe and the Comoros under field conditions are generally "local" outbreed races. Third, a population of viruses associated with an infected host can interact in a complex synergistic manner. Mixed infections of interactive variants of the same virus species [27], as well as mixed infections with members of different families of viruses [28], are known to potentially induce more severe symptoms as single infections (for review [29]).

In conclusion, we have demonstrated that the TbLCD on the Island of Grande Comore is likely caused by a complex consisting of members of at least three distinct African monopartite begomovirus species. Further, our agroinoculation experiments emphasise the complexity of TbLCD aetiology with symptom expression varying widely depending on both the virus and host combinations tested. Considering the high diversity of begomoviruses found in tomato in the South West Indian Ocean (SWIO) region and in cassava across Africa, more extensive sampling of symptomatic and asymptomatic tobacco plants seems essential and would also probably reveal a great diversity of begomoviruses.

Acknowledgments The authors thank M. Grondin for his excellent technical assistance, Andy Gallagher for his helpful English editing and the Tobacco Institute of Bergerac (France) for tobacco seeds. This study was funded by the CIRAD, the Conseil Régional de la Réunion, the French Ministère de l'Outre-Mer and the GIS Centre de recherche et de veille sanitaire sur les maladies émergentes dans l'océan Indien (N°PRAO/AIRD/CRVOI/08/03). M. Thierry is a recipient of a PhD fellowship from the Conseil Régional de La Réunion.

References

1. Seal SE, Jeger MJ, Van den Bosch F (2006) Begomovirus evolution and disease management. Adv Virus Res 67:297–316
2. Fauquet CM, Mayo MA (2001) The 7th ICTV report. Arch Virol 146(1):189–194
3. Paximadis M, Idris AM, Torres-Jerez I, Villarreal A, Rey ME, Brown JK (1999) Characterization of tobacco geminiviruses in the Old and New World. Arch Virol 144(4):703–717
4. Kitamura K, Murayama A, Ikegami M (2004) Evidence for recombination among isolates of Tobacco leaf curl Japan virus and Honeysuckle yellow vein mosaic virus. Arch Virol 149(6): 1221–1229
5. Moran YM, Ramos PL, Dominguez M, Fuentes AD, Sanchez Y, Crespo JA (2006) Tobacco leaf curl Cuba virus, a new begomovirus infecting tobacco (*Nicotiana tabacum*) in Cuba. Plant Pathol 55(4):570
6. Zhou XP, Xie Y, Zhang ZK (2001) Molecular characterization of a distinct begomovirus infecting tobacco in Yunnan, China. Arch Virol 146(8):1599–1606
7. Singh MK, Singh K, Haq QM, Mandal B, Varma A (2011) Molecular characterization of Tobacco leaf curl Pusa virus, a new monopartite Begomovirus associated with tobacco leaf curl disease in India. Virus Genes 43(2):296–306
8. Lefeuvre P, Martin DP, Hoareau M, Naze F, Delatte H, Thierry M, Varsani A, Becker N, Reynaud B, Lett JM (2007) Begomovirus 'melting pot' in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. J Gen Virol 88(12):3458–3468
9. Brown JK, Idris AM, Torres-Jerez I, Banks GK, Wyatt SD (2001) The core region of the coat protein gene is highly useful for establishing the provisional identification and classification of begomoviruses. Arch Virol 146(8):1581–1598
10. Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett JM (2005) South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. J Gen Virol 86(5):1533–1542
11. Lett JM, Lefeuvre P, Couston L, Hoareau M, Thierry M, Reynaud B, Martin DP, Varsani A (2009) Complete genomic sequences of Tomato yellow leaf curl Mali virus isolates infecting tomato and pepper from the North Province of Cameroon. Arch Virol 154(3):535–540

12. Briddon RW, Bull SE, Mansoor S, Amin I, Markham PG (2002) Universal primers for the PCR-mediated amplification of DNA beta: a molecule associated with some monopartite begomoviruses. Mol Biotechnol 20(3):315–318
13. Marie-Jeanne V, Ioos R, Peyre J, Alliot B, Signoret P (2000) Differentiation of poaceae potyviruses by reverse transcription-polymerase chain reaction and restriction analysis. J Pathol 148(3):141–151
14. Rojas MR, Gilbertson RJ, Russell DR, Maxwell DP (1993) Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis 77(4):340–347
15. Haible D, Kober S, Jeske H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. J Virol Methods 135(1):9–16
16. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680
17. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599
18. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59(3):307–321
19. Posada D (2006) ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Res 34(Web Server issue):W700–W703
20. Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P (2010) RDP3: A flexible and fast computer program for analysing recombination. Bioinformatics 26(19):2462–2463
21. Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77(12):7347–7351
22. Urbino C, Thebaud G, Granier M, Blanc S, Peterschmitt M (2008) A novel cloning strategy for isolating, genotyping and phenotyping genetic variants of geminiviruses. Virol J 5:135
23. Paximadis M, Rey ME (2001) Genome organization of Tobacco leaf curl Zimbabwe virus, a new, distinct monopartite begomovirus associated with subgenomic defective DNA molecules. J Gen Virol 82(12):3091–3097
24. Lett JM, Lefeuvre P, Naze F, Delatte H, Mohamed-Ali Y, Reynaud B (2006) First report of Tobacco leaf curl Zimbabwe virus affecting tobacco in the Comoros Archipelago. Plant Pathol 55(4):567
25. Paximadis M, Dusterwald G, Duyver L, Rey MEC (1997) Preliminary investigation of the causal agent(s) of a disease causing leaf curl of tobacco in South Africa. Plant Pathol 46:282–290
26. Patil BL, Fauquet CM (2009) Cassava mosaic geminiviruses: actual knowledge and perspectives. Mol Plant Pathol 10(5): 685–701
27. Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R (2006) Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature 439(7074): 344–348
28. Rochow WF, Ross AF (1955) Virus multiplication in plants doubly infected by potato viruses X and Y. Virology 1(1):10–27
29. Syller J (2011) Facilitative and antagonistic interactions between plant viruses in mixed infections. Mol Plant Pathol. doi:10.1111/j.1364-3703.2011.00734.x

Occurrence of the Israel strain of *Tomato yellow leaf curl virus* in New Caledonia and Loyalty Islands

F. Péréfarres[1,2], A. De Bruyn[1,2], S. Kraberger[3], M. Hoareau[1], F. Barjon[4], P. Lefeuvre[1], F. Pellegrin[5], P. Caplong[6], A. Varsani[3,7] and J.-M. Lett[1]*

(1) CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France
(2) Université de La Réunion, UMR PVBMT, Pôle de Protection des Plantes, 97410 Saint-Pierre, Ile de La Réunion, France
(3) Biomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
(4) Groupement de Défense des Cultures sous Abri (GDCSA), 98848 Nouméa, Nouvelle-Calédonie
(5) IRD, CBGP, Campus international de Baillarguet, 34988 Montferrier sur Lez, France
(6) Chambre d'Agriculture de la Nouvelle Calédonie, GDS-V, BP 111, 98845 Nouméa Cedex, Nouvelle-Calédonie
(7) Electron Microscope Unit, University of Cape Town, 7701, Cape Town, South Africa

*lett@cirad.fr

Accepted in New Disease Report

In 2007, severe symptoms of leaf curling and yellowing resembling those of tomato yellow leaf curl disease were observed for the first time on tomato plants (*Solanum lycopersicum*) with a high incidence in fields and greenhouses in the south western region (Nouméa) of the Pacific island of New Caledonia. Tomato samples with leaf curling and yellowing symptoms were collected in the south west and west of New Caledonia and also Ouvéa (Loyalty Islands), respectively, in November and December 2010 (Table 1). Samples were tested for the presence of begomoviruses using a polymerase chain reaction (PCR) assay with a set of degenerate primers designed to amplify genomic regions of the Old World begomovirus DNA-A component (Delatte *et al*., 2005). PCR products of the expected sizes were obtained for all the five samples from New Caledonia and for four of the six samples from Ouvéa suggesting the presence of an Old World monopartite begomovirus.

PCR positive samples were processed further and full-length viral genomes were successfully amplified from six samples (Table 1) by rolling-circle amplification, cloned using *Xmn*I restriction enzyme and sequenced (Shepherd *et al*., 2008). The complete DNA-A genome sequences obtained (EMBL-GenBank-DDBJ Accession Nos. HE603241-HE603246), showed the highest pairwise sequence

identity of 97.6 to 99.4% (BLAST, NCBI) with isolates of the Israel strain of *Tomato yellow leaf curl virus* (TYLCV-IL) from Spain ([SP:Alm], AJ489258) and Reunion ([RE:SGi:RE4:04], AM409201). The new sequences were aligned with representative sequences of TYLCV strains using MUSCLE (using default settings) in MEGA5 (Tamura et al., 2011). A maximum-likelihood (ML) phylogenetic tree (Fig. 1) was constructed from the full alignment using PHYML with GTR+G4 selected as the best model of sequence evolution by RDP3 (Martin *et al*., 2010).

Table 1: Geographical origin of samples and characterization of TYLCV isolates.

Date of sampling (dd/mm/yyyy)	Region	Island or district	Village#	GPS coordinates		TYLCV-IL isolates Acronym	EMBL accession N°
				Latitude	Longitude		
05/11/2010	New Cadeonia	Nouméa	La Coulée	-22.233952	166.588520	[NC:Nou1:10]	HE603241
05/11/2010	New Cadeonia	Nouméa	La Coulée	-22.233952	166.588520	[NC:Nou2:10]	HE603242
25/11/2010	New Cadeonia	Nouméa	La Coulée	-22.233612	166.596360	[NC:Nou3:10]	HE603243
18/11/2010	New Cadeonia	Nouméa	La Coulée	-22.233916	166.568569	[NC:Nou4:10]	HE603244
18/11/2010	New Cadeonia	La Foa	Focola	-21.719043	165.827997	[NC:LFo:10]	HE603245
15/12/2010	Loyalty Islands	Ouvéa	Saint-Joseph	-20.460901	166.603052	[NC:Ou:10]	HE603246

#each sample correspond to a distinct leaf from a different plant

The ML phylogenetic tree confirmed the relationship of New Caledonia and Ouvéa isolates of TYLCV-IL with the isolates of Spain [SP:Alm] and Reunion [RE:SGi:RE4:04] (Fig. 1). Surprisingly despite the proximity of New Caledonia and Ouvéa to Australia, the New Caledonian TYLCV isolates seem to have a different origin/source of introduction to those recently described from Australia [AU:Bri1:06] and [AU:Bun1:06] (Van Brunschot *et al*., 2010). This divergence suggests that the epidemic of TYLCD in New Caledonia and Loyalty Islands seems not directly associated with the introduction of TYLCV-IL in Australia where the first symptoms of TYLCD were described in 2006. To our knowledge, this is the first report of the Old World TYLCV implicated in yellow leaf curl disease on tomato in New Caledonia and Loyalty Islands. This description confirms the invasion and the dissemination of the Israel strain, also called "severe" strain, of TYLCV in the Pacific region, and represents a novelty of first importance for the regional management of crop emerging diseases and regulatory institutions.

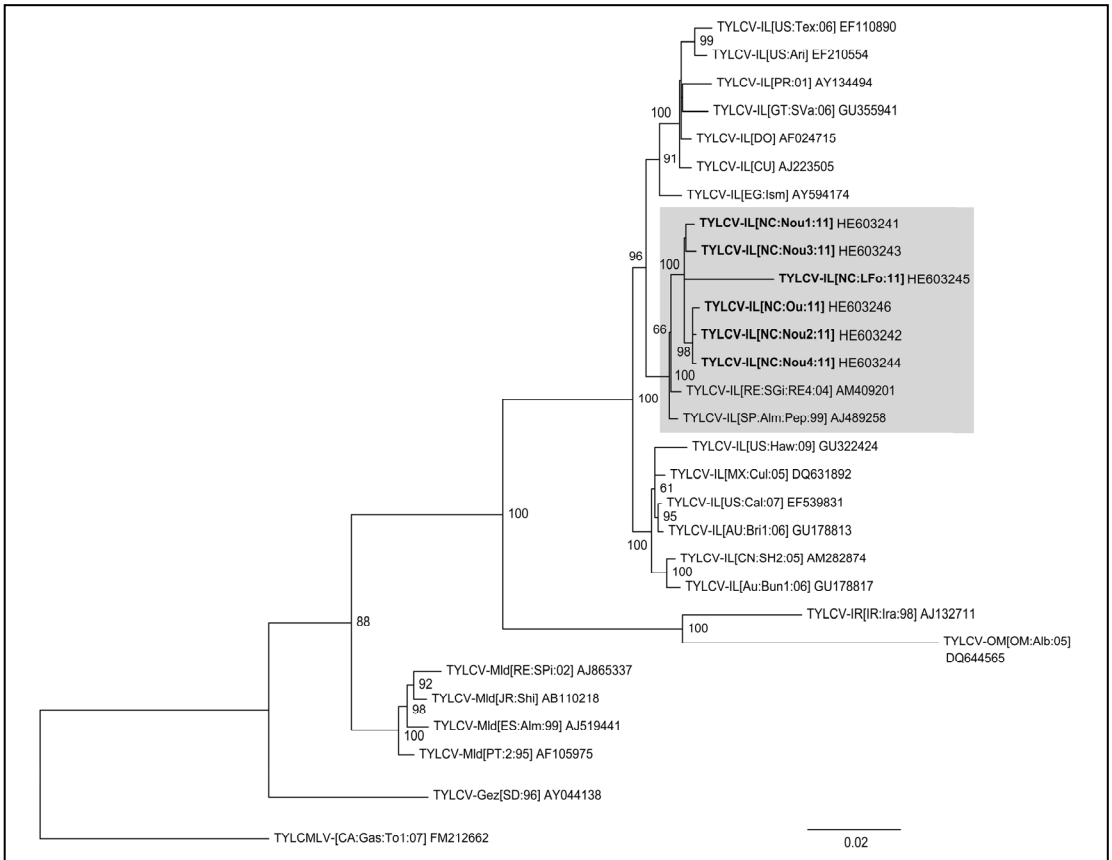


Figure 1: Maximum likelihood tree depicting the evolutionary relationships between New Caledonia and Loyalty Islands isolates of TYLCV (bold) and other representative isolates. The DNA-A tree was constructed using PHYML (Guindon & Gascuel, 2003, Systematic Biology 52: 696-704) and GTR+G4 selected as the best model of sequence evolution by RDP3 (Martin et al., 2010). The shaded area represents a clade including New Caledonia, Ouvéa, Spain and Reunion isolates of TYLCV-IL. The tree was rooted using Tomato yellow leaf curl Mali virus (TYLCMLV; Lett et al, 2009) as an outgroup. Bootstrap values were computed with 1000 replicates and only bootstraps above 60 are indicated.

Acknowledgements

This study was funded by the Conseil Régional de La Réunion, the European Union (FEDER) and the CIRAD. AV is supported by the Marsden fund of New Zealand.

References

Delatte H, Martin DP, Naze F, Goldbach R, Reynaud B, Peterschmitt M, Lett JM, 2005. South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group. *Journal of General Virology* **86**, 1533-1542.

Lett J-M, Lefeuvre P, Couston L, Hoareau M, Thierry M, Reynaud B, Martin DP, Varsani A., 2009. Complete genomic sequences of *Tomato yellow leaf curl Mali virus* isolates infecting tomato and pepper from the North Province of Cameroon. *Archives of Virology* **154**, 535-540.

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P, 2010. RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**, 2462-2463.

Shepherd DN, Martin DP, Lefeuvre P, Monjane AL, Owor BE, Rybicki EP, Varsani A, 2008. A protocol for the rapid isolation of full geminivirus genomes from dried plant tissue. *Journal of Virological Methods* **149**, 97-102.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-2739.

Van Brunschot SL, Persley DM, Geering ADW, Campbell PR, Thomas JE, 2010. *Tomato yellow leaf curl virus* in Australia: distribution, detection and discovery of naturally defective DNA molecules. *Australasian Plant Pathology* **39,** 412-423.

Epidemiology and sustainable control of *Begomovirus* on tomato

Emerging viruses are defined as those that have recently appeared or those whose populations have recently increased in prevalence, pathogenicity and/or geographical distribution. Among the phytoviruses, the genus *Begomovirus* (single-stranded circular DNA) transmitted by the whitefly *Bemisia tabaci* is responsible of numerous emerging diseases on many crops. The viral emergence is usually associated with environmental and molecular factors leading to new interactions virus - vector - plant - environment. Understanding these interactions and integrating the various components of this complex are prerequisites to the development of suitable control methods including breeding strategies.

Introduction of new viruses and/or vectors is the main cause of emergence. The development of a diagnostic tool based on original real-time PCR for the detection of begomoviruses present in the French overseas departments will ensure an early diagnosis to restrict the movement of infected plants and will be useful in epidemiological survey. The application of these real-time PCR also revealed important and original data on viral accumulation following bipartite and monopartite begomoviruses and for the first time between the two components of the bipartite begomovirus genomic components.

In Reunion, the accidental and successive introductions of two exotic and invasive strains of *Tomato yellow leaf curl virus* (TYLCV) gave us the opportunity to study the spatial and temporal evolution of this viral complex in a tropical and insular ecosystem. This study revealed a rapid displacement of TYLCV-Mld by the newcomer TYLCV-IL. Our work highlighted better ecological aptitudes of TYLCV-IL involved in this rapid spread. Our analysis also revealed complex interactions between these two strains in the colonization of their principal host and in the natural spread that allowed the maintenance of TYLCV-Mld in Reunion Island.

In the case of a viral infection, control methods are limited and mainly relied on the use of resistant cultivars. The main difficulty in developing cultivars resistant to begomoviruses is the great viral diversity to control. Our study explored the spectrum of action of the main resistance genes used in breeding programs. While resistance genes from *Solanum chilense* (ie *Ty-1*, *Ty-3*) confer a broad-spectrum resistance, *Ty-2* from *Solanum habrochaites* confers specific resistance to TYLCV-IL. Our results also suggest that the combination of resistance genes from *S. chilense* and *S. habrochaites* could effectively and sustainably control TYLCV. In a breeding program, the pyramiding of genes is a strategy commonly held to increase the level of resistance and extend its spectrum of action. The diversification of resistance sources aims to identify resistance mechanisms distinct from those controlled by the dominant factors already used by breeders. In this context, the recessive resistance to PYMV identified in *S. pimpinellifolium* 'LA2187-5' is particularly interesting. This work highlighted the broad spectrum as well as the monogenic recessive nature of this resistance. In addition to virus resistance, resistance to the vector represents a complementary option to increase the level and spectrum of resistance. We have demonstrated the interest of the *B. tabaci* resistant accession *S. habrochaites* 'LA1777' for the control of the two main strains of TYLCV (TYLCV-Mld and TYLCV-IL). In addition, the screening of near-isogenic lines derived from the interspecific cross of *S. lycopersicum* 'E6203' and 'LA1777' revealed viral quantitative resistance factors to TYLCV-IL and -Mld. Therefore, these lines could be of a great interest in the diversification of resistance sources.

This thesis characterized the biological diversity of viral populations involved in diseases caused by begomoviruses in tomato, the interactions between these populations, and the consequences on epidemiological dynamics. For the purpose of diversifying resistance factors, we explored alternative resistance sources that will contribute to ensure sustainable control of begomoviruses on tomato.

Epidémiologie et contrôle durable des *Begomovirus* chez la tomate

Les virus émergents sont définis comme ceux qui sont récemment apparus ou ceux dont les populations ont récemment augmenté en prévalence, en pathogénicité et/ou en répartition géographique. Parmi les phytovirus, le genre *Begomovirus* (ADN circulaire simple brin) transmis par l'aleurode *Bemisia tabaci* regroupe actuellement le plus grand nombre d'espèces virales émergentes d'importance économique. L'émergence virale est généralement associée à des facteurs écologiques et moléculaires aboutissant à de nouvelles interactions virus - vecteur - plante – environnement. Comprendre ces interactions et intégrer les différentes composantes de ce complexe sont des préalables nécessaires au développement de stratégies de contrôle et notamment à l'orientation des programmes de création variétale.

Les introductions de virus et/ou de vecteurs sont les premières causes du phénomène d'émergence. Le développement d'un outil de diagnostic de PCR quantitative pour les bégomovirus présents dans les départements de l'Outre-Mer français permettra d'assurer un diagnostic précoce, de limiter les mouvements de plants contaminés et de réaliser des veilles épidémiologiques. Cet outil a permis également de mettre en évidence des différences d'accumulation virale entre les bégomovirus monopartites et bipartites, ainsi qu'entre les deux molécules du génome des bégomovirus bipartites.

A La Réunion, l'introduction accidentelle et successive de deux souches exotiques et invasives de *Tomato yellow leaf curl virus* (TYLCV) nous a offert l'opportunité d'étudier l'évolution spatio-temporelle de ce complexe viral dans un écosystème tropical et insulaire. Cette étude a mis en avant un déplacement rapide du TYLCV-Mld par le TYLCV-IL. Nos travaux ont permis de mettre en évidence des aptitudes écologiques supérieures du TYLCV-IL. Nos analyses ont également mis en évidence des interactions fortes entre ces deux souches au niveau de la colonisation de l'hôte et de la dissémination naturelle qui ont permis le maintien du TYLCV-Mld à la Réunion.

Dans le cas d'une infection virale, l'éventail des méthodes de lutte demeure restreint et repose sur l'utilisation de cultivars résistants. La principale difficulté dans la création de cultivars résistants aux bégomovirus réside dans la grande diversité des virus à contrôler. Ce travail a permis de dessiner les contours de la spécificité de la résistance conférée par les principaux gènes de résistance utilisés dans les programmes d'amélioration variétale. Alors que les gènes issus de *Solanum chilense* (i.e. *Ty-1*, *Ty-3*) confèrent une résistance à spectre large, *Ty-2* originaire de *Solanum habrochaites* confère une résistance spécifique au TYLCV-IL. Nos résultats suggèrent que la combinaison des gènes de résistance provenant de *S. chilense* et *S. habrochaites* pourraient contrôler efficacement et durablement le TYLCV.

Le pyramidage de gènes est une stratégie efficace pour augmenter le niveau de la résistance et étendre le spectre d'action de celle-ci. La diversification des cibles pour la lutte génétique a pour objectif l'identification de mécanismes de résistance distincts de ceux contrôlés par les facteurs dominants déjà utilisés par les sélectionneurs. Dans ce contexte, la résistance récessive au PYMV identifiée chez *S. pimpinellifolium* 'LA2187-5' est particulièrement intéressante. Ce travail a permis de mettre en évidence le large spectre ainsi que le caractère monogénique récessif de cette résistance. En complément des résistances aux virus, la résistance au vecteur constitue une option avantageuse pour augmenter le niveau et le spectre de la résistance. Nous avons mis en avant l'intérêt de l'accession résistante à *B. tabaci S. habrochaites* 'LA1777' pour le contrôle des deux souches émergentes du TYLCV. De plus, le screening de lignées quasi-isogéniques de issues du croisement de *S. lycopersicum* 'E6203' et de 'LA1777' a mis en évidence des facteurs quantitatifs de résistance aux TYLCV-Mld et TYLCV-IL qui permettront également de diversifier les sources de résistance.

Ce travail de thèse a permis de caractériser biologiquement la diversité des populations virales, d'étudier les interactions entre ces populations et leurs conséquences dans les dynamiques épidémiologiques. Dans le second volet portant sur la recherche et la caractérisation de sources de résistance nous nous sommes attachés à caractériser des résistances complémentaires de celles déjà utilisées qui pourraient contribuer à un contrôle plus durable des bégomovirus sur la tomate.