THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'INSTITUT AGRO MONTPELLIER ET DE L'UNIVERSITE DE MONTPELLIER

En Mécanismes des Interactions parasitaires pathogènes et symbiotiques

École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Portée par
PHIM – Plant Health Institute of Montpellier

Caractérisation de déterminants biologiques et moléculaires de l'invasion d'un recombinant du *Tomato yellow leaf curl virus* sur des tomates résistantes Ty-1

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Le 14 Mars 2023

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UNIVERSITÉ DE MONTPELLIER

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Remerciements

Je tiens tout d'abord à remercier les membres de mon jury de thèse qui ont, sans hésiter, accepté d'évaluer mon travail. Merci à Emanuela Noris et Cécile Desbiez, qui ont bien voulu être rapportrice de mon mémoire de thèse et j'espère que cette lecture leur plaira. Mes remerciements vont également à Emmanuel jacquot et Nils Poulicard qui complètent ce jury en tant qu'examinateur.

Je suis également reconnaissante à toutes les personnes qui ont fait partie des membres de mes comités de thèse : Mathias Lorieux, mon référent, qui a pris son rôle à cœur, Jean-Luc Gallois, présent à tous mes comités et qui a apporté son expertise avec bienveillance, Jean-Benoit Morel, directeur de l'unité PHIM où j'ai réalisé ma thèse et qui a montré un vrai intérêt à discuter de mes résultats, et Thomas Kroj, Moussa Benhamed et Claire Neema, présents à mon premier comité.

Je voudrais particulièrement remercier Michel Peterschmitt, mon directeur de thèse, pour m'avoir donné l'opportunité de réaliser ce travail de thèse. Il s'est toujours rendu disponible pour répondre à mes questions et m'apporter son expertise sur le sujet.

J'exprime ma gratitude à mes autres encadrants de thèse. Tout d'abord à Cica Urbino de m'avoir initié avec patience aux techniques de laboratoire, un domaine qui m'était pratiquement inconnu au début de ma thèse. Elle a su m'accompagner, me soutenir et me conseiller avec bienveillance tout au long de ces trois années. Merci à Mikhaïl Pooggin, alias Misha, pour m'avoir apporté une aide précieuse dans l'analyse d'une partie de ma thèse grâce à sa grande expertise des mécanismes de gene silencing mais également sur tous les sujets touchant à la virologie. Enfin, merci à Clémence Plissonneau, de Gautier Semences, pour son aide sur les analyses transcriptomiques mais également pour ses conseils et ses encouragements tout au long de ma thèse.

A tous les membres de l'unité PHIM qui m'ont apporté une aide précieuse tout au long de ces trois années, un grand merci. A Martine Granier, pour son aide dans les expérimentations, elle m'a apporté toutes ses connaissances et sa grande expertise dans les techniques de laboratoire et a toujours été bienveillante à mon égard. A Nathalie Laboureau, pour son aide apporté au laboratoire pour certaines expérimentations et à Axel Cremades et Sophie Le Blaye pour leur aide technique en serre. A Gaël Thébaud et Yannis Michalakis pour leur conseil sur les analyses statistiques et à Victor Golyaev pour les extractions ARN et les analyses bio-informatiques des résultats de séquençage.

Je remercie également tous les membres des deux équipes dont j'ai fait partie durant ma thèse : l'équipe VIP dirigé par Stéphane Blanc, quand l'UMR BGPI existait encore, pour leur soutien, l'aide qu'ils ont pu m'apporter et la bonne ambiance qui régnait au sein de ce groupe, et l'équipe DefensiRNA dirigé par Mikhaïl Pooggin après la création de PHIM qui m'ont généreusement accueilli au sein de leur équipe. Une petite pensée à toute l'équipe CAMEPI dans laquelle j'ai failli atterrir qui était prête à m'accueillir et avec qui j'ai pu partager quelques réunions. Et merci à Marie-Carmen Martinez pour sa patience et sa disponibilité pour toutes les démarches administratives pour lesquelles elle m'a grandement aidé.

Un grand merci aux autres collègues avec qui j'ai moins collaboré au niveau professionnel mais avec qui j'ai partagé de très bons moments au labo et en dehors et qui m'ont permis de m'épanouir pleinement. Merci Michel Amphoux et Agnès pour toutes les randonnées dans lesquelles vous m'avez embarqué. Un immense merci aux camarades doctorants, post-doctorants, stagiaires, techniciens pour tous les moments de détente au labo (pause-café, partie de ping-pong…) et en dehors (sortie plage, restaurant, apéro, soirées…) que nous avons partagés.

Merci à mes amies de toujours, Assmina et Myriam qui, même si je suis loin, sont toujours là quand j'en ai besoin. J'adresse également une pensée à tous mes amis que j'ai rencontré pendant mon parcours, les copains de prépa et mes amis d'agrocampus ouest qui représentent un soutien sans faille.

Enfin, le meilleur pour la fin, je voudrais exprimer ton mon amour et ma gratitude à ma famille, que j'aime de tout mon cœur et qui m'a toujours soutenu dans tout ce que j'ai entrepris. A mes parents si exceptionnels, à mon frère Arthur que j'adore et qui, avec sa femme Pauline, m'ont offert le plaisir immense de devenir tata et marraine d'une adorable petite Gabrielle au cours de ma thèse.

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Résumé

La maladie du *tomato yellow leaf curl* (tylc) est l'une des maladies virales les plus dévastatrices des cultures de tomates. Elle est causée par un complexe d'espèces virales du genre *Begomovirus* (famille Geminiviridae). Les bégomovirus sont des virus circulaires à ADN simple brin, très recombinogènes et transmis par l'aleurode *Bemisia tabaci*. L'espèce virale la plus répandue du complexe est le *Tomato yellow leaf curl virus* (TYLCV). Dans le bassin méditerranéen, le *Tomato yellow leaf curl Sardinia virus* (TYLCSV) est l'autre espèce majeure associée au tylc. Pour lutter contre la maladie, l'utilisation de variétés résistantes est la solution la plus courante. Les variétés portant le gène Ty-1 sont les plus utilisées et ont largement remplacé les variétés sensibles. Les tomates Ty-1 ne présentent aucun symptôme et l'accumulation virale est environ dix fois plus faible que chez les plantes sensibles. Mais en 2010, dans le Souss, au sud du Maroc, des symptômes de tylc ont été détectés sur des variétés Ty-1, et l'analyse de ces plantes a révélé la présence d'un nouveau recombinant entre TYLCV-IL et TYLCSV appelé TYLCV-IS76. Son nom est inspiré du fragment de 76 nucléotides dans la région intergénique héritée du parent TYLCSV. Ce recombinant a pratiquement remplacé les virus parentaux dans le Souss et s'est étendu au nord du Maroc. Son émergence coïncide avec le remplacement des variétés sensibles par des variétés Ty-1. Son pouvoir invasif a pu être expliqué expérimentalement en montrant que dans les plantes Ty-1, TYLCV-IS76 s'accumule plus que les parents et qu'il affecte fortement l'accumulation de TYLCV-IL. L'objectif de cette thèse était d'affiner le scénario d'émergence de TYLCV-IS76 et de comprendre les mécanismes moléculaires qui sous-tendent son exceptionnelle compétitivité.

La compétitivité de TYLCV-IS76 a été testée dans des conditions contrôlées pour simuler des situations naturelles. Ainsi, TYLCV-IS76 a été coinoculé avec ses virus parentaux dans des plantes d'âges différents, ou surinoculé après les virus parentaux, dans des plantes où d'autres recombinants TYLCV/TYLCSV ont pu se former. La compétitivité de TYLCV-IS76 n'est pas altéré par l'âge de la plante, ni par la présence de virus parentaux ou d'autres recombinants avant son arrivée dans la plante. En testant la dynamique de surinfection avec d'autres clones viraux, nous avons montré pour la première fois un phénomène de prémunition entre TYLCV et ses variants avec des altérations dans la région intergénique, et démontré que TYLCV-IS76 est capable de la contourner. Enfin, nous n'avons pas détecté d'avantage du TYLCV-IS76 dans la transmission par vecteur.

Ty-1 code pour une ARN polymérase ARN-dépendante (RDRγ) impliquée dans l'amplification du *gene silencing*. Puisque l'avantage sélectif de TYLCV-IS76 se manifeste surtout sur plante résistante et non sur plante sensible isogénique, l'avantage est lié à l'action du gène Ty-1. Cependant, en absence d'un scénario crédible de *gene silencing*, nous avons adopté une approche sans a priori de séquençage à haut débit de transcrits et de petits ARNs. Nous avons montré que Ty-1 stimulait la production de siRNA viraux et que TYLCV-IS76 avait une meilleure transcription des gènes de la protéine de capside et d'un suppresseur de *silencing*, comparé à TYLCV-IL. Dans les plantes coinfectées par TYLCV-IL et TYLCV-IS76, le recombinant est plus compétitif pour la machinerie de réplication et de transcription et a un impact négatif sur la réplication et la transcription de TYLCV-IL. La production accrue de siRNA par Ty-1 ciblerait préférentiellement TYLCV-IL. Ensemble, ces résultats expliquent la diminution drastique de l'accumulation de TYLCV-IL en présence de TYLCV-IS76. L'analyse des gènes différentiellement exprimés des plantes résistantes et sensibles infectées par TYLCV-IL ou TYLCV-IS76 a révélé que le gène Ty-1 est surexprimé dans les plantes résistantes et donc que cette résistance est préétablie dans les plantes Ty-1.

Abstract

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating viral diseases of tomato crops. It is caused by a complex of viral species of the genus *Begomovirus* (family *Geminiviridae*). Begomoviruses are circular single-stranded DNA viruses, highly recombinogenic and transmitted by the whitefly *Bemisia tabaci*. The most widespread viral species of the TYLCD complex is *Tomato yellow leaf curl virus* (TYLCV). In the Mediterranean basin, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) is the other major species associated with TYLCD. To control the disease, the use of resistant varieties is the most common solution. Varieties carrying the Ty-1 gene are the most widely used and have largely replaced susceptible varieties. Ty-1 tomatoes show no symptoms and virus accumulation is about ten times lower than in susceptible plants. But in 2010, in the Souss, region of southern Morocco, TYLCD symptoms were detected in Ty-1 varieties, and analysis of these plants revealed the presence of a new recombinant between TYLCV and TYLCSV called TYLCV-IS76. Its name is inspired by the 76-nucleotide fragment in the intergenic region inherited from the TYLCSV parent. This recombinant has virtually replaced the parental viruses in the Souss and has spread to northern Morocco. Its emergence coincides with the replacement of susceptible varieties by Ty-1 varieties. Its invasiveness could be experimentally explained by showing that in Ty-1 plants, TYLCV-IS76 accumulates more than the parents and that it heavily affects the accumulation of TYLCV-IL. The objective of this thesis was to refine the emergence scenario of TYLCV-IS76 and to understand the molecular mechanisms underlying its exceptional competitiveness.

The competitiveness of TYLCV-IS76 was tested under controlled conditions to simulate natural situations. Thus, TYLCV-IS76 was co-inoculated with its parental viruses in plants of different ages, or over-inoculated after the parental viruses, in plants where other TYLCV/TYLCSV recombinants could be formed. The competitiveness of TYLCV-IS76 was not altered by the age of the plant, nor by the presence of parental viruses or other recombinants before its arrival in the plant. By testing the dynamics of superinfection with other viral clones, we showed for the first time a phenomenon of cross-protection between TYLCV and its genetic variants with alterations in the intergenic region, and demonstrated that TYLCV-IS76 is able to bypass it. Furthermore, we did not detect any advantage of TYLCV-IS76 in vector transmission.

Ty-1 encodes an RNA-dependent RNA polymerase (RDRγ) involved in gene silencing amplification. Since the selective advantage of TYLCV-IS76 is mainly manifested on resistant plants and not on isogenic susceptible plants, the advantage is related to the action of the Ty-1 gene. However, in the absence of a credible "gene silencing" scenario, we adopted a without-a priori approach of high-throughput sequencing of transcriptome and small RNAome. We showed that Ty-1 boosted the production of viral siRNAs and that TYLCV-IS76 had better transcription of the genes coding for the capsid protein and a silencing suppressor, compared to its parent TYLCV-IL. In plants coinfected with TYLCV-IL and TYLCV-IS76, the recombinant was more competitive for the replication and transcription machinery and had a negatively impact on the replication and transcription of TYLCV-IL. The increased siRNA production by Ty-1 preferentially targeted TYLCV-IL. Together, these findings explain the drastic decrease in accumulation of TYLCV-IL in the presence of TYLCV-IS76. Analysis of differentially-expressed genes of resistant and susceptible plants infected with TYLCV-IL or TYLCV-IS76 revealed that the Ty-1 gene is overexpressed in resistant plants and thus this resistance is pre-established in the Ty-1 plants.

Introduction générale

I. La maladie du tomato yellow leaf curl (tylc)

1. Description de la maladie

La maladie du *tomato yellow leaf curl* (tylc), aussi connue en français comme la maladie des feuilles jaunes en cuillère de la tomate, est l'une des maladies les plus dévastatrices de la tomate cultivée (*Solanum lycopersicum*). Elle est présente dans de nombreuses régions tropicales et subtropicales du monde (Moriones & Navas-Castillo, 2000). Elle entraine des pertes de rendement qualitatives et quantitatives importantes pouvant aller jusqu'à 100% de pertes de rendement si l'infection se produit à un stade précoce de développement de la plante (Picó et al., 1996).

La première description de la maladie remonte à la fin des années 1930, au Moyen-Orient, dans la vallée du Jourdain. Dans les années 1960, les cultures de tomates sont sévèrement touchées par la maladie dans les pays du Moyen-Orient (Cohen & Harpaz, 1964) et à la fin des années 70, toutes les régions de culture de tomates du Moyen-Orient sont touchées (Czosnek & Laterrot, 1997 ; Ioannou, 1985 ; Makkouk et al., 1992 ; Mazyad et al., 1979 ; Yassin & Nour, 1965).

A partir de la fin des années 1980, la maladie du tylc a connu une expansion géographique rapide et s'est étendue aux pays du bassin méditerranéen (Chypres (Ioannou, 1985), Italie (Credi et al., 1989 ; Accotto et al., 2003), Liban (Abou-Jawdah et al., 2006), Espagne (Moriones et al., 1993), Egypte (Nakhla et al., 1993), Tunisie (Gharsallah Chouchane et al., 2007), Maroc (Peterschmitt et al., 1999), Grèce (Avgelis et al., 2001). En France, la maladie a été ponctuellement détectée à partir de 1999 (Dalmon et al., 2000) et est présente dans les départements et régions d'outremer en Guadeloupe et Martinique (Urbino et al., 2003; Urbino & Tassius, 2003) et à la Réunion (Delatte et al., 2005).

La maladie est également présente dans le reste du monde et détectée sur tous les continents. En Asie, elle a été retrouvée à Taïwan (Green et al., 1987), en Chine (Wu et al., 2006), au Japon (Kato et al., 1998), en Azerbaidjan, au Turkmenistan et en Uzbekistan (Czosnek & Laterrot, 1997). En Afrique, elle a été reportée au Burkina Faso (Konaté et al., 1995), au Mali, au Nigeria et au Sénégal (D'Hondt & Russo, 1985). Elle a également gagné plusieurs pays du continent américain, notamment aux Etats-Unis (Valverde et al., 2001 ; Momol et al., 1999 ; Rojas et al., 2007 ; Polston et al., 1999), au Mexique (Ascencio-Ibáñez et al., 1999), au Guatemala (Salati et al., 2010), au Venezuela (Zambrano et al., 2007) et dans les Caraïbes, à Cuba (Martínez Zubiaur et al., 2002), en République Dominicaine (Nakhla et al., 1994 ; Polston et al., 1994) et en Jamaïque (McGlashan et al., 1994).

De nos jours, la maladie se propage toujours avec de récentes épidémies reportées dans de nouvelles régions comme au Costa Rica (Barboza et al., 2014) et à Trinité-et-Tobago (Chinnaraja et al., 2016).

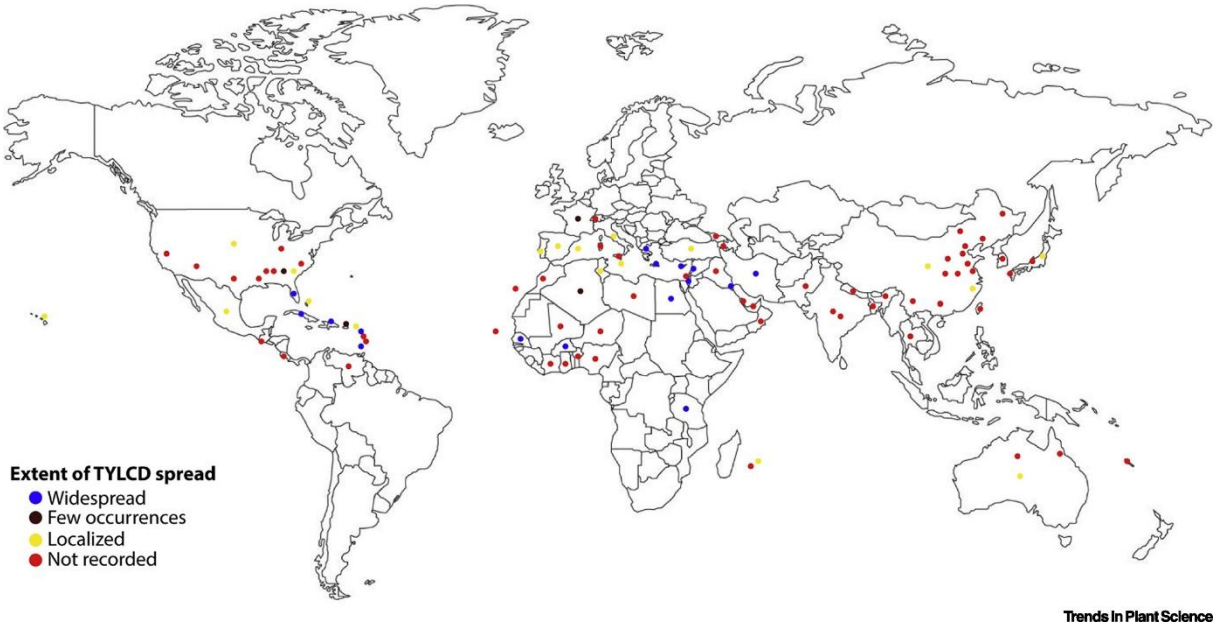

Figure 1 : Répartition de la maladie du tomato yellow leaf curl (tylc) dans le monde (Prasad et al., 2020)

La maladie du tylc sur tomate se caractérise par un retard sévère de la croissance des plantes, une réduction de la surface des feuilles accompagnée de leur jaunissement et de leur déformation avec un repliement des bords des folioles vers le haut (Cohen & Harpaz, 1964). On peut également observer des nécroses au niveau des feuilles âgées. Si les plantes sont infectées à un âge précoce, la maladie peut également provoquer un avortement des fleurs (Picó et al., 1996). Les premiers symptômes apparaissent en général entre 15 et 25 jours après l'infection et deviennent plus importants après deux mois.

Figure 2 : Symptômes causeés par la maladie du tylc (CABI.org)

2. Les virus responsables de la maladie

Bien que la maladie du tylc ait été identifiée pour la première fois dans les années 1930, il a fallu attendre les années 1960 pour que le premier virus causant la maladie soit identifié (Cohen & Harpaz, 1964), il a été appelé *Tomato yellow leaf curl virus* (TYLCV). Aujourd'hui, il est reconnu que la maladie du tylc est causée par un complexe d'espèces virales appartenant à la famille des Geminiviridae et au genre *Begomovirus*. Les symptômes de la maladie du tylc peuvent être causé par une douzaine d'espèce de bégomovirus outre le TYLCV.

Le TYLCV est considéré comme l'un des dix virus de plantes les plus importants économiquement et scientifiquement dans le monde et comme l'un des pathogène les plus dévastateurs chez la tomate, après le *Tobacco mosaic virus* (TMV) et le *Tomato spotted wilt virus* (Rybicki, 2015 ; Scholthof et al., 2011) et tout récemment le Tomato brown rugose fruit virus (ToBRFV, (Zhang et al., 2022)).

A. Classification

La famille des Geminiviridae est la plus grande famille de virus de plantes. Ce sont des virus à ADN simple brin circulaire, composé d'une (monopartite) ou deux (bipartite) molécules, contenues chacune dans une capside formée par l'union de deux parties jumelles icosaèdriques incomplètes (Zhang et al., 2001). Cette famille est divisée en 14 genres distincts en fonction de leurs hôtes, de leur insecte vecteur et de leur génome (Fiallo-Olivé et al., 2021; Zerbini et al., 2017) : *Becurtovirus, Capulavirus, Citlodavirus, Curtovirus, Eragrovirus, Grablovirus, Maldovirus, Mastrevirus, Mulcrilevirus, Opunvirus, Topilevirus, Topocuvirus et Turncurtovirus.* (https://ictv.global/report/chapter/geminiviridae/geminiviridae)

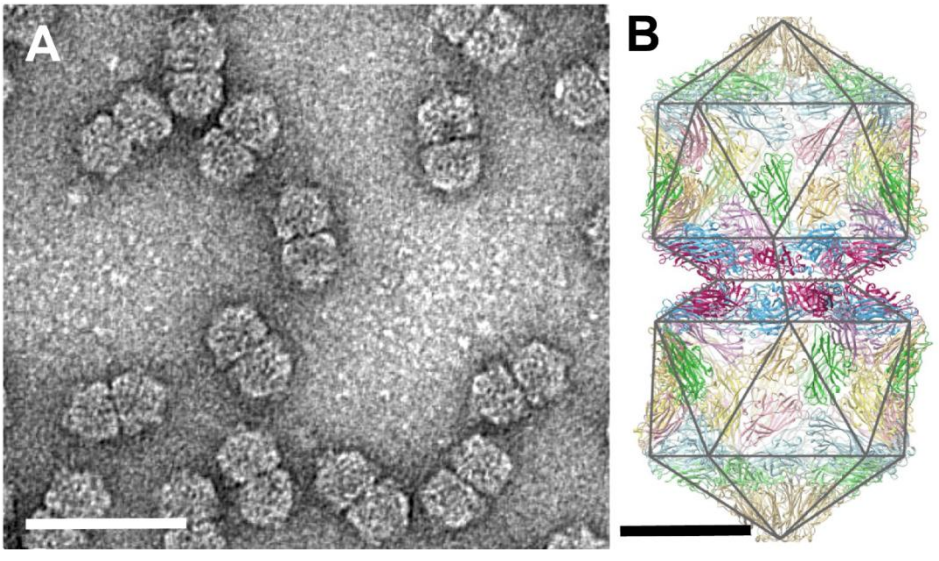

Figure 3 : A. Photo de virion du Maize streak virus (MSV) au microscope électronique (Zhang et al., 2001) et B. Modèle atomique de la capside du Ageratum yellow vein virus, AYVV (Hesketh et al., 2018)

Le genre *Begomovirus* est celui qui comprend le plus d'espèces (plus de 440 espèces). Ce sont des virus transmis par l'aleurode *Bemisia tabaci* (Hemiptera : Aleyrodidae), infectant principalement des plantes dicotylédones et ayant un génome monopartite ou bipartite (Fiallo-Olivé et al., 2021; Zerbini et al., 2017).

Pour le genre *Begomovirus*, selon les critères de démarcation de l'ICTV, les virus partageant au moins 94% de leur séquence nucléotidique sont considérés comme des souches d'une même espèce et ceux partageant moins de 91% d'identité sont considérés comme deux espèces différentes (Brown et al., 2015).

La souche Israélienne du TYLCV (TYLCV-IL) est le premier virus de ce complexe d'espèces à avoir été caractérisé et aussi le plus répandu dans le monde. C'est le virus associé à la maladie le plus répandu dans le monde. La souche mild du TYLCV (TYLCV-Mild) est également répandue dans le monde, alors que les autres virus associés à la maladie du tylc sont généralement restreints à certaines zones géographiques (Navas-Castillo et al., 2011).

B. Gamme d'hôtes du TYLCV

Le TYLCV a une gamme d'hôte très large, il a été détecté dans 49 espèces appartenant à 16 familles différentes rien qu'à Chypre (Papayiannis et al., 2011). La tomate est l'hôte principal du TYLCV, mais il peut également infecter plusieurs autres espèces cultivées comme le haricot (*Phaseolus vulgaris*, (Navas-Castillo et al., 1999)), le poivron (*Capsicum annuum* (Reina et al., 1999)) et l'aubergine (*Solanum melongena* (Fidan & Sarıkaya, 2020)). De nombreuses plantes adventices ont également été décrites comme hôtes naturels du TYLCV telles que l'*Amaranthus*, *Plantago*, *Mercurialis annua* (Abou-Jawdah et al., 1999), *Solanum nigrum* (Bedford et al., 1998), *Mercurialis ambigua*, *Solanum luteum* Mill. (Sánchez-Campos et al., 2000) ou encore *Datura stramonium* (Cohen & Harpaz, 1964; Cohen & Nitzany, 1966). Ainsi des plantes adventices peuvent jouer le rôle de réservoir de virus, en entretenant un inoculum viral entre deux saisons de cultures.

C. Transmission du TYLCV

Le TYLCV et ses virus associés sont, comme tous les begomovirus, transmis de façon vectorielle par l'aleurode *Bemisia tabaci* (Cohen & Harpaz, 1964). *B.tabaci* est un complexe d'espèces d'au moins 39 espèces cryptiques appartenant à l'ordre Hemiptera et qui, pris dans son ensemble, peut se nourrir sur plus de 600 espèces de plantes différentes (Alemandri et al., 2015). Les biotypes B et Q, aussi appelés Middle East Asia Minor 1 (MEAM1) et Mediterranean (MED) respectivement, transmettent efficacement le TYLCV, et tout particulièrement le biotype B qui s'est répandu partout dans le monde. Ces deux biotypes ont une très large gamme d'hôtes (Brown, 2007).

Les begomovirus sont transmis selon le mode persistant circulant (Cohen & Harpaz, 1964 ; Moriones & Navas-Castillo, 2000). Etant donné que les particules virales de géminivirus s'accumulent dans les noyaux, elles sont difficilement absorbées par l'insecte vecteur. Ce n'est que dans les tubes criblés formés de cellules sans noyau

et par lesquels le virus se propage dans toute la plante que les particules virales peuvent être absorbées par le vecteur. Du fait que le tube criblé est aussi le tissu le plus prisé des aleurodes pour leur alimentation, cette coïncidence fait que l'acquisition virale est potentiellement efficace. Donc pour acquérir le virus, le stylet de l'aleurode doit traverser les cellules de l'épiderme et du parenchyme avant de pénétrer dans les tissues vasculaires et atteindre les tubes criblés (Pollard, 1955). La durée minimale de contact entre l'aleurode et la plante pour réaliser une acquisition virale (*acquisiton access period*, AAP) est de 15 à 60 min, et celle de la période nécessaire à l'inoculation (*inoculation access period*, IAP) est de 15 à 30 min (Cohen & Harpaz, 1964 ; Mansour & Al-Musa, 1992 ; Mehta et al., 1994). Le contact minimum avec le phloème pour garantir le succès est de 1,8 min (Jiang et al., 2001).

Après l'ingestion, il y a une période de latence avant que les virus puissent être transmis à une autre plante par salivation. Pour cela, les virions doivent passer dans l'œsophage puis dans l'intestin. Les particules virales doivent alors traverser la paroi intestinale et passer dans l'hémolymphe de l'insecte pour enfin arriver dans les glandes salivaires. L'efficacité de transmission du virus dépend des protéines de type GroEL produites par des bactéries endosymbiotiques de l'insecte vecteur et interagissent avec les particules virales et les protègent de la dégradation dans l'hémolymphe (Morin et al., 1999, 2000). Le TYLCV a été détecté dans les glandes salivaires de l'insecte 7h après le début de l'AAP et l'insecte était capable de le transmettre seulement 1h après sa détection dans les glandes salivaires (Ghanim et al., 2001). Cependant, après avoir ingéré les virions, l'aleurode n'est pas forcément capable de transmettre le virus jusqu'à la fin de sa vie ; en effet, sa capacité d'inoculation diminue de façon constante dans le temps, jusqu'à devenir nulle entre 10 et 12 jours après acquisition. Pendant cette période, l'insecte est incapable d'acquérir de nouveau du virus et doit attendre de ne plus être capable de transmettre le virus pour pouvoir de nouveau acquérir le virus et être à nouveau virulifère (Cohen & Harpaz, 1964).

La capacité d'infection des insectes diminue avec le temps. En effet, les insectes plus âgés acquièrent moins de particule virale que les insectes jeunes (Rubinstein & Czosnek, 1997). Les insectes mâles transmettent moins bien le virus que les insectes femelles qui le transmettent 6 fois plus efficacement et qui ont de plus, une espérance de vie plus longue (Cohen & Harpaz, 1964). La présence de particule virale dans l'insecte diminue sa durée de vie d'environ 20% par rapport à des insectes non virulifères et diminue également leur fertilité (Rubinstein & Czosnek, 1997).

Le TYLCV peut se transmettre à la descendance par voie transovarienne sur au moins deux générations et les descendants sont capable de transmettre le virus sur tomate (Ghanim et al., 1998 ; Ghanim & Czosnek, 2000).

Il était généralement admis que les begomovirus ne sont pas capable de se répliquer dans l'insecte vecteur *B.tabaci* et une possible réplication du TYLCV dans son insecte vecteur a été fortement débattu quand de premiers indices d'une possible réplication ont été publiés (Czosnek et al., 2017). Ainsi, alors que certaines études ne révélaient aucune trace de réplication du TYLCV dans l'insecte vecteur (Becker et al., 2015 ; Sánchez-Campos et al., 2016), d'autres ont réussi à apporter des preuves que le TYLCV pouvait se répliquer dans l'insecte (He et al., 2020 ; Pakkianathan et al., 2015). Il semble également que la transcription des virus du TYLCV dans l'insecte vecteur soit possible (Sinisterra et al., 2005).

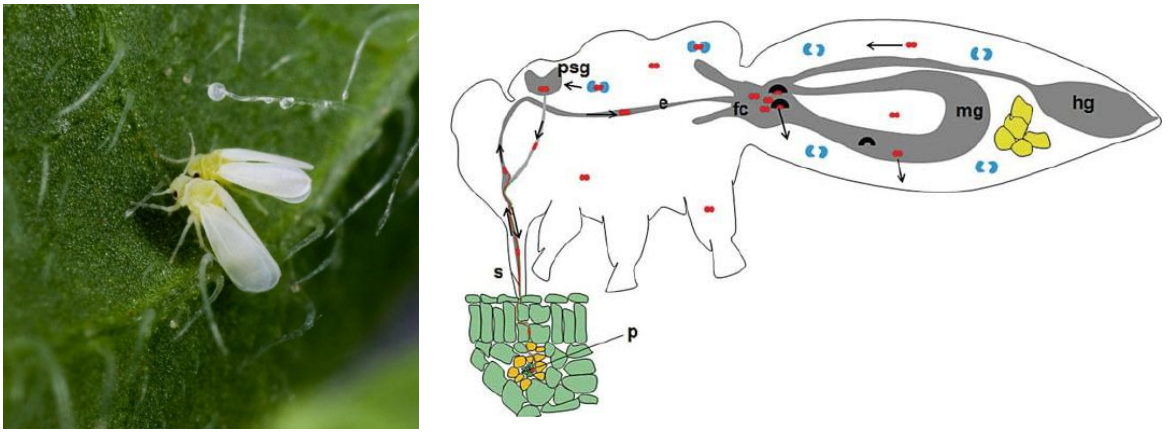

Figure 4 : Photo d'aleurode *B.tabaci* et schéma de la circulation du TYLCV (en rouge), grâce aux interactions avec les « heat shock protein 70» (HSP70, en noir) et les protéines GroEL (en bleu) produites des bactéries symbiotiques situées dans les bactériocytes (en jaune), à l'intérieur du vecteur *B.tabaci* (p : Phloème, s : stylet, e : œsophage, fc : filter chamber, mg : intestin moyen, psg : glandes salivaires primaires). (Kanakala & Ghanim, 2015)

D. Répartition géographique

Le Moyen-Orient est considéré comme le foyer d'origine du TYLCV. Le premier TYLCV y aurait émergé entre 1930 et 1950. La propagation de ces virus dans le monde aurait commencé dans les années 1980, après l'émergence de deux souches, le TYLCV-IL et le TYLCV-Mild qui sont les plus répandues dans le monde. Le bassin méditerranéen a été identifié comme le principal foyer de dissémination de ces virus (Lefeuvre et al., 2010).

La distribution mondiale de la maladie est étroitement liée au commerce international de plants (Czosnek & Laterrot, 1997), à l'augmentation de la population de son insecte vecteur dans le monde entier (Makkouk et al., 1979) et à l'évolution rapide de variants viraux.

L'un des facteurs majeurs ayant contribué à la propagation du virus est la mobilité géographique du biotype B du vecteur *B.tabaci* (MEAM1), du fait de sa gamme d'hôte très large et de la forte augmentation de sa population partout dans le monde (Bedford et al., 1994 ; Cohen & Harpaz, 1964 ; Varma & Malathi, 2003).

Aujourd'hui, les virus du tylc sont présents dans de très nombreuses régions du monde (Cf I.1)

L'introduction de ces virus dans de nouveaux environnements conduit à leur adaptation à de nouveaux hôtes et à son évolution conduisant à l'apparition de nouvelles souches par mutation ou recombinaison (Péréfarres et al., 2012).

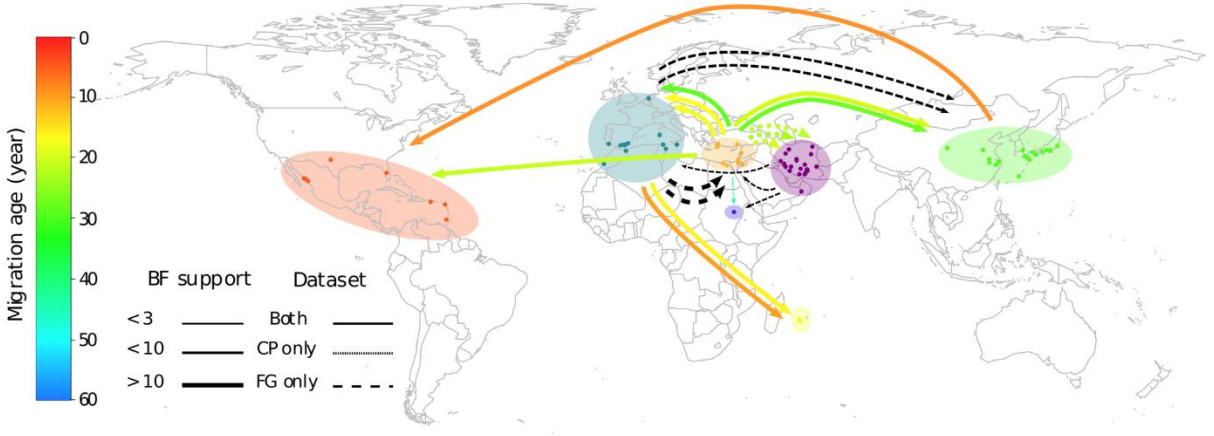

Figure 5 : Dissémination du TYLCV et des virus associés dans le monde (Lefeuvre et al., 2010)

E. <u>Génome des bégomovirus monopartites</u>

Les virus associés à la maladie du tylc sont, comme tous les géminivirus, des virus à ADN simple brin circulaire. Ils sont principalement monopartites, excepté certains comme le *Tomato yellow leaf curl Kanchanaburi virus* (TYLCKaV), le *Tomato yellow leaf curl Thailand virus* (TYLCThV) ou le Tomato yellow leaf curl New Delhi virus (TYLCNDV), originaires d'Asie, qui sont bipartites avec deux molécules d'ADN simple brin appelé ADN-A at ADN-B. Le génome des virus monopartites (équivalent de l'ADN-A des bipartites) mesure environ 2.8 kb et contient six gènes organisés dans deux directions transcriptionnelles séparées par une région intergénique d'environ 300 nucléotides (Kheyr-Pour et al., 1991 ; Navot et al., 1991).

Deux gènes sont transcrits dans le sens viral : les protéines V1 et V2 ; tandis que les quatre autres gènes (C1 à C4) sont transcrits dans le sens complémentaire.

La région intergénique de ces virus contient une structure en tige-boucles dont la tige est composée de deux séquences inverses répétées de 10 à 12 nts, riche en GC, et la boucle est formée de 12 à 14 nts contenant une séquence de neuf nucléotides, très conservée chez tous les géminivirus (5'-TAATATTAC-3') connues comme étant l'origine de réplication (Fontes, Gladfelter, et al., 1994 ; Hanley-Bowdoin et al., 1999 ; Laufs et al., 1995 ; Lazarowitz & Shepherd, 1992). La région intergénique contient également les promoteurs des gènes V1 et V2 du côté 3' de la tige boucle et les promoteurs des gènes C1 et C4 du côté 5' de la tige boucle. Également du côté 5' de la tige boucle, se trouve des séquences appelée itérons, qui permettent la fixation de la protéine Rep (Argüello-Astorga et al., 1994 ; Eagle et al., 1994 ; Fontes, Eagle, et al., 1994 ; Fontes, Gladfelter, et al., 1994 ; Jupin et al., 1995).

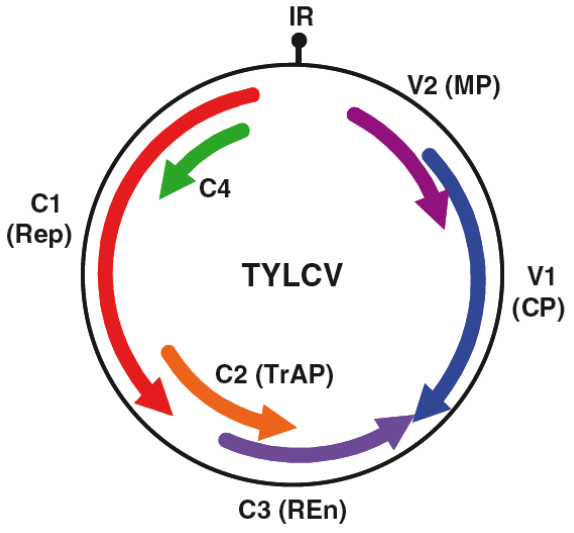

Figure 6 : organisation du génome du TYLCV (Gronenborn, 2007)

F. <u>Fonction des protéines</u>

Le gène V1 code pour la protéine de capside (coat proetin ,CP), la protéine V2 est parfois appelée pre-coat ou protéine de mouvement (MP), la C1 est la protéine initiatrice de la réplication (Rep), la C2 est la protéine activatrice de la transcription (*transcription activator protein*, TrAP), la C3 est la protéine activatrice de la réplication (*replication enhancer protein,* REn) et la C4 est la protéine déterminante pour l'expression des symptômes et la propagation du virus (Kheyr-Pour et al., 1991 ; Navot et al., 1991 ; Wartig et al., 1997). Le composant B des begomovirus bipartite contient les gènes BV1 codant pour la « nuclear shuttle protein » (NSP) et BC1 codant pour une protéine de mouvement (MP).

La protéine V1 (30.3 kDa, 260 acides aminés) est la seule protéine à constituer la capside dans laquelle l'ADN viral est encapsidé. En plus de son rôle d'encapsidation de l'ADN viral, la capside des géminivirus monopartite

est essentielle pour l'infection de la plante par le virus. En effet, elle joue un rôle dans le mouvement du virus, l'infection systémique du virus et son accumulation dans la plante (Wartig et al., 1997). La CP du TYLCV est capable de se lier à l'ADNsb, assurant ainsi l'entrée de ce dernier dans le noyau des cellules de la plante hôte durant le processus d'infection (Kunik et al., 1998, 1999 ; Palanichelvam et al., 1998). Elle permet aussi le mouvement systémique du virus dans la plante (Rojas et al., 2001). Elle joue également un rôle clé dans la transmission du virus par son insecte vecteur ; la protéine de capside est la seule protéine virale requises pour la transmission par l'insecte vecteur *B.tabaci* (Briddon et al., 1990). Une région critique pour la transmission par *B.tabaci* a été identifié entre les acides aminés 129 et 134 (Noris et al., 1998) qui serait localisés dans une structure formant une boucle qui pourrait être la zone d'interaction avec les protéines de l'insecte ou d'autres composants requis pour la transmission.

La protéine V2 (13.5 kDa, 116 aa) est une protéine cytoplasmique. Elle se situe à la périphérie du noyau et colocalise avec le réticulum endoplasmique (Hak et al., 2015). Cette protéine a toujours été considérée comme une protéine fondamentale au mouvement du virus car certaines mutations du gène V2 ont un impact négatif sur l'accumulation virale et la sévérité des symptômes (Fondong, 2013 ; Padidam et al., 1996 ; Wartig et al., 1997). Mais il a été montré par la suite que la V2 du TYLCV-IL n'était pas nécessaire dans le mouvement du virus (Hak et al., 2015) mais qu'elle jouait plutôt un rôle dans la suppression du *gene silencing*, un mécanisme de défense antivirale. La protéine V2 est capable de se lier à la protéine SGS3, qui est impliquée dans le mécanisme de *gene silencing*. Il a été suggéré que la liaison de la V2 à la protéine SGS3 permettrait l'inactivation de cette dernière, bloquant le processus de *silencing* (Glick et al., 2008 ; Sharma & Ikegami, 2010 ; Zrachya et al., 2007).

La protéine Rep (41kDa, 357 aa) est la protéine essentielle à la réplication chez les Géminivirus (Desbiez et al., 1995 ; Laufs et al., 1995). Elle possède une activité de ligation à l'ADN (Heyraud-Nitschke et al., 1995 ; Jupin et al., 1995) et une activité endonucléolytique. Elle possède également une activité hélicase (Clérot & Bernardi, 2006). Ces différentes activités lui permettent de jouer son rôle dans la réplication : la reconnaissance de l'origine de réplication, son clivage et le recrutement des différents composants du réplisome de la plante hôte pour l'initiation de la réplication du virus par la polymérase (Gutierrez, 1999). Elle permet aussi la ligation de l'ADN pour former l'ADN simple brin circulaire à la fin de la réplication. La protéine Rep peut aussi interagir avec les protéines du rétinoblastome (Kong et al., 2000), permettant la transition entre la phase G0/G1 à la phase S du cycle cellulaire et ainsi déclencher la production des protéines requises pour la réplication comme les facteurs appelés PCNA (*proliferating cell nuclear antigen*). Il a d'ailleurs été montré que la Rep du TYLCSV peut interagir directement avec les PCNA, des composants essentiels à la réplication (Castillo et al., 2003). Il a

été démontré que la protéine Rep jouerait aussi un rôle dans la suppression du *transcriptional gene silencing* (Rodríguez-Negrete et al., 2013).

La protéine TrAP (15,6 kDa, 135aa) est localisée dans le noyau des cellules (Dong et al., 2003 ; van Wezel et al., 2001) et intervient dans l'activation de la transcription des gènes en dirigeant les facteurs de transcription de l'hôte vers les promoteurs des gènes (Gronenborn, 2007). Mais elle joue aussi un rôle dans le pouvoir pathogène du TYLCV (van Wezel et al., 2001; Wartig et al., 1997) et dans la suppression du *gene silencing* en interagissant avec la calmoduline qui est un régulateur du *silencing* (Dong et al., 2003 ; Trinks et al., 2005 ; Vanitharani et al., 2004 ; van Wezel et al., 2002).

La protéine REn (15,9kDa, 134 aa) n'est pas essentielle pour la réplication virale mais elle intervient plutôt dans l'accumulation virale. Il a été montré qu'elle augmentait environ 50 fois l'accumulation de l'ADN viral, et intervient aussi dans le développement des symptômes (Settlage et al., 2005 ; Sunter et al., 1990). Elle permet l'amplification de la réplication en interagissant avec la Rep, les protéines PCNA et les protéines du rétinoblastome, qui sont impliquées dans la réplication et dans la régulation du cycle cellulaire respectivement (Castillo et al., 2003). De plus, la REn du *Tomato leaf curl virus* (ToLCV) peut interagir avec le domaine NAC des protéines SINAC1 de la tomate, des protéines souvent induites lors d'une infection par un pathogène. Cette interaction permet l'augmentation de l'accumulation de l'ADN viral (Selth et al., 2005). La protéine REn permettrait donc l'amplification de la réplication grâce à divers mécanismes ; en augmentant l'affinité de la Rep avec l'origine de réplication ou en interagissant avec d'autres protéines pour recruter les protéines nécessaires à la réplication de l'ADN viral.

Enfin, il a été montré que la protéine C4 (10,9 kDa, 98 aa) avait un rôle dans le mouvement du virus (Jupin et al., 1994 ; Rojas et al., 2001) puisqu'elle est localisée à la périphérie des cellules, au niveau des plasmodesmes (Rojas et al., 2001). Il a aussi été suggéré que la C4 jouait un rôle dans l'expression des symptômes et l'accumulation virale (Jupin et al., 1994 ; Krake et al., 1998 ; Rigden et al., 1994). Cette protéine pourrait également être impliquée dans la suppression du *gene silencing* selon des résultats obtenus pour d'autres géminivirus (Fondong et al., 2007 ; Rodríguez-Negrete et al., 2013 ; Vanitharani et al., 2004).

G. Cycle de vie

Après le dépôt de virus dans les cellules du phloème par la salivation des aleurodes vectrices, son génome doit se retrouver dans un noyau cellulaire pour initier sa réplication. Cette étape, encore mal connue, est supposée mobiliser des éléments du cytosquelette, des protéines de mouvement de l'hôte (*nuclear shuttle proteins*) et un passage dans les pores nucléaires (Gafni & Epel, 2002). Il est à noter qu'au moment de l'entrée du virus dans la cellule, aucune protéine virale n'est présente en dehors de la CP. Le mouvement du virus est donc

entièrement dépendant de la CP en plus des protéines de transport de l'hôte (Glick et al., 2009). Il a été démontré que la CP possède un signal de localisation nucléaire (*nuclear localization signal*, NLS) pouvant permettre à la CP d'entrer dans le noyau des cellules (Kunik et al., 1998). De plus, la CP a une nature karyophilique (Rojas et al., 2001) ce qui lui permet d'interagir dans la tomate avec une protéine homologue de la Karyophérine α (Kunik et al., 1999) ; la Karyophérine α est une *nuclear shuttle protein* (NSP), une protéine pouvant faire entrer les protéines possédant un NLS dans le noyau des cellules. De plus, la CP possède une capacité de liaison à l'ADN simple brin (Palanichelvam et al., 1998). Tous ces résultats permettent de considérer la CP comme une NSP pour le génome viral du TYLCV (Glick et al., 2009).

Dans le noyau, l'ADN simple brin circulaire viral est converti en ADN double brin circulaire grâce à la synthèse d'un brin complémentaire par les ADN polymérases de la plante hôte. Chez les *Begomovirus*, la synthèse du brin complémentaire serait initiée grâce à une amorce d'ARN (Saunders et al., 1992). Ensuite, des histones se lient aux doubles brins d'ADN viral pour former des minichromosomes. Ces minichromosomes sont les matrices de la transcription et de la réplication (Pilartz & Jeske, 1992, 2003).

Le génome du TYLCV est transcrit par les ARN polymérases de l'hôte (Pol II). La transcription est bidirectionnelle. Par analogie avec la cartographie des transcrits du composant A de l'Abutilon mosaic virus (AbMV) (Frischmuth et al., 1991) et du virus monopartite Tomato leaf curl virus (ToLCV, (Mullineaux et al., 1993), la transcription des gènes du TYLCV se ferait par trois transcrits polycistroniques, un transcrit dans le sens viral comprenant les deux gènes V1 et V2, et deux transcris dans le sens complémentaire, un premier comprenant les quatre gènes C1 à C4 et un deuxième comprenant seulement les gènes C2 et C3. Le promoteur du transcrit viral des gènes V1 et V2 est situé dans la région intergénique, du côté 3' de la tige-boucle, le promoteur du transcrit contenant les gènes C1 à C4 se trouve également dans la région intergénique mais du côté 5' de la tige boucle tandis que le promoteur du transcrit des gènes C2 et C3 est situé dans l'ORF C1. Les promoteurs sont tous situés entre 20 et 30 nts après un motif TATA box et l'extrémité 3' des transcrits se chevauche et se situe au niveau d'une séquence polyadénylée (Mullineaux et al., 1993). Dans un premier temps, La transcription précoce dans le sens complémentaire permet la formation des ARNm de la Rep et des autres protéines impliqués dans la réplication et la transcription. A un temps plus tardif, l'ADN est transcrit dans le sens viral pour former les ARNm de la CP et de la V2 (Shimada-Beltrán & Rivera-Bustamante, 2007 ; Shivaprasad et al., 2005).

La réplication du TYLCV dépend de la machinerie cellulaire de l'hôte. Afin de favoriser la réplication, les cellules sont reprogrammées par le virus pour permettre aux cellules d'être dans les bonnes conditions pour la réplication. Comme évoqué précédemment, les protéines Rep et REn peuvent se lier à des protéines du

rétinoblastome et aux PCNA, impliquées dans la régulation du cycle cellulaire, pour la production de protéines impliquées dans la réplication (Arguello-Astorga et al., 2004 ; Castillo et al., 2003 ; Gutierrez, 2000 ; Hanley-Bowdoin et al., 1999 ; Kong et al., 2000 ; Settlage et al., 2001, 2005).

Après sa transcription, sa traduction et son mouvement jusqu'au noyau, la protéine Rep se fixe dans la région intergénique du virus au niveau des séquences itérons (Fontes, Eagle, et al., 1994 ; Orozco & Hanley-Bowdoin, 1998) et coupe l'ADN viral au niveau de la séquence conservée (TAATATT↓AC), située dans la tige-boucle de la région intergénique (Akbar Behjatnia et al., 1998 ; Fontes, Gladfelter, et al., 1994 ; Laufs et al., 1995 ; Orozco & Hanley-Bowdoin, 1996), connue pour être l'origine de réplication. Cette coupure de l'ADN viral permet d'initier la réplication par un mécanisme appelé réplication en cercle roulant ou *Rolling circle replication* (RCR) (Stenger et al., 1991). Après coupure du brin viral par la Rep, il y a élongation de ce brin du côté 3'-OH par la polymérase de l'hôte le long du brin complémentaire. Pendant cette élongation, le brin viral déjà existant est décollé du brin complémentaire et la Rep reste liée à la polymérase et à l'extrémité 5' du brin viral d'origine (Laufs et al., 1995). Lorsque l'élongation est terminée, et que le complexe de réplication arrive au niveau de la tige-boucle, la Rep va couper et relier les deux extrémités du brin viral déplacé pour former une nouvelle copie d'ADN viral circulaire simple brin (Gutierrez, 2000 ; Hanley-Bowdoin et al., 1999 ; Laufs et al., 1995).

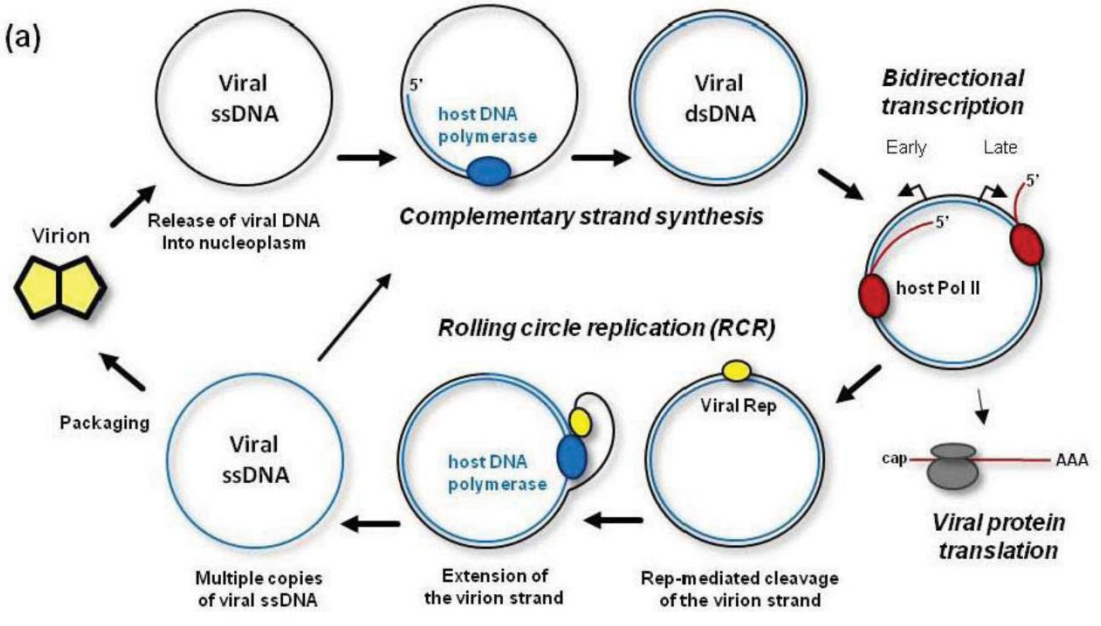

Figure 7 : La réplication des géminivirus par la RCR (Pooggin, 2013)

En plus de la réplication par RCR, le génome des géminivirus peut également être répliqué par un autre mécanisme appelé *recombination dependent replication* (RDR) (Jeske et al., 2001 ; Preiss & Jeske, 2003). La RDR

est un mécanisme de réplication à partir de molécules incomplètes d'ADN viral issues de perturbations lors de la réplication par RCR pouvant être un manque de nucléotides, une digestion de l'ADN ou encore une collision entre les complexes de transcription et de réplication. L'ADN double brin circulaire est ouvert par l'action d'une hélicase et l'ADN incomplet simple brin vient se fixer sur la région homologue du brin complémentaire de l'ADN double brin. L'ADN simple brin sert alors d'amorce à l'ADN polymérase pour son élongation le long de l'ADN complémentaire (la Rep n'est pas nécessaire pour l'initiation de ce mécanisme). Puis un brin complémentaire de cet ADN simple brin est synthétiser et il y a formation d'ADN double brin linéaire de différentes tailles (Jeske et al., 2001). Les longs ADN double brin linéaires formés, contenant au moins deux origines de réplication, peuvent être à nouveau répliqués, avec initiation de la réplication par la protéine Rep, formant de nouvelles copies d'ADN viral simple brin circulaire, ou peuvent être utilisés par la polymérase Pol II pour la transcription et former de nouveaux ARNm. La RDR explique pourquoi les geminivirus sont très sujet à la recombinaison. En effet, si une cellule est infectée par deux virus différents, ce mécanisme permet de produire aisément une grande variété de génome recombiné (Pooggin, 2013).

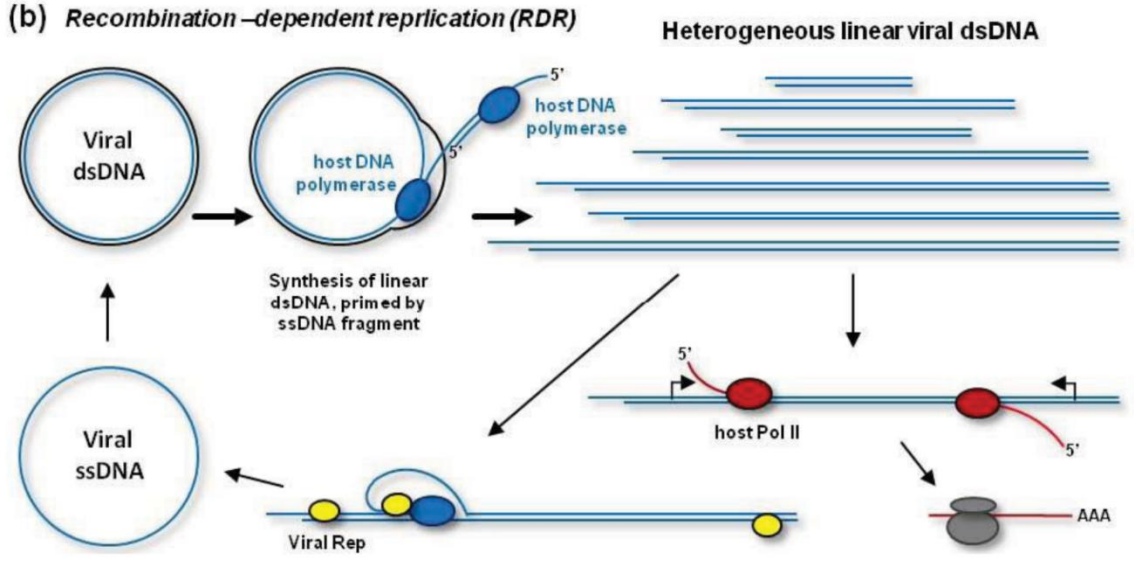

Figure 8 : La réplication des Geminivirus par la RDR (Pooggin, 2013)

Les nouveaux ADN simple brin circulaire viraux peuvent être utilisés pour un nouveau cycle de réplication ou être encapsidés pour former des virions à un temps plus tardif de l'infection, lorsque les protéines de capside ont été formées par la transcription.

L'export de l'ADN viral en dehors du noyau est probablement pris en charge par la CP tout comme son entrée (Gafni & Epel, 2002 ; Glick et al., 2009 ; Krichevsky et al., 2006 ; Kunik et al., 1998 ; Rojas et al., 2001). Après sa

sortie du noyau, le virus doit pouvoir se déplacer de cellule en cellule pour une infection efficace et généralisée de la plante. Le mouvement de cellule à cellule du TYLCV se ferait par l'intervention de la protéine C4 (Rojas et al., 2001) qui localise à la périphérie des cellules et interagit avec les plasmodesmes.

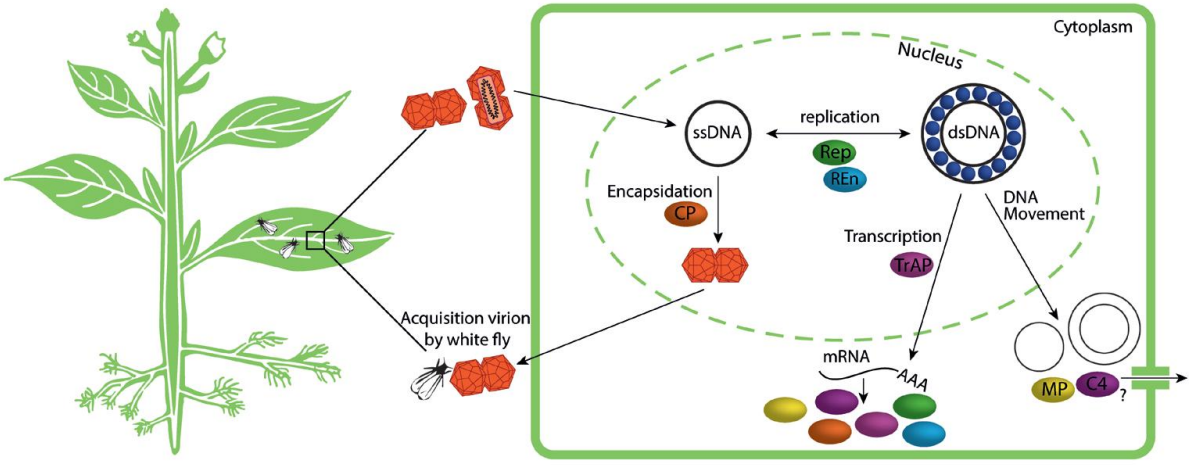

Figure 9 : Cycle de vie des Geminivirus dans la plante (Voorburg, 2021)

II. Mécanismes de résistance des plantes aux virus

Pour lutter contre leur infection par des pathogènes, les plantes ont développé différents mécanismes de résistance. En général, les plantes sont résistantes à la majorité des pathogènes et seulement une petite partie sont capables de causer une maladie (Dangl & Jones, 2001). Le premier obstacle que doit franchir un pathogène pour réussir son infection est une barrière physique, la cuticule des feuilles puis la paroi cellulaire. C'est la première protection de la plante. Certains pathogènes ne peuvent pas franchir cette paroi et sont donc incapables d'infecter la plante. Les virus sont capables de contourner cette première résistance grâce à des organismes vecteurs se nourrissant de plantes. D'autre mécanismes internes de la plante doivent donc prendre le relais pour lutter contre les attaques.

1. Résistances dominantes

La plupart des résistances connues chez les plantes sont des résistantes dominantes, pour lesquelles un seul gène de résistance (gène R) est suffisant et qui reposent principalement sur des mécanismes de reconnaissance du pathogène conduisant à l'induction de cascades de signalisation.

A. Mécanismes de détection à l'extérieur de la cellule

Au niveau extracellulaire, les plantes reconnaissent l'infection par un pathogène grâce à des récepteurs membranaires appelés PRR (*Pattern recognition receptor*). Ces récepteurs membranaires sont capables de détecter des motifs moléculaires conservés chez les pathogènes désignés par le terme PAMP (*pathogen-associated molecular patterns*). Les PRR possèdent un domaine extracellulaire permettant la reconnaissance de l'agent pathogène, une région transmembranaire et pour certains, un domaine kinase intracellulaire. Ils sont alors regroupés en deux sous-familles, les RLP (*receptor-like proteins*) et les RLK (*receptor-like kinase*) selon la nature de leur domaine protéique. Les PAMPs peuvent être de nature biochimique variées (protéines, glycoprotéines, polysaccharides, lipides…) (Dangl et al., 2013). La résistance issue de ces mécanismes de reconnaissance est nommée PTI (*PAMP-triggered immunity*, plus récent nommé *pattern-triggered immunity*).

Les PRR peuvent percevoir de manière directe la présence d'agents pathogènes par la reconnaissance de ce pathogène directement ou de manière indirecte par la détection de perturbation des protéines de l'hôte ciblées par certains effecteurs. Un exemple de perception directe est celui du récepteur RLK FLS2 chez Arabidopsis thaliana qui reconnait flg22, un des principaux composants du flagelle bactérien et va ensuite s'associer à la protéine BAK1 déclenchant des voies de signalisation et permettant la mise en place de

l'immunité (Chinchilla et al., 2007 ; Gómez-Gómez & Boller, 2000). Un exemple de perception indirecte est le récepteur RLP Cf-2 chez la tomate. L'effecteur Avr2 du champignon *Cladosporium fulvum* cible la protéine RCR3, une protéine de la tomate, et l'inhibition de l'activité de RCR3 qui suit cette interaction entraine l'activation de Cf-2, déclenchant les cascades de signalisation immunitaire (Rooney et al., 2005).

Les virus ayant un cycle de vie principalement intracellulaire, la détection par des protéines codés par des gènes de résistance se fait principalement par des protéines intra-cellulaires (décrit dans le paragraphe suivant). Pourtant, certaines études semblent bien indiquer un rôle des PRR dans la défense antivirale. Une protéine PRR d'*Arabidopsis thaliana,* BAK1, confère à la plante une résistance à trois virus à ARN différents, par la reconnaissance d'une molécule dérivée de l'infection virale (Kørner et al., 2013). En outre, le récepteur extracellulaire SERK1 peut reconnaitre les ARN double brin formé lors de l'infection par un virus et permet l'activation de défenses chez *Arabidospsis thaliana* et *Nicotiana benthamiana* (Niehl et al., 2016).

B. Mécanismes de détection intracellulaire

Les plantes possèdent également des récepteurs intracellulaires appelés NLR (*nucleotide-binding oligomerization domain* (*NOD*)-*like receptors*). Ces protéines sont codées par des gènes de résistance, ou gènes R. Elles détectent spécifiquement des protéines injectées par le pathogène dans la cellule végétale qu'on appelle des effecteurs. Les NLR sont tous composés d'un site central de liaison aux nucléotides (NB = *nucleotide-binding site*) et d'un domaine carboxy-terminal constitué de répétitions riche en leucine (LRR = *leucine-rich repeats*). Deux grandes classes de NLR existe en fonction de leur domaine N-terminal, ceux possédant un domaine TIR (*toll interleukine-1 receptor*) et ceux possédant un domaine en superhélice CC (*coiled-coil*). Ces NLR confèrent à la plante une résistance extrême (ER) ou une résistance associée à une réaction d'hypersensibilité (HR). La HR provoque l'apparition de nécroses engendré par une mort cellulaire programmée empêchant la propagation du pathogène. La résistance médiée par ces types de récepteur est appelée ETI (*effector-triggered immunity*) (Dangl & Jones, 2001).

Les NLR peuvent détecter les effecteurs de manière directe. Par exemple, les NLR L5, L6 et L7 du lin, codés par des allèles différents d'un même gène, reconnaissent directement l'effecteur AvrL567 du champignon *Melampsora lini* causant la maladie de la rouille du lin. Le polymorphisme du domaine LRR de ces trois NLR détermine la spécificité de reconnaissance de l'effecteur (Ravensdale et al., 2012).

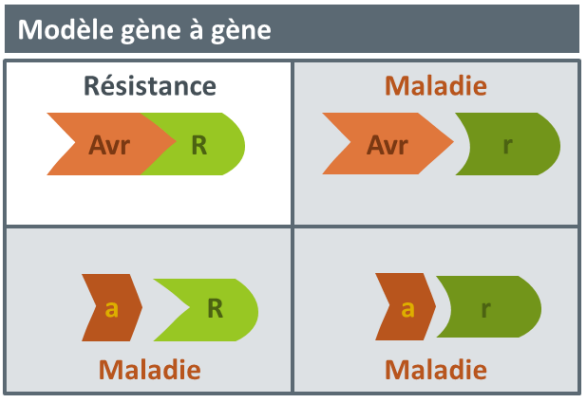

Figure 10 : Détection directe de l'effecteur par les protéines de résistances (Gauffier, 2015)

La perception des effecteurs par les NLR peut aussi être indirecte, lorsque le NLR reconnait les perturbations chez des composantes de l'hôte qui sont induites par des effecteurs. Parmi les perceptions indirectes, on distingue deux modèles, le modèle de garde et le modèle du leurre.

Dans le modèle de garde, les NLR surveillent l'intégrité des composantes de l'hôte, qui ont souvent des fonctions immunitaires importantes, et qui sont ciblés par des effecteurs (Dangl & Jones, 2001 ; Jones & Dangl, 2006). Chez Arabidopsis thaliana, la modification de la protéine RIN4 par les effecteurs AvrRpt2 ou AvrRPM1 active les récepteur NB-LRR RPS2 et RPM1 respectivement (Axtell & Staskawicz, 2003 ; Mackey et al., 2003).

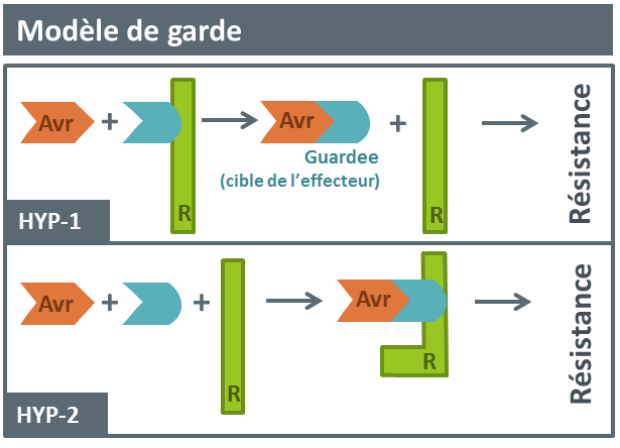

Figure 11 : Détection indirecte de l'effecteur par le modèle de garde (Gauffier, 2015)

Dans le modèle du leurre, la protéine « gardée » par le NLR n'est pas une réelle cible de l'effecteur du pathogène mais seulement une protéine imitant la structure des protéines véritablement ciblées par les effecteurs. Elles fonctionnent donc comme des leurres qui détourne les effecteurs de leur véritable cible (van der Hoorn & Kamoun, 2008). Un exemple connu de ce modèle du leurre est le complexe Pto/prf chez la tomate. La protéine Pto est une protéine kinase leurre qui détourne les effecteurs AvrPto et AvrPtoB de leur cible réelle BAK1. Lorsque Pto est ciblé par ces deux effecteurs le complexe formé de Pto et du récepteur NLR

Prf induit alors des réponses de défense (Ntoukakis et al., 2013). Un modèle de leurre intégré a également été proposé dans lequel la cible de l'effecteur a été dupliquée et fusionnée à une protéine R (Cesari et al., 2014).

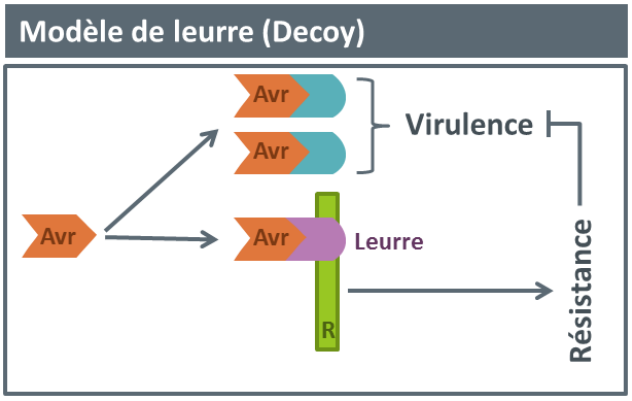

Figure 12 : Détection indirecte de l'effecteur par le modèle du leurre (Gauffier, 2015)

Les virus étant des pathogènes intracellulaires, de nombreux gènes codant pour des NLR et conférant une résistance à plusieurs genres viraux ont été identifiés dans différentes espèces de plantes (Gouveia et al., 2017). Par exemple, chez la tomate, le gène Tm-2 code pour des NLR associés à la résistance chez les *Tobamovirus*. Chez le piment, quatre allèles du locus L codent pour des NLR reconnaissant la CP des *Tobamovirus*. Chez les virus, les NLR semble reconnaitre principalement des protéines virales telles que la CP, la réplicase ou la protéine de mouvement (Gouveia et al., 2017). Dans certains cas, les NLRs reconnaissent directement ou indirectement les activités des suppresseurs viraux du *gene silencing* (Zvereva & Pooggin, 2012).

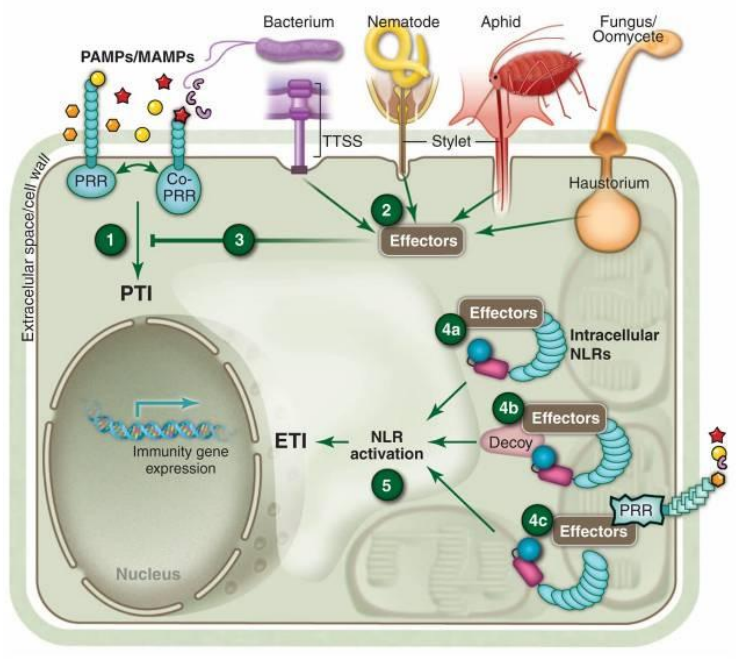

Figure 13 : schéma de l'immunité des plantes (Dangl et al., 2013)

C. Les voies de signalisation de défenses activées lors du PTI/ETI

Les voies de signalisation déclenchée lors du PTI et de l'ETI sont très similaires. La détection d'un agent pathogène par les récepteurs provoque un enchainement de cascades de signalisation coordonnées qui reprogramment le métabolisme de la plante pour mettre en place les réactions de défense (Bigeard et al., 2015).

Suite à la perception de la présence d'un pathogène dans la plante, il y a un flux d'ion calcique entrant dans la cellule grâce à l'activation des canaux calciques qui permettent le passage des ions Ca^{2+} extracellulaire vers le cytoplasme (Jeworutzki et al., 2010; Ranf et al., 2011). Le Ca^{2+} sert de second messager en liant les réponses membranaires à l'activité transcriptionnelle de la cellule grâce aux calmodulines (CaM) ou aux « calmodulines-like » à domaine kinase (CDPK) qui transmettent l'information par des interactions protéiques ou la phosphorylation de leurs cibles (Aldon et al., 2018). Le calcium semble jouer un rôle déterminant dans l'apparition de la HR (Jacob et al., 2021). Après la reconnaissance d'un agent pathogène, la plante produit également des formes réactives de l'oxygène (ROS). Les ROS sont produites par l'activité enzymatique des chloroplastes, des mitochondries et des peroxysomes (Qi et al., 2017). Ces molécules ont un effet toxique sur les agents pathogènes. Elles participent également à la régulation de plusieurs mécanismes de défense de la plante (Qi et al., 2017). Des protéines kinases, les MAPK (mitogen activated protein kinase) peuvent également être activées après la reconnaissance d'un agent pathogène. L'activation de ces protéines entraine une cascade de phosphorylation impactant notamment les voies de régulation des hormones végétales comme l'éthylène (ET), l'acide salicylique (SA) et l'acide jasmonique (JA). Ces hormones contrôlent l'expression des gènes de défense, notamment par la régulation de facteurs de transcription. L'augmentation de SA dans les plantes provoque l'activation de NPR1 (*Non expressor of pathogenesis-related gene 1*) qui va induire l'expression de gène de défense grâce à son interaction avec des facteurs de transcription (Vlot et al., 2009). Le JA permet quant à lui la levée de la répression des gènes de défense grâce à l'ubiquitination des facteurs de transcription JAZ (*jasmonate zim domain*) (Antico et al., 2012). Les voies hormonales JA et SA sont antagonistes et s'inhibent mutuellement (Spoel & Dong, 2008). D'autres hormones comme l'éthylène, l'acide abscissique ou encore l'auxine sont impliqués dans l'activation de gène de défense de la plante.

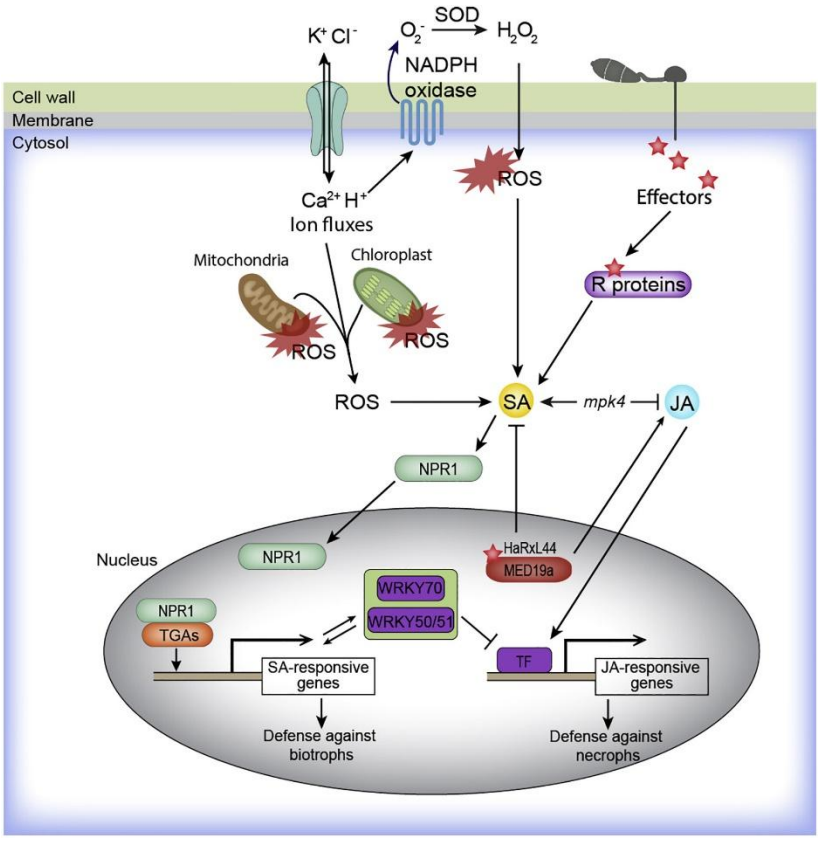

Figure 14 : Activation des voies de signalisation pour la défense des plantes contre les pathogènes (Kim et al., 2022)

D. <u>La résistance systémique acquise (SAR)</u>

La résistance systémique acquise (SAR, *systemic acquired resistance*) est le développement d'une résistance systémique de la plante à une large gamme de pathogènes après l'infection localisée par un pathogène. Ce phénomène a été mis en évidence lorsqu'il a été observé qu'une infection locale du TMV (*Tobacco mosaic virus*) permettait à la plante de développer une résistance à d'autres pathogènes dans d'autres tissus (Ross, 1961).

La SAR se caractérise par l'expression de gènes PR (pathogenesis-related genes). L'expression de ces gènes est induite par l'accumulation de l'acide Salicylique (SA) (Park et al., 2007).

2. Résistances récessives : résistance par perte de sensibilité

Certaines résistantes sont appelées résistances récessives car la résistance est induite par la perte de sensibilité de la plante aux pathogènes. Elle peut notamment résulter de mutations dans les composants des voies de signalisation. Par exemple, l'allèle dominant MLO code pour une protéine membranaire inhibant la mort cellulaire. Il a été montré que la perte de cette protéine confère une résistance à l'oïdium (Kusch & Panstruga, 2017).

Les virus sont très dépendants de la machinerie cellulaire de leurs hôtes pour compléter leur cycle de vie et infecter les plantes et il est supposé que la résistance récessive face aux virus se ferait par l'impossibilité du virus d'utiliser les composants de l'hôte dont il a besoin pour réaliser son cycle infectieux. Cela expliquerait pourquoi les résistances récessives sont plus fréquentes pour lutter contre les virus que contre les autres agents pathogènes. L'un des cas de résistance récessive chez les virus est celle démontrée contre des *Potyvirus*, faisant intervenir les protéines eIF4E et eIF4G. Ces protéines sont des facteurs d'initiation à la traduction. Des mutations dans des régions spécifiques de ces protéines les rendent incompatibles avec les protéines du virus chargées de les recruter. Les plantes possédant ces isoformes ne sont pas affectées dans leur développement mais ont perdu leur sensibilité au virus (Combe et al., 2005 ; Gallois et al., 2010 ; Nicaise et al., 2007 ; Robaglia & Caranta, 2006). La synaptotagmine (SYTA) est une protéine qui régule le trafic des vésicules d'endocytose et d'exocytose. Elle a été identifiée comme interagissant avec les protéines de mouvement de *Tobamovirus* et de *Begomovirus*. Chez *Arabidopsis thaliana*, des mutants de cette protéine retardent l'infection systémique des virus sans altérer le développement de la plante (Uchiyama et al., 2014).

3. Le *gene silencing* : mécanisme de défense antivirale

Le *gene silencing* (aussi nommé *RNA silencing* ou *RNA interference*) est un mécanisme jouant un rôle majeur dans la régulation de l'expression des gènes, et des séquences invasives du génome comme les transposons et transgènes. Mais il intervient également dans la défense des plantes contre les infections virales (Borges & Martienssen, 2015 ; Ding & Voinnet, 2007 ; Pooggin, 2018). Ce mécanisme fait intervenir de petits ARNs interférents qui ciblent le génome viral.

Suite à l'infection d'une plante par un virus, il y a formation de doubles brins d'ARN (ARNdb) qui sont les précurseurs de petits ARNs interférents. Ces ARNdb proviennent notamment des formes réplicatives virales lors d'une infection par un virus à ARN, ou de l'appariement des transcrits sens et antisens issus de la transcription des génomes des virus à ADN (Pooggin, 2018; Ramesh et al., 2017). Ces ARNdb sont reconnus par des protéines RNase III de la famille des DCL (*Dicer-like*) qui vont les couper en petits ARN double brin appelés siRNA (*small interfering RNA*) de 21, 22 ou 24 nucléotides. Un seul des deux brins de ces siRNA est chargé dans une protéine de la famille des Argonautes (AGO) ayant une activité endonucléase, et qui constitue le composant principal d'un complexe RISC (RNA-induced silencing complex). Ce complexe RISC peut cibler le génome de virus à ARN dont la séquence est complémentaire du siRNA chargé dans la protéine AGO. Le complexe RISC peut aussi cibler les ARNm pour empêcher leur traduction par leur clivage et leur dégradation, selon un processus appelé PTGS (*post-transcriptional gene silencing*). Enfin, la transcription de l'ADN viral peut aussi être ciblée par la méthylation des cytosines, selon un processus appelé TGS (*transcriptional gene*

silencing). Chez Arabidopsis thaliana, il existe 4 gènes codant pour des DCL distinctes, DCL4 génère des siRNA de 21nt, DCL2 génère des siRNA de 22nt et DCL3 génère des siRNA de 24nt. Les siRNA de 21 et 22nt sont impliqués dans le PTGS et les siRNA de 24nt sont impliqués dans le TGS. Chez *Arabidopsis thaliana*, 10 protéines AGO sont présentes, AGO1 et AGO2 s'associent préférentiellement avec les siRNA de 21 et 22 nt, tandis que AGO4 s'associe avec les siRNA de 24nt (Pooggin, 2017, 2018).

Le *gene silencing* est amplifié grâce à l'action de polymérases de l'hôte appelées RDR (*RNA-dependent RNA polymerase*). Les RDR reconnaissent les molécules d'ARN aberrantes, pouvant provenir notamment du clivage des ARNm lors du *gene silencing* primaire, et vont les convertir en ARNdb avec l'aide du cofacteur SGS3 (*suppressor of gene silencing 3*). Ce nouvel ARNdb peut alors être ciblé par les DCL pour produire des siRNA secondaires. Cette amplification contribue donc au renforcement de ce mécanisme de résistance des plantes mais permet également à la transitivité du *gene silencing* avec la production de siRNA ciblant de nouvelles régions du génome. La production de ces siRNA secondaires joue aussi un rôle clefs dans la propagation du *gene silencing*. En effet, le *gene silencing* peut devenir systémique grâce aux siRNA qui peuvent se propager dans le reste de la plante en empruntant les plasmodesmes et les vaisseaux conducteurs.

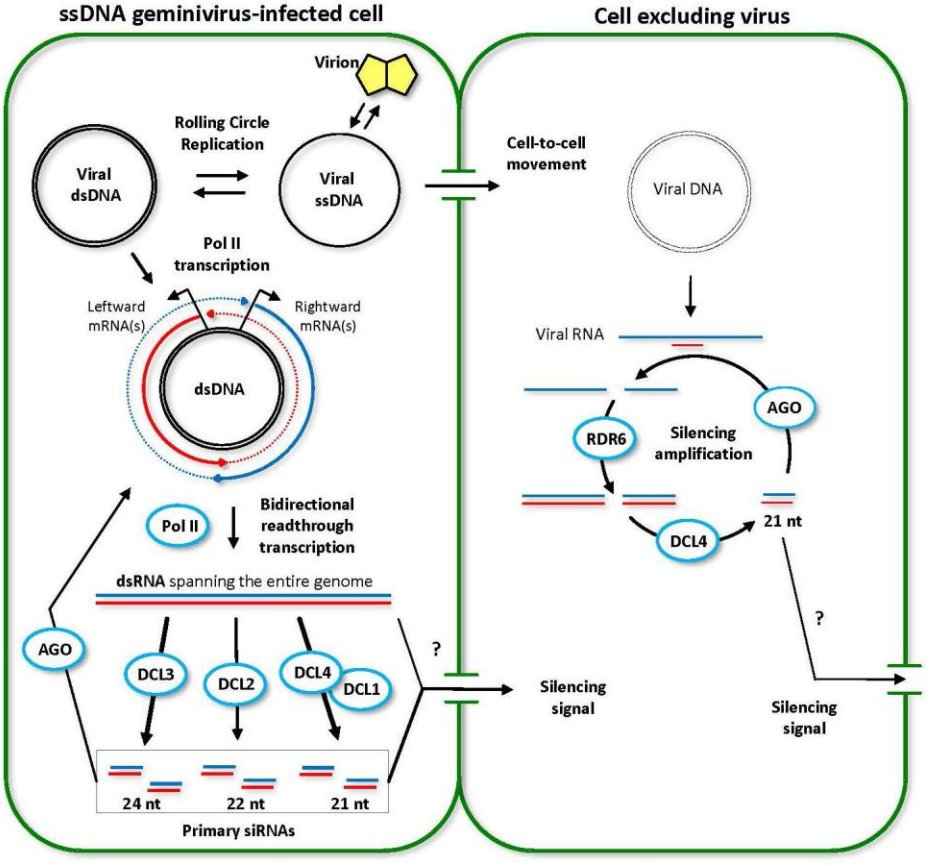

Figure 15 : Mécanisme du *gene silencing* dans une plante infectée par un geminivirus (Pooggin, 2018)

Les virus, pour contrer ce mécanisme de défense, ont développé des stratégies pour supprimer le *gene silencing*. Beaucoup de virus de plantes codent pour des protéines RSS (*RNA silencing suppressor*). Par exemple, la protéine P19 des *Tombusvirus* séquestre les siRNA pour empêcher leur chargement dans les protéines AGO (Vargason et al., 2013). La protéine HC-Pro des Potyvirus fonctionne de la même manière. D'autres RSS inhibent le *gene silencing* par la séquestration des protéines AGO comme la protéine 2B du CMV (*Cucumber mosaic virus*) ou par leur dégradation comme la protéine P0 du BWYV (*Beet western yellow virus*). D'autres encore agissent sur l'amplification du *gene silencing* en visant des RDR ou la protéine SGS3 (Okano et al., 2014).

Comme les autres géminivirus, le TYLCV est ciblé par les deux formes du *gene silencing* (TGS et PTGS). En effet, durant une infection par le TYLCV, les plantes produisent des siRNA de 21, 22 et 24nt (Aregger et al., 2012; Fuentes et al., 2016; Piedra-Aguilera et al., 2019). De nombreuses protéines RSS ont été identifiées chez les Geminivirus. La protéine V2 du TYLCV interagit avec la protéine SGS3 et inhibe l'amplification du signal de *silencing*, et celle du *Cotton leaf curl Multan virus* (CLCuMV) interagit directement avec les ARNdb qui ne peuvent donc plus être coupés par les DCL en siRNA (Amin et al., 2011 ; Glick et al., 2008 ; J. Zhang et al., 2012). La Rep et la C4 sont capables de séquestrer les siRNA (Vanitharani et al., 2005 ; Wang et al., 2014). Certaines protéines inhibent le PTGS. La protéine TrAP est connue pour amplifier la transcription de gènes de l'hôte qui inhibe le PTGS (Trinks et al., 2005). D'autres protéines ciblent également le TGS, notamment en ciblant les mécanismes de méthylation. La TrAP inhibe l'activité d'une ADK (*adenosine kinase*) importante dans le cycle de la méthylation (Buchmann et al., 2009 ; H. Wang et al., 2005) et d'une méthyltransférase ciblant les histones (Castillo-González et al., 2015). La Rep et la C4 inhibent l'expression de deux méthyltransférase (Rodríguez-Negrete et al., 2013).

4. Prémunition

La prémunition ou protection croisée est un phénomène naturel présent chez les plantes par lequel une plante infectée par un virus donné développe une résistance à un autre virus apparenté. La prémunition est principalement observée entre souches d'un même virus mais elle peut aussi parfois être observée entre deux espèces distinctes proches. Par exemple, l'infection par le *Citrus veine enation virus* permet de protéger la plante contre le *Citrus tristeza virus* (CTV) (Koizumi & Sasaki, 1980). La prémunition a été démontré pour la première fois en 1929 par McKinney (McKinney, 1929), avec le *Tobacco mosaic virus* (TMV, genre *Tobamovirus*), en montrant que les symptômes d'une première souche se caractérisant par une mosaïque jaune était réprimé dans des plantes déjà infectées par une autre souche. Après cette découverte, il a été montré qu'une souche avirulente du *Potato virus X* (PVX, *Potexvirus*) pouvait protéger la pomme-de-terre contre la surinfection d'une souche virulente du PVX (Salaman, 1933). Dans les années qui ont suivi ces

premières découvertes, de nombreux exemples de prémunition ont été étudiés pour des virus à ARN et à ADN, comme le *Cauliflower mosaic virus* (CaMV, (Tomlinson & Shepherd, 1978 ; Zhang & Melcher, 1989)), le *Cucumber mosaic virus* (CMV, (Dodds, 1982 ; Dodds et al., 1985), le *Plum pox virus* (PPV, (Ravelonandro et al., 2008)), le *Barley yellow dwarf virus* (BYDV, (Aapola & Rochow, 1971 ; Jedlinski & Brown, 1965)) et beaucoup d'autres (Pechinger et al., 2019 ; Ziebell & Carr, 2010).

Ce phénomène naturel a été utilisé comme méthode pour le contrôle de certaines maladies virales, en utilisant une souche peu virulente, provoquant peu de symptômes, pour les inoculer dans la plante et la protéger contre les dégâts provoqués par une souche plus virulente. Le premier exemple du contrôle d'une maladie virale par l'infection avec une souche peu virulente a été démontré avec le CTV (Costa & Grant, 1951) et le *Cacao swollen shoot virus* (CSSC, (Posnette & Todd, 1951, 1955)). Plus tard, quelques exemples de réussites de contrôle de maladie virale ont été décrit comme pour le CTV (Roistacher et al., 2010), le *Pepino mosaic virus* (PepMV, (Pechinger et al., 2019)), le *Zucchini yellow mosaic virus* (ZYMV, (Lecoq et al., 1991 ; Perring et al., 1995 ; Walkey et al., 1992 ; Yarden et al., 2000)).

Deux mécanismes majeurs ont été proposé pour expliquer la prémunition, la résistance induite par la protéine de capside (*Coat-protein-mediated resistance*) et le *gene silencing* (Gal-On & Shiboleth, 2006). La résistance induite par la CP a été démontré en premier lieu pour la prémunition entre souches du TMV. La plante *Nicotiana sylvestris* est protégée de l'infection d'une souche de TMV induisant des nécroses par l'infection première d'une autre souche de TMV mais pas lorsque seulement l'ARN, sans sa capside, du deuxième virus est surinfecté dans la plante pré-infectée (Sherwood & Fulton, 1982). Des observations similaires ont été faites pour la prémunition entre deux souches de CMV (Dodds et al., 1985). Des travaux avec des plantes transgéniques exprimant la protéine de capside ont montré que ces plantes étaient résistantes au virus comme le ferait des plantes prémunies (Abel et al., 1986 ; Beachy, 1999 ; Beachy et al., 1990 ; Lomonossoff, 1995 ; Nelson et al., 1987). En 1996, (Culver, 1996) a montré que c'était également vrai dans les plantes non transgéniques en utilisant un vecteur PVX exprimant la CP du TMV pour infecter les plantes et l'accumulation du TMV surinfecté était moins importante que dans les plantes non protégées. De plus, c'est l'expression de la protéine de capside qui permet la protection et pas seulement la présence du transcrit (Bendahmane et al., 1997 ; Lu et al., 1998 ; Powell et al., 1990). Le modèle proposé pour la prémunition induite par la CP est que la CP du premier virus interfère avec la désencapsidation du deuxième virus ce qui empêche sa réplication et sa transcription et traduction.

Cependant, ce mécanisme n'explique pas tous les cas observés de prémunition car des virus déficients pour la CP, les viroïdes et des satellites peuvent conférer de la prémunition (Gallitelli, 1991 ; Gerber & Sarkar, 1989 ; Montasser, 1991 ; Niblett et al., 1978 ; Sayama, 1993 ; Tien & Wu, 1991). Le *gene silencing* a été proposé

comme un autre mécanisme principal pouvant expliquer la prémunition. En effet, puisque ce mécanisme permet la résistance via des siRNA complémentaire au génome du virus, cela explique que la prémunition se fasse entre deux virus proches génétiquement. Le virus surinfectant la plante va donc entrer dans la cellule végétale où l'autre virus est déjà présent et va être directement ciblé par les siRNA produits lors de l'infection par le premier virus. Il peut également entrer dans une cellule non infectée par le premier virus mais où les siRNA se sont propagés et où le *gene silencing* est opérationnel et sera également ciblé par le complexe RISC. Enfin, il est proposé que les deux mécanismes agissent tous les deux dans les plantes protégées par prémunition et que le deuxième virus, s'il entre dans une cellule déjà infectée, soit ciblé à la fois par la résistance induite par la CP et par le *gene silencing* (Gal-On & Shiboleth, 2006).

5. Tolérance

La résistance, qu'elle soit complète ou partielle, se traduit par la diminution de la quantité de pathogène présent dans son hôte. Au contraire, la tolérance ne modifie pas la quantité de parasites présents mais réduit les dommages infligés par le parasite à son hôte. La tolérance chez les plantes infectées par les virus est fréquente (Pagán & García-Arenal, 2018). Cependant la tolérance a été très peu étudiée et les connaissances sur les mécanismes impliqués sont peu nombreuses. Un seul gène de tolérance est exploité agronomiquement, le gène Zym de la courgette conférant la tolérance au *Zucchini yellow mosaic virus* (ZYMV). Les variétés de courgette porteuse du gène zym présentes que des symptômes très faibles de la maladie sans altération de la qualité ou de la quantité des courgettes produites. Pourtant le virus s'accumule dans les plantes au même niveau que dans les autres variétés ne portant pas le gène de tolérance (Desbiez et al., 2003).

III. Les méthodes de lutte contre le TYLCV

De nombreuses méthodes pour protéger les cultures contre les infections des virus du tylc ont été développées depuis de nombreuses années. En raison de son importance, les principales informations sur les méthodes de lutte concernent le TYLCV. Les principales méthodes visent à protéger les plantes des infections virales par des stratégies d'échappement aux infections, des protections physiques, la protection contre le vecteur par des techniques physiques ou la lutte chimique et enfin par l'utilisation de variété résistantes.

1. Les méthodes prophylactiques

Des méthodes prophylactiques diverses ont été utilisées avec tout d'abord des pratiques culturales adaptées pour éviter les infections. Tout d'abord, les premiers moyens pour contrôler l'incidence de la maladie ont été les choix de la période et de la localisation des cultures. La culture de tomates à des périodes où les populations d'aleurodes sont moins nombreuses et donc l'incidence du TYLCV plus basse a été une des stratégies utilisées en Israël. Les cultures étaient plantées au champ entre Mars et Avril et récoltées trois mois plus tard afin d'éviter la période de fin d'été et d'automne où la propagation du TYLCV est la plus forte car les populations d'aleurodes culminent entre Septembre et Novembre. De plus, les nouvelles cultures ne doivent pas être placées proche d'anciennes cultures connues comme étant des hôtes du TYLCV ou de champs où les populations d'aleurodes ne sont pas contrôlées (Polston & Lapidot, 2007). De plus, la saison de production doit commencer par l'utilisation de plants sains non infectés. Respecter une période de deux mois sans cultures maraichères s'est montré efficace pour éliminer le TYLCV en Israël (Ucko et al., 1998). Il est également recommandé d'éliminer les anciennes plantes de tomates au champ directement après la récolte pour réduire la population d'aleurodes (Ioannou, 1987). Les plantes adventices présentes dans les champs mais également dans les zones non cultivées jouent un rôle de réservoir pour le TYLCV puisqu'un nombre important de ces plantes a été identifié comme hôtes du virus. Ce sont également des réservoirs pour les populations d'aleurodes. L'éradication de telles plantes permet de mieux contrôler la propagation du virus (Cohen et al., 1988). L'alternance de rang de culture de tomate avec d'autres cultures, qui sont de meilleurs hôtes pour les aleurodes et ayant un couvert plus important, s'est montré efficace pour limiter l'infection (Musa, 1982 ; Schuster, 2004). L'interdiction, pendant les 3 mois précédant la culture de tomate, de culture d'espèces hôte de *B. tabaci* a permis de réduire la prévalence du TYLCV dans les régions de production et de décaler d'environ un mois l'arrivée des contaminations sur les plantes de tomate en République dominicaine (Salati et al., 2002).

Une autre méthode pour limiter les attaques d'aleurodes sur les plantes de tomates a été d'utiliser des protections plastiques sur les cultures pour éviter que les aleurodes se posent sur les plantes. En Floride, des

protections plastiques réfléchissantes avec notamment de l'aluminium sont utilisées afin de refléter la lumière du jour et de désorienter les aleurodes. Cette technique a l'avantage de limiter l'incidence d'autres insectes vecteurs et de limiter l'incidence de plusieurs autres virus de tomates et elle est efficace même avec de fortes populations d'aleurodes. Cependant, cette méthode baisse en efficacité lorsque les plantes deviennent âgées et que le couvert végétal augmente (Csizinszky et al., 1997, 1999 ; Polston & Lapidot, 2007). En Israël, l'utilisation de protection plastique de couleur jaune est une pratique commune (Cohen & Berlinger, 1986 ; Cohen & Melamed-Madjar, 1978). En effet il a été démontré que la couleur jaune attiré les aleurodes (Mound, 1962). Les aleurodes sont donc attirés sur le plastique jaune et à cause des hautes températures de la protection plastique, meurent de déshydratation. Comme en Floride, cette méthode n'est efficace qu'au début de la croissance de la plante, jusqu'à 20 à 30 jours après plantation (Cohen, 1982).

Un autre moyen pour limiter l'incidence de la maladie est d'utiliser des barrières physiques pour empêcher les aleurodes d'envahir les cultures de tomates. Dans le bassin méditerranéen, des constructions comme des serres protégées par des filets à mailles fines ont été utilisés à partir des années 1990 pour protéger les cultures contre les attaques d'aleurodes et l'infection par le TYLCV (Berlinger et al., 2002 ; Berlinger & Lebiush-Mordechi, 1996 ; Cohen & Antignus, 1994). Mais l'utilisation de ces barrières physiques peuvent, en plus d'augmenter le coût de production, créer des températures excessives et des problèmes de ventilation.

2. Le contrôle des populations de *Bemisia tabaci*

Les aleurodes perçoivent les UV et les utilisent pour se repérer (Mound, 1962). Ces UV sont un fort stimulus pour le vol des aleurodes, et induisent notamment un comportement de migration. En Israël, l'application de plastique absorbant les UV sur les serres et les filet *insect-proof* a permis d'obtenir un certain niveau de protection des cultures contre le TYLCV (Antignus et al., 1998, 2001). Mais l'utilisation de ces écrans anti-UV peut augmenter la température et l'humidité à l'intérieur des serres.

L'utilisation de produits chimiques pour réduire les populations d'aleurodes est une méthode répandue. Différentes classes d'insecticides sont utilisés contre les aleurodes. Cependant, les aleurodes ont développé des résistances à de nombreux insecticides et leur efficacité a baissé dans le temps (Ahmad et al., 2002 ; Faria & Wraight, 2001 ; Mason et al., 2000 ; Palumbo et al., 2001 ; Polston & Anderson, 1997).

La plupart de ces méthodes de contrôle de la maladie repose principalement sur le contrôle de l'insecte vecteur *B.tabaci* mais les techniques utilisées ont de nombreux désavantages comme un coût de production plus élevé ou encore un manque d'efficacité en cas de forte incidence de l'insecte vecteur (Lapidot & Friedmann, 2002). De plus, l'utilisation des produits chimiques est peu respectueuse de l'environnement.

L'utilisation de variétés résistantes apparait donc comme l'alternative la plus efficace pour lutter contre le TYLCV.

3. L'utilisation de gènes de résistance dans le contrôle de la maladie du tylc

Les tomates cultivées (*Solanum Lycopersicum*) sont toutes sensibles au TYLCV. C'est la raison pour laquelle les sources de résistances ont dû être recherchées dans des espèces sauvages apparentées à la tomate. Il s'agit de *S. pimpinellifolium*, *S. peruvianum*, *S. chilense*, *S. habrochaites*, *S. cheesmaniae* (Banerjee & Kalloo, 1987 ; Friedmann et al., 1998 ; Hassan et al., 1984 ; Hassan & Abdel-Ati, 1999 ; Kasrawi, 1989 ; Kasrawi & Mansour, 1994 ; Lapidot et al., 1997 ; Vidavsky & Czosnek, 1998 ; Zakay et al., 1991). Six gènes de résistance ont été identifiés et sont disponibles pour introgression dans des programmes de création variétale, Ty-1 à Ty-6.

Ty-1 est le premier gène de résistance à avoir été cartographié (Zamir et al., 1994). Il est issu d'une accession de l'espèce sauvage *S.chilense* (LA1969) originaire du Nord-Est de l'Amérique du Sud. Des plantes de tomates portant le gène Ty-1 ont été trouvé hautement résistantes au TYLCV (Zakay et al., 1991). Ty-1 est situé sur le chromosome 6 de la tomate proche du centromère (Zamir et al., 1994). Le gène de résistance Ty-3 a été identifié également chez l'espèce sauvage *S.chilense* dans les accessions LA2779, LA1932 et LA1938. Il permet une résistance au TYLCV et au ToMoV (Ji et al., 2007). Au départ, *Ty-3* avait été considéré comme un gène différent de *Ty-1*, mais une cartographie fine de ces deux gènes a finalement montré qu'ils étaient en réalité deux allèles d'un même gène (Verlaan et al., 2011, 2013). Les plantes portant l'allèle Ty-3 présentent de faibles niveaux d'accumulation virale et des symptômes modérés. Les plantes Ty-1 montrent également une baisse d'accumulation virale (Belabess et al., 2016) comparée à des plantes sensibles mais ne présentent aucun symptôme visible (Michelson et al., 1994). Les allèles Ty-1 et Ty-3 codent pour une RDR (*RNA-dependent RNA polymerase*) de type γ possédant un motif atypique DFDGD dans leur domaine catalytique. Les RDRγ correspondent aux RDR 3, 4 et 5 connues chez *Arabidopsis thaliana* et diffèrent des RDRα correspondant au RDR 1, 2 et 6 (tous impliqué dans le *gene silencing*) par leur motif dans leur domaine catalytique (Verlaan et al., 2013). Contrairement aux RDRγ dont les fonctions sont peu connues, les RDRα ont été bien étudiées et sont connues pour leur rôle d'amplification du *gene silencing*. Récemment, des études ont permis de démontrer que le gène Ty-1 était également impliqué dans l'amplification du *gene silencing*. En effet, des plantes porteuses du gène Ty-1 infectées par le TYLCV présentent une quantité de siRNA viraux plus importante que dans des plantes sensibles (Butterbach et al., 2014). La proportion de siRNA de 22 et 24nt était plus importante pour les plantes résistantes que pour les plantes sensibles, un résultat qui suggère que les deux mécanismes de silencing, TGS et PTGS, sont concernés par l'amplification du signal (Voorburg et al., 2021). De plus, l'ADN viral présentait un niveau de méthylation plus important chez les plantes portant le gène Ty-1 confirmant que le gène Ty-1 permet

l'augmentation de la réponse de la plante par le mécanisme de TGS (Butterbach et al., 2014). Enfin, ces observations sont cohérentes avec le fait que la résistance contre le TYLCV est compromise lorsque les plantes Ty-1 sont coinfectées avec un agent possédant un fort suppresseur de *silencing* tel que le CMV ou un betasatellite (Butterbach et al., 2014 ; Voorburg et al., 2020).

Le gène Ty-2 provient de l'espèce sauvage *S. habrochaites* et son introgression dans la tomate a permis le développement de la lignée H24, résistante au TYLCV. Il est localisé sur le bras long du chromosome 11 et c'est un gène dominant. Le gène Ty-2 ne permet pas la résistance à toutes les souches de TYLCV ni aux begomovirus bipartites. Il est souvent introgressé dans des plantes possédant également une introgression de Ty-1 ou Ty-3 (Hanson et al., 2000 ; Ji, Scott, & Schuster, 2009). Le gène code pour une protéine de type NB-LRR (Yamaguchi et al., 2018). La protéine Rep a été identifiée comme étant l'effecteur de la protéine codée par Ty-2 (Shen et al., 2020).

Ty-4 a été identifié dans l'accession LA1932 de *S.chilense* et est localisé sur le bras long du chromosome 3. La résistance apportée par ce gène est moins forte que celle apportée par les autres gènes de résistance avec une diminution des symptômes de seulement 16%. Son mode d'action est encore inconnu (Ji et al., 2009).

Le gène Ty-5 provient de l'espèce sauvage *S.peruvianum*. Il est localisé sur le chromosome 4. Il permet une réduction des symptômes de plus de 40% (Anbinder et al., 2009). La lignée TY172 dérivant de plusieurs accessions de *S.peruvianum* possède ce gène de résistance et ne présente pas de symptômes et l'accumulation virale est fortement réduite. Ce gène est partiellement dominant (Friedmann et al., 1998). Il code pour un homologue d'une protéine responsable du recyclage des ribosomes durant la synthèse des protéines. Il a été proposé que Ty-5 coderait pour une telle protéine mais dysfonctionnelle ce qui limiterait la synthèse des protéines virales et, par ce fait diminuerait l'accumulation virale. Une autre hypothèse serait que la protéine codée par Ty-5 interagirait avec les protéines impliquées dans la réplication virale (Lapidot et al., 2015).

Le gène Ty-6 est localisé sur le chromosome 10 de la tomate. Il est efficace contre le TYLCV et le ToMoV. Ce gène correspond à une dominance incomplète avec une résistance intermédiaire dans les plantes hétérozygotes mais son mode d'action est encore inconnu (Gill et al., 2019).

Le pyramidage de ces différentes régions associées à des résistances contre le TYLCV améliore le degré de résistance au TYLCV. Bien que l'utilisation de variétés résistantes pour lutter contre le TYLCV reste la meilleure option pour contrôler la maladie, elle reste difficile pour les sélectionneurs car de nouveaux virus émergent constamment, notamment par l'expansion constante des populations de *B.tabaci* et la fréquence importante de recombinaison chez les géminivirus (Padidam et al., 1999). De plus, la pression de sélection exercée par les

cultivars résistants sur les virus fait émerger de nouveaux variants potentiellement capables de compromettre ces résistances.

IV. Emergence d'un recombinant invasif du TYLCV au Maroc

Le Maroc fait partie des plus gros exportateurs de tomate dans le monde. La majorité de la production se concentre dans la région du Souss situé au Sud-Ouest du Maroc (Sippel, 2015). La maladie du tylc est observée au Maroc depuis la fin des années 1990 et le TYLCV et le TYLCSV ont été identifiés comme les deux agents causant la maladie (Monci et al., 2000 ; Peterschmitt et al., 1999). A cette époque les variétés cultivées étaient sensibles à la maladie et de nombreuses méthodes de contrôle ont été appliquées afin de limiter l'impact sur la production, les plus importantes étant l'utilisation massive d'insecticides et la construction de serres *insect-proof.* Puis, les variétés sensibles ont été massivement remplacées par des variétés résistantes au début des années 2000, principalement par des variétés portant le gène Ty-1 qui bloque l'expression des symptômes de la maladie et diminue la charge virale mais sans la supprimer.

Cependant, en 2010, des variétés de tomate résistantes portant le gène Ty-1 présentent des symptômes spécifiques de la maladie dans la région du Souss. Il a été montré que ces plantes Ty-1 symptomatiques étaient toutes infectées par un recombinant entre le TYLCV et le TYLCSV qui présentait un profil de recombinaison atypique (Belabess et al., 2015). En effet contrairement aux recombinants qui avaient été décrits précédemment, ce recombinant n'avait hérité du parent TYLCSV qu'un très court fragment de 76 nucléotides situés dans la partie 3' de la région intergénique. Selon les critères taxonomiques de l'ICTV, ce recombinant est une souche du TYLCV qui a été nommé TYLCV-IS76. Un échantillonnage très large de tomate de diverses variétés, sensibles et résistantes, et d'autres espèces de plantes cultivées et non cultivées, a montré que ce nouveau recombinant avait envahi la région du Souss en remplaçant presque entièrement les virus parentaux, et qu'il se propageait vers le Nord du pays (Belabess et al., 2015).

En condition contrôlée au laboratoire, il a été démontré que ce recombinant avait un avantage sélectif dans les plantes résistantes Ty-1. En effet, contrairement aux plantes sensibles dans lesquelles son accumulation est équivalente à celle de ces virus parentaux, dans les plantes résistantes Ty-1 son accumulation est supérieure à celles des virus parentaux. De plus, lorsqu'il est inoculé en infection mixte avec le TYLCV-IL, son accumulation reste inchangée alors que celle du TYLCV-IL décroit, notamment dans les plantes Ty-1, ou la baisse de l'accumulation virale de TYLCV-IL est très forte et il finit par ne plus être détectable dans les plantes (Figure 16, (Belabess et al., 2016)).

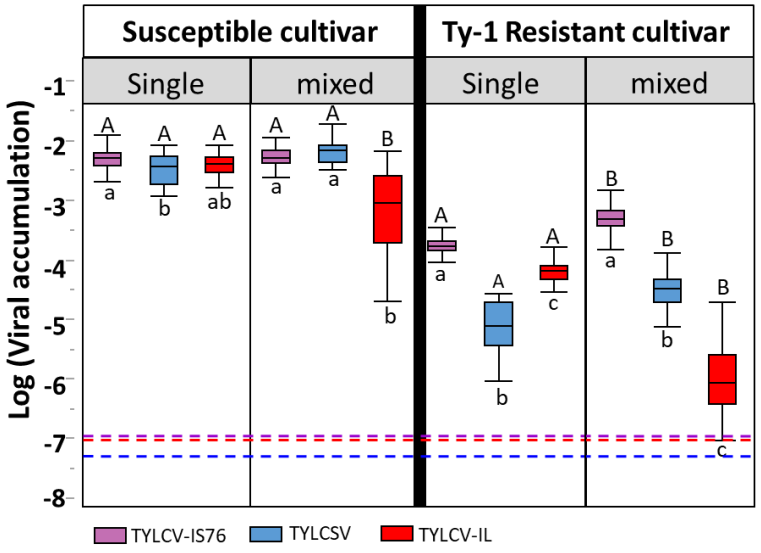

Figure 16 : Accumulation virale du TYLCV-IL, TYLCSV et TYLCV-IS76 en infection simple ou mixte dans les plantes résistantes Ty-1 et des plantes isogéniques sensibles (Belabess et al., 2016).

Le détail de la découverte de TYLCV-IS76, son scénario d'émergence et les différentes hypothèses sur ce qui lui confère cet avantage sélectif sont détaillés dans un chapitre de livre que j'ai cosigné avec mes encadrants. Ce chapitre s'intitule « Invasive Tomato yellow leaf curl virus recombinants challenge virus diagnosis and disease management » et a été publié en 2022 dans un livre édité par Academic Press chez Elsevier « Geminivirus : detection, diagnosis and management ».

Comme j'ai participé à la rédaction de ce chapitre d'ouvrage, je l'ai inclus dans mon introduction générale pour décrire la problématique de mon sujet de thèse et introduire les objectifs.

Chapter 31

Invasive tomato yellow leaf curl virus recombinants challenge virus diagnosis and disease management

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31.1 Overview

Recombination is a key mechanism in the generation of virus diversity and the evolution of viral populations. The development of high-throughput sequencing and the improvement of computer programs designed for the detection of recombination events have triggered abundant literature showing that recombination participates in virus evolution in most viral families and genera. However, a dynamic picture of the evolutionary process, from the generation of a new recombinant genome to its emergence and spread, including the characterization of major emergence drivers, was rarely reported. This gap was filled with the detection of an unusual recombinant virus that emerged in tomato plants selected for resistance to tomato yellow leaf curl virus (TYLCV).

TYLCV is a circular single-stranded DNA virus belonging to the genus *Begomovirus* of the family *Geminiviridae*. Geminiviruses replicate via rolling circle and recombination-dependent mechanisms and therefore are prone to recombination, as evidenced by the numerous recombination breakpoints detected in genomes of sequence databases. Recombination propensity is also revealed by the ease with which recombinant genomes are generated in inoculated plants, and particularly between TYLCV and related begomoviruses like tomato yellow leaf curl Sardinia virus (TYLCSV).

TYLCV-IS76 is an unusual TYLCV/TYLCSV recombinant, detected 10 years ago in resistant tomato plants exhibit-ing tomato yellow leaf curl disease (TYLCD) symptoms in the South of Morocco. It is distinguished by a very short length of its TYLCSV inherited genome fragment—76 nucleotides (nt) adjacent to the origin of replication—much shorter than that of previously reported TYLCV/TYLCSV recombinants.

The emergence of TYLCV-IS76 coincided with a region-wide replacement of susceptible tomato cultivars by TYLCD-resistant plants bearing the resistance gene *Ty-1*. This observation, together with experimental studies showing that *Ty-1*-resistant plants positively select TYLCV-IS76, strongly supports that the emergence was resistance-driven. The most typical feature of the selective advantage of TYLCV-IS76 is the strong negative impact on the accumulation of the TYLCV parent when they are coinoculated in *Ty-1*-resistant plants. This result, compiled with the monitoring of TYLCV-IS76 prevalence over time and space in comparison to that of TYLCV and TYLCSV parental type viruses, and the estimation of its most probable generation time using BEAST, made it possible to reconstruct an emergence scenario.

The fitness advantage of TYLCV-IS76 sheds new light on the highly variable genomic region of geminivirus genomes corresponding to the 76-nt sequence inherited from TYLCSV. TYLCSV does not exhibit the fitness phe- notype of TYLCV-IS76, indicating that the 76-nt sequence is not by itself the determinant of the fitness advantage. Several hypotheses were proposed to identify determinants of potentially new molecular interactions triggered by the recombination event and some of them were tested. As the emergence of TYLCV-IS76 was driven by the

Geminivirus: Detection, Diagnosis and Management. DOI: https://doi.org/10.1016/B978-0-323-90587-9.00004-3

deployment of *Ty-1*-resistant plants, we presume that the recombination event does not only modify intra- and inter-virus interactions but also virus plant interactions. These later interactions are expected to be associated with RNA interference (RNAi) and gene silencing because *Ty-1* encodes an RNA-dependent RNA polymerase (RDR), related to other RDR components of the silencing machinery. In the absence of any strong scenario that may explain how TYLCV-IS76 may trigger gene silencing that would be deleterious for TYLCV accumulation but not for its own accumulation, an RNAomics study is recommended because of its systemic approach that does not need any prior hypothesis to be tested.
The management of the invading TYLCV recombinants will need the discovery of new resistance genes and prefera- bly genes that block virus replication to prevent the emergence of resistance-breaking viruses and particularly recombinants.

31.2 Background

31.2.1 Importance of tomato yellow leaf curl disease

TYLCD is one of the most important viral diseases affecting tomato crops in the world. In the Mediterranean Basin, it is caused by two monopartite begomoviruses, namely, TYLCSV of Western Mediterranean origin, and TYLCV of Middle Eastern origin (Lefeuvre et al., 2010). Their main vectors in Mediterranean countries are whiteflies from two cryptic species of *Bemisia tabaci* Gennadius, Middle Eastern Asia Minor 1 (MEAM1) and Mediterranean (MED). To mitigate the important damages caused by TYLCV and TYLCSV, various techniques have been deployed including prophylactic measures, the instauration of crop-free periods, as well as physical- and chemical-based techniques. Unfortunately, *B. tabaci* exhibits a high propensity to develop resistance to insecticides (Cahill et al., 1994, 1996; Gauthier et al., 2014; Horowitz et al., 2005). The efficiency of chemical controls decreased dramatically, encouraging more frequent treatments and with higher insecticide concentrations which in turn were highly damaging to the environ- ment. Thereafter, management strategies were tightened by extending the physical protection of the nursery plants over the whole growing season. In the 2000s, when resistant cultivars were made available, they became the gold standard of TYLCD control.

31.2.2 *Ty-1* resistance is associated with gene silencing mechanisms

Among the 6 genes identified in *Solanum* accessions to confer resistance to TYLCD, the *Ty-1/Ty-3* alleles of a gene derived from *Solanum chilense* (Verlaan et al., 2011; Zamir et al., 1994) were the most widely introgressed in commer- cial cultivars, because they blocked TYLCD symptoms. The *Ty-1*/*Ty-3* gene, subsequently abbreviated *Ty-1* for conve- nience, maps to chromosome 6 of tomato (Zamir et al., 1994). It was shown to code for an RDR of the γ-clade defined by a DFDGD catalytic domain, similar to the RDRs 3, 4, and 5 of *Arabidopsis thaliana* for which no function has been described yet (Verlaan et al., 2013; Wassenegger & Krczal, 2006). Unlike the γ-clade RDR, the RDR of the α-clade (similar to *Arabidopsis* RDRs 1, 2, and 6) were reported to be involved in RNAi and gene silencing pathways (Willman et al., 2011). RNAi is a conserved gene regulation mechanism in which double-stranded RNA is processed by Dicer family proteins into small interfering RNAs (siRNAs) that get associated with Argonaute family proteins to target, by sequence-specific recognition, complementary nucleic acid sequences for inhibition of gene expression.
In plants, siRNAs silence gene expression at the posttranscriptional level by cleavage and degradation of target mRNA or inhibition of its translation (posttranscriptional gene silencing, PTGS), and at the transcriptional level by cytosine methylation of target DNA and chromatin modifications (transcriptional gene silencing, TGS) (Borges & Martienssen, 2015). The α-clade RDRs are essential for the production of double-stranded RNA precursors of second- ary siRNAs from various single-stranded RNA templates, which amplifies and reinforces gene silencing. RNAi plays a major role in plant defenses against RNA and DNA viruses and therefore, for successful infection, viruses should sup- press or evade antiviral RNAi (Csorba et al., 2015; Pooggin, 2013). In geminivirus-infected *Arabidopsis* plants, the α-clade RDRs are not required for viral siRNA biogenesis and Dicers generate 21-, 22-, and 24-nt viral siRNAs from double-stranded RNA precursors arising from bidirectional sense and antisense transcription of viral DNA (Aregger et al., 2012; Blevins et al., 2006).
Since γ-clade RDRs are likely nonfunctional in *Arabidopsis*, their potential role in antiviral defense could not be addressed in this model plant (Aregger et al., 2012). The fact that the tomato *Ty-1* gene codes for a γ-clade RDR raises a possibility that this protein mediates the amplification of secondary viral siRNAs that may contribute to TYLCV resistance. Consistent with this hypothesis, the accumulation of TYLCV-derived siRNAs was found to be

increased in *Ty-1*-resistant plants, compared to susceptible tomato plants (Butterbach et al., 2014; Voorburg et al., 2021). Moreover, the increase in siRNAs accumulation coincided with elevated levels of cytosine methylation in the TYLCV *V1* promoter region, suggesting that *Ty-1* would have a role in TGS. Furthermore, the resistance against TYLCV was compromised in *Ty-1*-plants coinfected with cucumber mosaic virus (Butterbach et al., 2014) or a bego- moviral betasatellite (Voorburg et al., 2020), two pathogens that are known to express strong suppressors of silenc- ing. Although no functions have been described for the γ-clade RDRs, these results suggest that silencing mechanisms are associated with the *Ty-1* gene.

The Achilles heel of the *Ty-1*-resistance gene is that virus replication is not blocked (Michelson, 1994), which is exposing *Ty-1*-resistant cultivars to the risk of emergence of resistance-breaking variants (García-Andrés et al., 2009), as reported for other viral diseases.

31.2.3 Emergence of TYLCV/TYLCSV recombinant viruses

Despite their DNA genome being replicated by plant polymerases via rolling circle and recombination-dependent mechanisms (Hanley-Bowdoin et al., 1999; Jeske et al., 2001), the mutation rate of geminiviruses is relatively high, probably because of the lack of proofreading activity (Duffy & Holmes, 2008; Ge et al., 2007; Urbino et al., 2008). Moreover, geminiviruses are highly prone to recombination (García-Andrés et al., 2007a; Padidam et al., 1999; Urbino et al., 2013), which altogether underpins the risk of resistance breaking.

Following the encounter of TYLCV and TYLCSV in Mediterranean countries, 6% 75% of plants, positive for TYLCD viruses, were coinfected with TYLCV and TYLCSV in Israel and Jordan (Anfoka et al., 2014), Portugal (Fiallo-Olivé et al., 2019), Spain (Navas-Castillo et al., 1999; García-Andrés et al., 2007a; Sanchez-Campos et al., 1999), Morocco (Belabess et al., 2015), Italy (Davino et al., 2008; García-Andrés et al., 2007a), and Tunisia (Mnari- Hattab et al., 2014; Pellegrin et al., 2008). The TYLCV/TYLCSV recombinants detected in fields (Davino et al., 2008; García-Andrés et al., 2007a) exhibited recombination profiles characterized by one recombination breakpoint (RB) in the conserved stem-loop of the intergenic region carrying the origin of replication (SLOR), and at least another RB within genes between nucleotide positions 900 and 2400. Similar recombination profiles were detected in tomato plantscoinfected in experimental conditions (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007b). Interestingly, the virion sense genes side region starting from the SLOR always derives from the TYLCSV parent.

Two recombinants were frequently detected in Spain, tomato yellow leaf curl Malaga virus (TYLCMalV) (Monci et al., 2002) and tomato yellow leaf curl Axarquia virus (TYLCAxV) (Garcia-Andres et al., 2006) (Fig. 31.1).

TYLCMalV is a recombinant between TYLCV-Mld and TYLCSV. It was more prevalent in beans than in tomatoes and the symptoms it induced in susceptible tomatoes were much milder than those induced by TYLCV-Mld and TYLCSV (Monci et al., 2002). It exhibits a hybrid phenotype because unlike TYLCSV, it was infectious in beans, and unlike TYLCV-Mld, it was infectious in two noncultivated Solanaceous species. TYLCAxV, a recombinant between TYLCV-IL and TYLCSV, was found to be more virulent than TYLCV-IL or TYLCSV in tomato and *Solanum nigrum*, but less virulent than TYLCV-IL in bean (Garcia-Andres et al., 2006). TYLCAxV was also detected in Italy, Tunisia, and Jordan (Anfoka et al., 2016; Davino et al., 2009; Mnari-Hattab et al., 2014).

The population structure of TYLCV/TYLCSV recombinants generated in *Ty-1*-resistant tomato plants, coinoculated with TYLCV and TYLCSV, were characterized with RFLP and compared to that generated in nearly isogenic suscepti- ble plants (García-Andrés et al., 2009). Slight differences in restriction profiles were detected between resistant and sus- ceptible plants. Similar results were obtained with a PCR/sequencing analysis which scanned the most frequent RB along the viral genome (Belabess et al., 2018). The RBs detected from resistant plants were scattered on a larger region of the genome than the RBs from susceptible plants. The most striking difference was between nucleotide positions 141 and 962, where 18 RBs were detected from 10 resistant plants and only 3 from 10 susceptible plants. Most importantly, this study showed that recombinants bearing a RB at position 141 were positively selected in *Ty-1*-resistant plants but not in the nearly isogenic susceptible plants (Belabess et al., 2018; Urbino et al., 2020). Interestingly, the positive selec- tion of this type of recombinant named TYLCV-IS141 was fully confirmed from field surveys. Indeed, TYLCV-IS141 recombinants were identified from symptomatic *Ty-1*-resistant plants collected in different locations in Italy (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018).

The positive selection exerted by *Ty-1*-resistant plants on recombinants containing short TYLCSV fragments wasalso supported with TYLCV-IS76, an unusual recombinant detected in 2010 in Morocco. Monitoring the prevalence of TYLCV-IS76 over time has allowed the reconstruction of its emergence scenario as described below.

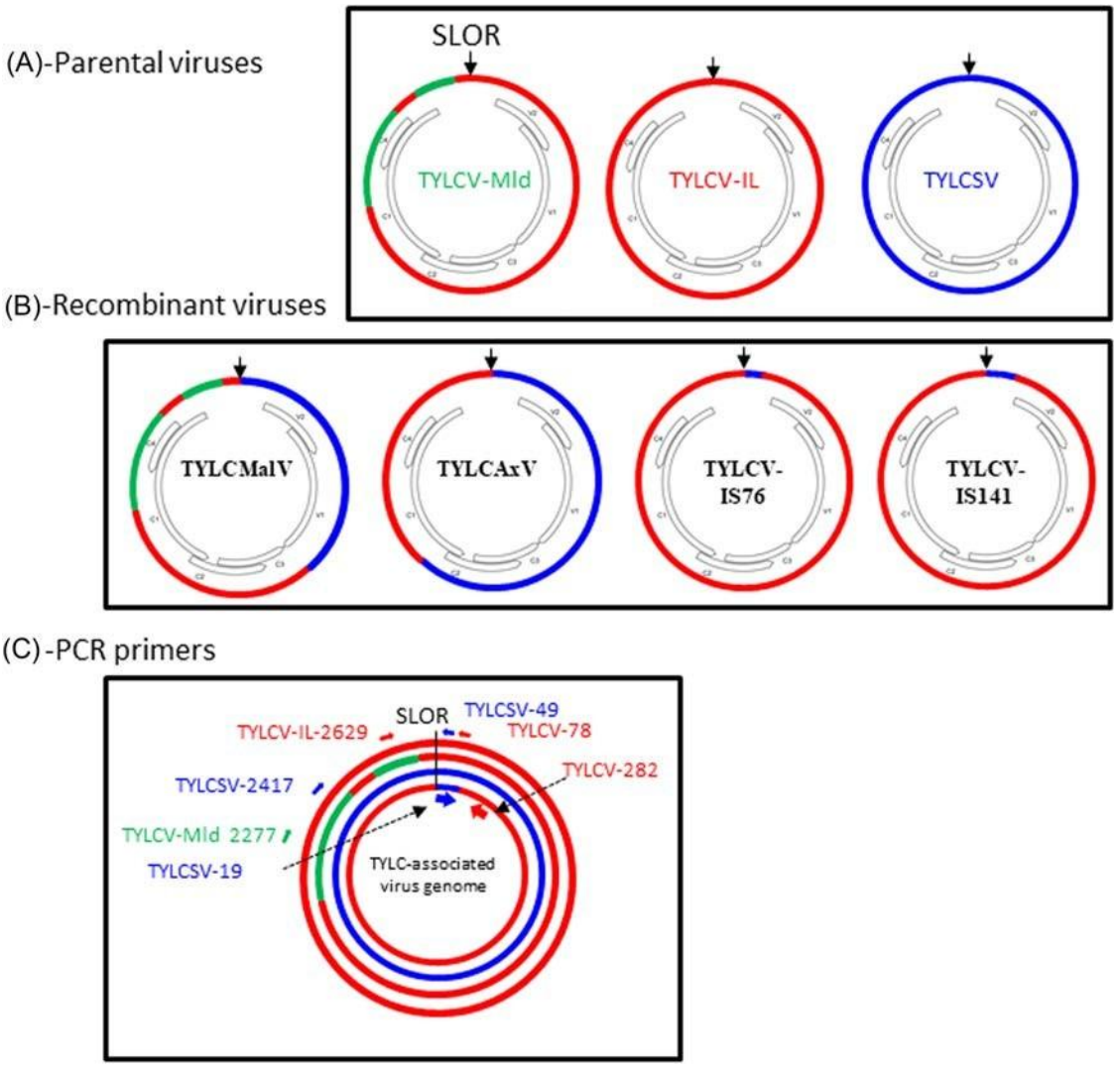

FIGURE 31.1 Recombination profiles of Mediterranean TYLCV/TYLCSV recombinants and PCR primer positions for their detection and distinction from parental viruses. (A) Parental genomes: TYLCV-IL genome is presented in red and TYLCSV in blue; TYLCV-Mld genome is presented in red and green because it was generated by recombination between a TYLCV-IL ancestor (*red*) and an unknown virus (*green*). (B) The recombinant genomes are presented with the color code of the parental viruses in A. (C) Positions and specificity of PCR primers. The color indicates their specificity to viral genomes according to the color code in A. Forward primers TYLCV-Mld-2277, TYLCV-IL-2629, and TYLCSV-2417 were used with either TYLCSV-49 (multiplex PCR1) or TYLCV-78 (multiplex PCR2), for detection of parental and recombinant viruses, as described in Belabess et al. (2015). Primers TYLCSV-19 and TYLCV-282 (thick arrows inside the circles) were used to specifically detect TYLCV-IS76-type recombinants in samples that tested positive for TYLCV/TYLCSV recombinants (multiplex PCR1). The black vertical arrow indicates the position of the conserved stem-loop containing the origin of replication of begomoviruses (SLOR). Positions of the virion (V1, V2) and complementary sense (C1, C2, C3, C4) open reading frames are indicated in A and B.

31.3 The emergence scenario of the resistance-driven recombinant TYLCV-IS76

31.3.1 Discovery and monitoring of the prevalence of TYLCV-IS76

Unexpected TYLCD symptoms have been observed in 2010 on *Ty-1*-resistant tomato plants in the Souss, oneof the main tomato-producing areas of Morocco (Belabess et al., 2015). These plants were found to be infectedwith a TYLCV/TYLCSV recombinant bearing a TYLCSV-derived fragment which was much shorter than that of previously reported recombinants. It exhibits two RBs, one within SLOR, and one within a region of six nondiscriminating nucleotides, between nucleotide positions 74 and 79 of the TYLCSV genome (Fig. 31.1). The recombinant was named TYLCV-IS76, in which "I" stands for the IL strain of TYLCV, "S" for TYLCSV,

and "76" for one of the two median positions of the 74 79 RB position range, arbitrarily selected for naming the recombinant (Fig. 31.1).

PCR and RFLP tests developed previously to identify TYLCV/TYLCSV recombinants (Davino et al., 2008, 2012; Garcia-Andres et al., 2006; García-Andrés et al., 2007a, 2007b; Monci et al., 2002) were not specific enough to identify TYLCV-IS76 as a recombinant genome. Indeed, due to the unusually short TYLCSV-derived fragment, TYLCV-IS76 would have been detected as a TYLCV genome. Therefore a new test had to be designed to specifically detect TYLCV-IS76. A two-step procedure was worked out to identify the infection profile of plants potentially infected with TYLCD-associated viruses (Belabess et al., 2015) and monitor the prevalence and emergence dynamic of TYLCV-IS76 in Morocco. Plant samples were first tested with two multiplex PCR tests targeting the SLOR recombination hotspot to discriminate between TYLCV, TYLCSV, and TYLCV/TYLCSV recombinants (Fig. 31.1). Noteworthy, the PCR tests were worked out to fill another shortcoming of previous tests, i.e., the distinction between the two common TYLCV strains in Mediterranean countries, TYLCV-IL and TYLCV-Mld. The test distinguishes also the TYLCV strains when they are recombined with TYLCSV. Samples detected positive for the presence of recombinants were tested further for the presence of TYLCV-IS76-type recombinants with primer pairs encompassing the 76 RB (Fig. 31.1).

A total of 879 plants were sampled throughout Morocco from 1998 to 2003 and from 2010 to 2014. Samples detected positive for TYLCV-IS76 were obtained only among plants sampled from 2010. These samples revealed that TYLCV-IS76 has completely displaced its parental viruses by 2012 in the Souss and that it was spreading northwards up to the Mediterranean coast. TYLCV-IS76 remained dominant in the Souss until the last sampling carried out in 2014 (Belabess et al., 2015). Noteworthy, TYLCV-IS76 is the first TYLCV/TYLCSV recombinant that completely displaced its parental viruses.

31.3.2 Resistance-driven emergence of TYLCV-IS76

According to a probabilistic approach implemented in the computer program BEAST, the most probable date of generation of TYLCV-IS76 was in the 90s (Belabess et al., 2015). It was not detected before the deployment of *Ty-1*-resistant cultivars, suggesting that resistant cultivars triggered its emergence. This hypothesis was fully confirmed with experimental studies showing that the accumulation level of TYLCV-IS76 is higher than that of parental viruses in *Ty-1*-resistant tomato cultivars without paying any fitness cost in susceptible cultivars (Fig. 31.2).

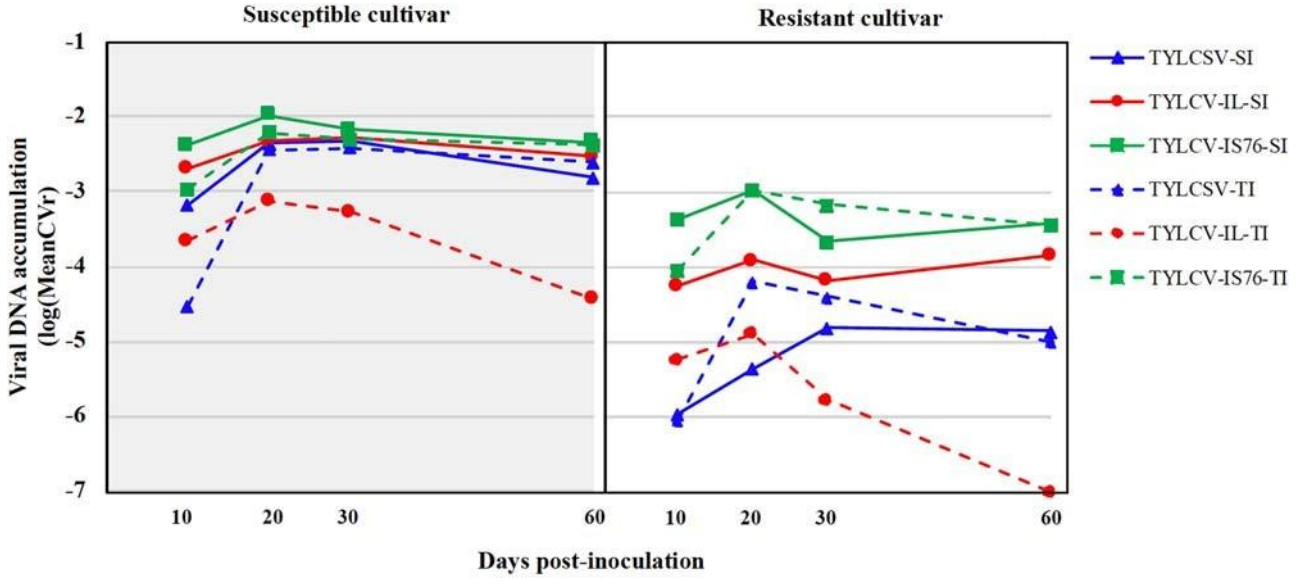

FIGURE 31.2 DNA accumulations of TYLCV-IL, TYLCSV, and TYLCV-IS76 in tomato plants of the *Ty-1*-resistant cv. Pristyla (*Ty-1/ty-1*) and a susceptible nearly isogenic cultivar (*ty-1/ty-1*). Viruses were agroinoculated alone (single infection, SI) or in coinfection with the two other viruses (triple infection, TI). Viral DNA was quantified by qPCR from at least 26 plants per treatment sampled at 10, 20, and 30 days postinoculation (dpi), and from at least eight plants sampled at 60 dpi. The logarithm of the Mean of Calibrated qPCR fluorescence values (logMeanCVr) reflects viral DNA accumulation. For the sake of clarity, standard deviations were not added to the curves. The distribution of accumulation values and statistics are available in Fig. 2 of Belabess et al. (2016).

To demonstrate that the recombination event but not point mutations is the major determinant of the fitness advan- tage of TYLCV-IS76, a mutated TYLCV-IS76 infectious clone (called IS76") was engineered in which its TYLCV- and TYLCSV-derived sequences are 100% identical to, respectively, TYLCV and TYLCSV infectious clones used in competition tests (Belabess et al., 2016).

The most conspicuous feature of the fitness advantage of TYLCV-IS76 is detected when *Ty-1*-resistant plants are coinfected with TYLCV-IL, the most competitive parent. The accumulation level of TYLCV-IL in such coinfected plants is drastically lower than its accumulation in plants infected only with TYLCV-IL (Fig. 31.2). It is about 10 times lower at 10 days postinfection (dpi) and the deleterious effect triggered by TYLCV-IS76 is maintained over time, the content of TYLCV-IL decreasing further until it drops below the qPCR detection threshold at 90 dpi (Belabess et al., 2016). Noteworthy, TYLCV-IL could not be transmitted to tomato plants by insect vectors from such coinfected plants at 60 dpi (Belabess et al., 2016). This competitive advantage is consistent with the decrease of TYLCV-IL incidence in Morocco. Interestingly, the negative impact of TYLCV-IS76 on TYLCV-IL accumulation is also detected in susceptible plants but to a lesser extent. The effect was presumably not strong enough to eliminate TYLCV-IL before the advent of *Ty-1*-resistant cultivars (Fig. 31.2).

Unlike TYLCV-IL, TYLCSV benefits from coinfection with TYLCV-IS76 as determined in controlled conditions (Belabess et al., 2016) (Fig. 31.2). Thus, although TYLCSV's disappearance coincided with that of TYLCV in natural conditions, mechanisms were different. The disappearance of TYLCSV is thought to be a direct effect of the *Ty-1* gene because its negative impact on virus content is 10 times more effective with TYLCSV than TYLCV (Belabess et al., 2016; García-Andrés et al., 2009) (Fig. 31.2).

31.3.3 Generation and preemergence of TYLCV-IS76

The generation and preemergence of TYLCV-IS76 were tested experimentally with *Ty-1*-resistant tomato plants agroinfected with representatives of the parental TYLCV-IL and TYLCSV. Considering the high propensity of parental viruses to recombine (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007b) and the selective advantage of TYLCV-IS76 in *Ty-1*-resistant plants (Belabess et al., 2016), it was thought that TYLCV-IS76-type recombinants would be generated among the naturally generated progeny of TYLCV/TYLCSV recombinants and would be positively selected in resistant plants. Intriguingly, whereas this prediction was confirmed for TYLCV-IS141, the recombinant reported from Italy, it was not confirmed for TYLCV-IS76. Although recombinants bearing the RB at position 76 were detected, they remained at an extremely low concentration up to the end of the experiment at 365 dpi (Belabess et al., 2018). If TYLCV-IS76 was generated in tomato, it may have required an unlikely and possibly multistep process involving vector transmission. Another possibility is that it has preemerged in a nontomato species exerting different selection pressures on de novo generated recombinants.

The contrasted selection of TYLCV-IS141 and TYLCV-IS76 in controlled conditions is fully consistent with paral-lel emergences of TYLCV-IS141 recombinants in natural conditions (Urbino et al., 2020), and the phylogeny results of TYLCV-IS76 which are in favor of a unique generation (Belabess et al., 2015).

31.3.4 Emergence scenario of TYLCV-IS76

Based on field observations and experimental results, a scenario of TYLCV-IS76 emergence can be reconstructed (Fig. 31.3).

According to Bayesian inferences, the recombinant TYLCV-IS76 was generated in the 90s (Belabess et al., 2015),very close to the first detections of TYLCD (1997), and its parental viruses, TYLCV-IL (1998) and TYLCSV (1999) in Morocco (Monci et al., 2000; Peterschmitt et al., 1999; Tahiri et al., 2006). TYLCV-IS76 was not detected in tomato samples collected before 2004, presumably because the number of samples available for the tests was too low to detecta recombinant supposed to be at low incidence and low concentration, at least in tomato. Indeed, it is possible that its prevalence was higher in nontomato species where it may have preemerged. The deployment of *Ty-1*-resistant cultivars has positively selected TYLCV-IS76 due to its selective advantage as demonstrated in controlled conditions. The accumulation of TYLCV-IS76 being higher than that of parental viruses in coinfected plants, its transmission by whitefly vectors was assumed to be relatively higher, in accordance with a previous report showing a correlation between viral DNA accumulation and transmission efficiency of TYLCD-associated viruses (Lapidot et al., 2001). Preliminary transmission tests confirmed this prediction (Belabess et al., 2016).

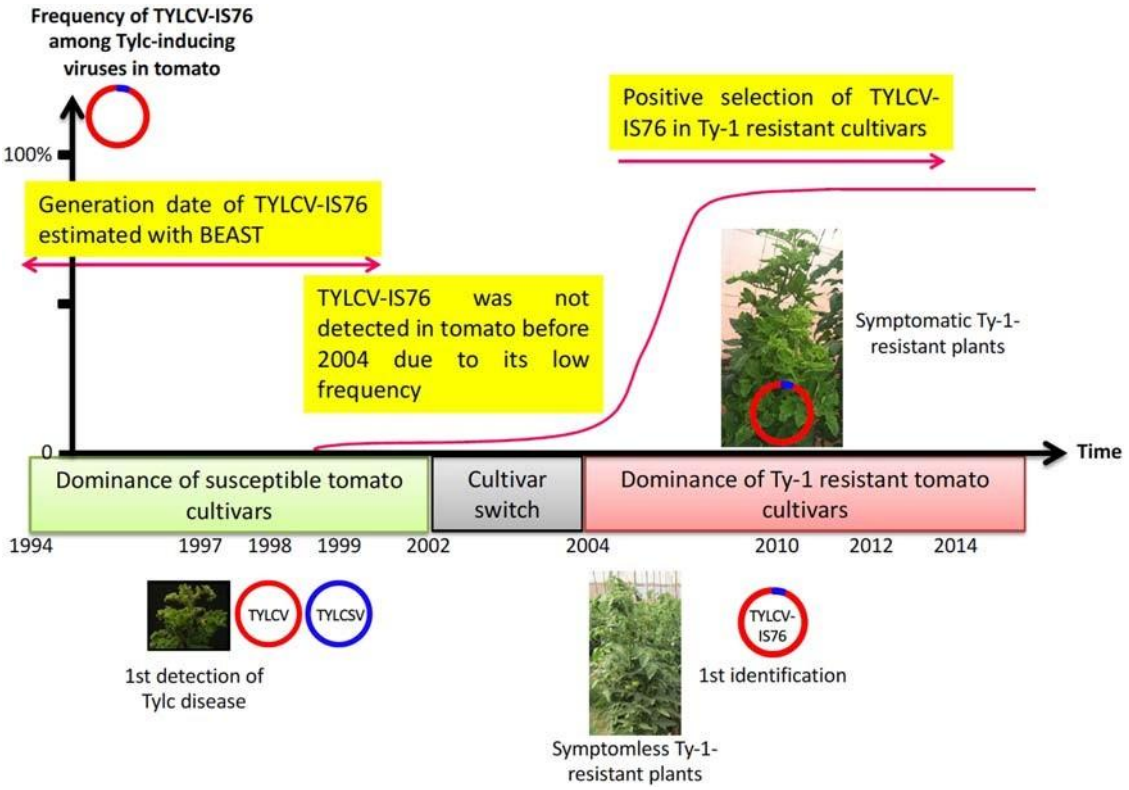

FIGURE 31.3 Invasion scenario of the recombinant TYLCV-IS76 in Southern Morocco (Souss). Consistently with the first detection of TYLCV-IL and TYLCSV in Morocco, the most probable generation date of the TYLCV-IS76 recombinant was estimated between 1994 and 2003. TYLCV-IS76 probably remained at a low frequency in the Souss region until the deployment of *Ty-1*-resistant tomato cultivars. Its frequency increase coincided with the replacement of susceptible cultivars by resistant cultivars which were experimentally shown to select positively TYLCV-IS76. Symptomatic *Ty-1*-resistant plants initially revealed the emergence of TYLCV-IS76. The tomato samples collected in the Souss after 2010 revealed that parental viruses were replaced by TYLCV-IS76.

31.4 An invading TYLCV recombinant shed new light on an underexplored region of the viral genome

31.4.1 Intragenomic interactions

The dramatic success of TYLCV-IS76 is triggered by the 76-nt fragment inherited by TYLCV from TYLCSV. It is composed of a 58-nucleotide region distinguishing TYLCV and TYLCSV, hereafter named intergenic region 58 (IR58). IR58 is framed with nondiscriminating 5^0 and 3^0-end sequences containing the RBs (Fig. 31.4).

Thus, once a TYLCV genome inherited IR58 from TYLCSV by recombination and emerged following a very improbable process (Belabess et al., 2018), it acquired the ability to displace its progenitor TYLCV at the plant level and progressively at the regional level. The fact that TYLCSV was not able to displace TYLCV indicates that the dis- placing phenotype is not determined by IR58 itself but by molecular interactions modified by the recombination event. This conclusion is consistent with the fact that the displacing phenotype was observed with TYLCV-IS76 and TYLCV- IS141 irrespective of the recombination site (76 or 141) and the parental TYLCV or TYLCSV genomes (Urbino et al., 2020). Noteworthy, the previously reported TYLCV/TYLCSV recombinants comprising TYLCSV sequences extending beyond position 900 did not replace their parental viruses at a regional level (Garcia-Andres et al., 2006; Monci et al., 2002). Therefore it is thought that they do not have the typical displacing phenotype of TYLCV-IS76 and TYLCV- IS141. Taken together these results suggest that the molecular modifications that determine the displacing phenotype are determined by intragenomic interactions between IR58 from TYLCSV and the *V1* and *V2* genes of TYLCV (posi-tion 141 to 900) or their products.

In a previous study on TYLCV recombinants generated *in planta*, Martin and colleagues (Martin et al., 2011) showed that recombination tended to preserve pairwise nucleotide associations between genomic regions. Whereas

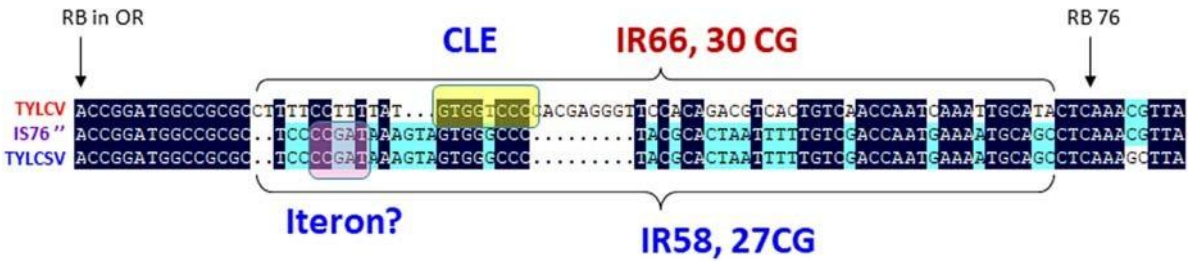

FIGURE 31.4 Alignment of viral sequences in the short region of Tomato yellow leaf curl Sardinia virus, (TYLCSV-ES[MA: Aga5a: 12], LN846598) inherited by the recombinant TYLCV-IS76" (IS76") IS76" is an engineered representative of TYLCV-IS76 derived from TYLCSV and Tomato yellow leaf curl virus (TYLCV-IL [RE:STG4:04], AM409201). The aligned sequences are located between the two recombination breakpoints of IS76" (RB, represented by arrows). Sequences common to the three genomes are on a black background, whereas those common to two genomes are on a blue background. The region differentiating IS76" from TYLCV-IL comprises 66 nucleotides (IR66), including 30 C or G nucleotides, and contains the typical conserved late element (CLE, GTGGTCCC highlighted in yellow). The sequence of TYLCSV and TYLCV-IS76 homologous to IR66 comprises only 58 nucleotides, including 27 C or G nucleotides (IR58). The CLE sequence of IR58 (GTGGGCCC) is different from that of IR66. Moreover, IR58 contains the sequence CCGAT (highlighted in pink) which is part of the reverse complementary sequence of the TYLCV-IL iteron sequence (AATCGGTGTC).

some of these associations corresponded to reported intragenomic interactions, other detected associations, like those between the *V1* gene or its product and the virion-sense-gene side of the intergenic region, did not correspond to any reported interactions. Interestingly, this latter type of association is consistent with the intragenomic interactions that are suspected to determine the displacing phenotype of TYLCV-IS76 and TYLCV-IS141.

The comparison between TYLCV-IS141 and TYLCV-IS76 revealed a trade-off. Indeed, whereas TYLCV-IS141does not displace TYLCV as efficiently as TYLCV-IS76 at 30 dpi (Urbino et al., 2020), TYLCV-IS141 emerged more frequently than TYLCV-IS76 which presumably emerged only once. This suggests that the determinism of the optimal displacement phenotype may also involve interactions between IR58 and the TYLCV genome sequence between posi- tions 76 and 141, a region containing the conserved TATA-box of the *V1/V2* promoter. Moreover, the probability of emergence of TYLCV/TYLCSV recombinants seems to be positively correlated with the length of the TYLCSV inher- ited fragment.

31.4.2 Molecular features of the recombinant region IR58

IR58 is located upstream of the TATA-box of the *V1/V2* genes promoter. IR58 comprises two conserved elements known as transcription regulating elements, i.e., a CAAT-box reported in many begomoviruses but also in plant promo- ters (Sunter & Bisaro, 2003), and the so-called Conserved Late Element (CLE; GTGGTCCC) present only in some begomoviruses (Fig. 31.4). Although CAAT-box is present in TYLCV and TYLCSV, the different sequence contexts may induce contrasted interactions with cellular transcription factors. CLE is involved in the transactivation activity of TrAP protein (*C2* gene product) and thereby in TrAP-mediated transactivation of the expression of both the capsid pro- tein (*V1* gene product) and the silencing suppressor protein encoded by the *V2* gene. CLE is highly conserved in TYLCV genomes but its sequence differs in one position in TYLCSV (GTGGGCCC) (Fig. 31.4). So far, it cannot be excluded that this single mutation introduced by recombination in TYLCV-IS76 and TYLCV-IS141 participates in the determination of their typical phenotype (Fig. 31.4).

The non-coding IR of begomovirus contains repeated DNA elements called "iterons" which are key elements for the recognition of the replication origin and the initiation of the replication (Fontes et al., 1994; Laufs et al., 1992).The IR iteron of TYLCV-IL located on the complementary sense gene side of the SLOR is "AATCGGTGTC". Interestingly, IR58 of TYLCSV but not its TYLCV homologous region (IR66) contains a 5 nucleotide sequence CCGAT which is reverse complementary to a sequence contained in the iteron (ATCGG) (Fig. 31.4). It cannot be excluded that the acqui- sition of this partial TYLCV iteron by TYLCV-IS76 and TYLCV-IS141 may boost their replication efficiency and thus increase their fitness.

31.4.3 Testing IR58 for fitness determinants

The TYLCSV IR58 is 8 nt shorter than TYLCV IR66 and has only 27C/G instead of 30 for TYLCV (Fig. 31.4). We speculate that a shorter sequence may induce a higher replication efficiency and a lower CG content may reduce

cytosine methylation level and in turn transcriptional silencing. This latter hypothesis is consistent with the nature of the *Ty-1*-resistance gene, i.e., an RDR potentially involved in RNAi and DNA methylation as described above (Butterbach et al., 2014). It is also consistent with reports showing that the region homologous to IR66 is highly tar- geted by siRNAs in the A component of bipartite begomoviruses, namely, mungbean yellow mosaic India virus (MYMIV) (Yadav & Chattopadhyay, 2011), tomato leaf curl New Delhi virus (ToLCNDV) (Sahu et al., 2014), and pepper golden mosaic virus (PepGMV) (Rodr´ıguez-Negrete et al., 2009).

The length hypothesis was tested by engineering TYLCV-Del8T and TYLCV-Del8CG, two TYLCV-IL variants in which 8T or 8C or G of IR66 were deleted, respectively (Fig. 31.5).

TYLCV-Del8CG was also used to test the CG content hypothesis. The variants were constructed with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's recom- mendations. To test if these modifications are associated with the typical phenotype of TYLCV-IS76 and TYLCV- IS141, the variants were coagroinoculated with TYLCV-IL in *Ty-1*-resistant plants, and virus accumulation was assessed by qPCR in samples collected at 30 dpi. Plants agroinoculated with single viruses were used as controls. Experimental procedures were as previously described (Belabess et al., 2016). In single infections, the accumulation of TYLCV-IL was significantly lower than that of TYLCV-Del8CG, but similar to TYLCV-Del8T (Fig. 31.5A). According to previous reports showing that methylation has a negative effect on virus replication (Brough et al., 1992; Ermak et al., 1993), the higher accumulation of TYLCV-Del8CG may be explained by a lower exposure to methylation and hence to a lower inhibition of virus replication. In coinfection with TYLCV-IL, the accumulation of

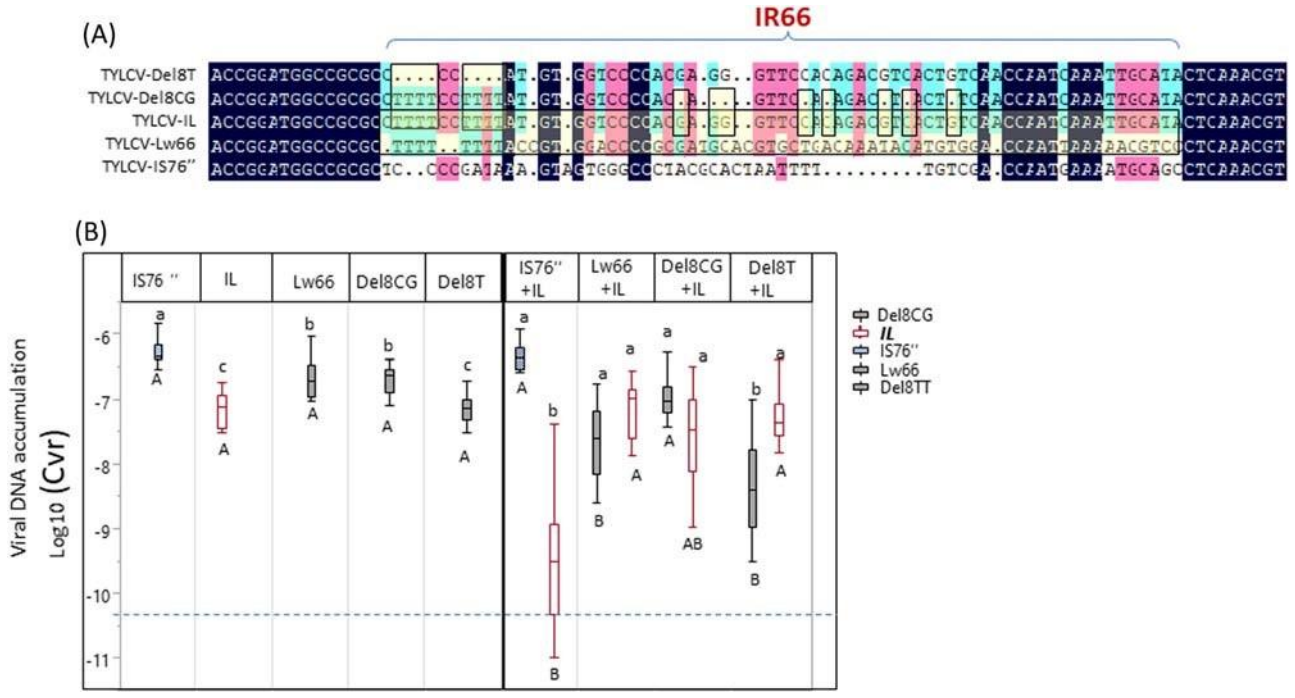

FIGURE 31.5 Comparison of the DNA accumulations of TYLCV-IS76" (IS76"), TYLCV-IL, and three variants of TYLCV-IL modified in IR66 for their length, CG content, or sequence composition in *Ty-1*-resistant tomato plants. (A) Alignment of IR66 of the wild type clone TYLCV-IL (AM409201) (IL) with its engineered variants and the homologous sequence of TYLCV-IS76" (IR58). TYLCV-Del8T (Del8T) is an IL variant inwhich 8T were deleted (framed T on a yellow background). Likewise, TYLCV-Del8CG (Del8CG) is an IL variant in which 8G or C were deleted. TYLCV-Lw66 (Lw66) is an IL variant in which IR66 was replaced by the homologous IR66 of tomato leaf curl Liwa virus (AF195782). (B) DNAaccumulations of IL, IS76," Del8T, Del8CG, and Lw66 clones in agroinfected tomato plants of the *Ty-1*-resistant cv. Pristyla. The infection status of plants is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected at 30 dpi from 15 plants pertreatment. The logarithm of the Calibrated qPCR fluorescence Value (logCVr) reflects viral DNA accumulation. Within the boxes, the horizontal line indicates the median value (50% quantile), the box itself delimits the 25% and 75% quantiles, and the vertical lines represent the normal range of the values. The dotted line represents the threshold of positive detection determined with the mean of logCVr values obtained with mock-inoculated plantstested with IL, IS76," Del8T, Del8CG, and Lw66 specific primers. Box plots with different letters indicate significant differences in viral DNA accu- mulations (Tukey's test, P 5 0.05): small letters correspond to comparisons between single-infected viruses or between different viruses in coinfected plants, whereas capital letters correspond to the comparisons of the same virus between treatments.

TYLCV-Del8CG was similar to its accumulation in single infection, whereas the accumulation of TYLCV-Del8T was significantly lower than its accumulation in single infection (Fig. 31.5B). When TYLCV-IL is coinfected with the recombinants, its accumulation was not significantly lower than that in single infection, which shows that none of the engineered modifications was able to recreate the typical TYLCV-IS76 phenotype (Fig. 31.5B).
Another possibility would be that IR66 is a burden for TYLCV-IL fitness. In this case, swapping this region wouldbe a relief and the fitness advantage might be obtained with a homologous sequence inherited from a non-TYLCSV donor begomovirus. Tomato leaf curl Liwa virus (ToLCLwV), was selected as a donor virus because it is monopartite like TYLCV, and its genomic region homologous to IR66 is also 66-nt long with 30 CG (Genbank Accession HF912280, Fig. 31.5A). This last feature was also considered to avoid confounding effects potentially induced by the length of the recombinant region. The variants were constructed with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), as described (Belabess et al., 2016). TYLCV-Lw66, the TYLCV-IL variant bearing the IR66 of ToLCLwV, was tested in parallel with TYLCV-Del8CG and TYLCV-Del8T as described above. In single infection, TYLCV-Lw66 content is significantly higher than TYLCV-IL content. However it has no deleterious effect on TYLCV-IL accumulation (Fig. 31.5B). Although none of the three hypotheses could be validated, i.e. burden, length, or CG content of IR66, these results revealed at least that IR66 is a genomic region that can be substantially modified with no major effect on virus accumulation.

31.4.4 Plant—virus interactions potentially associated with fitness advantage

The negative impact of TYLCV-IS76 on TYLCV-IL is far milder in susceptible plants than in *Ty-1*-resistant plants (Fig. 31.2), which shows that the *Ty-1* gene is a key player in virus interactions. Thus, the scope of the studies on the selective advantage of TYLCV-IS76 and TYLCV-IS141 should be extended to plant virus interactions and their poten- tial modifications by recombinants. As *Ty-1* codes for a γ-clade RDR likely involved in RNAi machinery, it was thought in a first approach that IR58 is less targeted by transcriptional silencing than the homologous region of TYLCV-IL. However, such a difference does not explain its negative impact on TYLCV-IL. Another possibility would be that TYLCV-IS76 triggers a higher RNAi response than TYLCV-IL, but the mechanism by which it would specifi- cally affect TYLCV-IL accumulation is not clear. To further test the possible involvement of RNAi and gene silencing in the selective advantage of TYLCV-IS76 and TYLCV-IS141, an RNAomics study is recommended because of its sys- temic approach that does not need any prior hypothesis to be tested. RNAomics of TYLCV was previously performed in susceptible tomato plants (Piedra-Aguilera et al., 2019) but also in comparison with *Ty-1*-resistant tomato plants (Voorburg et al., 2021). In this later study, Voorburg and colleagues confirmed that *Ty-1* enhances the production of virus-derived siRNAs (vsiRNA). Although the IR was the region with the less vsiRNA in both susceptible and *Ty-1* resistant cultivars, the IR was relatively more targeted in *Ty-1* tomato plants. Interestingly, the distribution of vsiRNA from IR revealed two sub-regions relatively more targeted including the region homologous to the short TYLCSV- derived region of TYLCV-IS76 (IR58). These results are consistent with a possible involvement of the RNAi machinery in the competitiveness of TYLCV-IS76 and emphasize the relevance of a further RNAomics study with recombinant and parental type viruses in susceptible and *Ty-1*-resistant plants.
An indirect effect of *Ty-1* is also possible via other plant genes or regulatory elements important for the virus. An example of a plant element involved in TYLCD resistance was recently discovered in a tomato cultivar bearing the *Ty- 2*-resistance gene (Yang et al., 2019). It consists of a long noncoding region (lncRNA) produced by tomato plants and involved in the normal development of plants and in resistance against TYLCV. In susceptible plants, this lncRNA is silenced by viral siRNA derived from a 25-nt sequence located in IR of TYLCV, on the complementary sense gene side of SLOR. The silencing is associated with stunting and leaf curling symptoms and increase of TYLCV accumula-tion. However, when the lncRNA exhibits a 14-nt deletion in the region targeted by viral siRNAs, as observed in a *Ty- 2*-resistant cultivar, lncRNA is not silenced and TYLCV accumulation is negatively affected. Similar plant virus interactions involving IR58 may occur in *Ty-1*-resistant plants.

31.4.5 Insect—virus interactions potentially associated with fitness advantage

Considering the extent of the replacement of the parental viruses by the recombinant TYLCV-IS76, the recombination event could have also induced a higher transmission efficiency by the whitefly vector *B. tabaci*. So far, only the plant host was considered for transmission advantage, because TYLCV-IS76 and its most competitive parent TYLCV are100% identical in their coat protein, the unique determinant of geminivirus transmission specificity (Briddon et al., 1990). However, based on the recent results showing that TYLCV replicates in the vector and that replication enhances

transmission efficiency and persistence (He et al., 2020), the vector should also be considered as a competition area. Indeed, if TYLCV-IL and TYLCV-IS76 replicate in the vector, TYLCV-IS76 may have the capacity to induce and evade the insect antiviral RNAi and gene silencing and thereby be transmitted more efficiently than TYLCV-IL.

31.5 Perspective on detection and management of tomato yellow leaf curl virus recombinants

Growing virus-resistant plants is considered to be the best method to control virus diseases. It is particularly true for begomoviruses for which the chemical control of vectors is highly detrimental to the environment. Indeed, the high propensity of *B. tabaci* to develop insecticide resistance (Cahill et al., 1994, 1996; Gauthier et al., 2014; Horowitz et al., 2005) often encourages increased frequency and dose of insecticide treatments.

31.5.1 Risk of emergence of resistance-breaking viruses

One of the major concerns when using resistant cultivars is the emergence of new variants that may potentially break the resistance. Such a risk is particularly high when the resistance is not strong enough to block virus replication or spread, like in *Ty-1*-cultivars. Moreover, as *Ty-1* is virtually the only gene introduced in the commercial cultivars deployed in the Mediterranean Basin, high and specific selection pressure was potentially exerted on TYLCD-inducing viral populations. The significantly higher prevalence of TYLCV in *Ty-1*-resistant plants as compared to that of TYLCSV (García-Andrés et al., 2009) was the first confirmation of this prediction. Subsequently, the emergence of the recombinant TYLCV-IS76 and the parallel displacement of the parental TYLCV and TYLCSV further confirmed the strong selection pressure exerted by *Ty-1*-cultivars.

The emergence of more competitive variants does not necessarily create an agronomical problem if their virulence is not higher than that of the progenitor. The detection of TYLCV-IS76 and its sister recombinant TYLCV-IS141 on symptomatic *Ty-1*-resistant plants in field conditions suggested that they are potentially more virulent than other TYLCD-associated viruses. However, TYLCD symptoms were not induced when recombinants are agroinoculated to *Ty-1*-resistant plants in growth chambers, and *Ty-1*-plants detected positive for TYLCV-IS76 in the field were not always symptomatic. From trials carried out to evaluate resistance levels for different genotypes, it was concluded that conditions that may occur in summer, such as high inoculum pressure, plant age, high temperatures, and high light intensity may favor the expression of the disease symptoms (Lapidot et al., 2006; Pico et al., 1996). High temperature tested in controlled conditions (over 30°C) did not trigger symptom expression. We also thought that symptom expression may be induced in natural conditions because the rootstock frequently used for preparing the grafted tomato plants is from a susceptible cultivar. However, this hypothesis was not supported experimentally (Belabess et al., 2016). The discrepancy in symptom expression between field and laboratory conditions was also observed with TYLCV-Mu15, an emerging TYLCV variant detected in symptomatic *Ty-1*-resistant tomato cultivars grown in Spain. Noteworthy, according to phylogenetic studies, TYLCV-Mu15 is a TYLCV parental type generated from TYLCV-IS76 following back recombination (Torre et al., 2018).

31.5.2 Prevalence of tomato yellow leaf curl virus recombinants in the Mediterranean Basin

At present, several TYLCV variants exhibiting resistance-breaking features were reported from *Ty-1*-resistant plants in Morocco (Belabess et al., 2015), Italy (Panno et al., 2018), and Spain (Torre et al., 2018, 2019). Routine diagnostic tools need to be adapted to the detection of these recombinants to assist the management strategy of TYLCD. Considering that virtually all TYLCV-resistant cultivars in the Mediterranean Basin rely on the *Ty-1*-resistance gene, the management of TYLCD is increasingly challenging. The increasing whitefly populations associated with climate warming and increasing areas for vegetable cultivation (Gilioli et al., 2014; Mudereri et al., 2021) are conducive to rapid dissemination of these recombinants in the Mediterranean Basin. Even if the recombination event at the origin of TYLCV-IS76 seems to be very rare, such a risk should not be neglected in view of the high invasiveness of such recombinants. Indeed, TYLCV-IS76 invaded Morocco up to the Mediterranean boarder and was detected in Spain (Torre et al., 2019). TYLCV-IS141 has emerged more frequently but its prevalence has not been studied yet.

Although TYLCV and TYLCSV were reported from several Mediterranean Basin countries, and *Ty-1*-resistant culti- vars are intensively cultivated in all these countries, TYLCV-IS76 was detected only in Morocco and TYLCV-IS141 only in Southern Italy. This puzzle may be explained by conducive conditions specific to some regions as for example the genetic background of resistant cultivars or a possible involvement of TYLCD-susceptible non- tomato hosts in the

emergence process. Unlike TYLCV-IS76 and TYLCV-IS141, recombinants with a TYLCSV fragment of more than 900 nts were reported from all countries where TYLCV/TYLCSV recombinants were studied. However, it is not known yet if they have a selective advantage in *Ty-1*-resistant plants like TYLCV-IS76 or TYLCV-IS141.

31.5.3 Testing different allelic combinations of the *Ty-1*-resistance gene

Apart from *Ty-1*, other resistance genes were introgressed into tomato from wild tomato species (reviewed in Yan et al., 2018) to mitigate TYLCD symptom expression. Unfortunately, none of them blocked virus replication or movement. Here, we compared four different allelic combinations of the *Ty-1* gene for their resistance level against TYLCV-IL and TYLCV-IS76. Tomato plants were agroinfected with TYLCV-IL, TYLCV-IS76, or both and further processed as described (Belabess et al., 2016). Accumulation levels of each virus were estimated with real-time PCR at 30 dpi (Fig. 31.6).

Accumulations of TYLCV-IL and TYLCV-IS76 were significantly reduced with all allelic combinations in compari-son with susceptible plants. The resistance of *Ty-1*-homozygous plants tends to be higher but not significantly different from that of *Ty-1*-heterozygous plant, but the resistance of *Ty-3*-heterozygous plants was significantly lower than that of *Ty-1*-plants, irrespective of *Ty-1* status; in comparison to the susceptible cultivar, the mean accumulation values of TYLCV-IL were 10 and 100 times lower in the *Ty-3* and *Ty-1* genotypes, respectively. These results confirm previous fundings showing the lower efficiency of the *Ty-3* allele (Butterbach et al., 2014). As previously observed (Belabess et al., 2016), the *Ty-1* resistance is less effective on TYLCV-IS76 than on TYLCV-IL. Indeed, whereas the reduction of the mean accumulation of TYLCV-IL was 30 times with *Ty-1/ty-1* and 50 times with *Ty-1/Ty-1*, the reduction of TYLCV-IS76 was only 12 times with *Ty-1/ty-1* and 24 times with *Ty-1/Ty-1*. TYLCV-IS76 exhibits its typical negative impact on TYLCV-IL accumulation irrespective of the allelic combination of the *Ty-1* gene. Interestingly the intensityof the negative impact is positively correlated with the efficiency of the allelic combinations to reduce virus accumula- tion (Fig. 31.6).

Identifying new resistance gene and combining different resistance genes are needed to reduce virus replication and in turn to increase the durability of resistance to TYLCD-associated viruses.

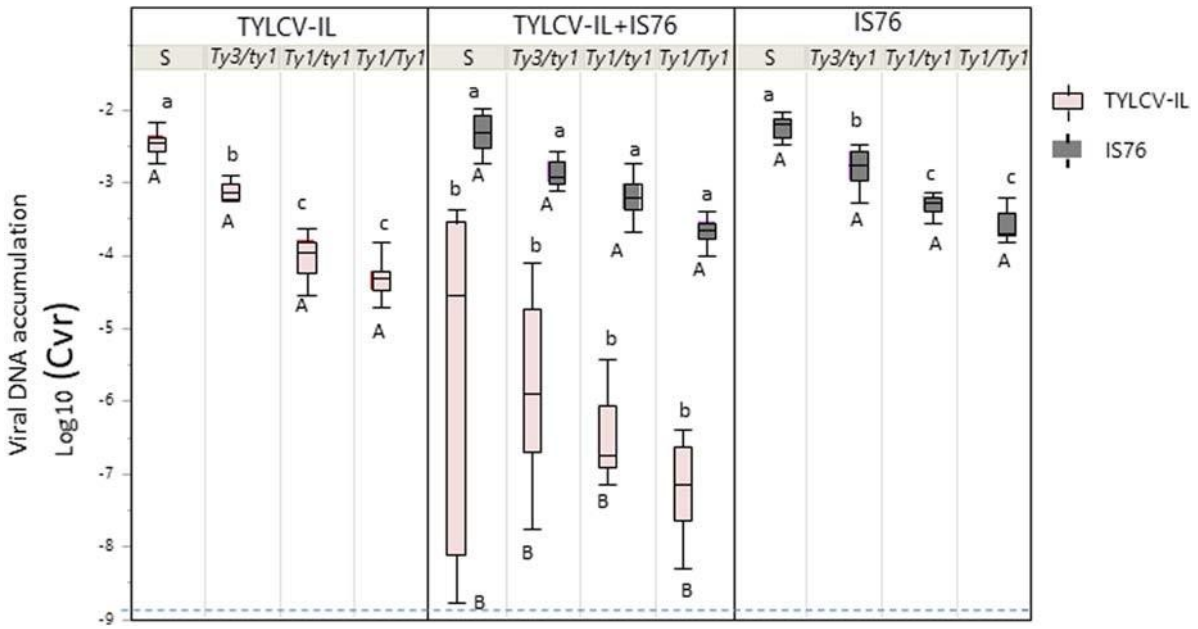

FIGURE 31.6 DNA accumulations of TYLCV-IL and TYLCV-IS76 (IS76) clones in agroinfected tomato plants having different allelic combina- tions at the *Ty-1/Ty-3* locus. *Ty-1* and *Ty-3* are resistance alleles, whereas *ty-1* stands for a susceptibility allele. *Ty-1/ty-1* corresponds to cv. Pristyla and *ty-1/ty-1* corresponds to a susceptible nearly isogenic cultivar (S). The infection status of plants is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected at 30 dpi from 15 plants per treatment. Representation of DNA accumulations, box plots, negative thresholds, and statistics as in Fig. 31.5.

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Objectifs de la thèse

Cette thèse comporte deux objectifs principaux.

Le premier objectif était de décrire plus finement le processus d'invasion de TYLCV-IS76 depuis sa génération jusqu'à son établissement dans la région du Souss. Des simulations de ce processus étaient nécessaires car aucun échantillon de plantes n'avait été récolté entre 2003 où le recombinant n'avait pas encore été détecté et 2010, quand sa présence était déjà généralisée dans le Souss. Parmi les situations ayant pu se produire sur le terrain et dans lesquelles la progression de TYLCV-IS76 pouvait être entravé ou ralenti, nous avons testé plusieurs scénarios. Sachant que la résistance d'une plante peut augmenter avec l'âge et qu'une plante peut être visitée par des vecteurs virulifères à tout âge, nous avons tester si l'âge de la plante au moment de son infection pouvait avoir un impact sur la compétitivité du recombinant. Un autre scénario que nous avons souhaité reproduire est l'arrivée du recombinant sur des plantes déjà infectées par des virus parentaux, une situation qui a dû se produire fréquemment en début d'émergence quand la prévalence de TYLCV-IS76 était encore relativement faible par rapport à celles des virus parentaux. Dans ce dernier cas où des recombinants TYLCV/TYLCSV néoformés occupaient déjà la plante à côté des virus parentaux à l'arrivée de TYLCV-IS76, nous avons aussi pu tester les interactions de TYLCV-IS76 avec ces recombinants résidants. Toutes ces simulations sont réalisées sur les plantes infectées artificiellement par la méthode dite d'agroinoculaiton des plantes. Pour tester une potentielle composante vecteur de la compétitivité du recombinant, nous avons comparé son efficacité de transmission à celle du parent TYLCV-IL, le parent connu pour être le plus efficacement transmis par *Bemisia tabaci*. Les résultats issus de ces simulations sont décrits dans le chapitre 1.

Des résultats préliminaires sur la compétitivité de TYLCV-IS76 dans des plantes déjà infectées par les virus parentaux avait montré que TYLCV-IS76 n'était pas impacté par la pré-infection des virus parentaux. Pourtant, selon le principe de prémunition, une plante infectée par un virus donné est potentiellement protégée contre l'infection d'une autre souche de ce même virus ou d'un autre virus proche génétiquement. Même si le phénomène de prémunition a été rarement établi pour les géminivirus, on ne peut exclure que la prémunition existe pour le TYLCV dans la tomate mais que TYLCV-IS76 soit capable de contourner ce phénomène. Selon la littérature la proximité du TYLCV avec TYLCV-IS76 serait théoriquement suffisante pour manifester un effet protecteur. Dans un premier temps il s'agissait de tester l'existence d'un phénomène de prémunition pour le TYLCV en utilisant des variants qui ne se distingue que par quelques SNP permettant de les distinguer par qPCR. Si la prémunition était avérée pour le couple TYLCV-tomate, le 2e objectif serait alors de tester si la prémunition reste efficace pour un recombinant présentant le même profil de recombinaison que TYLCV-IS76 et présentant la même distance génétique avec TYLCV-IL. Les résultats de cette étude font l'objet du chapitre 2.

Le deuxième objectif de la thèse est d'étudier les mécanismes moléculaires impliqués dans l'avantage sélectif de TYLCV-IS76. Cet avantage de TYLCV-IS76 étant principalement observé dans les plantes résistantes portant le gène Ty-1 et pas dans des plantes isogéniques sensible. C'est donc autour du gène Ty-1 qu'il a fallu développer une approche expérimentale. Le gène Ty-1 code pour une RDR dont la présence induit une augmentation de la méthylation des génomes viraux et de la production de petits ARNs impliquées dans le *gene silencing* (Butterbach et al., 2014 ; Voorburg et al., 2021). Il était cependant difficile de trouver un scénario crédible qui puisse expliquer comment TYLCV-IS76 pourrait déclencher une réaction de défense de type *gene silencing*, sans en être affecter soi-même. En effet comme le *gene silencing* est par définition une réaction de défense séquence spécifique, on ne comprend pas bien comment deux virus présentant 97% d'identité de séquence puisse se soustraire au *gene silencing* de son homologue. En l'absence d'un scenario solide à tester, nous avons entrepris une approche RNAomique sans a priori. Il s'agira de comparer le profil des petits ARNs et des transcrits dans des plantes de tomate Ty-1 et isogéniques sensible inoculé selon différentes modalités. Cette étude comporte deux volets, l'un consacré à l'analyses de petits ARNs et des transcrits complémentaires des génomes viraux, décrit dans le chapitre 3, et l'autre consacré à l'analyse des transcrits complémentaires au génome de la plante. Ce deuxième volet vise à connaitre les différences de réaction des plantes sensibles et résistantes pouvant intervenir lors de l'infection par le TYLCV-IL et le TYLCV-IS76 et potentiellement observée des différences entre ces deux virus qui pourrait expliquer la plus forte compétitivité de TYLCV-IS76. Cette étude transcriptomique fait l'objet du chapitre 4.

CHAPITRE 1

Article publié dans Virology :

Refining the emergence scenario of the invasive recombinant Tomato yellow leaf curl virus IS76 (TYLCV-IS76)

Virology 578 (2023) 71–80

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Refining the emergence scenario of the invasive recombinant Tomato yellow leaf curl virus -IS76

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ARTICLE INFO

Keywords:
Begomovirus
Geminivirus
Resistance-breaking virus
Bemisia tabaci
Invasive virus
Quantitative PCR

ABSTRACT

TYLCV-IS76, a unique recombinant between tomato yellow leaf curl virus (TYLCV) and tomato yellow leaf curl Sardinia virus (TYLCSV), has replaced its parental viruses in southern Morocco. To refine its emergence scenario, its fitness was monitored experimentally in conditions aiming at reproducing natural situations, i.e. superin- fection of plants already infected with parental viruses and competition with other TYLCV/TYLCSV recombinants (LSRec) automatically generated in plants coinfected with TYLCV and TYLCSV. TYLCV-IS76 accumulated significantly more than parental viruses regardless of plant age and superinfection delay. Although TYLCV-IS76 and LSRec both accumulated more than parental viruses in laboratory conditions, LSRec were displaced by TYLCV-IS76 in nature like parental viruses were. TYLCV-IS76 did not exhibit any vector transmission advantage over LSRec and TYLCV the most competitive parental virus. Thus, it is apparently only in the plant compartment that the recombination event that generated TYLCV-IS76, induced the competitiveness advantage by which the last became first.

1. Introduction

Besides mutation, recombination is a key driver of virus diversity (García-Andrés et al., 2007b; García-Arenal et al., 2001; Tromas et al., 2014) and thereby of virus evolution and adaptation (Abrantes et al., 2020; Belabess et al., 2016; Fernández-Cuartero et al., 1994; Lefeuvre and Moriones, 2015; Martin et al., 2011; Padidam et al., 1999). One of the most documented shift of virus populations involving recombination is with begomoviruses (genus *Begomovirus*, family *Geminiviridae*) infecting tomato (*Solanum lycopersicum*) in Morocco: tomato yellow leaf curl virus (TYLCV) and tomato yellow leaf curl Sardinia virus (TYLCSV) (Belabess et al., 2016, 2015; Urbino et al., 2022, 2020). TYLCV and TYLCSV co-circulated in southern Morocco in the 2000s when tomato cultivars susceptible to tomato yellow leaf curl disease (TYLCD) were progressively replaced with TYLCD-resistant tomato cultivars bearing the Ty-1 resistance gene. In 2012, TYLCV-IS76, a recombinant of TYLCV and TYLCSV had virtually replaced the parental type viruses and resis- tant plants exhibited TYLCD symptoms. The co-occurrence of the replacement of tomato cultivars and the shift of virus populations, as well as the experimental demonstration of the fitness advantage of TYLCV-IS76 over the parental viruses in Ty-1 resistant plants (Belabess et al., 2016), strongly support a selection-driven emergence of TYLCV-IS76 and confirmed recombination to be a key driver of adap- tation to a new environment. The typical fitness advantage of TYLCV-IS76 is its strong negative impact on the accumulation of TYLCV, the most competitive parental virus in Ty-1 resistant plants. A similar shift of virus populations associated with emerging recombinant viruses was reported with the rabbit haemorrhagic disease virus (RHDV), family *Caliciviridae*, genus *Lagovirus* (Abrantes et al., 2020). Like TYLCV-IS76, it virtually replaced the former viruses (Le Gall-Reculé et al., 2013) and compromised virus control measures, in this case, vaccinations (Dalton et al., 2014; Le Gall-Reculé et al., 2011).

The dynamics of the replacement of parental viruses by TYLCV-IS76 in southern Morocco could not be monitored because of the lack of to- mato sampling between 2004 and 2011 when TYLCD decreased with the deployment of Ty-1 resistant cultivars; before 2004, infected samples tested all negative for TYLCV-IS76, whereas after 2012 virtually all samples tested positive for TYLCV-IS76. From 2004 to 2011, only the four tomato samples on which TYLCV-IS76 was discovered in 2010 are available (Belabess et al., 2015). In an attempt to reconstruct the emergence scenario, the competitiveness of TYLCV-IS76 was further analyzed in controlled conditions.

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https://doi.org/10.1016/j.virol.2022.11.006
Received 5 August 2022; Received in revised form 2 November 2022; Accepted 15 November 2022
Available online 22 November 2022

To explain the displacement of parental viruses over time, we have already shown that the fitness of TYLCV-IS76 is higher than that of parental viruses both in single and mixed infected resistant tomatoes(Belabess et al., 2016). However, as plants were simultaneously coin-fected with the competitors, these competition tests are only partially representative of natural conditions. Indeed, as TYLCV-IS76 emerged in tomato fields that were already infected by parental viruses, superin- fection was probably the most common competition situation. More- over, as the initiation of the competition may have occurred at any plant age, the effect of plant age needs to be investigated; only 15 day-old plants were used in previous competition tests. A potential effect of plant age cannot be excluded according to reports showing that older plants were less conducive to infection than younger ones (Gibson, 1991; Hu and Yang, 2019; Lindblad and Sigvald, 2004; Venekamp and Beemster, 1980). Noteworthy, as the recombination event that led to TYLCV-IS76 emergence is thought to be unique (Belabess et al., 2015; Urbino et al., 2020), the colonization of tomato fields is supposed to be driven only by successive secondary infections from an initial source plant, which further emphasizes the need of high superinfection competitiveness.

The specific and presumably most challenging case of superinfection was with plants infected with both parental viruses, an infectious status that was relatively common in Morocco during the pre-emergence period (1998–2003) (Belabess et al., 2015). In such plants, recombi- nants are automatically generated (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2006, 2007b, 2009). The frequency of these TYLCV recombinants at 4–5 months post-infection (mpi) was estimated to be around 50% of the total viral genomes (García-Andrés et al., 2007b). The most abundant TYLCV/TYLCSV recombinants are typically different from TYLCV-IS76 because their fragment inherited from TYLCSV is much longer (Davino et al., 2009, 2012; García-Andrés et al., 2006, 2007b, 2009; Monci et al., 2002) than that inherited by TYLCV-IS76 (76 nts); they are further referred to as LSRec, i.e. **L**ongTYLC**S**V fragment **Rec**ombinants. As coinfection of plants with TYLCVand TYLCSV implies mechanically the generation of LSRec, LSRec necessarily participated to the competition with TYLCV-IS76.

Like other begomoviruses, TYLCV and its related viruses are trans- mitted by the whitefly *Bemisia tabaci*. Thus, host plants may not be the only arena where fitness advantage has been gained by the recombi- nation event. A more efficient whitefly transmission may have contrib- uted to the replacement of parental viruses. A preliminary test showed that TYLCV-IS76 was the only virus transmitted from Ty-1 resistant to- mato plants co-infected with TYLCV and TYLCV-IS76 (Belabess et al.,2016). This result is consistent with the drastically lower virus accu-mulation of TYLCV induced by the deleterious effect of TYLCV-IS76. However, it is not known if TYLCV-IS76 has also a transmission advantage from plants infected individually with each virus. It was initially presumed that TYLCV-IS76 and TYLCV are transmitted with similar efficiency because TYLCV-IS76 inherited its capsid protein (CP) from TYLCV, CP being the protein that determines transmission speci- ficity (Briddon et al., 1990). However, with the compelling results recently reported in favour of TYLCV replication in whiteflies (He et al., 2020), it cannot be excluded that the recombination event may have modified virus-vector interactions that may increase transmission efficiency.

The objective of the study was to explore further the fitness of TYLCV-IS76 and reconstruct its emergence scenario. To reproduce what occurred at the beginning of its emergence when its prevalence was relatively low, we tested if TYLCV-IS76 is able to superinfect resistant tomato plants at 1 month after parental virus infection, or later, at 4 mpi, when LSRec recombinants have built up in co-infected plants. The encounter with LSRec was studied further by natural means of inocu- lation using whitefly vectors. The influence of plant age on competi- tiveness was addressed by co-inoculating viruses in 15, 45- and 135-day- old resistant tomato plants. Finally, the efficiency for vector trans- mission was compared between TYLCV and TYLCV-IS76 using the most common *B. tabaci* species in the Mediterranean, Middle East Asia Minor1 (MEAM1) and Mediterranean (Med).

Our results show that infection profile and competitiveness were not affected by plant age. TYLCV-IS76 is more competitive than parental viruses in any of the competition situations tested, including when it is superinfected with 4-month delays. In superinfection, the competitive- ness of TYLCV-IS76 is similar to that of LSRec but following coinfection with whitefly vectors, it is relatively higher. Finally, we showed that the recombination event that produced TYLCV-IS76 did not provide any detectable benefit for transmission efficiency. Based on these results, the reconstruction of the emergence scenario was refined, and the puzzling disappearance of highly competitive LSRec is discussed.

2. Material and methods

2.1. Biological material

The resistant *Solanum lycopersicum* tomato cultivar "Pristyla" car- rying the Ty-1 resistance gene in a heterozygote state (*Ty-1/ty-1*) (Gautier Semences, France) and a nearly isogenic susceptible cultivar (*ty-1/ty-1*) (Belabess et al., 2016) were used for all the experiment except the transmission efficiency test between TYLCV and TYLCV-IS76. For this latter test, the susceptible tomato cultivar Monalbo (INRAE, France) was used as source plants and plants of *Datura stramonium* were used as recipient plants. Plants were grown in containment growth chamber with 14 h light at 26 °C and 10 h dark at 24 °C. They were watered with a solution containing 15:10:30 NPK fertilizer and oligoelements.

Three agroinfectious clones were previously constructed (Belabess et al., 2016, 2015) with the following viruses: TYLCSV-ES [MA: Aga5a:12] (GenBank accession number LN846598), hereafter referred to as TYLCSV; TYLCV-IS76 [MA:SouG8:10] (GenBank accession number LN812978); and TYLCV-IL [RE:STG4:04] (GenBank accession number AM409201), hereafter referred to as TYLCV.

Vector inoculation of viral clones was performed with two pop- ulations of *B. tabaci*. They were both collected in France and identified with microsatellite markers (Dalmon et al., 2008). Thus, the population collected in Tarascon was identified as belonging to the cryptic species Mediterranean (Med, also referred to as biotype Q) and the population collected in Nice to the cryptic species Middle East Asia Minor 1 (MEAM1, also referred to as biotype B). The two populations were reared on eggplants (*Solanum melongena*).

2.2. Agroinoculation

Virus agroinfiltration or co-agroinfiltration of 15-day old plants were performed with agrobacteria preparations as described in Belabess et al. (2016). Older plants of 45 and 135 days, were agroinoculated with a needle at 5 different levels along the stem using a total of 0.5–1 ml of agrobacteria preparation per plant.

Plants used as negative controls were agroinfected with a prepara- tion of bacteria of the C58 MP90 strain of *Agrobacterium radiobacter* var. *tumefaciens* containing an empty pCAMBIA2300 plasmid.

2.3. Competition between TYLCV-IS76 and LSRec recombinants following vector transmission

Fifteen-day-old susceptible isogenic tomato plants were co- agroinoculated with TYLCV and TYLCSV. After at least 4-month post inoculation, when LSRec recombinants became dominant according to qPCR estimations (see below), they were used as LSRec source plants for whitefly transmission; to maximize LSRec transmission, we used only plants in which the LSRec content was higher than that of parental vi- ruses. Source plants infected with TYLCV-IS76 were growned in parallel and used with the same delay after agro-inoculation.

Adult Med whiteflies collected from synchronised rearing on

eggplants were given an acquisition access period (AAP) of 2 days on LSRec or TYLCV-IS76 source plants. Whiteflies from both source plants were then transferred together to 15-day old resistant tomato plants fora 5-day inoculation access period (IAP). The experiment was performed three times; number of source plants, test plants and insects used in each experiment are described in Table 1.

2.4. Transmission efficiency of TYLCV and TYLCV-IS76

Adult Med and MEAM1 whiteflies collected from synchronised rearing on eggplants were given a 3-day AAP on tomato plants of the cultivar Monalbo at one month after their agroinoculation with TYLCV or TYLCV-IS76. Female whiteflies were then transferred to 18-day old datura plants, 5 whiteflies per recipient plant, for a 4-day inoculation access period (IAP); datura plants were used rather than tomato plants, because under the small cages used for IAP, there is less whitefly mor- tality with datura. Twenty recipient plants were used for each of the four virus/whitefly species combination. MEAM1 whiteflies collected at the end of the first IAP were transferred for a second 4-day IAP to 10 datura plants for TYLCV-IS76 and 11 for TYLCV, 5 whiteflies per plant. The transmission success was assessed by the leaf curl symptoms induced by the viruses. The symptom-based diagnostic was validated by a virus- specific PCR test on a set of symptomatic and non-symptomatic plants.

2.5. Sampling and DNA extraction

Leaf samples from each plant consisted of five 4-mm diameter leaf discs, collected on the youngest leaf for which five leaflets were visible; one disc per leaflet. DNA was extracted with the protocol of Dellaporta et al. (1983) modified as follows: leaf tissue was ground in 400 μL extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 10% SDS, 0.8% w/v Na_2SO_3, and 100 mg/ml RNase), incubated at 65 °C for 10 min and centrifuged (16,000 g 10 min). One volume of cold isopropanol was added to 300 μL of the supernatant and nucleic acids were precipitated by centrifugation (16,000 g, 20 min); the pellet was washed with ethanol 70% and then resuspended in 250 μL sterile bidistilled water. DNA extracts were used for virus quantification, or stored at —20 °C for further use.

2.6. Quantification of viral DNA

The level of viral accumulation in each plant was estimated by quantitative real-time PCR using the LightCycler 480 SYBR Green I qPCR master mix (Roche, Germany). The qPCR reaction was performed in a final volume of 10 μL containing 2 μL of total DNA extract diluted 1:20, the Mastermix of Roche, and primers. Primers used for quantification of TYLCV, TYLCSV and TYLCV-IS76 are those described in Belabess et al. (2016). TYLCV/TYLCSV recombinants for which the fragment inherited from TYLCSV was longer than 178 nt (LSRec), were quantified with a pair of heterologous primers (TYLCV/TYLCSV) as shown in Fig. 1. These primers do not detect TYLCV-IS76, the recombinant that typically exhibit a shorter TYLCSV derived fragment. The tomato 25S rRNA gene was used to standardize virus quantification with respect to plant DNA content. Primers are described in Table 2 and Fig. 1. Two technical repeats were performed for each DNA extract.

The qPCR was run in 384-well plates using a LightCycler 480 (Roche, Germany) with the following cycling conditions: 95 °C for 10 min followed by 40 cycles consisting each of a 10s denaturation step at 95 °C, a hybridization step detailed in Table 1, and a 15s elongation step at 72 °C.

The qPCR results were analyzed with the LinReg computer program (Ruijter et al., 2009), which calculate the initial concentration N_0 for each sample, expressed in fluorescence units. The copy number of the target were interpolated from the N_0 values using the appropriate standard curve obtained for each virus with 10 fold serial dilutions of virus-specific PCR products containing the qPCR target. The dilutions ranged between 10^1 and 10^8 copies. Mock-inoculated plants were tested in parallel as negative controls. Detection thresholds were calculated for each viral target as the mean of the theoretical copy number corresponding to N_0 values obtained with mock-inoculated plants.

Table 1
Experimental conditions of three virus transmissions using Med Bemisia tabaci. The source plants were agro infected with TYLCV-IS76 (IS76) or coagroinfected with TYLCV-IL and TYLCSV-ES (IL+ES). The transmission success is reported in Table 3 and virus accumulations in recipient plants is presented in Fig. 6. Tests 2 and 3 were conducted in parallel; viruliferous whiteflies were from the same source plants.

	Number of source plants/virus source	Number of insects/virus source/recipient plant	Number of recipient plants
Test1	2	2–3	68
Test2			
Test3	2 (IS76); 3 (IL+ES)	5	73
	2 (IS76); 3 (IL+ES)	>10	14

2.7. Statistical analysis

All statistical analyses were performed using R Studio software, version 4.1.2 (R_Development_Core_Team, 2010). Viral DNA accumu- lations were compared between or within plants using the log trans- formation of viral accumulation data. ANOVA, Kruskal-wallis or Wilcoxon tests were used to compare viral accumulation between different infection status and sampling dates in each experiment. The R package "car" was used for Anova and the package "rstatix" was used for Kruskal-wallis and Wilcoxon test. Differences were considered statistically significant when P-value < 0.05.

3. Results

3.1. Plant age did not affect the competitiveness of TYLCV-IS76

The competitiveness of TYLCV-IS76 was determined to be higher than that of parental viruses when 15 day-old resistant tomato plants were infected (Belabess et al., 2016). To test a potential effect of plant age at the time of inoculation, the test was performed again with plants co-inoculated with TYLCV, TYLCSV and TYLCV-IS76 at 15, 45 and 135 days after seed sowing. Plants were sampled at one and four mpi and virus content was measured by qPCR.

Consistently with previous results (Belabess et al., 2016), when plants are co-inoculated at 15 days, TYLCV-IS76 exhibited the highest accumulation followed by TYLCSV and TYLCV in a decreasing order (Fig. 2). A similar accumulation profile was detected with the plants co-inoculated at 45 or 135 days after sowing (Fig. 2), indicating that plant age at virus inoculation has no significant effect on virus accumulations. In particular, the deleterious effect of TYLCV-IS76 on TYLCV accumulation is detected irrespective of plant age and the duration of infection period.

3.2. LSRec, a pool of underground competitors

Due to the high recombination propensity of geminiviruses, competition between geminiviruses are expected to be influenced by non-inoculated competitors, i.e. the *de novo* generated recombinants between coinoculated viruses. It is presumed that the competition by which TYLCV-IS76 has replaced parental viruses included LSRec as additional competitors because plants coinfected with TYLCV and TYLCSV were relatively common in Morocco during the pre-emergence period (1998–2003) (Belabess et al., 2015). Consistent with previous results (Belabess et al., 2018), LSRec were detected by qPCR in tomato plants coinoculated with TYLCV and TYLCSV, irrespective of the plant cultivar, Ty-1 resistant or susceptible (Fig. 3). In both cultivars, LSRec

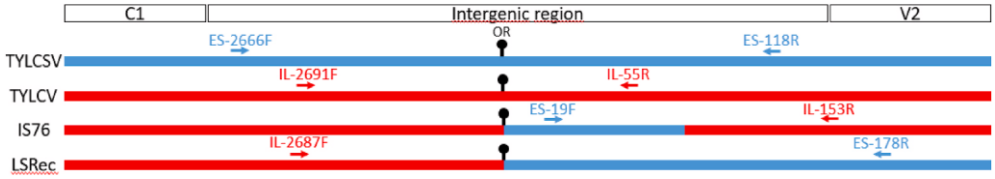

Fig. 1. Positions of qPCR primers on the targeted viral genomes, i.e. TYLCSV-ES (ES), TYLCV-IL (IL), TYLCV-IS76 and LSRec. The genome fragment presented is between the 5′ ends of C1 and V2 ORFs framing the intergenic region. IL sequences and IL specific primers are in red and those of ES are in blue. OR, origin of replication.

Table 2
Description of the primers used for qPCR quantifications of viral DNA and a control plant gene. Primer names specifies the nucleotide position of the targeted genome to which the terminal 5' nucleotide of the primer is designed to hybridize. F and R designate forward and reverse primers. The forward primers for viral DNA amplification are the primers identical to the viral strand.

qPCR target	Primer name	Primer sequence	Primer concentration	Hybridization
TYLCV-IL	IL-2691F	5′-AATGGCTATTTGGTAATTTCG-3′	800 nM	63 °C - 40s
	IL-55R	5′-CGTCTGTGGAACCCTCG-3′		
TYLCSV-ES	ES-2666F	5′-AGATTGGTAGCTCTTATATACTTG-3′	800 nM	60 °C - 40s
	ES-118R	5′-GAAGCCAAGTTTATAACAAAGT-3′		
TYLCV-IS76	ES-19F	5′-CCGATAAAGTAGTAGGCCCTACGCA-3′	300 nM	63 °C - 20s
	IL-153R	5′-AGTGGGTCCCACATATTGCAAGAC-3′		
LSRec	IL-2687F	5′-CCTAATGGCTATTTGGTAATTTCG-3′	600 nM	52 °C - 30s
	ES-178R	5′-CTGAATCTGGAAATTCATTTAATAAAGGA-3′		
25S rRNA tomato gene	25S–1137F	5′-AGAACTGGCGATGCGGGATG-3′	300 nM	60 °C - 20s
	25S-1297R	5′-GTTGATTCGGCAGGTGAGTTGT-3′		

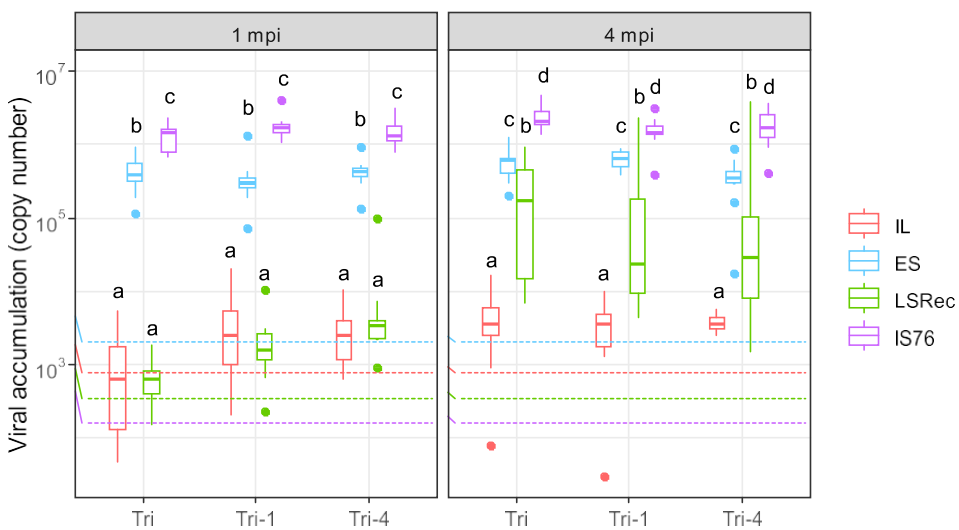

Fig. 2. DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES), TYLCV-IS76 (IS76) and LSRec in Ty-1 resistant tomato plants (cultivar Pristyla) co-agroinoculated with IL, ES and IS76 at two week (Tri), one month (Tri-1) or four months (Tri-4) after sowing. Viral DNA was quantified by real-time PCR from leaf samples collected at one and four month post inoculation (mpi). The viral accumulation is expressed in number of viral copies and the ordinate axis is in logarithm scale. The boxes delimit the 25% and 75% quantiles, the horizontal lines in the boxes represent the medians, the vertical lines below and above the boxes indicate the range of the values and the points outside the boxes correspond to outliers. The dotted lines indicate the mean values obtained with 35 mock-inoculated plants (15 susceptible and 20 resistant) tested with the primers targeted to the genomes of TYLCV-IL (in red), TYLCSV-ES (in blue), TYLCV-IS76 (in purple) and LSRec (in green). Letters indicate if virus contents are statistically different within plants; different letters indicate significant differences (P ≤ 0.05).

were detected from 1 mpi, and the average content of LSRec at 5 mpi was significantly higher than that of the parental viruses. Consistent with the 10 times higher accumulation of parental viruses in susceptible tomato plants compared to resistant ones, the LSRec amount in sus- ceptible plants was 10 times higher as well which may reflect the replication-driven generation of recombinants (Jeske et al., 2001).

3.3. Delayed infection of TYLCV-IS76 does not impede its dominance over parental viruses

To reconstruct the emergence scenario of TYLCV-IS76, its competitiveness was also tested in experiments aiming at reproducing pre-emergence situations when parental viruses were more frequent than TYLCV-IS76. At that time, we presume that TYLCV-IS76 was inoculatedin plants that were already infected with parental viruses. We previously showed that TYLCV-IS76 is fitter than parental viruses when inoculated simultaneously (Belabess et al., 2016). Here, we tested the competitiveness of TYLCV-IS76 in superinfection. To do this, 15 day-old resistant tomato plants were co-agroinoculated with TYLCV and TYLCSV and subsequently at 1 or 4 mpi the infected plants were super-inoculated with TYLCV-IS76. Some of the plants coinfected with TYLCV and TYLCSV that were not super-inoculated and plants coinoculated simul- taneously with TYLCV, TYLCSV, and TYLCV-IS76 were used as controls. Leaf samples were collected over time and viral DNA loads were measured by qPCR. Virus combinations, date of sampling and plant age are summarized in Fig. 4.

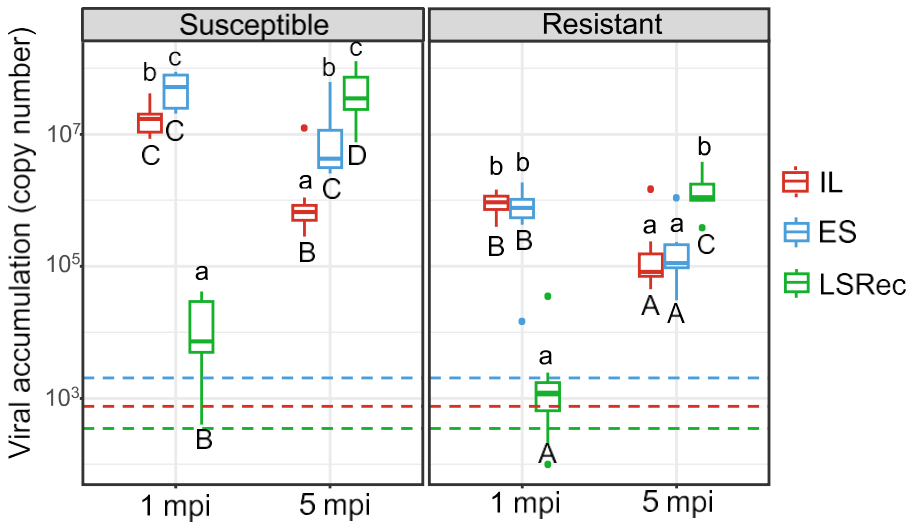

Fig. 3. DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES), and LSRec in susceptible and Ty-1 resistant tomato plants (cv. Pristyla) co- agroinoculated at two weeks after sowing with IL and ES. Viral DNA was quantified by real-time PCR from leaf samples collected at one and five month post inoculation (mpi). Viral accumulations, box plots and positive thresholds as in Fig. 2. Letters indicate if virus contents are statistically different. Small letters correspond to comparisons of different viruses within plants, whereas capital letters corre- spond to comparisons of the same virus between plants of different cultivars and collected at different sampling times; different letters indicate significant differences ($P \leq 0.05$)

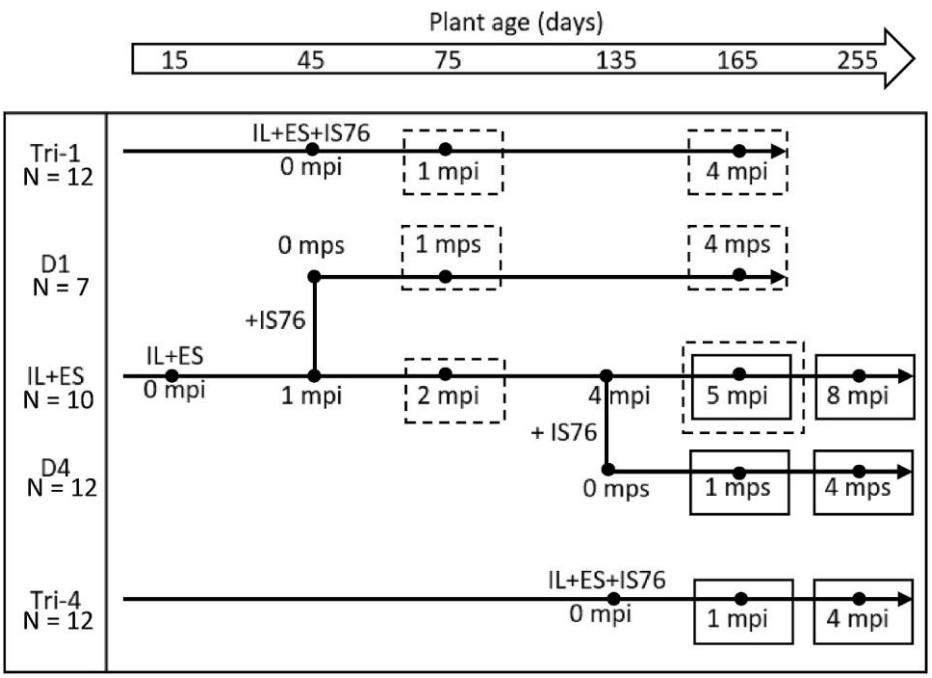

Fig. 4. Schematic representation of the agro-inoculation and sampling schedules of super-infected tomato plants (D1, D4) and of non-super-infected plants used as controls (IL + ES, Tri-1, Tri-4). IL + ES: fifteen-day-old Ty-1 resistant to- mato plant (cv. Pristyla) were co-agroinoculated with TYLCV-IL (IL) and TYLCSV-ES (ES) and then sampled at different month post inoculation (mpi). D1: Some IL + ES plants were super-agroinoculated with TYLCV-IS76 (IS76) at 1 mpi and sampled at 1 and 4 month post superinfection (mps). D4: same as D1 except that IS76 was super-agroinoculated at 4 mpi. Tri-1: 45 day-old Pristyla plants were co-agroinoculated with IL, ES and IS76 and sampled at 1 and 4 mpi. Tri-4: same as Tri-1 except that plants were co-agroinoculated at 135 days. The dotted boxes specify the sampling dates of D1 and those of the corresponding controls, i.e. IL + ES and Tri-1. The full line boxes specify the sampling dates of D4 and the corresponding controls, i.e. IL + ES and Tri-4.

The delayed inoculation with TYLCV-IS76 did not impede its infec- tivity. Indeed, all the super-inoculated plants were detected positive for TYLCV-IS76, irrespective of the superinfection delay. Moreover, what- ever the delay, the accumulation of the superinfected TYLCV-IS76 wasin the same range as that of parental viruses at 1 month post superin- fection (mps) and significantly higher at 4 mps (Fig. 5A, D1; Fig. 5B, D4). TYLCV-IS76 reached similar accumulation levels regardless of its inoc- ulation timing, together with parental viruses or with delays (Fig. 5A, Tri-1; Fig. 5B Tri-4). In addition, the deleterious effect on TYLCV accu- mulation, the hallmark of TYLCV-IS76, was also observed in superin-fection conditions. Noteworthy, an initial test with the same experimental design was performed (Table S1, Fig. S1), in which plants were in a very poor condition due to a fertilization defect. In spite of this serious defect, similar results were obtained, i.e., TYLCV-IS76 accumu- lated more than parental viruses and its deleterious effect on TYLCV was observed regardless of superinfection delays.

3.4. TYLCV-IS76 and LSRec exhibit mostly neutral interactions

When TYLCV-IS76 was super-inoculated in the plants that were co-inoculated 1 or 4 months before with TYLCV and TYLCSV, TYLCV- IS76 had to cope not only with the parental viruses but also with the de-novo generated TYLCV/TYLCSV recombinants, i.e. LSRec.

In plants superinfected with one-month delay (Fig. 5A), when theamount of LSRec was significantly lower than the amount of parental viruses (Fig. 5A, 1mps), LSRec was not the major competitor. Unex- pectedly, the build-up of LSRec as observed in plants infected by parental viruses only (IL ES) (Fig. 5A, IL ES, 4 mps), was not hampered by the superinfection of TYLCV-IS76 (Fig. 5A, D1, 4 mps); indeed, the amount of LSRec was similar between D1 and IL ES plants at 4 mps. The significantly lower LSRec accumulation in plants where parental viruses and TYLCV-IS76 were simultaneously coinoculated (Fig. 5A, Tri1) is likely due to an indirect effect of TYLCV-IS76 mediated by its negative impact on TYLCV accumulation. Thus, with less TYLCV

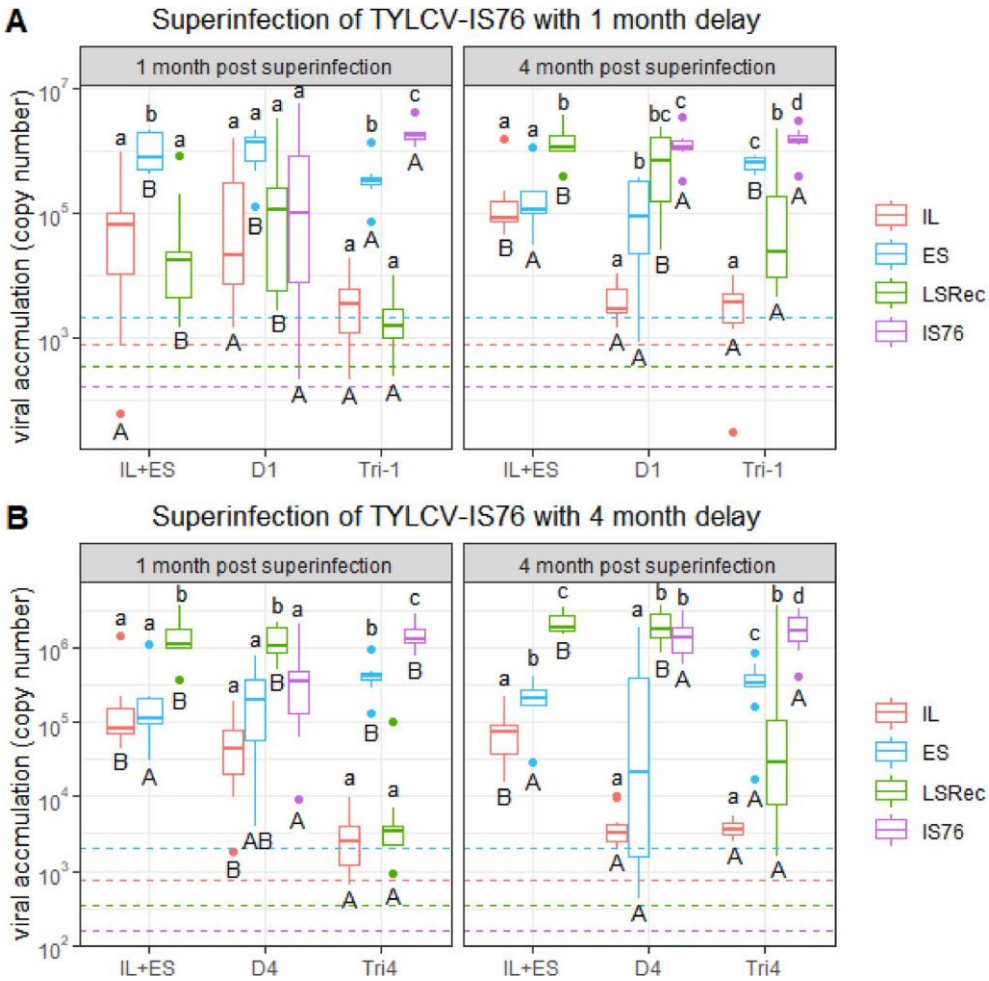

Fig. 5. DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES), TYLCV-IS76 (IS76) and LSRec in Ty-1 resistant tomato plants (cv. Pristyla) following the various agro-inoculation schemes described in Fig. 4. "IL + ES" corresponds to plants co-agroinoculated with IL and ES. "Tri" corresponds to plants co-agroinoculated with IL, ES and IS76. "D" corresponds to IL + ES plants in which IS76 was super-agroinoculated. [A] DNA accumulations in IL + ES plants that were super-agroinoculated with IS76 with one-month delay (D1). Viral DNA contents were monitored on leaf samples collected on D1 plants at one- and four-month post superinfection and compared with viral contents of relevant controls (IL + ES and Tri-1) as described in Fig. 4. [B] Same as [A] except that the super-agroinoculation was performed with a four-month delay. Viral DNA was quantified by real-time PCR. Viral accumulations, box plots and positive thresholds as in Fig. 2. Letters indicate if virus contents are statistically different. Small letters correspond to comparisons between different viruses within plants, whereas, capital letters correspond to comparisons of the same virus between treatments at the same sampling date; different letters indicate significant differences ($P \leq 0.05$).

parent, the interspecies recombination is apparently less frequent.

In plants superinfected with four months delay, when the amount of LSRec was significantly higher than the amounts of parental viruses (Fig. 5B, 1 mps), LSRec is potentially a major competitor. However, in spite of the dominance of LSRec, the accumulation of TYLCV-IS76 in superinfected plants at 4 mps (Fig. 5B, D4, 4 mps) was not significantly different from plants in which viruses were simultaneously inoculated (Fig. 5B, Tri4, 4 mps). At 4 mps, TYLCV-IS76 accumulated up to the level of LSRec (Fig. 5B, D4); thus, like in the D1 plants (Fig. 5A, D1, 4 mps), the amount of TYLCV-IS76 was not significantly different from that of LSRec at 4 mps.

3.5. *TYLCV-IS76 is more competitive than LSRec following whitefly vector transmission*

TYLCV-IS76 and LSRec exhibited similar competitiveness in superinfection tests (Fig. 5). However, it cannot be excluded that the results may be skewed because of the contrasted routes in which protagonists entered into the competition, i.e. agro-inoculation of TYLCV-IS76 versus de-novo generation of LSRec. To avoid this potential bias, the compet- itors were both co-inoculated into recipient tomato plants using viru- liferous whitefly vectors that were fed separately on plants coinfected with IL, ES and LSRec and plants infected with IS76 alone. The plants on which whiteflies were given AAPs were compared for viral

Table 3
Description of the infectious status of Ty-1 resistant tomato plants (cv. Pristyla) inoculated by whitefly vectors. Recipient plants were co-inoculated with whiteflies from two types of source plants, tomato plants agroinfected with TYLCV-IS76 (IS76) and tomato plants co-agroinfected by TYLCV-IL (IL) and TYLCSV-ES (ES). As the source plants were used several months after their agroinfection - 5 months for test 1 and 9 months for tests 2 and 3 - the IL + ES co-infected plants contained LSRec naturally generated over time during infection. The source plants were from the susceptible isogenic cultivar.

	Number of plants of each infection status/number of recipient plants							Number of virus-positive plants/number of recipient plants			
	IS76	LSRec	LSRec +ES	LSRec +IL	LSRec +ES +IS76	IS76 +ES	LSRec	IS76	LSRec	IL	ES
Test 1	11/68 (16%)	6/68 (8.8%)	2/68 (2.9%)	2/68 (2.9%)	1/68 (1.5%)	0/68 (0%)	1/68 (1.5%)	13/68 (19%)	12/68 (18%)	2/68 (2.9%)	3/68 (4.4%)
Test 2	2/73 (2.7%)	2/73 (2.7%)	3/73 (4.1%)	0/73 (0%)	1/73 (1.4%)	2/73 (2.7%)	2/73 (2.7%)	7/73 (9.6%)	8/73 (11%)	0/73 (0%)	7/73 (9.6%)
Test 3	0/14 (0%)	0/14 (0%)	2/14 (14%)	0/14 (0%)	1/14 (7.1%)	1/14 (7.1%)	7/14 (50%)	9/14 (64%) (71%)	10/14	0/73 (0%)	10/14 (71%)
All tests	**13**	**8**	**7**	**2**	**3**	**3**	**10**	**31%**	**33%**	**1%**	**28%**

accumulations (Supplementary Fig. S2). LSRec accumulated at a higher level than parental viruses. Moreover, LSRec accumulated more than TYLCV-IS76 with a ratio ranging from 1.05 to 3.35.

The infection status of the vector inoculated plants is presented in Table 3. The transmission efficiency of TYLCV-IS76 and LSRec was similar in each test (Table 3). Thirteen recipient plants were qPCR positive only for TYLCV-IS76 and eight were positive only for LSRec. The qPCR measurements of these single infected plants indicate that the accumulation of TYLCV-IS76 is significantly higher than that of LSRec (Fig. 6). Thus, the slightly higher accumulation of LSRec in the source plants did not result in any higher accumulation in recipient plants. As only three plants exhibited the expected TYLCV-IS76/LSRec pattern, the relative accumulation of the competitors had to be statistically compared in the 10 plants which were infected not only with the competitors but also with TYLCSV. In those 10 plants, the accumulation of TYLCV-IS76 was significantly higher than that of LSRec. To disentangle the potential effect of TYLCSV on this accumulation pattern, it is worth noting that the accumulation level of LSRec was similar irrespective of the treatments: LSRec, LSRec + TYLCSV, LSRec + TYLCSV + TYLCV-IS76. On the contrary, the accumulation of TYLCV-IS76 tended to be higher in co-infected plants (Kruskal-Wallis, P-value = 0.054) than in single infected plants. The higher accumulation of TYLCV-IS76 in coinfected plants may be due to a synergism between TYLCSV and TYLCV-IS76 as previously reported (Belabess et al., 2016) or by a synergism between TYLCV-IS76 and LSRec.

3.6. Transmission efficiency of TYLCV-IS76 and TYLCV are similar

To test if a higher transmission efficiency has contributed to the emergence of TYLCV-IS76 in Morocco, we compared its transmission efficiency with that of TYLCV, previously reported to be the most competitive parental virus in Ty-1 resistant tomato plants and the most efficiently transmitted parental virus (Belabess et al., 2016; García-Andrés et al., 2009; Monci et al., 2002; Sánchez-Campos et al., 1999). Source plants used for whitefly acquisitions of TYLCV and TYLCV-IS76 were shown to exhibit similar virus accumulations. With MEAM1 whiteflies, TYLCV-IS76 was transmitted to 18 plants and TYLCV to 20 plants. With Med whiteflies, TYLCV-IS76 was transmitted to 9 plants and TYLCV to 10 plants. The lower transmission efficiency ob- tained with Med whiteflies was correlated with a lower survival during IAP; at the end of the 5 day-IAP the average number of living whiteflies per plant was 3 for MEAM1 and 1.5 for Med. There were enough living whiteflies of the MEAM1 species to compare the persistence of virusinfectivity between TYLCV-IS76 and TYLCV. TYLCV-IS76 was transmitted to 10 plants out of 10 and TYLCV to 11 plants out of 11 after the second IAP. Altogether, these results did not reveal any advantage of TYLCV-IS76 over TYLCV regarding transmission features under our laboratory conditions.

4. Discussion

4.1. TYLCV-IS76, the last becoming first

The selection-driven emergence of the recombinant TYLCV-IS76 in Morocco is a clear illustration of the potential impact of homologous recombination on virus evolution (Belabess et al., 2016, 2015). Before 2004, TYLCV-IS76 was not detected, and after 2011, it replaced its parental viruses in southern Morocco. Based on the above-described and previous findings, the dynamics of TYLCV-IS76 can be summarized as follow: "the last shall be first", i.e., the variant that arrived last, is finally first regarding prevalence. Noteworthy, this summary applies to the population dynamics at both the agroecosystem level and the plant level. Indeed, although TYLCV-IS76 was inoculated the last in plants pre-infected with parental viruses (Fig. 5), TYLCV-IS76 became the first regarding intra-plant frequency. The same result was observed with both superinfection delays (1 and 4 months), which indicate that this intra-plant population dynamics is robust, and thus, may have been a driver of the emergence dynamics observed at the agroecosystem level. According to previous results suggesting a positive correlation between TYLCV accumulation in tomato plants and transmission efficiency with whiteflies (Lapidot et al., 2001), it is thought that a relatively higher intra-plant frequency of TYLCV-IS76 in co-infected plants has induced a relatively higher transmission probability and in turn a higher invasiveness. In agreement with this result, only TYLCV-IS76 was transmitted by *B. tabaci* whiteflies from TYLCV/TYLCV-IS76 co-infected plants containing 2000 times more TYLCV-IS76 than TYLCV (Belabess et al., 2016).

4.2. A non-aggression pact between LSRec and TYLCV-IS76

The LSRec recombinants monitored in this study are similar to the TYLCV/TYLCSV recombinants usually detected from tomato plants coinoculated with TYLCV and TYLCSV (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007b, 2009). LSRec were often detected from the field where plants coinfected with TYLCV and TYLCSV are relatively frequent, as reported from Spain (García-Andrés et al., 2007a; Monci et al., 2002), Italy (Davino et al., 2012, 2009), Tunisia (Mnari-Hattab et al., 2014) as well as Morocco (Belabess et al., 2015).The viral environment in which TYLCV-IS76 emerged contained parental viruses as well as LSRec (Belabess et al., 2015). Indeed, before

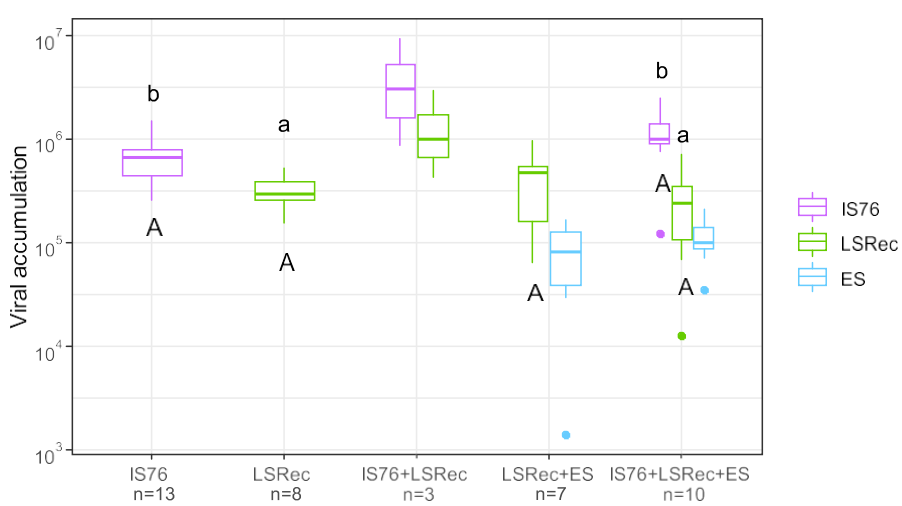

Fig. 6. DNA accumulations of TYLCV-IS76 (IS76), TYLCSV-ES (ES) and LSRec in Ty-1 resistant tomato plants (cv. Pristyla) coinoculated with whiteflies that were given an AAP on plants agroinfected with IS76 and other whiteflies on plants co-agroinfected with ES and TYLCV-IL. Viral DNA was quantified by real-time PCR from leaf samples collected at one month post infection (mpi). Viral accumulations and box plots as in Fig. 2. Fifteen Ty-1 resistant mock inoculated plants were used as negative controls; the mean values obtained with these control plants are between 10^2 and 10^3 on the viral accumulation scale. Letters indicate if virus contents are statisti- cally different. The small letters correspond to comparisons between IS76 and LSRec in the mono-infected plants or in the co-infected plants whereas the capital letters correspond to comparisons of the same virus (IS76 or LSRec) between the different infection status; different letters indicate significant differences ($P \leq 0.05$).

2004, when TYLCV-IS76 was not yet detected, LSRec was detected in 7 out of 46 tomato plants testing positive for TYLC disease viruses. Thus, TYLCV-IS76 has presumably encountered LSRec at certain stages of the emergence period. According to the qPCR test designed here to quantify LSRec load, their accumulation was significantly higher than that of parental viruses at 5 mpi (Fig. 3), suggesting that LSRec had the po-tential to be a major rival of TYLCV-IS76. In previous competition tests, in which TYLCV-IS76 and parental viruses were simultaneously inocu- lated (Belabess et al., 2016), the potential effect of LSRec was probably minor because under these experimental conditions, LSRec accumulated less than parental viruses (Fig. 2). However, when TYLCV-IS76 is superinfected in plants already infected with parental viruses, the effect of LSRec could be assessed (Fig. 5). Indeed, when superinfection occurred with 4-month delay, the accumulation of LSRec was signifi- cantly higher than that of parental viruses. The qPCR monitoring of virus accumulations showed that the resident viruses and particularly LSRec did not reduce the infectivity of TYLCV-IS76 and most importantly, did not prevent the increase of its content above the content of parental viruses. Thus, in spite of their high accumulation, LSRec did not affect the fitness of TYLCV-IS76. Likewise, as shown in the experiment with 1-month superinfection delay, TYLCV-IS76 did not affect the accumu-
lation of LSRec. Altogether, the results indicate neutral interactions between TYLCV-IS76 and LSRec.

4.3. LSRec, the last becoming first but only at the plant level

Unlike TYLCV-IS76 which was inoculated as a clone, LSrec, gener-ated *de novo* during plant infection, is a pool of recombinants exhibiting a large range of recombination profiles (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007b). The LSRec targeted with the qPCR primers, are TYLCV/TYLCSV recombinants that inherited TYLCSV genomic fragments beyond the nucleotide position 178 (Fig. 1). In spite of their different nature (clonal versus heterogeneous) and distinctive recombination breakpoints (position 76 versus multiple positions beyond position 178), LSRec and the superinfected TYLCV-IS76 exhibit similar accumulation dynamics characterized by loads significantly exceeding the loads of parental viruses (Fig. 5). These increases of viral loads are all the more remarkable since both LSRec and TYLCV-IS76 arrived last after parental viruses. In spite of their similar behaviour at the plant level, their fate at the agroecosystem level in southern Morocco is completely different. Indeed, whereas TYLCV-IS76 became dominant, LSRec was displaced like parental viruses in resistant plants (Belabess et al., 2015). After the emergence of TYLCV-IS76, only two plants tested positive for LSRec out of 131 tomato and non-tomato plants sampled between 2012 and 2014. This result reflects a concomitant decline of LSRec and parental viruses which is consistent with previous results from Italy showing that LSRec were always present in plants coinfected with parental viruses and were never found alone in plants (Davino et al., 2009). Before 2004, when TYLCV-IS76 was not detected, a similar situation was observed in Morocco. However at an early stage of the emergence that could be monitored in non-Souss regions of Morocco when parental viruses were still present, 12 plants tested positive for LSRec and TYLCV-IS76 but tested negative for parental viruses (Belabess et al., 2015). Taken together, our results suggest that LSRec survived for a while during the emergence process but finally declined for at least two reasons: (i) the significant lower accumulation of LSRec compared to that of TYLCV-IS76 (Fig. 6) which in turn may result in a lower transmission efficiency and (ii) a reduction of the *de novo* generated LSRec, induced by the decrease of parental viruses; TYLCV content is decreasing in the presence of TYLCV-IS76 (Fig. 5 and Belabess et al., 2016) and TYLCSV is the parental virus which is the most affected by the Ty-1 induced resistance (Garcia Andres et al., 2009). The corollary of this second reason is that the major determinant of the high intra-plant accumulation of LSRec is rather the extremely high propensity of TYLCV and TYLCSV to recombine than a relatively higher fitness compared to parental viruses. Finally, the disappearance of LSRec suggest that TYLCV-IS76 could not replace TYLCV as donor of TYLCV fragments although TYLCV-IS76 genome is made of 97% of TYLCV sequences, and notably within the genomic region where LSRec genomes derive from the TYLCV parent. The propensity of TYLCV-IS76 to recombine may be relatively low, a presumption supported by the results presented in Fig. 5. Indeed, when plants are simultaneously inoculated with TYLCV, TYLCSV and TYLCV-IS76, the LSRec amount is lower than in plants only inoculated with parental viruses. Moreover, according to preliminary results, LSRec amount was lower in plants coinoculated with TYLCV-IS76 and TYLCSV than in plants coinoculated with TYLCV and TYLCSV (data not shown).

4.4. Vector transmission may not be a selective factor in the emergence scenario

For viruses infecting annual plants like tomato begomoviruses, transmission between plants is a crucial step for their survival and dissemination in the agroecosystem. Thus, it could not be excluded that contrasted transmission rates may have contributed to the emergence of TYLCV-IS76 or to the decline of LSRec. However, none of our experi-mental results supported these hypotheses. The transmission rate of TYLCV-IS76 was similar to that of TYLCV, the parental virus that was previously reported to be transmitted the most efficiently by whiteflies (Sánchez-Campos et al., 1999). Noteworthy, the TYLCV inherited region of the TYLCV-IS76 clone exhibit 27 mutations compared to the cognate region of the TYLCV clone, including 4 non-synonymous mutations in the CP. Our results suggest however that none of these differences was critical for transmission efficiency. Transmission efficiency was also similar between LSRec and TYLCV-IS76. Indeed, the vector transmission that was performed to compare their competitiveness revealed similar transmission rates with 31% for TYLCV-IS76 and 33% for LSRec (Table 3).

4.5. Conclusion and prospects

The results obtained in this study expand our understanding of the competitiveness of TYLCV-IS76 and refine the reconstruction of its emergence scenario. The accumulation of TYLCV-IS76 was higher than that of parental viruses in all experimental conditions, including the one in which it is infected 4 months after them. Due to the stringency of this last condition, we presume that our results are representative of most natural infection scenarios. LSRec were additional players during the emergence of TYLCV-IS76. However, although their load can be higher than that of parental viruses, they did not affect the accumulation dy- namics of TYLCV-IS76 in resistant plants. According to our transmission results with the whitefly vector, the recombination event that created TYLCV-IS76 did not provide any advantage for vector transmission.

According to previous reports (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007b), the DNAs from which the LSRec qPCR target was amplified correspond to a pool of highly diverse recombinants. Although it was simplistic to consider LSRec as a generic "recombinant entity", this approach revealed that their generation and/or accumulation dynamics can be influenced by experimental conditions. Indeed when TYLCV-IS76 is coinfected with parental vi- ruses, the generation and/or accumulation of LSRec are hampered. Based on previous results showing that the dominant recombination patterns of TYLCV recombinants evolve during the infection cycle of the plant (Urbino et al., 2013), and are influenced by the host genotype (Belabess et al., 2018), it is expected that the composition of the LSRec population may be influenced by the duration of the infection cycle, viral environment, and particularly the presence of TYLCV-IS76. This needs to be further investigated.

When TYLCV-IS76 was superinfected in plants that were already infected with TYLCV and TYLCSV, its infectivity and accumulation dy- namics did not substantially differ from that of a simultaneous infection. Considering that cross protection were reported with geminiviruses (Dai

et al., 2022; Reddy et al., 2012), it is surprising that TYLCV, with a genome sequence that is 99% identical to the genome of TYLCV-IS76, did not induce some cross protection against TYLCV-IS76. Further studies are needed to determine if this result reflects a fitness featurethat is typical to TYLCV-IS76, or if it is a general feature of begomovi- ruses in our experimental system.

Investigation of the determinants of the competitive advantage of TYLCV-IS76 is at a very early stage. TYLCV variants modified in the region homologous to the 76 nts recombinant region of TYLCV-IS76 were constructed to test if genome length, susceptibility to methyl- ation, or relief of a potential burden, may explain competitiveness (Urbino et al., 2022). However, none of the variants reproduced the competitiveness of TYLCV-IS76. Viral small interfering RNA (vsiRNA) were profiled in susceptible and Ty-1 resistant plants infected with TYLCV. Although the vsiRNA of TYLCV complementary to the 76 nts region were more abundant in the Ty-1 resistant cultivar (Voorburg et al., 2021), this region did not exhibit an increased level of cytosine methylation (Butterbach et al., 2014). Further studies are needed to understand the competitiveness of TYLCV-IS76.

Credit authorship contribution statement

Margaux Jammes: performed the experiments, analyzed the data, wrote the paper. **Cica Urbino and Michel Peterschmitt:** conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper. **Mame Boucar Diouf:** performed the experi- ments, analyzed the data.

Declaration of competing interest

The authors declare that they have no known competing financialinterests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements:

The study was supported by ANR through the PRIMA project "Pre- vention and control of new and invasive geminiviruses infecting vege- tables in the Mediterranean", by the Key Initiative Montpellier University on Risks & Vectors (KIM-RIVE 2021), by Gautier Semences (France), and CIRAD (https://www.cirad.fr/). The PhD grant of Mar- gaux Jammes was funded by the ANR project. We are thankful to François Bertaux for the supply of the MEAM1 population of *Bemisia tabaci*, and to Nathalie Laboureau, Martine Granier, Sophie Le Blaye and Axel Cremades for technical assistance. Mikhail Pooggin and Clémence Plissonneau are acknowledged for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2022.11.006.

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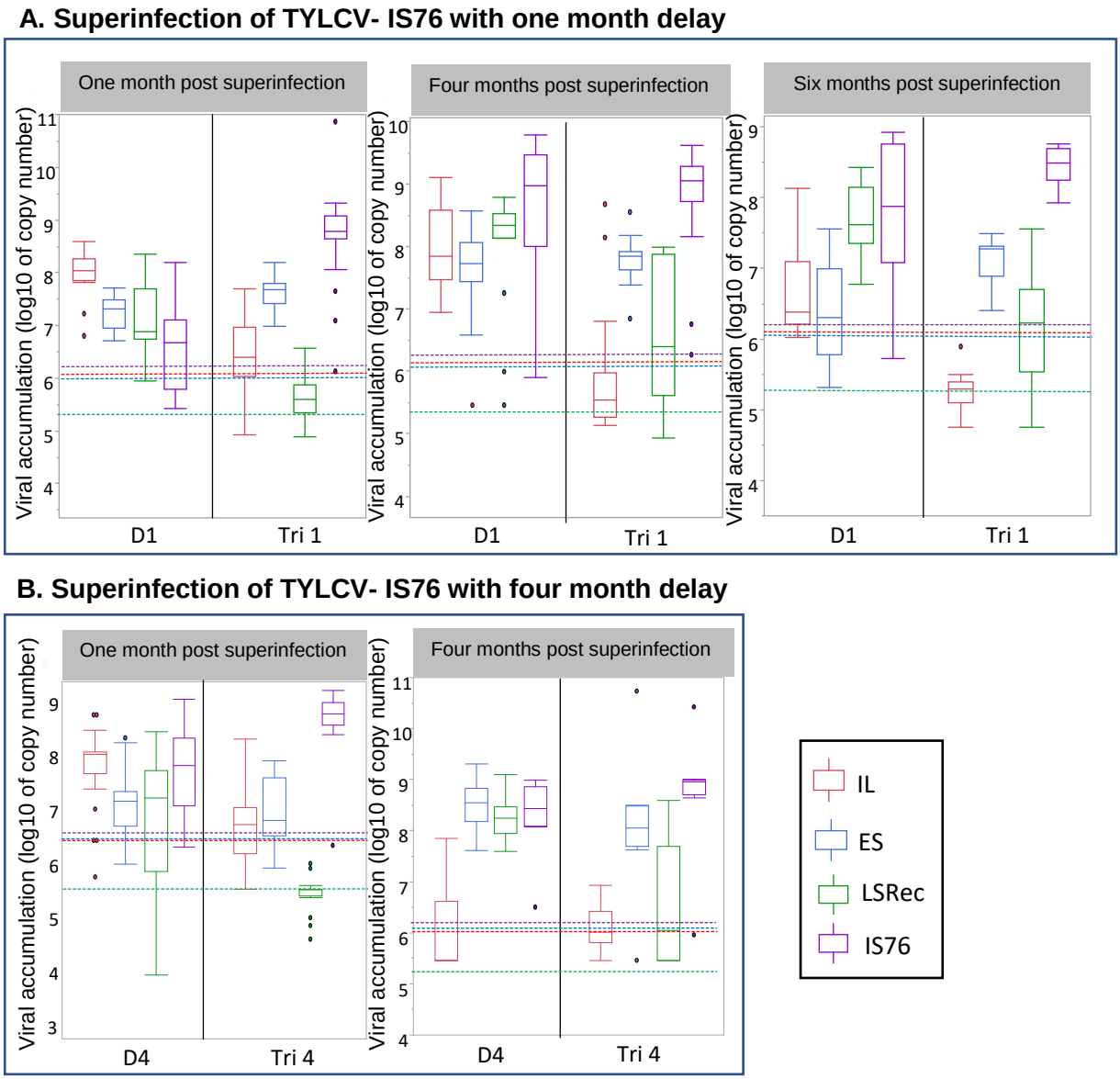
Supplementary Material

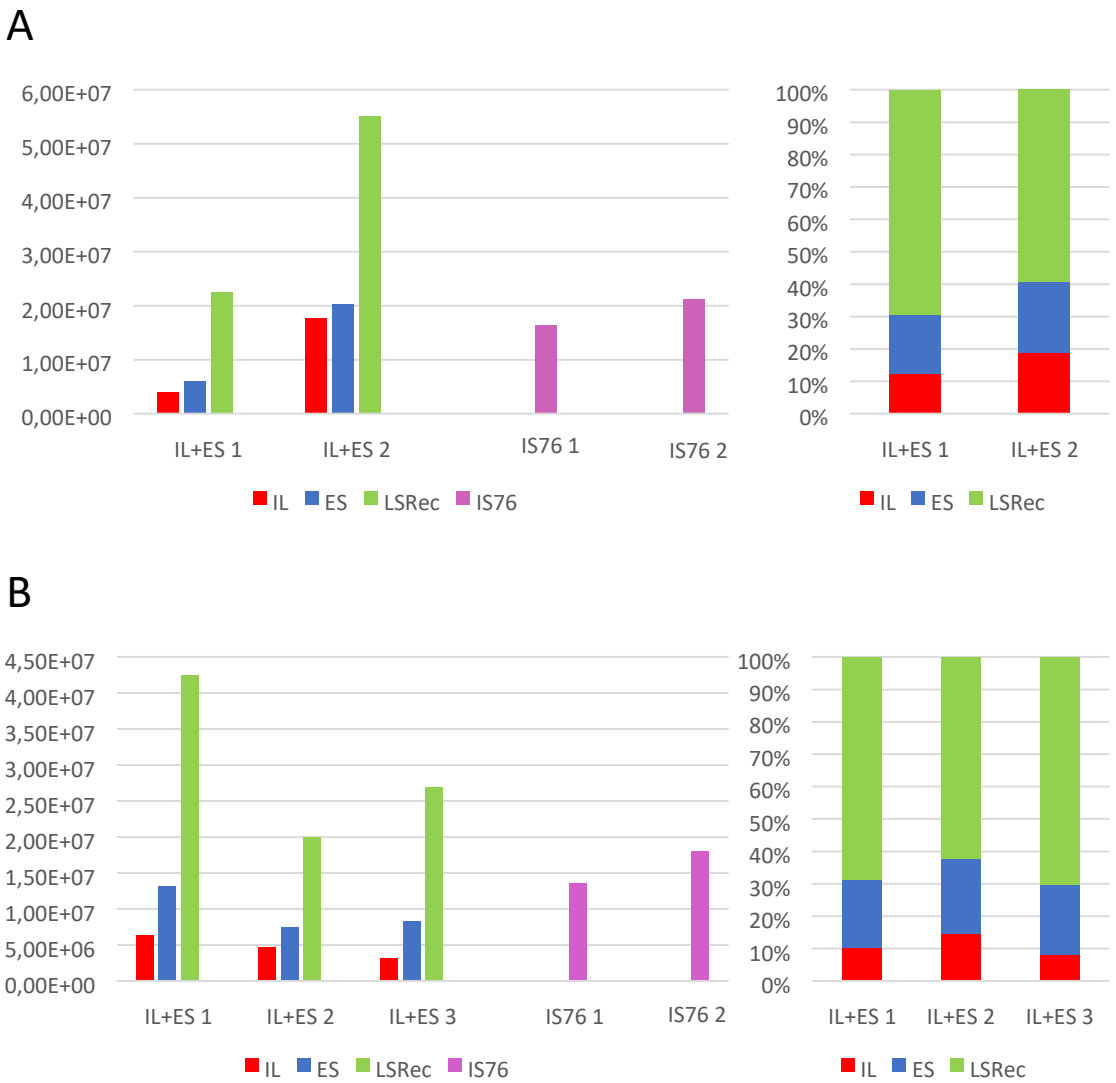
Supplementary Table S1: Description of the initial superinfection experiment (Experiment 0) that was repeated in this study (Experiment 1) to confirm the accumulation dynamics of the superinfected TYLCV-IS76. Experiment 0 was repeated because test plants suffered from a fertilization defect which severely affected their growth. The table indicate the number of plants tested in each treatment and from each sampling time. "Tri 1" and "Tri 4" correspond to Ty-1 resistant tomato plants (cv. Pristyla) co-agroinoculated with TYLCV-IL (IL), TYLCSV-ES (ES), and TYLCV-IS76 (IS76) at 45 or 135 days after sowing, respectively. "D1" and" D4" correspond to plants co-agroinfected with IL and ES, in which IS76 was super-agroinoculated with one-month delay and four months delay, respectively. The accumulation dynamics of viruses are presented in Fig. S1.

Treatment	Number of plants at 1 month post infection	Number of plants at 4 months post infection	Number of plants at 6 months post infection
Tri 1	30	17	10
D1	16	15	10
Tri 4	17	8	-
D4	23	9	-



Supplemental Figure S1: DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES), TYLCV-IS76 (IS76) and LSRec in Ty-1 resistant tomato plants (cv. Pristyla) following different agroinoculation conditions as described in Table S1. [A] Viral DNA contents were monitored on leaf samples collected on D1 plants collected at one, four and six-month post superinfection and compared with viral contents of Tri1 plants. [B] Viral DNA contents were monitored on leaf samples collected on D4 plants collected at one- and four-months post superinfection and compared with viral contents of Tri4 plants. The viral accumulation is expressed in the log10 of the number of viral copies. The boxes delimit the 25% and 75% quantiles, the horizontal lines in the boxes represent the medians, the vertical lines below and above the boxes indicate the range of the values and the points outside the boxes correspond to outliers. The dotted lines indicate the mean values obtained with mock inoculated plants tested with the primers targeted to the genomes of TYLCV-IL (in red), TYLCSV-ES (in blue), TYLCV-IS76 (in purple) and LSRec (in green). The numbers of plants tested in each treatment and at each time point are indicated in Table S1.

In spite the poor conditions of the test plants (fertilization defect), the superinfection success and the accumulation dynamics of TYLCV-IS76 is quite similar to that observed in Experiment 1 (Fig. 5). Indeed, the super-inoculation of TYLCV-IS76 in plants already infected with parental viruses was successful for 16 plants out of 17 for D1 and for 17 plants out of 23 for D4, and its accumulation was not hindered by the resident viruses (IL, ES) including the LSRec naturally generated by recombination before the superinfection. Thus, the accumulation of TYLCV-IS76 at 4- or 6-months post superinfection (mps) was similar between treatments regardless of its time of infection, either simultaneously with parental viruses (Tri) or with delay (D). Like in Experiment 1, the final accumulation level of LSRec was similar to TYLCV-IS76 confirming that their accumulation is not hindered by TYLCV-IS76. Finally, it can be noted in the D1 superinfection treatment, that the negative impact of TYLCV-IS76 on TYLCV-IL accumulation is delayed in Experiment 0 as comparted to Experiment 1. Indeed, whereas it is detectable at 4 mps in Experiment 1, it was detected only at 6 mps in Experiment 0.



Supplemental Figure S2: DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES), TYLCV-IS76 (IS76) and LSRec in tomato plants, infected with IS76 or co-infected with IL and ES (IL+ES). These infected plants were used as source plants for three tests in which the accumulations of LSRec and IS76 were compared following vector transmissions with B. tabaci (Tables 1 and 3, Fig 6). The histograms on the left side indicate for each source plants, the viral DNA copy numbers of each virus. The histograms on the right side represent the frequency of each virus derived from the copy numbers of viral DNA determined in the coinfected source plants. [A] Source plants of Test 1, with two plants for IL+ES and two plants for IS76. [B] Source plants of Tests 2 and 3, with three plants for IL+ES and two plants for IS76. The source plant were of the nearly isogenic susceptible cultivar (ty-1/ty-1).

CHAPITRE 2

Evidence of cross-protection between geminiviruses in tomato and on its breakdown by the invasive Tomato yellow leaf curl virus -IS76

Evidence of cross-protection between geminiviruses in tomato and on its breakdown by the invasive Tomato yellow leaf curl virus -IS76

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Abstract

Unlike ssRNA genomes for which an extensive literature is describing and analysing cross protection phenomena, there are few data for geminiviruses. Using tomato yellow curl virus (TYLCV) and variants as protecting or challenging viruses, we have shown that a first infection of tomato plants with TYLCV protects these plants against a second infection after a one-month interval, with TYLCV variants carrying modifications in the intergenic region. This result was confirmed in Ty-1 resistant tomato genotypes and a near isogenic susceptible line, using agroinoculation or insect vector inoculation or with TYLCV variants differing by 8 or 31 SNPs in the intergenic region. Unlike TYLCV-IS76, an invasive recombinant, which was shown to infect efficiently resistant tomato plants pre, infected at one-month interval with TYLCV and tomato yellow curl Sardinia virus, its parental viruses, the DNA accumulation of TYLCV-Liwa, a variant differing from TYLCV-IS76 only by the recombinant sequence was significantly reduced in the same conditions. These results indicates a possible breakdown of the cross protection by the invasive recombinant TYLCV-IS76, and shed new light on the particular feature of TYLCV-IS76 and on potential interactions prevailing in plants co-infected with begomoviruses.

1. Introduction

Thanks to the immune system, vertebrate animals and humans can be protected from viruses following a first infection. Vaccination is based on this natural phenomenon and one of the major challenges is to produce vaccines with the widest cross-protection range. Although plants do not produce antibodies, cross-protection was also detected in plants as a natural phenomenon, also known as pre-immunization or superinfection exclusion. Thus, a virus-infected plant can be protected against a subsequently inoculation of a similar virus. Cross-protection was first reported by McKinney in 1929, with Tobacco mosaic virus (TMV), a positive-sense single-stranded RNA (ssRNA+) virus, genus *Tobamovirus*, family *Virgaviridae*); the yellow mosaic symptoms caused by a particular TMV strain were repressed in a plant already infected by another strain causing light green symptoms. A few years later, the phenomenon was reported with Potato virus X (PVX) apositive sense ssRNA virus of the genus *Potexvirus* (family *Alphaflexiviridae*). Thus, potato plants infected with an avirulent strain of PVX were protected against superinfection with a virulent strain (Salaman, 1933, 1938). The subsequent report of cross protection effects in a highly diverse range of RNA and DNA viruses belonging to several genera and families (reviewed in Pechinger et al., 2019) revealed that it is a general phenomenon in plant viruses. Among RNA viruses, it includes viruses with both positive- and negative-sense ssRNA genomes. Virus architecture was also diverse, since cross protection was detected with viruses having spherical, bacilliform, rod shaped or flexuous particles and even with an enveloped virus, i.e. Tomato spotted wild virus, the negative-sense ssRNA virus of the genus *Orthotospovirus*, family *Tospoviridae* (Wang & Gonsalves, 1992).

In spite of the considerable diversity of cross protecting viruses, a common feature emerged which is that cross protection occurs only between related virus isolates. Different mechanisms were proposed to explain cross protection, which seems consistent with the contrasted biology of viruses exhibiting this phenomenon. Considering that cross protection is only effective between related virus isolates, RNA silencing was the mechanism that gained the most interest due to its sequence homology-based process. One of the supports of this mechanism is that viral mutants defective in their silencing suppressor gene provide efficient protection against challenging viruses as observed with a Tobacco mosaic virus (genus *Tobamovirus*, family *Virgaviridae*, Kubota et al., 2003) and Zucchini mosaic virus (genus *Potyvirus*, family *Potyviridae,* Lin et al., 2007). However, although the mechanism of RNA-mediated cross protection between viruses was clearly demonstrated for tobacco rattle virus genus *Tobravirus*, family *Viragviridae*) using reporter genes (Ratcliff et al., 1999), RNA silencing does not account for all cross-protection phenomena. For example, in the case of a mutant derivative of cucumber mosaic virus (CMV, genus *Cucumovirus,* family *Bromoviridae*) lacking its

silencing suppressor 2b gene, effective protection was observed only when the challenge virus is inoculated in the same leaf as the protector virus (Ziebell et al., 2007). Thus, as the protector virus was not able to trigger a strong systemic silencing signal directed against CMV-specific RNA, the authors suspected alternative mechanisms such as superinfection exclusion at the host cell level, a mechanism which was clearly demonstrated with potyviruses and potexviruses expressing fluorescent proteins (Dietrich & Maiss, 2003). Before the discovery of the RNA silencing mechanisms, cross protection was mainly explained by a mechanism involving the capsid protein as initially proven with strains of TMV (Sherwood & Fulton, 1982). The implication of the CP protein of TMV in the cross-protection phenomenon was further supported with its expression in a PVX vector (Lu et al., 1998) or in transgenic plants (Bendahmane et al., 1997).

Unlike ssRNA+ genomes (closteroviruses, tobamoviruses, potexviruses, tobraviruses, potyviruses and cucumoviruses), for which an extensive literature is describing and analysing cross protection phenomena, for geminiviruses with ssDNA genomes (family *Geminiviridae*) the literature is apparently limited to only three publications on species of the genus *Begomovirus*. Owor et al (2004) have reported a cross protective effect of a mild strain of East African cassava mosaic virus (EACMV, a bipartite member of the genus *Begomovirus*) against naturally infecting bipartite begomoviruses in field grown cassava plants. Reddy et al (2012) have reported complex interactions between African cassava mosaic virus (ACMV) and East African cassava Cameroon mosaic virus (EACMCV), two distinct bipartite begomoviruses in coagroinfected *Nicotiana benthamiana* plants. Whereas ACMV was inducing mild symptoms with a recovery phenomenon, EACMCV was inducing severe symptoms. The accumulation of EACMCV in co-infected plants was delayed and heavily decreased compared to single infection. As coinfected plants exhibited ACMV like symptoms, the plants were considered as cross protected by ACMV against the severe symptoms of EACMCV and the DNA-A component of ACMV was found to be responsible for eliciting cross-protection. The third paper about cross protection is from Dai et al (2022). Based on a more conventional cross protection experimental design with a sequential inoculation of protector and challenge strains, they have shown that a C4 gene mutated clone of Ageratum yellow vein virus (AYVV; a monopartite begomovirus) exhibiting attenuated leaf curl symptoms protects *N. benthamiana* plants against the wild type AYVV clone.

To extend the scarce cross protection results available for geminiviruses, we tested the existence of this phenomenon with a monopartite begomovirus Tomato yellow leaf curl virus (TYLCV), an economically important virus worldwide. Unlike previous cross protection studies with geminivirus clones conducted on the commonly used laboratory plant, *N. benthamiana*, the experiments were performed on tomato, the natural host of TYLCV. Moreover, to increase the significance of the study, plant infections were performed both with the artificial agroinoculation mean as well as with the

natural whitefly vector, *Bemisia tabaci*. The test of potential cross protective effects was performed by monitoring accumulations of the sequentially inoculated clones by qPCR. The results confirm and extend the results of Dai et al (2022) by showing that a first infection of a tomato plant with TYLCV protects it against a second infection with TYLCV variants carrying modifications in the intergenic region, regardless of inoculation means (agroinoculation or insect vector inoculation), tomato genotypes (TYLCV-resistant or non- resistant), and the extent of the nucleotide divergence between the sequential inoculated viral clones (8 or 31 SNPs). The results presented here together with our previous results on the invading TYLCV-IS76 recombinant, demonstrate for the first time the existence of a cross protection phenomenon in tomato against TYLCV, and indicated the possibility of its breakdown by the invasive recombinant TYLCV-IS76 (Jammes et al., 2023).

2. Material and methods

2.1. Biological Material

Plant material

Resistant tomato cultivar "Pristyla" carrying the Ty-1 resistance gene in a heterozygote state (*Ty-1/ty-1*) and a near isogenic susceptible line (Belabess et al., 2016) were used in this study. Plants were grown in containment growth chamber with 14h light at 26°C and 10h dark at 24°C. They were watered with a 15:10:30 NPK fertilizer + oligoelements solution.

Virus isolates

Four agroinfectious clones were used: a clone of TYLCV-IL [RE:STG4:04] (GenBank accession number AM409201), a clone of TYLCSV-ES [MA:Aga5a:12] (GenBank accession number LN846598), TYLCV-GC, a clone of TYLCV-IL deleted of 5 guanines and 3 cytosines in the IR at positions 40, 42, 43, 55, 61 and 48, 50, 57, respectively and TYLCV-Liwa (TYLCV-LW), a clone differing from TYLCV-IL only by the region between nucleotides 16 and 85. This region, homologous to the 76 region of TYLCV-IS76 was replaced by the homologous sequence of tomato leaf curl Liwa virus (ToLCLwV, Genbank accession number AF195782), inducing 30 SNPs between TYLCV-IL and TYLCV-LW (Urbino et al., 2022).

Agroinfectious constructs of TYLCSV and TYLCV-IL were described in Belabess et al (2015) and Belabess et al (2016) respectively. For TYLCV-GC and TYLCV-Liwa clones, tandem constructs were obtained as described in Urbino et al (2020).

2.2. Inoculation

Agroinoculation

Tomato plants were agroinoculated 14 days after sowing or 30 days after this first agroinoculation (superinoculation). Agroinoculation of 14-day old plants was performed as described in Belabess et al (2016) for single or mixed infections. For the superinoculation, 1 ml of the agrobacteria preparation was injected along the stem of the plant at 5 different levels between the collar and the last plant node. Plants used as negative controls were agroinfiltrated with a preparation of bacteria of the C58 MP90 strain of *A. radiobacter var. tumefaciens* containing an empty pCAMBIA2300 plasmid (PC).

Vector transmission

B. tabaci individuals of the putative species Med (Mediterranean, Q1 biotype) originating from Tarascon, (south of France) were used. Adults collected from synchronised rearing on eggplant (*S. melongena*) were given an acquisition access period (AAP) of 2 days on a Ty-1 resistant source plant infected with either TYLCV-IL or TYLCV-GC for 30 days. At the end of the AAP, 350 insects were transferred from each source plant per batch of 5 Ty-1 resistant tomato recipient plants pre-infected 30 days before with either TYLCV-IL, TYLCV-GC (10 plants) or PC (5 plants). This inoculation access period (IAP) lasted 5 days. The details of the vector transmission are described in Table 1.

The DNA accumulation of TYLCV-IL and TYLCV-GC were estimated in individual recipient plant 30 days after the beginning of the IAP by qPCR.

Table 1: Vector transmission experimental design

AAP			IAP			
Infection status of source plants	number of plants	number of days	Infection status of recipient plants	number of plants	number of insects	number of days
GC	1	2	IL	10	700	5
			PC	5	350	5
IL	1	2	GC	10	700	5
			PC	5	350	5

2.3. Experimental design of cross protection experiments

Four experiments have been conducted to test the cross protection between begomovirus isolates. In each experiment, 14-day old tomato plants were agroinoculated with either PC or a virus inoculum (preventing virus); thirty days later, plants were super inoculated with the second inoculum as described above (challenging virus(es)). The details of each experiment are described in Table 2.

Cross protection between TYLCV-IL and TYLCV-GC was tested in susceptible tomato plants (Experiment 1). Plants infected with TYLCV-IL or TYLCV-GC were superinoculated with TYLCV-GC (IL/GC) or TYLCV-IL (GC/IL) respectively. Controls were plants agroinoculated with the empty pCAMBIA2300 plasmid (PC) and superinoculated with either TYLCV-IL (PC/IL), TYLCV-GC (PC/GC) or

TYLCV-IL+TYLCV-GC (PC/IL+GC). The same experimental protocol was performed on Pristyla Ty-1 resistant plants with the same TYLCV isolates (Experiment 2), and with TYLCV-IL and TYLCV-LW (Experiment 3).

In Jammes et al (2023), we have shown that the invasive recombinant TYLCV-IS76 could infect resistant tomato plants pre infected with TYLCV+TYLCSV for 30 days, suggesting that the mixed infection did not protect plants against TYLCV-IS76. AS TYLCV-IS76 and TYLCV-LW differed from TYLCV-IL only by 30 or 31 SNPs distributed in the different recombinant sequences in the IR, a cross competition experiment with TYLCV-LW as challenging virus was conducted in the same condition tested in Jammes et al (2023), to distinguish between the nature of the recombinant region and the presence of TYLCSV in the preinfecting viruses (Experiment 4). Resistant plants infected with TYLCV-IL+TYLCSV were superinoculated with TYLCV-LW (IL+SAR/LW) in the same conditions as described above. Controls were plants agroinoculated with the empty pCAMBIA2300 plasmid (PC) and superinoculated with TYLCV-IL+TYLCSV+TYLCV-LW (PC/IL+SAR+LW).

Table 2: Description of cross-protection experiments.

Exp	Plant variety	Treatment	First inoculation	Superinfection	Nb of plants superinfected	Type of super-inoculation	Date of super-inoculation	Sampling date
1	Susceptible	IL/GC	TYLCV-IL	TYLCV-GC	15	agro-inoculation	30 dpi	30 dps 60 dps
		GC/IL	TYLCV-GC	TYLCV-IL	13			
		PC/GC	PC	TYLCV-GC	7			
		PC/IL	PC	TYLCV-IL	7			
		PC/IL+GC	PC	TYLCV-IL+ TYLCV-GC	18			
2	Resistant	IL/GC	TYLCV-IL	TYLCV-GC	15	agro-inoculation	30 dpi	30 dps 60 dps
		GC/IL	TYLCV-GC	TYLCV-IL	11			
		PC/GC	PC	TYLCV-GC	7			
		PC/IL	PC	TYLCV-IL	7			
		PC/IL+GC	PC	TYLCV-IL+ TYLCV-GC	18			
3	Resistant	IL/Liwa	TYLCV-IL	TYLCV-Liwa	10	agro-inoculation	30 dpi	30 dps 60 dps
		Liwa/IL	TYLCV-Liwa	TYLCV-IL	13			
		PC/IL	PC	TYLCV-IL	7			
		PC/Liwa	PC	TYLCV-Liwa	7			
		PC/IL+Liwa	PC	TYLCV-IL+ TYLCV-Liwa	16			
4	Resistant	IL+SAR/Liwa	TYLCV-IL+TYLCSV	TYLCV-Liwa	16	agro-inoculation	30 dpi	30 dps 60 dps
		PC/IL+SAR+Liwa	PC	TYLCV-IL+ TYLCSV+ TYLCV-Liwa	17			

Dps : days post superinfection

2.4. Sampling and DNA extraction

Plant samples were collected just before the superinfection of the plants to retain only those infected with the preventing viruses, and at 30 dpi (days post the first inoculation), and 30- and 60-days post superinfection (dps) to estimate the accumulation of each viral DNA. Samplings and DNA extractions were conducted as described in Jammes et al (2023).

2.5. Viral DNA Quantification

The level of viral accumulation in each plant was estimated by quantitative real-time PCR as described in Jammes et al (2023). Primer pairs and condition of amplification are described in table 3.

2.6. Data analysis

The number of viral copies for each viral isolate was standardized between plant DNA extracts with the quantification of the 25S RNA gene as described in Jammes et al (2023). All statistical analyses were performed using R Studio software, version 4.1.2 (R_Development_Core_Team, 2010). Viral DNA accumulations were compared within or between plants using the log transformation of viral accumulation data. Kruskal-Wallis or Wilcoxon tests were used. The package “rstatix” was used for Kruskal-Wallis when two variables were compared and Wilcoxon test when more than two variables were compared. Differences were considered statistically significant when P-value < 0.05.

Table 3: qPCR primers description

qPCR target	Primer name	Primer sequence	Primer concentration	Hybridization
TYLCV-IL	IL-2691F IL-55R	5'-AATGGCTATTTGGTAATTTCG-3' 5'-CGTCTGTGGAACCCTCG-3'	800 nM	63°C - 40s
TYLCSV	ES-2666F ES-118R	5'-AGATTGGTAGCTCTTATATACTTG-3' 5'-GAAGCCAAGTTTATAACAAAGT-3'	800 nM	60°C - 40s
TYLCV-GC	GC-33F IL-153R	5'-TCCCCACAGTTCAAGACTAC-3' 5'-AGTGGGTCCCACATATTGCAAGAC-3'	300 nM	60°C - 20s
TYLCV-Liwa	Lw-38F IL-127R	5'-GATGCACGTGCTGACAA -3' 5'-GGGACCAAGTATATAAAGACAAATGA -3'	300 nM	60°C - 20s
25S rRNA tomato gene	25S-1137F 25S-1297R	5'-AGAACTGGCGATGCGGGATG-3' 5'-GTTGATTCGGCAGGTGAGTTGT-3'	300 nM	60°C - 20s

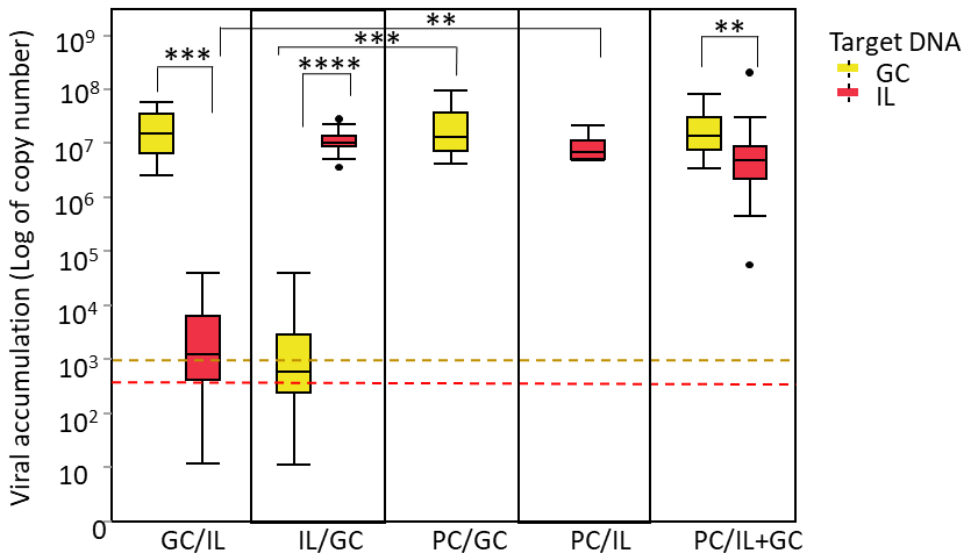

Figure 1. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in susceptible tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging inoculum (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/ the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation (dps). The viral accumulation is expressed in number of viral copies and the ordinate axis is in logarithm scale. The boxes delimit the 25% and 75% quantiles, the horizontal lines in the boxes represent the medians, the vertical lines below and above the boxes indicate the range of the values and the points outside the boxes correspond to outliers. The dotted lines indicate the mean values obtained with 3 mock-inoculated plants tested with the primers targeted to the genomes of TYLCV-IL (in red) or TYLCV-GC (in yellow). Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (**P ≤ 0.01, *** P ≤ 0,001 or **** P ≤ 0,0001).

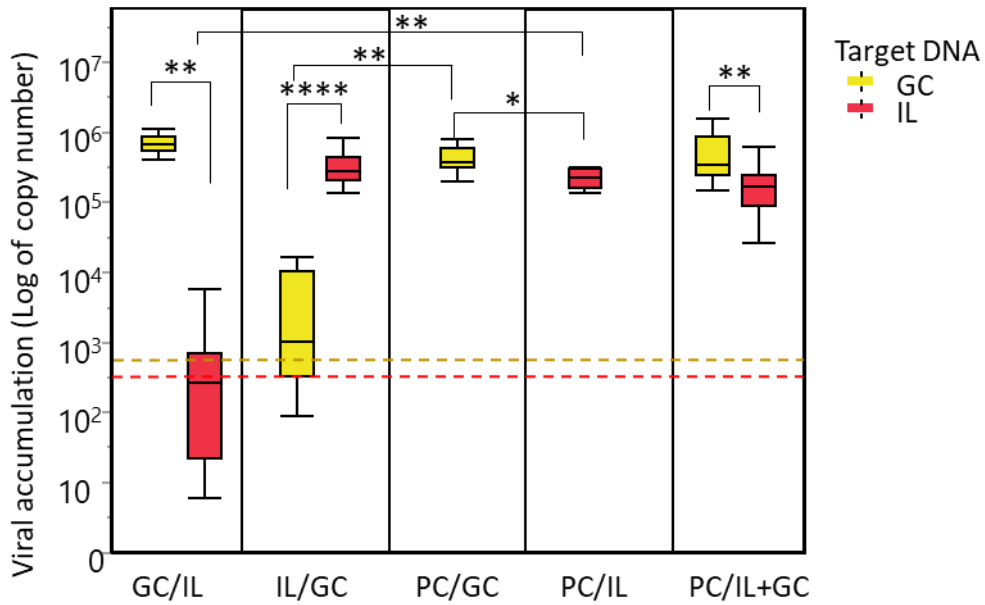

Figure 2. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging virus (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (*P ≤ 0.05 **P ≤ 0.01, or **** P ≤ 0,0001)

3. Results

Cross-protection between TYLCV closed related strains is observed in susceptible tomato plants

In order to test if cross-protection was possible between TYLCV isolates in susceptible tomato plants, TYLCV -IL and TYLCV-GC, a mutant variant differing only by 8 nts in the intergenic region (IR) were used. Plants pre-infected with TYLCV-IL or TYLCV-GC for 30 days were superinoculated at 30 dpi with TYLCV-GC and TYLCV-IL respectively and DNA accumulation of each virus isolates was estimated at 30 and 60 dps. DNA accumulations of protecting and challenging viruses were compared to each other and with those of the same virus inoculated after a pre-infection with an empty pCambia vector (Figure 1).

In mixed infection (PC/IL+GC), both variants were able to coinfect plants and even though TYLCV-GC accumulated 3 times more than TYLCV-IL at 30 dps, there was no strong competition between them, even at 60 dps (Suppl fig 1). Moreover, they accumulated at same level in singly-infected plants (PC/GC, PC/IL). However, when plants were superinoculated 1 month after the establishment of a first infection (GC/IL, IL/GC), the accumulation of the challenging virus remained at a low level (1000 and 3000 times lower than the protecting virus for GC/IL and IL/GC respectively) with around half of the plants having viral DNA levels below the detection threshold. The low DNA accumulation of the challenging virus cannot be attributed to the age of plants at the time of superinoculation, as each virus isolate was shown to accumulate at high level when it was inoculated after the empty plasmid (PC/GC, PC/IL). The same results were observed at 60 dps, confirming the cross-protection against a second TYLCV variant in pre-infected plants (Suppl fig 1). Collectively, a previous infection of susceptible tomato plants with a TYLCV variant affects the DNA accumulation of a closely related TYLCV variant inoculated 30 days later (Figure 1).

Cross-protection is also effective between closely related variants of TYLCV in resistant plants

The *Ty-1* gene is known to slow down the replication of TYLCV; indeed, TYLCV accumulates at least 10 times lower in resistant than in susceptible plants (Belabess et al., 2016). In order to check if this lower replication of TYLCV could have an impact on the cross-protection resistance, cross protection was tested in *Ty-1* resistant plants in the same conditions as described for the susceptible cultivar.

In the resistant plants that were superinfected by agroinoculation, the infection of the challenging variant was hampered by the preinfecting variant, TYLCV-IL or TYLCV-GC (Figure 2 and a repeated experiment in suppl. Figure 2) and this was still effective at 60 dps (Suppl. Figure 3). Indeed, the accumulation levels of the super inoculated viruses were much lower than the accumulation level of the first infecting variant. Moreover, for each TYLCV variant, DNA accumulation levels were

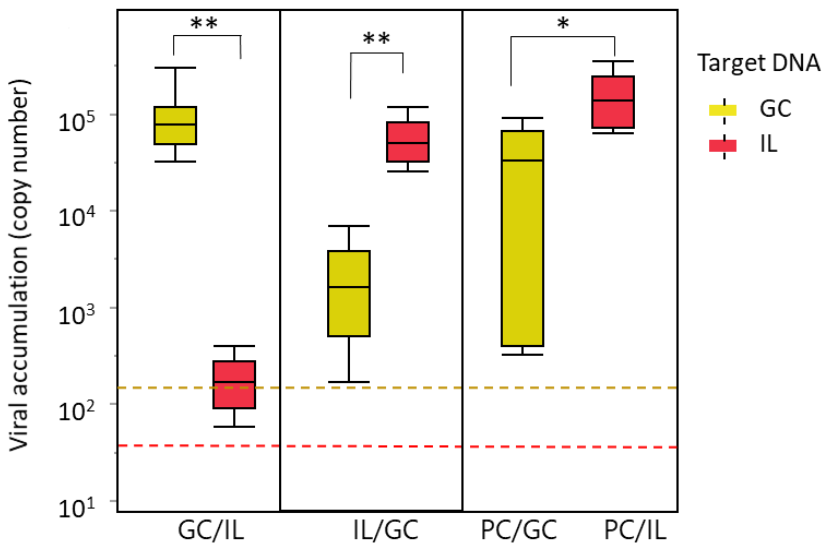

Figure 3. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in Ty-1 resistant tomato plants Following various inoculation schemes. Whiteflies that were given a 2 days acquisition access period on resistant tomato plants agroinfected for 30 days with IL or GC were transferred on resistant tomato plants previously agroinoculated 14-days after sowing with IL, GC or an empty plasmid (PC) for an inoculation access period of 5 days (350 individuals per batches of 5 plants). Each treatment was defined as following: "the first inoculum provided by agroinoculation/the second inoculum provided by viruliferous vectors". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation by vector (dps). Viral accumulations, box plots and positive thresholds as in Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (*P ≤ 0.05 **P ≤ 0.01).

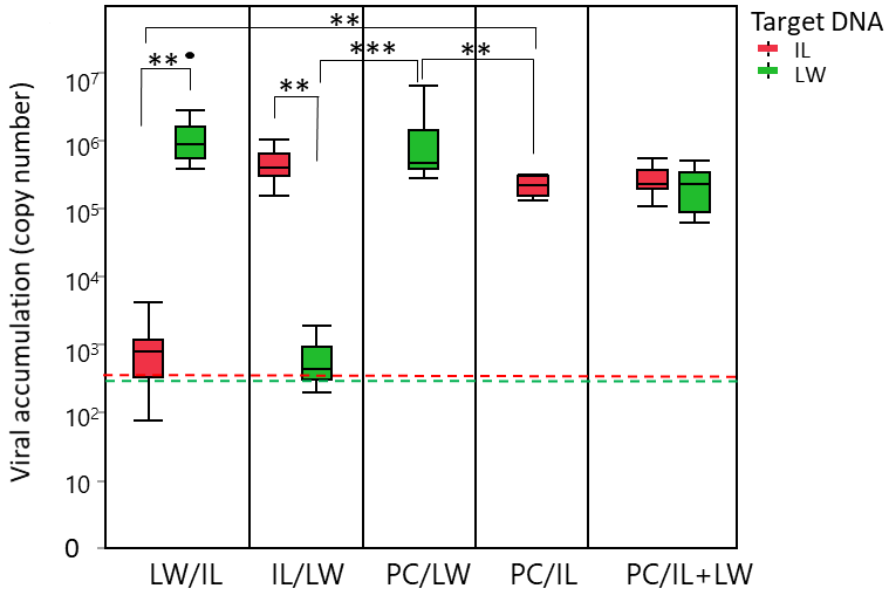

Figure 4. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging virus (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (*P ≤ 0.05 **P ≤ 0.01, or **** P ≤ 0,0001).

significantly higher when they were superinfected after an empty plasmid than after a pre-infecting virus, suggesting, as before, that the late superinfection do not affect the virus replication.

In order to mimic natural conditions, cross protection was also tested in *Ty-1* resistant plants with the same TYLCV isolates, when the challenging isolate was inoculated by vector inoculation. At 30 dps, the challenging virus accumulated at a much lower level than the protecting one in both treatments GC/IL and IL/GC (Figure 3). As observed before, the DNA accumulations of TYLCV-IL was not significantly different between IL/GC and PC/IL treatments. The result was not clear for TYLCV-GC for which DNA accumulation was not significantly different between the two treatments (GC/IL and GC/PC). This could be explained by the observation that two of the five plants of the PC/GC treatment, presented 100 times less DNA accumulations levels of GC (although higher than the detection threshold) than the 3 others.

Thus, the cross-protection mechanism was observed in Ty-1 resistant plants regardless the inoculation method used for the challenging virus, suggesting that neither the inoculum pressure, the accumulation level of the first infecting virus or the resistance mechanism provided by the *Ty-1* gene impacted the protection in tomato plants.

<u>Cross-protection occurs between TYLCV-IL and its variant differing by 30 SNPs in the intergenic region</u>

We have shown that cross-protection was effective between closely related TYLCV-IL variant differing by 8 nts in the intergenic region in both susceptible plants and resistant plants. In order to test the impact of the genetic distance on the establishment of cross-protection between viral strains, TYLCV-Liwa (TYLCV-LW), a variant differing from TYLCV by 30 nucleotide positions in the intergenic region was used as infecting or challenging virus in Ty-1 resistant plants in the same conditions as described above.

As previously, the challenging virus accumulated at very low level in IL/LW and LW/IL treatments at both 30 and 60 dps (Figure 4 and Supp Figure 4), with DNA loads being significantly lower than those in the control plants. Indeed, when TYLCV-Liwa was superinfected after TYLCV-IL, its accumulation was much lower than in the control plants PC/LW and PC/IL+LW and in some plants, the viral loads were below the detection threshold. The same results were obtained when TYLCV-IL was the challenging virus.

<u>TYLCSV does not interfere with cross-protection between TYLCV variants</u>

We have shown that TYLCV-Liwa is affected by the cross-protection phenomenon in Ty-1 resistant plants pre infected with TYLCV-IL. However, Jammes et al (2023) observed that TYLCV-IS76 which has a similar genetic distance with TYLCV-Liwa from TYLCV-IL (31 SNPs) and in the same genomic region could accumulate at high level when it was inoculated at 30 or 120 days after the combination of

TYLCV-IL and TYLCSV (SAR) in *Ty-1* resistant plants. This result suggested that either TYLCV-IS76 evades the cross-protection mechanism induced by coinfection of TYLCV-IL and TYLCSV, or the presence of TYLCSV in the preinfecting infection could have interfered with a cross-protection mechanism established by TYLCV-IL for TYLCV-GC and TYLCV-LW. In order to test this later hypothesis, we repeated the experiment described in Jammes et al (2023) but with TYLCV-Liwa as challenging virus: resistant plants already infected with TYLCV-IL and TYLCSV from 30 days were superinfected with TYLCV-LW (IL+SAR/LW). Control consisted of plants superinfected with TYLCV-IL, TYLCSV and TYLCV-Liwa, 30 days after an initial inoculation with the empty vector.

All three viruses coinoculated at the same time (PC/IL+SAR+LW) were able to coinfect the Ty-1 resistant plants with high DNA accumulation levels for the three viruses even though LW DNA accumulation was slightly lower than those of IL and SAR. In the contrary, when TYLCV-Liwa was inoculated 30 days after TYLCV-IL and TYLCSV, its viral accumulation stayed at very low levels at 30 and 60 dps, significantly much lower than its accumulation in the control plants. (Figure 5 and Supp Figure 5). The use of TYLCV-IL or TYLCV-IL + TYLCSV as preventing viruses has the same impact on the DNA accumulation of TYLCV-LW suggesting that TYLCSV does not interfere with the cross-protection against TYLCV-Liwa. So, the presence of TYLCSV in the first inoculation cannot explain by itself the capacity of TYLCV-IS76 to superinfect resistant tomato plants (Jammes et al., 2023) in the resistant plants pre-infected with TYLCV-IL and TYLCSV.

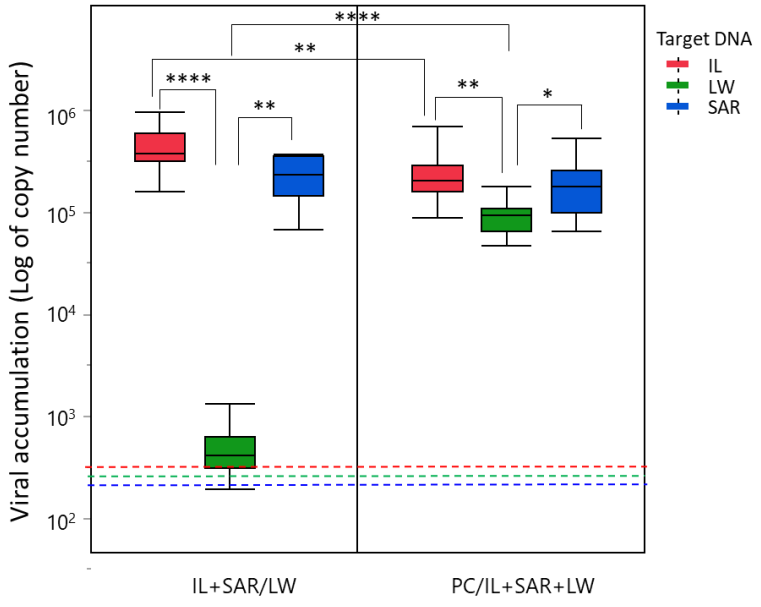

Figure 5. DNA accumulations of TYLCV-IL (IL), TYLCSV (SAR) and TYLCV-Liwa (LW) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, LW or an empty plasmid (PC) and super inoculated with a challenging inoculum (LW or IL+SAR+LW) one month after the first inoculation. Each treatment was defined as following : "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (*P ≤ 0.05 **P ≤ 0.015, or **** P ≤ 0,0001).

4. Discussion

We have shown that cross-protection phenomenon was effective between TYLCV variants differing by 8 or by 31 SNPs in the intergenic region. The demonstration was done in Ty-1 resistant plants as well as in susceptible isogenic plants and the strong protection against the challenging virus was shown to be still effective after 60 dps, or when the challenging virus was inoculated by the insect vector. Cross-protection has been previously reported to occur between geminiviruses (Dai et al., 2022; Owor et al., 2004; Reddy et al., 2012), but in contrast to our study, it was not verified by measuring directly the viral accumulation of the protecting and the challenging virus in the natural host of the virus (as, in our case, tomato) and with all the appropriate controls. Indeed, antagonism between cassava mosaic begomoviruses was considered as evidence of cross protection. This conclusion was inferred from coinfection experiments showing that the severity of symptom caused by a severe strain were delayed and modulated by the presence of a mild strain in cassava plants (Owor et al., 2004) and in *N. benthamiana* (Reddy et al., 2012). Concerning AYVV mutants (Dai et al., 2022) the protection with a mild strain against the expression of severe symptoms caused by a severe strain was also shown in *N. benthamiana* after sequential inoculation of viral isolates and lasted 34 days. In both models, the conclusions were deduced from the symptom observations and the experimental designs did not include all the controls showing that the superinfection after a mock inoculation was efficient or that the preventing and the challenging strains did not compete with each other in mixed infections. In our study, the quantification of preventing and challenging viruses in different conditions of inoculation confirmed the capacity of each viral strain to accumulate at similar level when inoculated at the 14-day old state or 30 days later.

The cross protection observed in our experiments was established when inoculation of preventing and challenging viruses was done at one-month intervals, while most of the studies on cross protection use an interval of 7 days. A minimum of time seems to be necessary for the defence to be efficient. Testing the condition of establishment of cross protection between potato virus X mutants, Cong et al (2019) have shown that 5 days were not enough because the level of accumulation of the challenging virus was not sufficient and the infection was not systemic yet, although these conditions were reached after an interval of 10 days.

Cross-protection has been principally reported between closely related viruses, probably because sequence specific cross-protection (based on RNA silencing) may not be possible when the genetic distance exceeds a certain threshold. However, there are some examples where distinct virus species can protect against each other, due to other mechanism (Huss et al., 1989; Nakazono-Nagaoka et al., 2009). The reported case with ACMV and EACMCV would imply that cross-protection is also possible between two different species from the same genus of geminiviruses. The question of cross

protection between TYLCV and TYLCSV, two begomovirus species sharing 82% nucleotide identity and frequently co-infecting tomato plants in the Mediterranean Basin, could help to understand the mechanism involved in cross protection among begomoviruses.

Interestingly, all TYLCV variants used in this study have similar infection rates and induced similar symptoms on susceptible tomato plants, indicating that cross protection could exist between strains with the same level of severity. These results, together with previous ones, do not support the hypothesis that the facility of recombination of geminiviruses could be explained by the absence of cross protection in this virus family (Saleem et al., 2016). Although the maintenance within plants of begomovirus recombinant genomes closely related to parental viruses has been observed (Davino et al., 2012; García-Andrés et al., 2007; Martin et al., 2011; Urbino et al., 2013), this does not suggest that there is no cross protection between geminiviruses. Indeed, as recombinants are generated continuously during the coinfection, it is not possible to differentiate competition, selection, genetic distance effects between recombinants and parental viruses which could also exclude cross protection mechanisms.

Cross protection is effective in susceptible plants and in resistant plants bearing the resistance gene *Ty-1*, which means that *Ty-1* does not compromise the protection of the first virus by reducing its viral accumulation. Moreover, as *Ty-1* codes for an RNA-dependant RNA polymerase of gamma clade (RDRγ) (Verlaan et al., 2013) which was shown to reinforce the RNA interference in the plants (Butterbach et al., 2014; Voorburg et al., 2021), we could have hypothesized that this gene is responsible of the establishment of the cross-protection, and that gene silencing is involved in the resistance. However, it is not the case, since cross-protection is also observed in susceptible plants, which means that the basal gene silencing of the plants is sufficient for the establishment of cross-protection, or that other mechanisms are involved.

We have shown previously that TYLCV-IL, TYLCV-GC and TYLCV-Liwa accumulated at similar levels in resistant plants (Urbino et al., 2022) and this was confirmed in this study. Thus, there is no strong competition between those variants and a difference in competitivity is not involved in the cross-protection phenomenon observed between those variants. It has been shown before that the probability for each TYLCV-like species, TYLCV and TYLCSV, to infect *Nicotiana benthamiana* or tomato cells was the same. Therefore, they could infect the same cell ruling out a superinfection cell exclusion mechanism (Morilla et al., 2004) but it seems to be different between closer related TYLCV variants and a superinfection cell exclusion can be hypothesized between our TYLCV variants. The cell exclusion can be explained by different mechanisms, such as the gene silencing or other defence mechanisms induced by the first virus which could impede the multiplication of the second virus, the

hijacking by the first virus of all the cell resources for its replication cycle hampering the replication of the second virus, or the CP-mediated resistance first proposed as a cross-protection mechanism.

As the mutations of the clones used for the test were located in the IR, our results suggest that mutations in this particular region do not impede the cross-protection between TYLCV variants. Moreover, the presence of a second virus, TYLCSV, in the first infection, did not compromised the efficiency of the cross-protection against TYLCV-Liwa, a virus with a similar recombination profile with TYLCV-IS76, and differing from TYLCV-IL, as this later, by 31 nts in the IR. However, in the same experimental conditions, TYLCV-IS76 was able to overcome the cross-protection phenomena initiated by the coinfection with TYLCV+TYLCSV. Indeed, the Ty-1 resistant plants previously infected with TYLCV-IL and TYLCSV were not protected against TYLCV-IS76 inoculated one month later. In this condition, this recombinant accumulated at similar level as when it was inoculated together with its parental viruses. It also kept its competitiveness against TYLCV-IL (Jammes et al., 2023). Evidences of breakdown of cross-protection have been reported before: Powell et al (2003), reported a breakdown of the cross-protection for citrus tristeza virus (CTV) isolates due to the introduction of a new insect vector, the brown citrus aphid; also, the breakdown of cross-protection between CTV isolates was reported when the protecting virus lacked the functional protein p33 (Folimonova, 2012). In our case, the protecting virus did not lack any protein but it is the new recombinant genomic sequence, gained by the challenging virus (TYLCV-IS76), which provided a strong competitiveness in resistant Ty-1 plants (Jammes et al., 2023).

Ty-1 could be responsible of the breakdown of cross-protection of TYLCV-IS76. Indeed, it has been shown that TYLCV-IS76 has a strong selective advantage in *Ty-1* resistant plants; it accumulates at higher level than its parental viruses in *Ty-1* plants in single infection, contrary to what is observed in susceptible plants where the viruses accumulate at same level. Moreover, in mixed infection with TYLCV-IL, TYLCV-IS76 has a deleterious effect on its parent accumulation. This deleterious effect is slight in susceptible plants but very strong in resistant plants with a rapid decrease of TYLCV-IL accumulation and its total disappearance after 120 dpi (Belabess et al., 2016). Such an advantage in Ty-1 resistant plants could explain why TYLCV-IS76 was able to break the resistance of the cross-protection. It will be interesting to test the potential of cross-protection between TYLCV-IL and TYLCV-IS76 in susceptible plants where TYLCV-IS76 does not outcompete TYLCV-IL.

The absence of the cross-protection phenomena against TYLCV-IS76 but not against TYLCV-Liwa used as challenging viruses, suggest that the small fragment inherited from TYLCSV in the IR plays a role in this difference. As suggested before, new interactions between the TYLCSV genome fragment inherited in TYLCV-IS76 and its TYLCV-IL genome seem to be responsible of the selective advantage

of TYLCV-IS76 and may be also responsible of the evasion of cross-protection. It would be interesting to test the capacity of the recombinant region inherited from TYLCSV in other begomovirus genomes to see if they will also help to evade cross-protection mechanism.

Acknowledgments

The study was supported by ANR through the PRIMA project "Prevention and control of new and invasive geminiviruses infecting vegetables in the Mediterranean", by Gautier Semences (France), and CIRAD (https://www.cirad.fr/). The PhD grant of Margaux Jammes was funded by the ANR project and the internship of Julie Balland by Gautier Semences. We are thankful to Martine Granier, and Axel Cremades for technical assistance.

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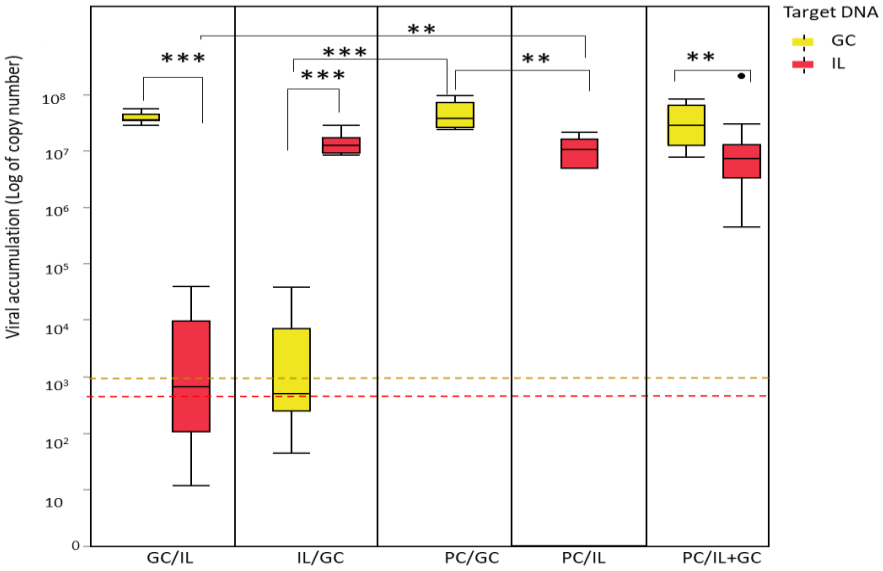
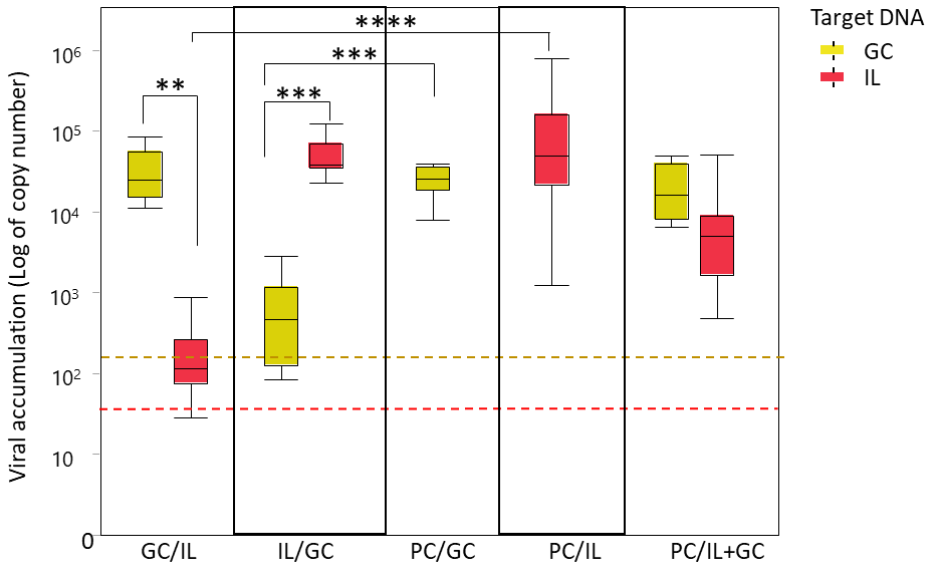
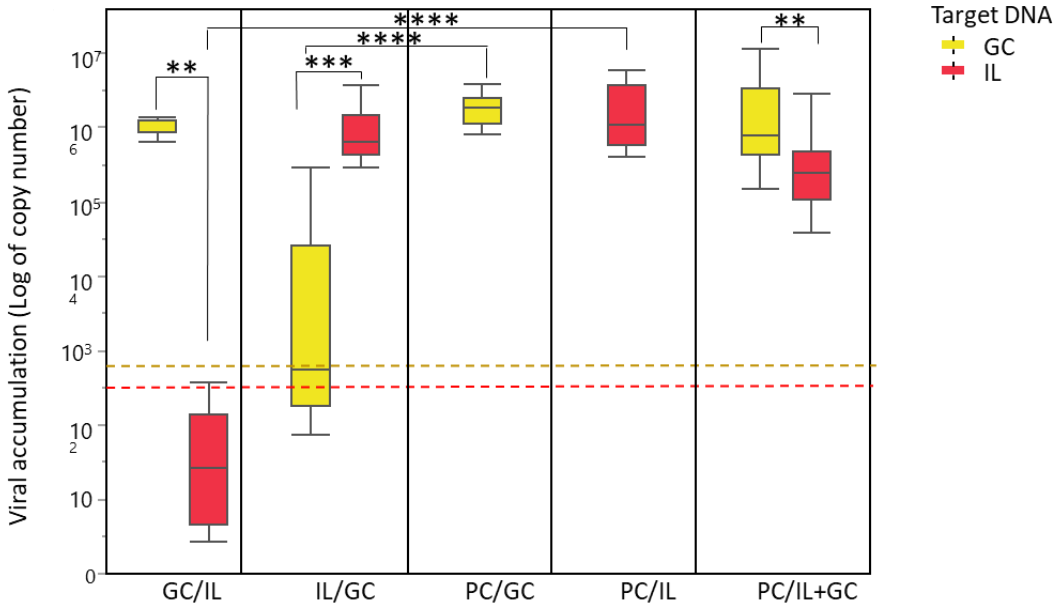
Supplementary data



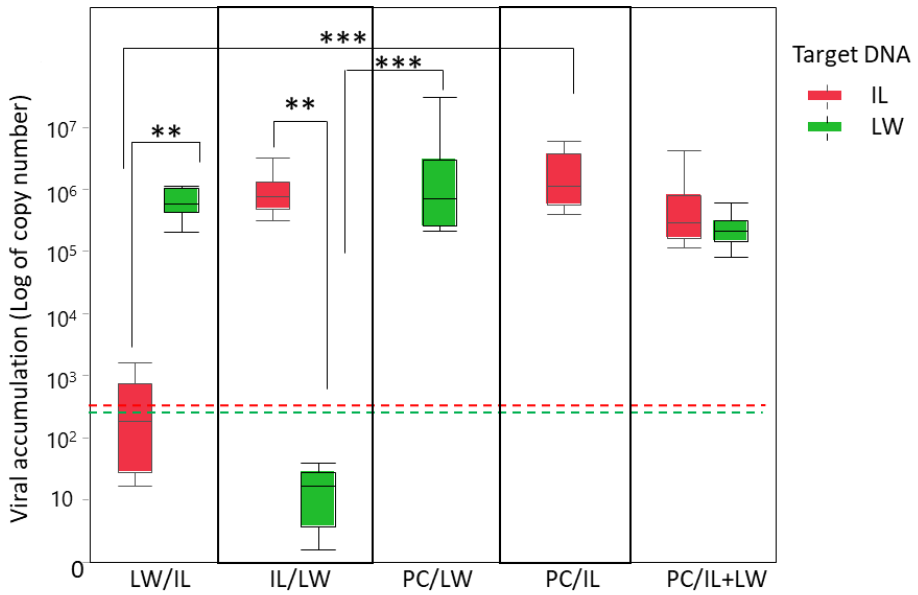
Suppl. Figure 1. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in susceptible tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging inoculum (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/ the second inoculum".Viral DNA was quantified by real-time PCR from leaf samples collected 60 days post superinoculation (dps). The viral accumulation is expressed in number of viral copies and the ordinate axis is in logarithm scale. The boxes delimit the 25% and 75% quantiles, the horizontal lines in the boxes represent the medians, the vertical lines below and above the boxes indicate the range of the values and the points outside the boxes correspond to outliers. The dotted lines indicate the mean values obtained with 3 mock-inoculated plants tested with the primers targeted to the genomes of TYLCV-IL (in red) or TYLCV-GC (in yellow). Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (**P ≤ 0.01 or *** P ≤ 0,001).



Suppl. Figure 2. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging virus (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 30 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Suppl. Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (**P ≤ 0.01, *** P ≤ 0,001 or **** P ≤ 0,0001).



Suppl. Figure 3. DNA accumulations of TYLCV-IL (IL), and TYLCV-GC (GC) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, GC or an empty plasmid (PC) and super inoculated with a challenging inoculum (IL, GC or IL+GC) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 60 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (**P ≤ 0.01, ***P ≤ 0,001 or ****P ≤ 0,0001).



Suppl. Figure 4. DNA accumulations of TYLCV-IL (IL), and TYLCV-Liwa (LW) in Ty-1 resistant tomato plants agroinoculated 14 days after sowing with IL, LW or an empty plasmid (PC) and super inoculated with a challenging inoculum (IL, LW or IL+LW) one month after the first inoculation. Each treatment was defined as following: "the first inoculum/the second inoculum". Viral DNA was quantified by real-time PCR from leaf samples collected 60 days post superinoculation (dps). Viral accumulations, box plots and positive thresholds as in Suppl. Fig.1. Asterisk indicated significant differences between DNA accumulations of viruses in a treatment or between treatments (**P ≤ 0.01, or ***P ≤ 0,001).

CHAPITRE 3

Transcriptome and small RNAome profiling uncover how a recombinant begomovirus evades RDRγ-mediated silencing of viral genes and outcompetes its parental virus in mixed infection

Transcriptome and small RNAome profiling uncover how a recombinant begomovirus evades RDRγ-mediated silencing of viral genes and outcompetes its parental virus in mixed infection

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Abstract

Tomato yellow leaf curl virus (TYLCV) and TYLCV-like viruses transmitted by whiteflies cause a severe disease of cultivated tomato. They belong to the genus *Begomovirus* of the family *Geminiviridae* whose circular single-stranded genomic DNA replicates via rolling-circle and recombination-dependent mechanisms, frequently generating recombinants in mixed infections. Circular double-stranded intermediates of replication also serve as templates for Pol II-mediated bidirectional transcription of viral mRNAs. IS76 is a recombinant derivative of TYLCV with a short sequence in the bidirectional promoter/origin-of-replication region acquired from of a related begomovirus. IS76 outcompetes TYLCV in mixed infection and breaks disease resistance in tomato cultivars carrying the resistance gene *Ty-1* that encodes a γ-clade RNA-dependent RNA polymerase (RDRγ) implicated in the biogenesis of small interfering (si)RNAs directing gene silencing. To investigate molecular mechanisms underlying evasion of RDRγ activity by IS76 and its competitive advantage, we profiled transcriptome and small RNAome of *Ty-1* resistant and control susceptible plants infected with TYLCV, IS76 or their combination at early and late infection stages. We found that RDRγ strongly enhances production rates of 21, 22 and 24 nt viral siRNAs from the entire viral genome, presumably by promoting its bidirectional readthrough transcription generating siRNA precursors, and modulates Dicer activities towards 22 and 24 nt siRNAs. Compared to parental TYLCV, IS76 undergoes faster transition to the replication phase favouring rightward transcription of silencing suppressor (V2) and coat protein (V1) genes and thereby facilitates its DNA accumulation and evades RDRγ activity in both single and mixed infections. In coinfected *Ty-1* plants, IS76 competes for transcription-replication machineries, thereby impairing both leftward and rightward transcription of TYLCV and forcing its elimination associated with further increased siRNA production. Taken together, the IS76 recombination facilitates replication and favours rightward transcription of viral silencing suppressor and coat protein genes, which helps the recombinant virus to evade the negative impact of RDRγ-promoted production of viral siRNAs directing transcriptional and posttranscriptional gene silencing.

1. Introduction

Tomato yellow leaf curl disease (TYLCD) causing leaf yellowing and curling, plant stunting and flower abortion is a severe threat for tomato cultivation worldwide. In the Mediterranean basin, the disease is mainly caused by tomato yellow leaf curl virus (TYLCV), tomato yellow leaf curl Sardinia virus (TYLCSV) and their recombinants, which belong to the genus *Begomovirus* of the family *Geminiviridae* (known as geminiviruses).

Geminiviruses are single-stranded (ss)DNA viruses that replicate in the plant cell nucleus via rolling-circle and recombination-dependent mechanisms and encapsidate circular ssDNA products of rolling-circle replication into geminate virions (reviewed in Hanley-Bowdoin et al., 1999 and Pooggin, 2013). A circular double-stranded (ds)DNA intermediate of both replication mechanisms also serves as a template for Pol II-mediated transcription of viral genes. As typical monopartite begomoviruses, TYLCV and TYLCSV possess six genes transcribed by Pol II bidirectionally from the virion and complementary strands of circular dsDNA of 2.8 Kbp. The rightward (virion strand) genes V2 and V1 encode the coat protein (CP/V1) (Hallan & Gafni, 2001) and the strong silencing suppressor (V2) also implicated in movement (Glick et al., 2008; Luna et al., 2012; Rojas et al., 2001; B. Wang et al., 2018; L. Wang et al., 2020; Zrachya et al., 2007). The leftward (complementary strand) genes (C1-to-C4) encode the replication initiator protein (Rep/C1) (Desbiez et al., 1995), the transcriptional activator and silencing suppressor (TrAP/C2) (Dong et al., 2003; Noris et al., 1996), the replication enhancer (REn/C3) (Settlage et al., 2005) and the silencing suppressor (C4) also implicated in movement (Jupin et al., 1994; Luna et al., 2012; Rojas et al., 2001; Xie et al., 2013). The intergenic region contains the origin of replication and the bidirectional promoter driving Pol II transcription of respectively the C1-C4 mRNA from which Rep/C1 and C4 are translated and the V2-V1 mRNA from which V2 and CP are translated. An additional monodirectional promoter drives Pol II transcription of the C2-C3 mRNA from which TrAP/C2 and REn/C3 proteins are translated and which is 3'-coterminal with the C1-C4 mRNA (Frischmuth et al., 1991; Mullineaux et al., 1993; Shivaprasad et al., 2005).

Geminiviruses are transmitted by phloem feeding insect vectors such as whiteflies, aphids and leafhoppers in a persistent circulative manner. Begomoviruses are transmitted exclusively and efficiently by the whitefly *Bemisia tabaci*. Due to its small size, high biotic potential, large host range and propensity to develop insecticide resistance, *B. tabaci* is a very difficult pest to control. That is why breeding for plant resistance has so far been the most effective strategy to prevent and control begomoviral diseases. In the case of TYLCD, six resistance genes (*Ty-1* to *Ty-6)* available from the wild *Solanum* species were introgressed into cultivated tomatoes (*Solanum lycopersicum*) and *Ty-1* resistant plants are the most cultivated (Zakay et al., 1991; Zamir et al., 1994).

The tomato *Ty-1* gene codes for an RNA-dependent RNA polymerase from the γ-clade (RDRγ) similar to the RDRγ genes of the model plant *Arabidopsis thaliana* (RDR3, RDR4 and RDR5) for which no function have been demonstrated yet (Verlaan et al., 2013). The α-clade RDRs of *A. thaliana* (RDR1, RDR2 and RDR6) are known to be involved in RNA interference (RNAi), an evolutionary conserved mechanism that regulates gene expression and defends against invasive nucleic acids such as transposons, transgenes and viruses in most eukaryotes. RNAi is directed by small interfering (si)RNAs which are produced by Dicer or Dicer-like (DCL) family proteins from double-stranded (ds)RNA precursors and get associated with Argonaute (AGO) family proteins forming RNA-induced silencing complexes. In plants, the dsRNA precursors of siRNAs can be generated by sense and antisense transcription or transcription of inverted repeats as well as by the activity of RDR1, RDR2 or RDR6 synthesizing complementary strands on their specific single-stranded RNA templates (Borges & Martienssen, 2015). DCLs process their respective dsRNA substrates into 21 nt (DCL4), 22 nt (DCL2) and 24 nt (DCL3) siRNAs which are then sorted by AGOs based mostly on their size and 5'-termninal nucleotide identity (Fang & Qi, 2016). In *A. thaliana* infected with a bipartite begomovirus, DCL4, DCL2 and DCL3 produce respectively 21, 22 and 24 nt viral siRNAs from both strands of the entire virus's genome (Aregger et al., 2012; Blevins et al., 2006). Notably, the majority of begomoviral siRNAs are produced independently of the activities of RDR1, RDR2 or RDR6 or the plant-specific DNA-dependent RNA polymerases Pol IV and Pol V (Aregger et al., 2012; Blevins et al., 2006), suggesting that begomoviral siRNA precursors are produced by Pol II-mediated bidirectional readthrough transcription of circular viral dsDNA (Aregger et al., 2012; Pooggin, 2013). Nonetheless, small amounts of 21-nt begomoviral siRNAs are generated by a RDR6- and DCL4-dependent pathway and those so-called secondary siRNAs are involved in cell-to-cell spread of RNAi (Aregger et al., 2012; Blevins et al., 2006). In *A. thaliana*, the RDRγ genes RDR3, RDR4 and RDR5 are located adjacent to each other and their functionality (if any) in siRNA biogenesis, gene silencing or antiviral defense remains unknown (Wassenegger & Krczal, 2006). In rice (*Oryza sativa*), RDR3 is involved in the regulation of transposons and other repeat-rich genomic regions generating 21 and 24 nt siRNAs and biochemical evidence shows its polymerase activities on both ssRNA and ssDNA templates similar to the activities reported for α-clade RDRs (Jha et al., 2021).

Solanum lycopersicum plants susceptible to infection with TYLCV accumulate 21, 22 and 24 nt siRNAs derived from both strands of the entire virus genome (Fuentes et al., 2016; Piedra-Aguilera et al., 2019; Voorburg et al., 2021) indicating that antiviral RNAi is mediated by at least three tomato DCLs. The tomato *Ty-1* gene-encoded RDRγ mediates resistance against TYLCV by enhancing production of virus-derived 22 and 24 nt siRNAs on expense of 21 nt siRNAs (Voorburg et al., 2021) and increasing cytosine methylation of viral DNA (Butterbach et al., 2014), suggesting its involvement in 24 nt

siRNA-directed transcriptional silencing of viral genes and possibly posttranscriptional silencing of viral mRNAs directed by 22 nt siRNAs (Voorburg et al., 2021). This hypothesis is consistent with the findings that the resistance against TYLCV is compromised in *Ty-1* plants co-infected with cucumber mosaic virus or a begomoviral betasatellite, which are known to express suppressors of posttranscriptional and transcriptional gene silencing (Voorburg et al., 2020).

In Morocco, an invasive recombinant between the IL strain of TYLCV (TYLCV-IL) and TYLCSV was detected in 2010, in *Ty-1* resistant plants exhibiting typical symptoms of TYLCD (Belabess et al., 2015). In this atypical recombinant, called TYLCV-IS76, a short sequence of the intergenic region of TYLCV-IL between position 1 (origin-of-replication and known recombination break-point) and position 84 was replaced with the homologous although slightly shorter sequence of TYLCSV (1-76). Extended surveys conducted from 2012 revealed that TYLCV-IS76 had almost totally replaced its parental viruses in the Souss region of Morocco where it probably originated from. Interestingly the invasion of TYLCV-IS76 coincided with the deployment of *Ty-1* resistant tomato cultivars in this country (Belabess et al., 2015). Under laboratory conditions, TYLCV-IS76 is positively selected in the *Ty-1* plants where it accumulates at higher levels than its parental viruses and, more intriguing, has a strong deleterious effect on TYLCV-IL, leading to disappearance of this parental virus at late stages of coinfection (Belabess et al., 2016; Jammes et al., 2023). The molecular mechanisms underlying evasion of RDRγ-mediated resistance by TYLCV-IS76 and its strong deleterious impact on TYLCV-IL in mixed infection of *Ty-1* plants are unknown. In this study we began to uncover these mechanisms by comparative transcriptome and sRNA-ome profiling of susceptible vs *Ty-1* resistant tomato plants infected with TYLCV-IL, TYLCV-IS76 or combination thereof at early and late stages of infection.

2. Material and methods

2.1. Plant material

Solanum lycopersicum cultivar "Pristyla" carrying the *Ty-1* resistance gene in a heterozygous state (*Ty-1*/*ty-1*) (Gautier Semences, France) and a nearly isogenic susceptible cultivar (*ty-1*/*ty-1*) (Belabess et al., 2016) were used. Seeds were sown in a nursery pot and young seedlings were transplanted in individual pots. Plants were grown in a S3 containment growth chamber with 14 h light at 26°C and 10 h dark at 24°C. They were watered with a solution containing 15:10:30 NPK fertilizer and oligoelements.

2.2. Viral infectious clones

Two agroinfectious clones were previously constructed (Belabess et al., 2015, 2016) with the following viruses: TYLCV-IS76 [MA:SouG8:10] (GenBank accession number LN812978); and TYLCV-IL [RE:STG4:04] (GenBank accession number AM409201).

2.3. Agroinoculation

Virus agroinfiltration or co-agroinfiltration of 14-day old seedlings were performed with agrobacteria preparations as described in Belabess et al (2016). Two groups of tomato plants were agroinfected, one for the 10-day post inoculation (dpi) sampling and another one for the 30 dpi sampling.

The following plants were sampled at 10 dpi. For susceptible plants, 14 seedlings were agroinoculated with TYLCV-IL, 14 with TYLCV-IS76 and 16 were co-agroionoculated with TYLCV-IL and TYLCV-IS76. For *Ty-1* resistant plants, 13 seedlings were agroinoculated with TYLCV-IL, 15 with TYLCV-IS76 clone and 18 were co-agroionoculated with TYLCV-IL and TYLCV-IS76.

The following plants were sampled at 30 dpi. For susceptible plants, 8 seedlings were agroinoculated with TYLCV-IL, 8 with TYLCV-IS76 clone and 12 were co-agroionoculated with TYLCV-IL and TYLCV-IS76. For *Ty-1* resistant plants, 7 seedlings were agroinoculated with TYLCV-IL, 8 with TYLCV-IS76 clone and 11 were co-agroionoculated with TYLCV-IL and TYLCV-IS76.

As negative controls, 3 seedlings of each cultivar and at each sampling date were agroinoculated with a preparation of bacteria of the C58 MP90 strain of *Agrobacterium tumefaciens* containing an empty pCAMBIA2300 plasmid.

2.4. Sampling procedures

At 10 and 30 dpi, the youngest leaves from the apex of each plant were immediately frozen in dry ice after collection, and then stored at -80°C. Samples were pooled in two biological replicates for each sampling dates (3 to 6 plants per replicate), based on quantitative (q)PCR analysis of viral DNA loads (see below).

Viral DNA loads of each plant in the 10 and 30 dpi group were assessed by qPCR analysis of five 4-mm diameter leaf discs cut from the youngest leaf for which five leaflets were visible (one disc per leaflet). Leaf discs were collected at 18 dpi and 30 dpi from the plants of the 10 dpi and 30 dpi groups, respectively, and stored at -20°C until use.

2.5. Quantification of viral DNA loads

The total DNA from the leaf disc samples was extracted using the protocol of Dellaporta et al (Dellaporta et al., 1983) modified as described in Jammes et al (2023).

The viral DNA load in each plant sample was estimated by quantitative real-time PCR using the LightCycler 480 SYBR Green I qPCR master mix (Roche, Germany). The qPCR reaction was performed in a final volume of 10 µL containing 2 µL of total DNA diluted 1:20, the Mastermix of Roche, and primers. Primers used for quantification of TYLCV and TYLCV-IS76 are those described in Belabess et al (2016), at 800 nM and 300 nM respectively. The tomato 25S rRNA gene was used to normalize virus quantification with respect to plant DNA. The 25S ribosomal RNA genes-specific primers, described in Belabess et al (2016), were used at a concentration of 300 nM. Two technical repeats were performed for each DNA sample. The qPCR was run in 384-well plates using a LightCycler 480 (Roche, Germany) with the following cycling conditions: 95°C for 10 min followed by 40 cycles consisting each of a denaturation step at 95°C for 10 sec, a hybridization step at 63°C for 40 sec for TYLCV-IL or 20 sec for TYLCV-IS76, and an elongation step at 72 ◦C for 15 sec. The qPCR results were analyzed with the LinReg computer program (Ruijter et al., 2009), which calculates the initial concentration N0 for each sample, expressed in fluorescence units. This N0 value was normalized by the plant DNA concentration and the amplicon size.

2.6. Choice of leaf samples for pooling

The pooling of the plant samples was performed according to the viral load of each plant estimated by qPCR. The objective was to create for each condition, two pools of plant samples with similar viral loads. For single infection, the plants with the most similar and representative (close to mean) viral loads were selected and homogenously divided in two batches; to do this, the selected samples were ranked from the sample exhibiting the lowest virus concentration to the one with the highest concentration and the selected alternatively to form the two pools. In mixed infection with TYLCV-IL and TYLCV-IS76, the criteria used for homogeneity was the ratio of the viral loads between TYLCV-IS76 and TYLCV-IL. The plants with the most similar ratio were selected and divided homogenously after ranking and alternative selection as described above. Due to the contrasted weight of available leaves to be collected at 10 and 30 dpi, the leaf samples collected at 10 dpi were from 6 plants, while the samples collected at 30 dpi, were from 3 plants.

For pooling the samples, samples of each batch were ground together in liquid nitrogen. The powder obtained was stored in -80°C before being processed for total DNA and RNA extraction.

2.7. RNA extraction

RNA extraction was performed on the pooled samples with a CTAB-LiCl method as described previously in Golyaev et al (2019).

2.8. DNA extraction

The total DNA from the pooled leaf samples was extracted using the CTAB method of Doyle (1990). A volume of 0.5 ml of 2% CTAB buffer was preheated at 60°C, and added to 0.1 g of leaf tissues ground in liquid nitrogen; the CTAB buffer is composed 100 mM Tris pH 8.0, 1.4 M NaCl, 50 mM EDTA pH 8.0, 2% CTAB, and 0.2% mercaptoethanol added before use). The mixture was incubated at 60°C for 1h and then centrifugated for 10 min at 10,000 rpm at room temperature. The supernatant was mixed with equal volume of chloroform:isoamylalcohol (24:1). The mix was shacked for 3 min and centrifuged for 10 min at 5,000 rpm at room temperature. The supernatant was transferred to a new tube and 0.66 volume of cool isopropanol was added. The tubes were stored at 4°C overnight and then centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was discarded and 0.5 ml of washing buffer (76% ethanol; 10 mM ammonium acetate) was added and the tubes were incubated at room temperature for 20 min and centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was air dried. Then 100 µl of H_2O and 1 µl of RNase (10 mg/ml) were added and the tubes were incubated for 1 h at 37°C. Two volumes of H_2O were added, and the DNA precipitated with 0.3 volumes of 3M sodium acetate and 2.5 volumes of cold absolute ethanol. The tubes were stored 15 min at -80°C and then centrifugated at 10,000 rpm during 10 min at room temperature. The supernatant was discarded and the pellet was air dried at room temperature. The pellet was resuspended with 50 µl of H_2O and the tubes stored at -20°C.

2.9. Illumina sequencing and bioinformatic analysis of viral mRNAs and viral sRNAs

Illumina sequencing was performed at Fasteris AG (www.fasteris.com) using the same total RNA extracts for library preparations with the Illumina stranded mRNA and the Illumina TruSeq small RNA protocols.

The mRNA libraries were multiplexed and sequenced in two flowcells of NovaSeq 6000, one flowcell with the samples from the plants collected at 10 dpi and the other one with the samples from the plants collected at 30 dpi, yielding 25’921’135 to 45’871’986 and 20’213’119 to 40’363’645 100 nt paired-end reads, respectively and Q30 = 89.36 to 91.87 and Q30 = 89.85 to 92.00 respectively.

The sRNA libraries were multiplexed and sequenced in two flowcells of NovaSeq 6000. The first flowcell with the samples from the plants collected at 10 dpi were sequenced with 50 nt paired-end reads yielding 28’814’844 to 54’420’088 reads with Q30 = 74.02 to 74.31. The Q30 is outside specifications mainly due to a drop of quality during the sequencing of read 2 but quality of read 1, from which the inserts were extracted for our follow-up analysis, was within specifications (Q30 = 96.46 to 96.83). Because of the low quality of the second read for this sequencing method, the

second flowcell with the samples from the plants collected at 30 dpi were sequenced with 75nt single-end, yielding 37’719’583 to 50’968’605 reads with Q30 = 96.86 to 97.43.

In all cases, the libraries were de-multiplexed, followed by adapter trimming with Trimmomatic. The resulting reads were mapped using Burrow-Wheeler Aligner (BWA) 0.7.12 (Li & Durbin, 2010), onto the reference sequences of TYLCV-IL and TYLCV-IS76 with and without mismatches. Mapped viral reads were sorted by polarity (forward, reverse) and, in the case of sRNAs, by size (15 to 34 nts).

To quantify viral mRNA and viral sRNA loads, we used the reads aligned without mismatches. The viral read counts in each library were normalized in reads per millions (RPM) of total (viral + plant) reads.

In mixed infection, the number of reads derived from each virus was calculated using reads aligned with mismatches at the SNP positions present along the genome of TYLCV-IS76 and TYLCV-IL. Noteworthy, to distinguish the TYLCV-IS76 RNAs from the parental TYLCV-IL RNAs in mixed infected plants, we purposely used the wild type TYLCV-IS76 infectious clone but not the laboratory generated one, TYLCV-IS76’ that has the same competitiveness properties (Belabess et al 2016), because unlike TYLCV-IS76’, the wild type recombinant can be distinguished from TYLCV-IL not only by the SNPs and indels in the recombination region but also by 28 SNPs scattered along the viral genome. Thus, the number of reads derived from each virus was counted at each SNP using MISIS-2 (Seguin et al., 2016) and a percentage of reads derived from each virus was calculated. The average percentage derived from all SNPs of a complete viral genome or a selected region of the viral genome was applied on the parts of the genome (or selected region of the genome) containing no SNPs to estimate the number of reads derived from the entire genome (or selected part of the genome) of each virus or its selected part.

For viral mRNA, a production rate for each viral mRNA was calculated dividing the mRNA counts in RPM by the DNA loads (measured by qPCR) and by the length of the mRNA.

For viral sRNAs, a production rate of sRNAs in the size range from 20 to 25 nts was calculated dividing the sRNA loads in RPM by the viral DNA load and, in the case of selected regions of the virus genome, by the length of each region.

Mean values with standard deviations of all those loads and rates were calculated for the two biological replicates in each condition.

Figure 1: Total viral DNA, mRNA and small (s)RNA accumulation in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76, or their combination (IL+IS76) at 10- and 30-days post inoculation (dpi).

3. Results and Discussion

In this study, we aimed at exploring the molecular mechanism(s) which have driven the emergence of the recombinant TYLCV-IS76 in Morocco and the displacement of its major parent TYLCV-IL. To this end, we analysed 14-days old tomato seedlings of a *Ty-1* resistant (R) cultivar and an isogenic susceptible (S) one, infected with viral clones of TYLCV-IL isolate RE4 (hereafter IL), TYLCV-IS76 (hereafter IS76) or their combination (IL+IS76). Viral DNA loads were assessed by qPCR, while loads, production rates and profiles of viral mRNAs and virus-derived siRNAs were analyzed by Illumina sequencing from systemically infected leaf tissues collected at 10- and 30- dpi. Two biological replicates were analysed for each condition.

Consistent with the previous studies (Belabess et al., 2016, 2018; Butterbach et al., 2014; Jammes et al., 2023; Urbino et al., 2020), the *Ty-1* resistance gene encoding RDRγ had a negative impact on viral DNA accumulation. Indeed, following single virus infection at both 10 and 30 dpi, the loads of viral DNA measured by qPCR in R plants carrying the functional RDRγ were much lower than those in nearly isogenic S plants lacking a functional RDRγ (Fig. 1A). Notably, whereas the ratio of viral loads between S and R plants was ~20 for IL, it was only ~5 for IS76 at both time points, indicating that the recombinant IS76 was able to evade the defence mediated by RDRγ better than its parent IL. Notably, DNA loads at 10 dpi were higher for IS76 than IL ~2 times in S plants and ~8 times in R plants (Fig. 1A). By 30 dpi, IS76 and IL accumulated their DNA at similar levels in S plants, whereas in R plants the DNA loads were ca. 4 times higher for IS76. Taken together, our results reveal that in R plants IS76 is consistently accumulating at a higher level than IL, thus supporting the above notion that the recombinant virus can evade RDRγ-mediated resistance better than its parent.

In mixed infection (IL+IS76) of S and R plants at 10 dpi, the DNA loads of IL were respectively 5.1 and 10 times lower than in singly infected S and R plants, whereas the DNA loads of IS76 were respectively 3.3 and 2.5 times lower than in singly-infected S and R plants (Fig. 1A). By 30 dpi, the DNA loads of IL were respectively 3.2 and 286 times lower than in singly-infected S and R plants. In sharp contrast, the DNA loads of IS76 by 30 dpi were similar between single and mixed infection of both S and R plants (Fig. 1A). Thus, mixed infection reduced the accumulation of both viruses but the effect was much less pronounced for IS76 for which coinfection did not affect its load by 30 dpi when the DNA load of IL became much lower in S plants, compared to 10 dpi, and barely detectable in R plants. These findings confirm the most remarkable fitness feature of the recombinant IS76, i.e., its strong negative impact on its parent IL in mixed infections eventually leading to elimination of the parental virus in R plants (Belabess et al., 2016; Jammes et al., 2023; Urbino et al., 2020).

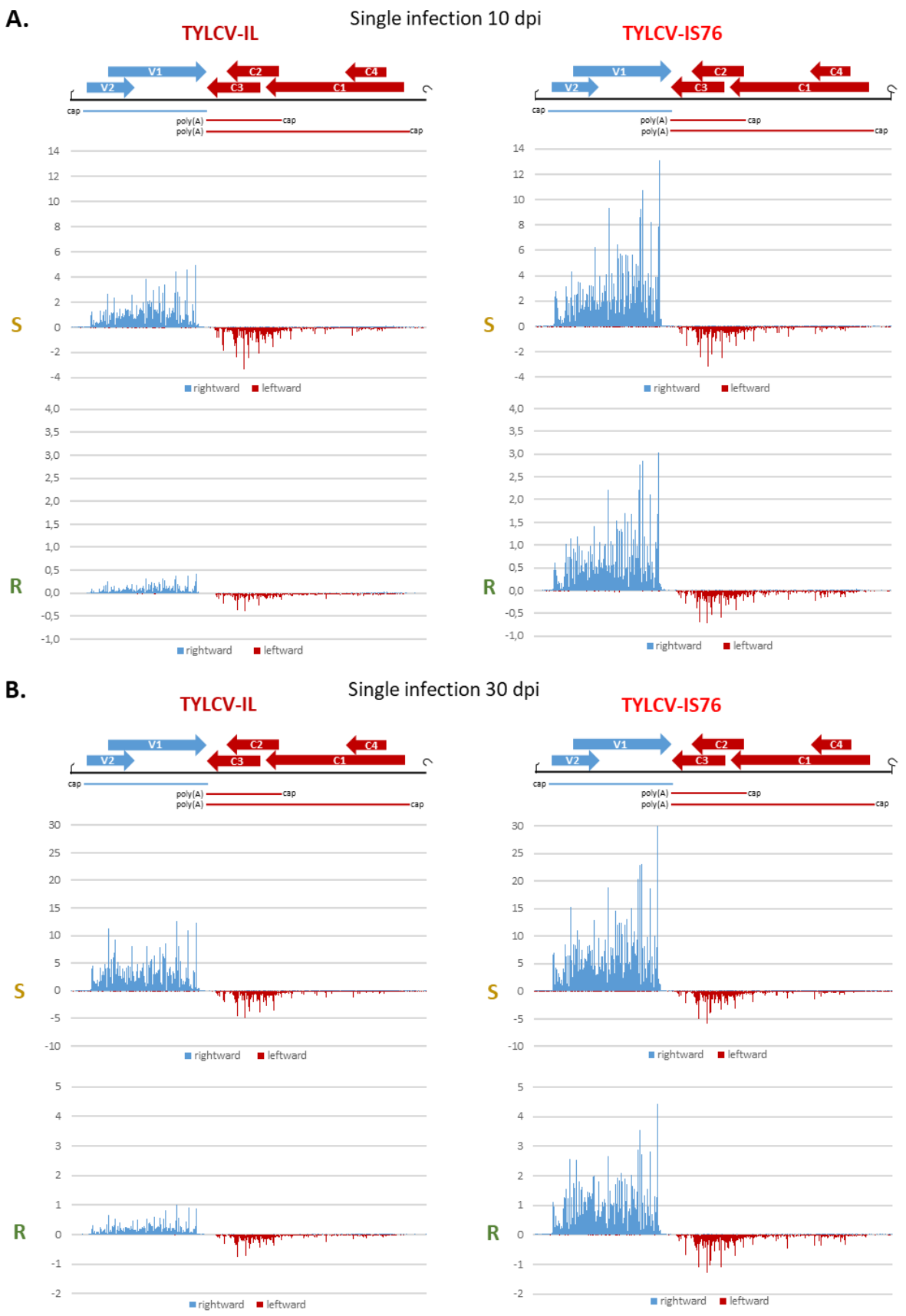

Figure 2. Single-nucleotide resolution maps of viral mRNA reads in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL or its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

3.1. RDRγ strongly enhances production rates of viral sRNAs and only slightly modulates production rates of viral mRNAs

Using the Illumina sequencing data, we first measured the collective loads of viral mRNAs and viral sRNAs in reads per million (RPM) of total (plant+viral) mRNA and total (plant+viral) sRNA reads, respectively. Whereas viral mRNA loads overall correlate relatively well with viral DNA loads, viral sRNA loads do not correlate with viral DNA loads (Fig. 1B vs Fig. 1A,) The most evident discrepancy with sRNA load correlation is between S and R plants (Fig. 1C vs Fig. 1A). Thus, the functional RDRγ has a much more profound effect on production rates of viral sRNAs rather than viral mRNAs.

3.1.1. mRNA

Despite the apparent correlation between viral DNA loads and viral mRNA loads, calculation of the production rates of viral mRNAs (i.e., viral mRNA loads divided by viral DNA loads) did reveal differences between S and R plants and between the two time-points. At 10 dpi, the viral mRNA production rates (mPR) in singly infected S plants were similar for IL (mPR = 7.1) and IS76 (mPR = 6.8), while in singly infected R plants the rates were slightly higher for both IL (mPR = 12) and IS76 (mPR = 9.5). Thus, both viruses appear to compensate in part the strong negative impact of RDRγ on viral DNA replication by increased production of viral mRNAs. By 30 dpi, the mRNA production rates dropped down for both viruses in both S and R plants and the compensatory effect was observed only for IL (mPR = 3.3 and 5.4, respectively) but not for IS76 (mPR = 4.8 and 4.7, respectively).

In mixed infections at 10 dpi, the rates of mRNA production from IL were similar between S (mPR = 7.2) and R (mPR = 7.5) plants and comparable to those in S plants singly infected with IL or IS76. Hence, in the presence of IS76, IL was not able to cope with the negative impact of RDRγ by increasing its mRNA production rate. In contrast, the presence of IL did not have any negative impact on the rates of mRNA production from IS76 which became even higher in S (mPR = 13) plants, compared to the plants singly infected with IS76. By 30 dpi, the rates of mRNA production from IS76 dropped down in both S (mPR = 4.4) and R (mPR = 5.3) plants as compared to 10 dpi. Compared to single infections with IS76 at this time-point, the presence of IL had only a slight negative impact on IS76 in S plants (mPR = 4.4 vs 4.8), while a slight positive impact of IL on IS76 was observed in R plants (mPR = 5.3 vs 4.7). In contrast, the presence of IS76 had a substantial negative impact on IL in both S (mPR = 1.0 vs 3.3) and R (mPR = 3.4 vs 5.4) plants.

3.1.2. sRNA

Comparison of the production rates of viral sRNAs (i.e., viral sRNA loads divided by viral DNA loads) revealed not only drastic differences between S and R plants at both time-points but also a substantial difference between IL and IS76 in R plants. In singly infected S plants, the sRNA production rates (sPR)

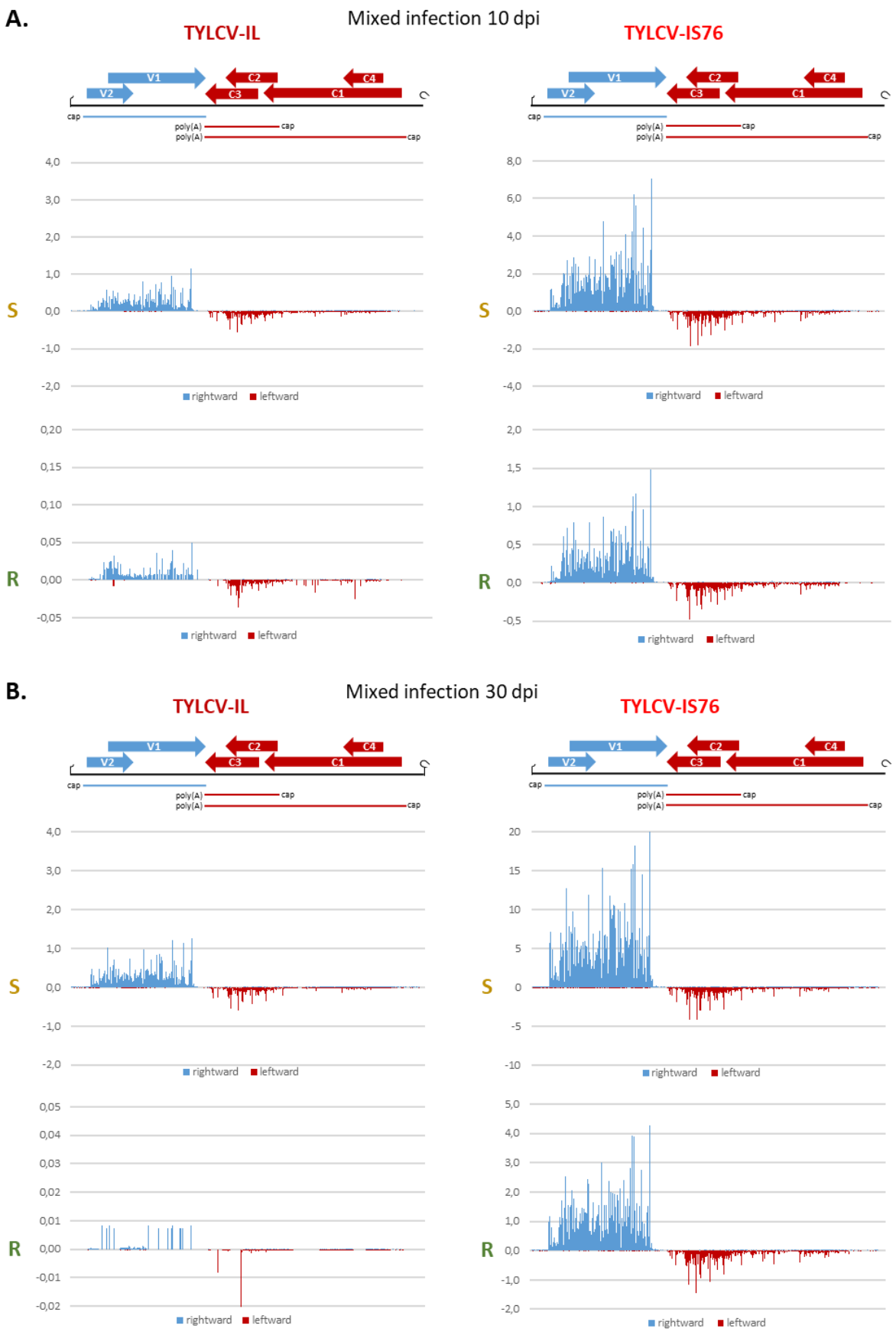

Figure 3. Single-nucleotide resolution maps of viral mRNA reads in susceptible (S) vs Ty-1 resistant (R) tomato plants coinfected with TYLCV-IL and its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

were slightly higher for IL (sPR = 65) than IS76 (sPR = 47) at 10 dpi and comparable for both viruses (sPR = 79 and 77, respectively) at 30 dpi. Notably, the slightly increased production rate of viral sRNAs observed for each virus at the later time-point coincides with the decreased production rates of viral mRNAs. Compared to S plants, in singly-infected R plants at 10 and 30 dpi the sRNA production rates were much higher for both IL (sPR = 1050 and 1097, respectively) and IS76 (sPR = 472 and 636, respectively) (Fig. 0). This strong enhancement of the sRNA production rates mediated by RDRγ, independent of virus identity, does not appear to have any negative effect on the production rates of viral mRNAs which become even higher for IL at both time points and for IS76 at 10 dpi, or remain unaffected for IS76 at 30 dpi (Fig 1C vs Fig 1B). Nonetheless, in R plants infected with IS76, the production rate of viral sRNAs becomes 1.3 times higher at 30 dpi, compared to 10 dpi, which coincides with 2 times decrease in the production rate of viral mRNAs. This is not the case in R plants infected with IL where 2.2 times decrease in the production rate of viral mRNAs between 10 and 30 dpi does not coincide with a reciprocal increase in the production rate of viral sRNAs. Mixed infection did not result in any drastic alterations in the rates of sRNA production from IL or IS76 in S plants at any time-point, although a slight negative effect of IS76 on IL could be observed at 30 dpi (sPR = 50 vs 79). On the other hand, mixed infection of R plants resulted in more pronounced and contrasting effects of IS76 on the rate of sRNA production from IL which was 1.4 times lower at 10 dpi (sPR = 725 vs 1050) but 6.3 times higher at 30 dpi (sPR = 6971 vs 1097), compared to single infection at the respective time-points. The strong increase in the rate of sRNA production from IL in mixed infection of R plants by 30 dpi coincides with a strong decrease in its DNA load (5.7 times, compared to 10 dpi in mixed infection, and 285 times, compared to 30 dpi in single infection), but does not result in a comparable decrease in its mRNA production rate which is decreased only 2.2 times, compared to 10 dpi in mixed infection, or only 1.6 times, compared to single infection at 30 dpi. In the case of IS76, mixed infection of R plants only slightly decreased its sRNA production rate at 10 dpi (sRPR = 419 vs 472 in single infection) and had an opposite effect by 30 dpi (sRPR = 706 vs 636 in single infection). These alterations did not correlate with alterations in the rates of mRNA production from IS76 (Fig 1C vs Fig. 1B).

Taken together, RDRγ strongly enhances overall production rates of viral sRNAs but only slightly modulates overall production rates of viral mRNAs for both viruses in both single and mixed infections at both time-points. To further investigate the role of RDRγ and the selective advantage of the recombinant IS76 over its parent IL, we analysed the relative abundance of individual viral mRNAs as well as the relative abundance, size profiles and polarity of viral sRNAs derived from the Pol II transcription units of viral mRNAs and from the intergenic regions of IL and IS76 in single and mixed infections of S and R plants.

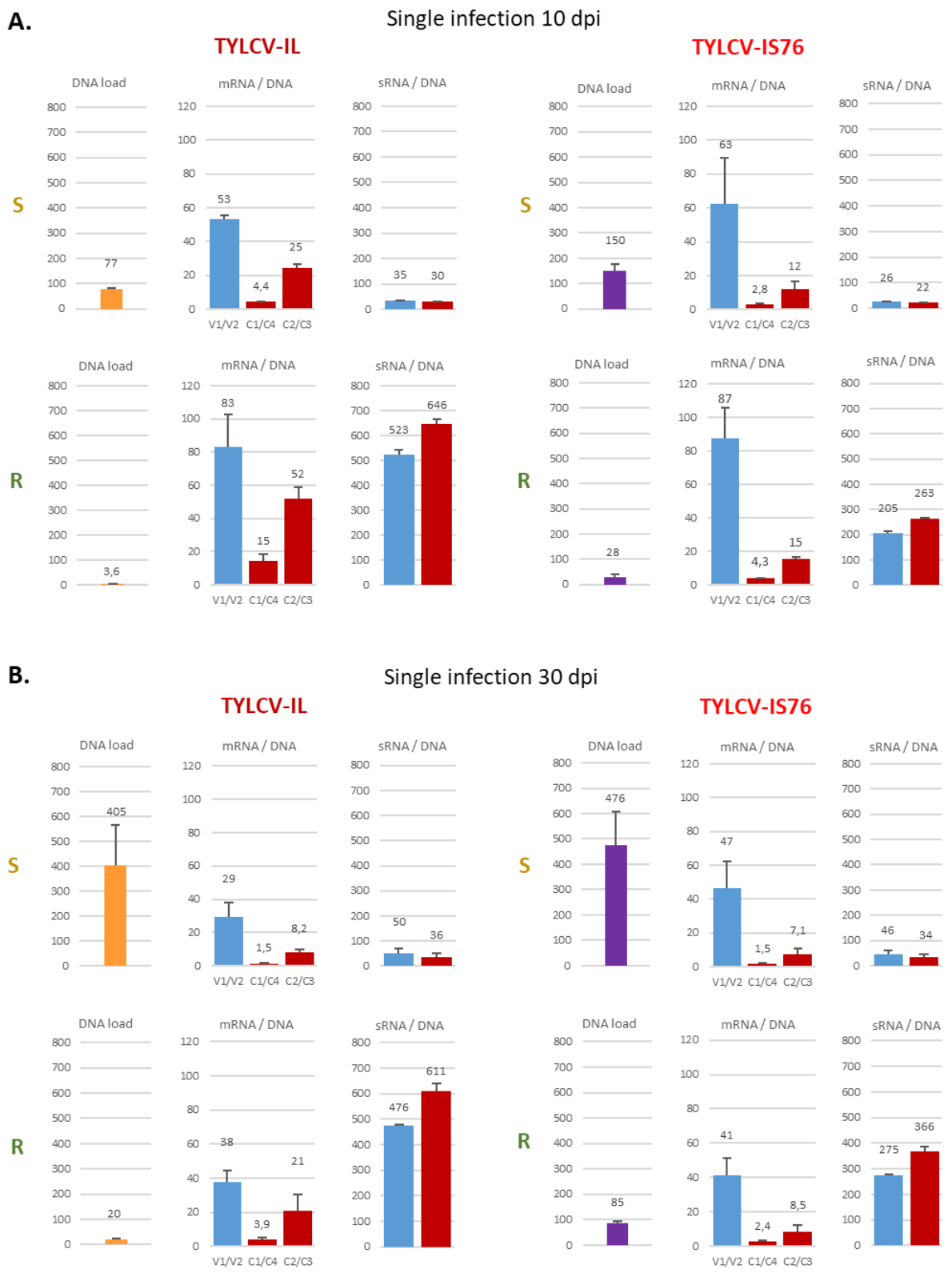

Figure 4. Production rates of viral mRNAs and of viral sRNAs in susceptible (S) vs Ty-1 resistant (R) tomato plants singly infected with TYLCV-IL and its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

3.2. IS76 recombination modifies viral mRNA transcription in favour of the rightward genes, independent of RDRγ activity or infection time-point

3.2.1. The virion-strand mRNA reads are more abundant than the complementary strand mRNA reads

Mapping of Illumina mRNA-seq 100 nt paired-end reads (enriched in those representing polyadenylated mRNAs produced by Pol II) on the reference genomes of IL and IS76 revealed three Pol II transcription units, one rightward (virion strand) unit for the V2-V1 mRNA and two leftward (complementary strand) units for 3'-coterminal the C1-C4 and C2-C3 mRNAs (Figs 2 and 3). Consistent with a previous study (Piedra-Aguilera et al., 2019), reads representing these three viral mRNAs were not homogeneously distributed along the length of each mRNA, likely due to sequence-specific biases in Illumina library preparation and sequencing protocols leading to either underrepresentation or overrepresentation of certain sequences: indeed, the map patterns are reproducible for each viral mRNA between S and R plants and between 10 and 30 dpi. The relative abundance of all reads (in RPM) representing each viral mRNA differed substantially, showing that V2-V1 mRNA is the most abundant for both viruses in all conditions, followed by the second most abundant C2-C3 mRNA and the least abundant C1-C4 mRNA (Figs 2 and 3; Supplementary Fig. S1). Similar mRNA profiles have been previously observed for TYLCV-IL (isolate Almeria) in susceptible tomato (cv. Moneymaker) at 7, 14 and 21 dpi (Piedra-Aguilera et al., 2019).

3.2.2. Virion versus complementary strand production rate of mRNAs is higher in IS76 than IL

3.2.2.1. Single infection

The most striking difference between IS76 and IL in both S and R plants at both 10 and 30 dpi is that IS76 accumulates its V2-V1 mRNA at relatively higher levels, compared to IL (Figs 2 and 3; Supplementary Fig. S1). This suggests that the recombination region of IS76 contains cis-element(s) modulating activities of the bidirectional promoter in favour of rightward transcription. Since the recombination region overlaps a CAAT box of the core promoter driving V2-V1 mRNA transcription, a direct effect of alterations in the sequences surrounding this element (19 SNPs and 3 indels of 2, 3 and 9 nucleotides) on the V2-V1 promoter would be more likely than indirect effects on the C1-C4 and C2-C3 promoters. However, calculation of the production rate of each viral mRNA (i.e., the mRNA load in RPM divided by the mRNA length in nucleotides and then by the viral DNA load) revealed that IS76 only slightly exceeds IL in the production rate of V2-V1 mRNA in both S and R plants singly infected with the respective viruses at both time points (Fig. 4). In contrast, IL greatly exceeds IS76 in the production rates of both C2-C3 and C1-C4 mRNAs in singly infected S and R plants at 10 dpi and in R (but not S) plants at 30 dpi (Fig. 4). Nonetheless, the ratio of the production rates of V2-V1 mRNA vs

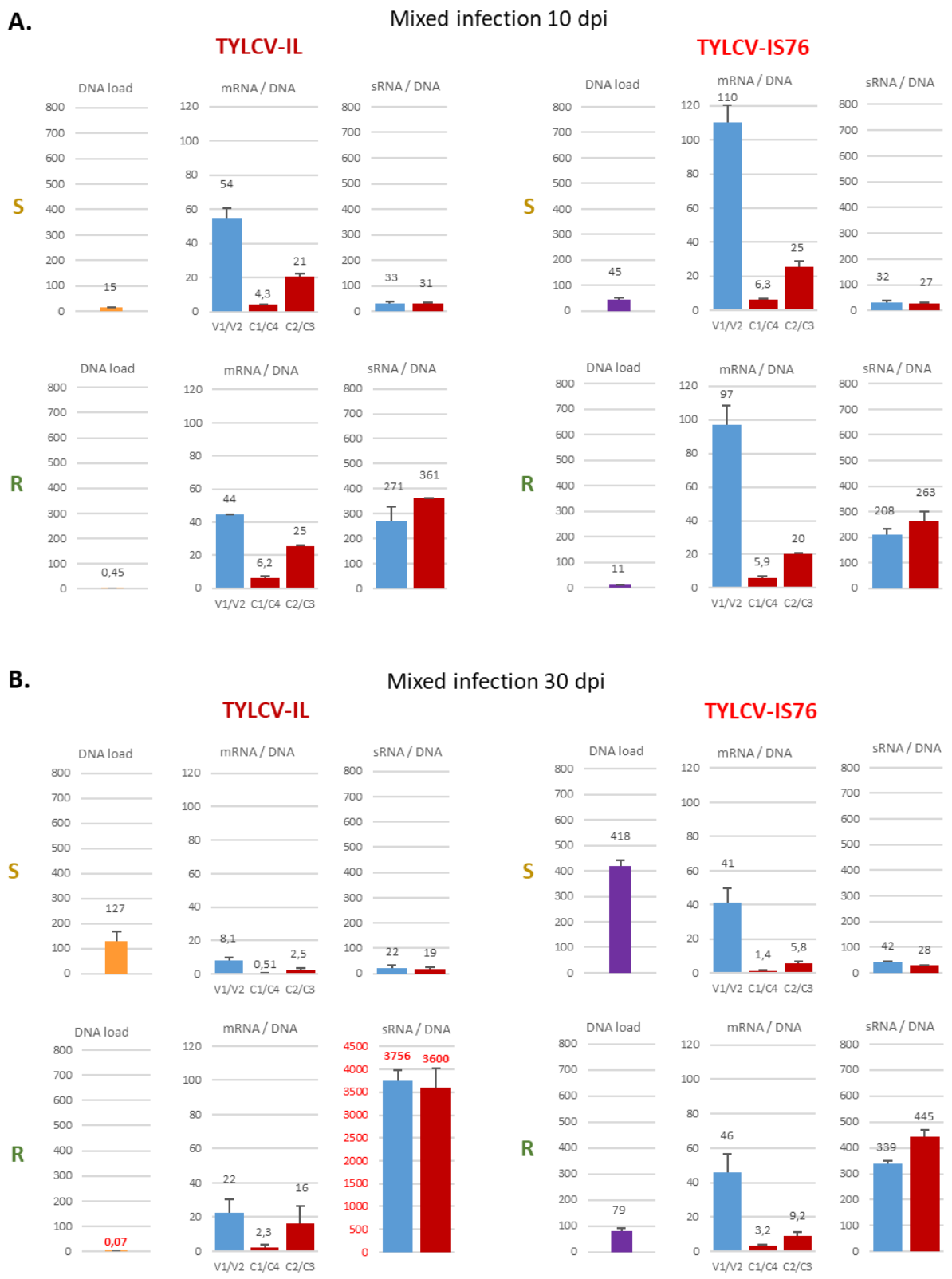

Figure 5. Production rates of viral mRNAs and of viral sRNAs in susceptible (S) vs Ty-1 resistant (R) tomato plants coinfected with TYLCV-IL and its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

C1-C4 mRNA is 1.9 times higher for IS76 than IL in singly-infected S plants at 10 dpi; by 30 dpi, this ratio increases for both IS76 and IL about 1.4 and 1.6 times, respectively, and thus still remains 1.6 times higher for IS76. The difference observed for both viruses between 10 and 30 dpi may reflect transition from the earlier to the later stages of infection when the encapsidation of viral DNA mediated by the viral CP translated from V2-V1 mRNA begins to prevail over the rolling-circle replication of viral DNA mediated by the viral Rep translated from C1-C4 mRNA. The higher V2-V1/C1-C4 mRNA production rate ratio for IS76 at both 10 and 30 dpi would then be consistent with the fact that IS76 replicates its DNA faster than IL (Fig 1A) and hence undergoes a faster transition from replication to encapsidation. This transition is likely controlled by the intergenic region-based cis-elements of the origin of replication and of the bidirectional promoter and, in particular, by the short-iterated sequence repeats (iterons) that bind the viral Rep, leading to inhibition of C1-C4 mRNA transcription as demonstrated for a bipartite begomovirus (Eagle et al., 1994). Rep-mediated inhibition of C1-C4 mRNA transcription would favour transcription of V2-V1 mRNA in the other direction and thereby promote production of viral CP required for encapsidation. Consistent with this hypothesis, in S plants infected with IL or IS76 the production rate of V2-V1 mRNA drops down between 10 and 30 dpi less pronouncedly than that of C1-C4 and C2-C3 mRNAs. Interestingly, a C2-C3/C1-C4 mRNA production rate ratio is somewhat higher for IL than IS76 at both 10 dpi (mPR = 5.7 vs 4.2) and 30 dpi (mPR = 5.4 vs 4.7), possibly due to indirect and differential effects of the recombination region-based cis-elements on the C1-C4 and C2-C3 promoters driving transcription of the respective mRNAs.

In R plants infected with IL or IS76 at 10 dpi the production rate of each viral mRNA was higher than in respective singly-infected S plants, suggesting that both viruses try to overcome the negative impact of RDRγ on viral replication by increasing transcription of each viral mRNA. However, IS76 and IL differ strikingly in that the production rates of C1-C4 and C2-C3 mRNAs are elevated much more pronouncedly for IL (mPR = 15 vs 4.4 and 52 vs 25, respectively) than for IS76 (mPR = 4.3 vs 2.8 and 15 vs 12, respectively), while the production rate of V2-V1 mRNA is elevated to comparable degrees for both IL (mPR = 83 vs 53) and IS76 (mPR = 87 vs 63). In other words, the rightward-to-leftward ratio of viral mRNA production rates drops down much more pronouncedly for IL than IS76 in R plants, compared to S plants. In fact, the V2-V1/C1-C4 ratio of IL is 2.2 times lower in R plants (mPR ratio = 5.5) than in S plants (mPR ratio = 12), whereas this ratio is only slightly reduced for IS76 (mPR ratio = 20.2 vs 22.5). Likewise, the C2-C3/C1-C4 ratio is more pronouncedly reduced for IL (mPR ratio = 3.5 vs 5.7) than IS76 (mPR ratio = 3.4 vs 4.2). The differences in individual mRNA production rates between IS76 and IL may reflect faster replication of IS76 and hence its faster transition to the phase favouring V2-V1 mRNA transcription even in R plants where its replication is inhibited by RDRγ (this

inhibition, however, is much less pronounced than that observed for IL). Similar to S plants, by 30 dpi in R plants

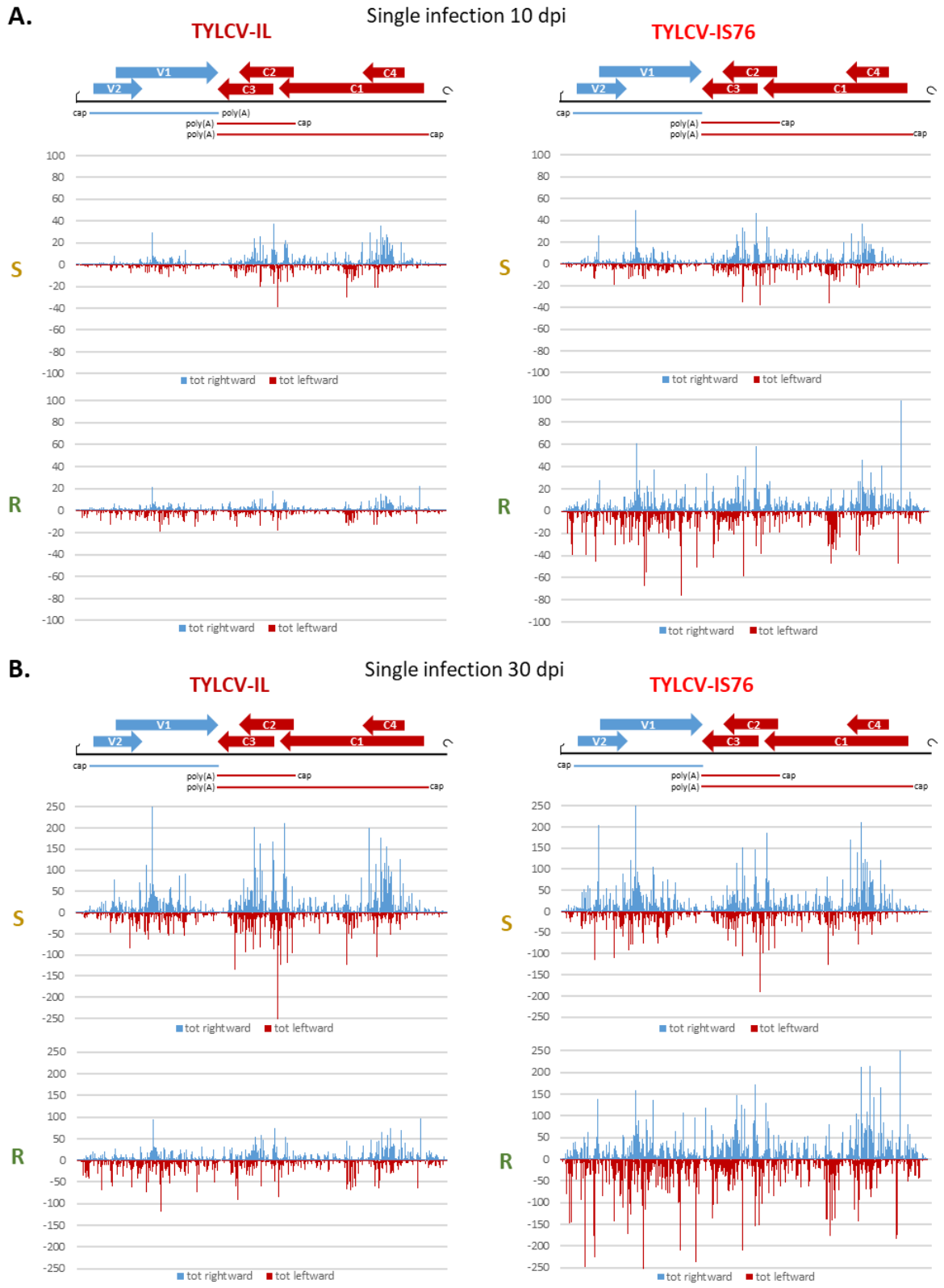

Figure 6. Single-nucleotide resolution maps of viral 20-25 nt small (s)RNAs in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL or its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

the individual production rates of all viral mRNAs dropped down substantially for both viruses. At the same time the V2-V1/C1-C4 mRNA production rate ratio remained much higher for IS76 (mPR ratio = 17.6) than for IL (mPR ratio = 9.7), although the difference is less pronounced than at 10 dpi (mPR ratio = 20.2 vs 5.5). This further supports that, in the presence of RDRγ slowing down replication of both viruses, IS76 is able to keep replicating faster than IL and thereby undergo much faster transition to the phase favouring the rightward transcription of V2-V1 mRNA over the leftward transcription of C1-C4 and C2-C3 mRNAs. In addition to the recombination region-based cis-elements, the kinetics of this transition is also modulated by RDRγ differentially for each virus. Indeed, RDRγ dramatically slows down this transition for IL as only by 30 dpi in R plants this virus could reach a rightward-to-leftward mRNA production rate ratio close to that achieved by this virus in S plants already by 10 dpi. By contrast in the case of IS76, the rightward-to-leftward mRNA production rate ratio is only slightly lower in R plants than in S plants at 10 dpi, possibly due to evasion of RDRγ-mediated defences by this recombinant virus already at early stages of infection. It is worth noting that the V2 ORF-encoded protein is a strong suppressor of antiviral defences based on RNAi and gene silencing (see references in Introduction). Favouring expression of this protein via enhanced transcription of V2-V1 mRNA at early stages of infection would help the recombinant IS76 better suppress antiviral silencing and, in particular, to counteract the action of viral sRNAs whose production rates are strongly enhanced by RDRγ for both viruses at both 10 and 30 dpi (Fig 1C). Interestingly, between 10 and 30 dpi in R plants the rightward-to-leftward mRNA production rate ratio is elevated only for IL but not for IS76 where it becomes slightly lower. This is unlike S plants where this ratio is elevated for both viruses by 30 dpi compared to 10 dpi. This correlates with the increased production of viral sRNAs in R plants for IS76 compared to IL whose sRNA production rate does not change between 10 and 30 dpi (Fig. 1C and see below).

3.2.2.2. Mixed infection

<u>10 dpi</u>

In mixed infection of S and R plants, the selective advantage of IS76 becomes apparent as IS76 effectively competes with IL for the host transcriptional machinery at both time points. At 10 dpi in S plants, the rate of mRNA production from IS76 is about 2 times higher than that from IL, mostly due to enhanced rightward transcription of V2-V1 mRNA (Fig. 5). Remarkably at this time point, the individual production rates of IL mRNAs in coinfected S plants are similar to those observed in S plants singly infected with IL and the presence of IL results in a ca. 2 times increase in the individual production rate of each mRNA of IS76. On the other hand, in coinfected R plants at 10 dpi the individual production rates of IS76 mRNAs are slightly higher than those observed in R plants singly infected with IS76, and

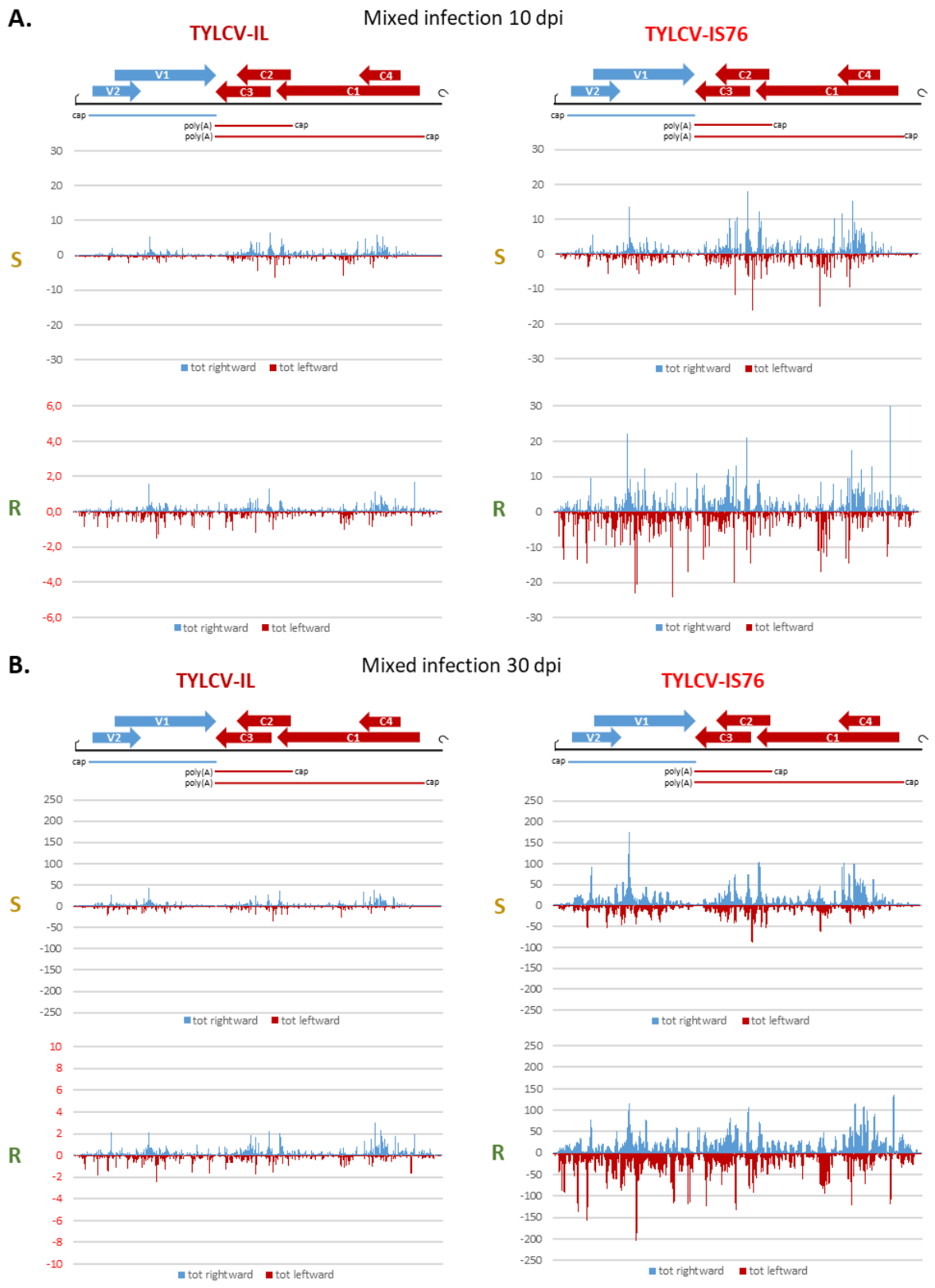

Figure 7. Single-nucleotide resolution maps of viral 20-25 nt small (s)RNAs in susceptible (S) vs Ty-1 resistant (R) tomato plants co-infected with TYLCV-IL and its recombinant derivative TYLCV-IS76 at 10- and 30-days post inoculation (dpi).

the presence of IS76 results in a ca. 2 times decrease in the individual production rate of each mRNA of IL. Thus, IS76 exerts a negative effect on mRNA production from IL at the early infection only in the presence of functional RDRγ, while IL does not interfere and even promotes mRNA production from IS76 especially in the absence of functional RDRγ.

30 dpi

At 30 dpi the negative effect of IS76 on mRNA production from IL becomes evident in both S and R plants. In coinfected S plants, the individual production rates of all IL mRNAs are proportionally decreased, each about 3 times, compared to singly infected S plants, which is concomitant with a 3.2 times decrease in IL DNA accumulation. In contrast, the individual mRNA production rates of IS76 are comparable in singly and coinfected S plants at 30 dpi, with its viral DNA accumulation being also unaffected by IL. In coinfected R plants, where only residual amounts of IL DNA were detected by 30 dpi, the individual production rates of IS76 mRNAs were comparable to those observed in singly infected R plants and DNA accumulation of IS76 almost reached the levels observed in singly infected R plants. On the other hand, IS76 impacted negatively the individual production rates of IL mRNAs in R plants, although to a lesser extent than in S plants. Notably, mixed infection did not have any drastic effect on the rightward-to-leftward mRNA production rate ratios of IS76 and IL, although this ratio was a somewhat lower for IS76 at 10 dpi, compared to single infection. The latter is consistent with our finding that IL interferes with IS76 replication at early stages of viral infection and thereby slows down its transition to the phase favouring the rightward transcription.

It should be noted that we cannot rule out that the viral mRNA production rates calculated here for the two distinct viruses may not represent the actual Pol II transcription rates at the respective transcription units, because the relative proportion of circular dsDNA (the template for Pol II-mediated transcription of viral mRNAs) in the total (ss+ds) viral DNA we measured by qPCR may differ at different conditions (S vs R plants, 10 vs 30 dpi). Southern blot hybridisation analysis with strand-specific probes did not, however, reveal any drastic difference in the ratio of circular ssDNA-to-circular dsDNA between IL and IS76 in S or R plants, while this ratio is somewhat higher in S plants for both viruses (Data not shown), consistent with the negative impact of RDRγ on replication of both viruses. Furthermore, several SNPs that distinguish each viral mRNA between IL and IS76 (18 SNPs in V2-V1 mRNA, 3 SNPs in C2-C3 mRNA and 7 SNPs in C1-C4 mRNA) may result in differential stability. Nonetheless, the fact that total viral DNA loads measured by qPCR correlate with total viral mRNA loads (Fig 1) gives us confidence that the mRNA production rates we calculated here do reflect the actual Pol II transcription rates at the respective transcription units and that the respective promoters are regulated accordingly by the recombination region-based cis-elements and the action of RDRγ.

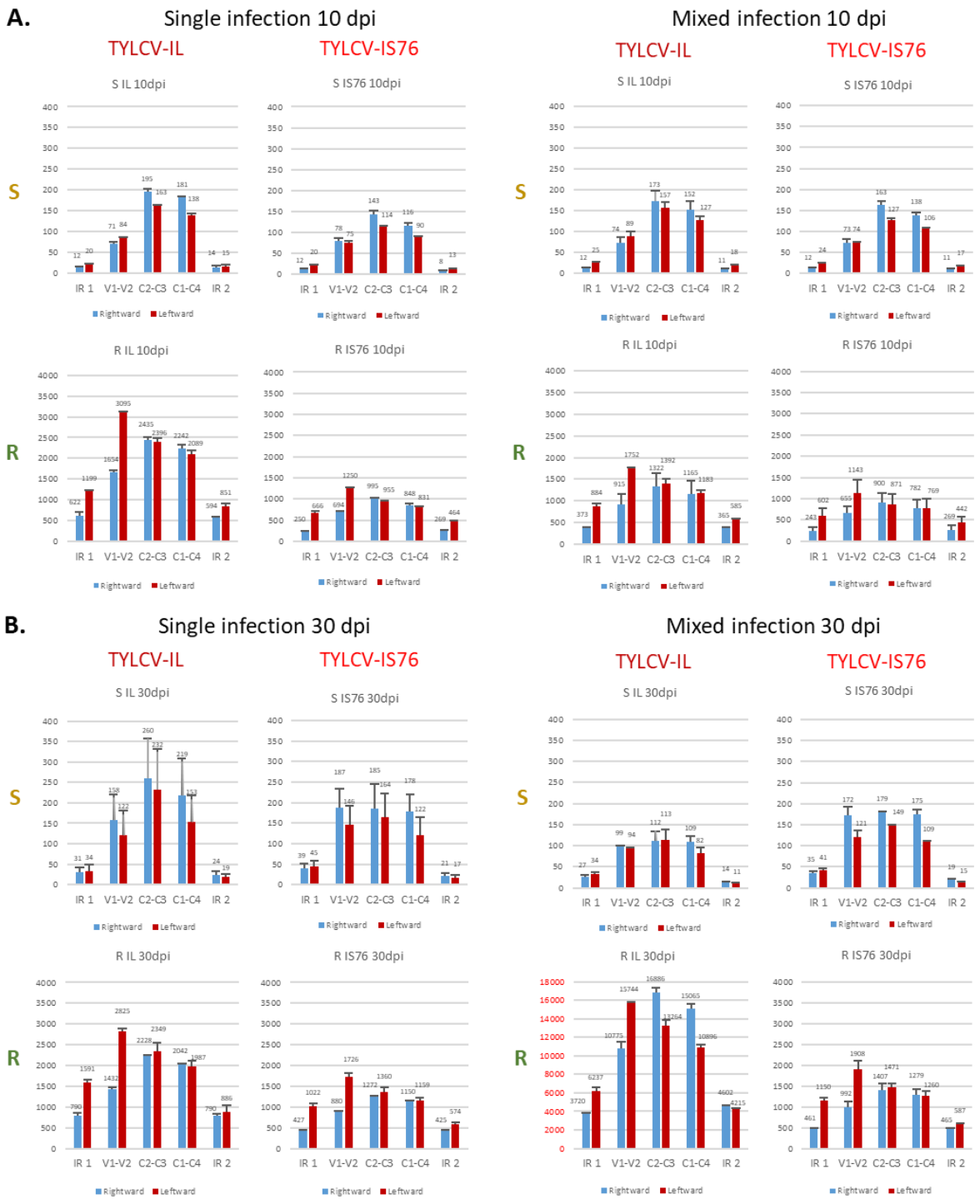

Figure 8. Production rates of viral sRNA reads in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

3.3. RDRγ boosts production rates of viral siRNAs of three major size classes from both strands of the entire virus genome and specifically promotes biogenesis of 22 and 24 nt siRNAs

The transcription rate as well as the posttranscriptional stability and translation of viral mRNAs can potentially be affected by antiviral RNAi mediated by virus-derived siRNAs including 24-nt siRNAs that direct transcriptional gene silencing as well as 21-nt and 22-nt siRNAs that direct posttranscriptional gene silencing. We therefore analysed the size, polarity and hotspot profiles of viral sRNAs whose total loads or total production rates did not correlate with total production rates of viral mRNAs (Fig 1).

Mapping Illumina sRNA reads on the reference genomes of IL and IS76 revealed that viral sRNAs are derived from both strands of the entire virus genomes in both S and R plants and at both time-points (Figs 6-8; Supplementary Fig. S2). In both singly-infected and coinfected S plants at both 10 and 30 dpi, the hotspots of viral sRNAs of sense and antisense polarities are concentrated within the Pol II transcription units and are underrepresented within the intergenic region (IR) with the V2-V1 and C1-C4 promoters, the poly(A) sites-containing Pol II terminator region and to a lesser extent the C2-C3 promoter region (between C2 and C4 ORFs). By contrast in R plants singly-infected or coinfected with IL and IS76 at both 10 and 30 dpi, the viral sRNA hotspots are more evenly distributed along the entire virus genome including the IR and the terminator region. These results are generally consistent with the previous studies profiling TYLCV-IL sRNAs at different time points in susceptible tomato plants (Fuentes et al., 2016; Piedra-Aguilera et al., 2019) and comparing the sRNA profiles of TYLCV-IL (isolate Almeria) in susceptible (cv. Moneymaker) and *Ty-1* resistant (cv. Tygress) plants (Voorburg et al., 2021). Production of viral sRNAs from both strands of the entire virus genome including the IR suggests that dsRNA precursors of viral siRNAs are generated by Pol II-mediated readthrough transcription beyond the poly(A) signals in both leftward and leftward directions as proposed earlier for a bipartite begomovirus (Aregger et al., 2012; Blevins et al., 2006). In support of this hypothesis, we found low-abundance reads likely representing remnants of the presumptive readthrough transcripts covering the antisense strands of both rightward and leftward transcription units and both strands of the IR of IL and IS76 (Supplementary Figure S2). These presumptive leftward and rightward readthrough transcripts that cover the entire virus genome could potentially form dsRNA precursors of viral sRNAs by pairing to the viral pre-mRNAs and/or mature mRNAs of opposite polarity and/or to each other. The latter events could generate dsRNA precursors of viral siRNAs derived from the IR outside of the Pol II transcription units. The observed enrichment of viral sRNA hotspots of sense and antisense polarities in the IR of both viruses in R plants, compared to S plants, would suggest that RDRγ could promote the presumptive bidirectional readthrough transcription of

the entire virus genome. Alternatively, dsRNA precursors of IR-derived sRNAs in S plants could be produced by other functional RDRs of alpha-clade

Figure 9. Size profiles of total viral sRNAs derived from forward and reverse strands of the viral genome in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

(RDR1, RDR2, and/or RDR6) using single-stranded RNAs generated either by Pol II-mediated readthrough transcription or by Pol V or Pol IV transcription. However, the previous studies in *A. thaliana* and its RDR1/2/6- and Pol IV/Po lV-deficient mutants infected with a bipartite begomovirus have ruled out involvement of these RNA polymerases in the biogenesis of bulk amounts of viral sRNAs of the three major size-classes (Aregger et al., 2012; Blevins et al., 2006). Importantly, Illumina sRNA-seq analysis of viral sRNAs derived from the bipartite begomovirus in *A. thaliana* wild type and *rdr1/2/6* triple mutant plants have revealed no substantial differences in the viral sRNA size, polarity and hotspot profiles (Aregger et al., 2012) which resemble the respective profiles we observed for IL and IS76 in S plants in that the bidirectional promotors and the Pol II terminator regions are depleted in siRNA hotspots. Thus, the viral sRNA hotspots concentrating in the mRNA transcription units cannot be explained by potential activities of RDR1, RDR2 or RDR6 converting viral mRNAs (or pre-mRNAs) into dsRNA substrates for DCLs (see below).

To further evaluate and quantify changes in viral sRNA biogenesis deduced from comparative analysis of the single nucleotide resolution maps of IL- and IS76-derived sRNAs at different conditions (Figs 6 and 7) we calculated the production rates of sRNAs derived from each strand of the Pol II transcription units, each unit from its cap site to its poly(A) site, and two parts of the intergenic region, one part (IR1) from the C1-C4 mRNA cap site to the origin-of-replication nick site (containing the leftward promoter) and another (IR2) from the nick site to the V2-V1 mRNA cap site (containing the rightward promoter). For this, the counts (in RPM) of viral sRNA reads derived from each viral sequence at each condition (IL or IS76 in single or mixed infection of S or R plants at 10 or 30 dpi) were divided by the length of each sequence and the viral DNA load. Comparison of the resulting sRNA production rates for each region and each strand revealed their dramatic increase in R plants, compared to S plants, for both viruses in both single and mixed infections at both time-points (Fig 8), indicating that RDRγ-dependent enhancement of viral sRNA production affects all the regions and strands of the virus genome. This enhancement is much more pronounced in both parts of the IR than in the Pol II transcription units. This finding would support our hypothesis that RDRγ could promote bidirectional readthrough transcription of the entire viral genome generating dsRNA precursors of viral sRNAs. Hypothetically, RDRγ could also be involved in conversion of viral RNAs produced by Pol II readthrough transcription (or by Pol IV or Pol V transcription) into dsRNA precursors of viral sRNAs. It should be noted that the observed changes in the viral sRNA hotspot profiles in R vs S plants are not consistent with viral mRNAs being direct targets of RDRγ (albeit the viral pre-mRNAs might be targets of RDRγ in the nucleus before completion of the processes of capping and polyadenylation by the Pol II complex and transport of the resulting mature mRNAs to cytoplasm for productive translation).

Figure 10. Size profiles of viral sRNAs derived from the recombinant part of intergenic region (IR-1) in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

In singly-infected S plants at 10 dpi, the highest sRNA production rates are observed in the C2-C3 and C1-C4 units, followed by the V2-V1 unit, without any substantial forward or reverse strand biases. However, while the V2-V1 unit produces sRNAs at similar rates in IL- vs IS76-infected S plants, both C1-C4 and C2-C3 units of IL produce sRNAs at higher rates than those of IS76 (Fig. 8A). This coincides with comparable production rates of V2-V1 mRNA and higher production rates of both C1-C4 and C2-C3 mRNAs in the respective plants (Fig. 4). Thus, viral sRNAs produced at higher rates from the leftward transcription units do not appear to interfere with rightward transcription of V2-V1 mRNA. In line with this notion, in both viruses the viral sRNAs of both polarities are produced at comparable rates from the C1-C4 vs C2-C3 units that produce the respective mRNAs at drastically different rates. Furthermore, the production rates of sRNAs from both parts of the IR are comparable in S plants singly infected with IL vs IS76 and, for both viruses, they are slightly higher from the IR 1 part containing the rightward promoter (Fig 8A). Since the estimated rate of the rightward transcription of V2-V1 mRNA is much higher than that of the leftward transcription of C1-C4 mRNA (Fig. 4), viral sRNAs produced form the IR-based bidirectional promoters do not appear to regulate Pol II-mediated transcription driven by these promoters. By 30 dpi in singly-infected S plants, the rates of viral sRNA production were slightly increased for both viruses. This increase was almost proportional (ca. 1.5 times) for each region of the IL genome in each polarity, whereas it was disproportional for IS76 in which both IR and V2-V1 unit produced sRNAs at ca. 2-3 times higher rates, while its leftward units at ca. 1.2-1.5 times higher rates (Fig 8B vs 8A). Similar to 10 dpi, at 30 dpi the relative production rates of viral sRNAs from different regions of IL and IS76 (Fig 8B) do not correlate with the relative production rates of respective viral mRNAs (Fig 4B), although the overall increase in viral sRNA production rates between 10 and 30 dpi does coincide with the overall decrease in viral mRNA production rates. Following our hypothesis, increased production of viral sRNAs at later stages of infection in S plants might be due to increased readthrough transcription generating dsRNA precursors of viral siRNAs.

Single infection

In singly-infected R plants at 10 dpi, the production rates of viral sRNAs derived from each region of the IS76 genome were almost proportionally (ca. 2-to-2.5 times) lower than those of the IL genome, indicating that IS76 can better compromise the RDRγ activity boosting sRNA production. In contrast to S plants, both viruses produced relatively more abundant sRNAs from the complementary (reverse) strand of the viral genome, especially within the V2-V1 transcription unit and to a lesser extent in other regions (Fig. 8A). Strand biases in viral sRNA profiles along the viral genome can be explained by differential sequence-specific stability of sRNAs produced by DCLs in a form of duplexes from longer dsRNA precursors and then sorted by AGO proteins. In particular, AGOs form a stable

complex with a guide strand of the sRNA duplex, and discard at passenger strand, leading to degradation of the latter.

Figure 11. Size profiles of viral sRNAs derived from the non-recombinant part of intergenic region (IR-2) in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

As the guide strand is selected by AGOs based on the 5´-nucleotide identity and other sequence features, differences in the nucleotide composition in dsRNA precursors of siRNAs can results in local hotspots and strand biases. It is conceivable that RDRγ-promoted readthrough transcription increases dsRNA production from the IR and the terminator region and simultaneously decreases dsRNA production from other regions, thereby resulting in alterations in viral sRNA hotspot profiles at both strands and in turn leading to alterations in viral sRNA forward-to-reverse strand ratios along the viral genome. By 30 dpi in singly-infected R plants, the sRNA production rates from each region of IL genome were slightly altered, with a slight increase being observed in both strands of the IR, concomitant with a slight increase in both strands of all the three transcription units. In the case of IS76, the sRNA production rates were slightly increased in both strands of each region of the virus genome but still remained lower than those in the regions and strands of IL genome. The viral sRNA strand biases also remained comparable in each region of each virus between 10 and 30 dpi. This indicates that the RDRγ activity boosting sRNA production constantly targets both viruses in the course of infection and that IS76 evades this activity better than IL. Similar alterations in sRNA hotspot profiles and strand bias were observed previously by Voorburg et al (2021) in TYCLV-infected *Ty-1* resistant vs susceptible plants sampled at a single time-point.

Mixed infection

In mixed infection of S plants at 10 dpi, the sRNA production rates are only slightly altered for each virus, compared to single infections. The most evident changes are observed in the C2-C3 and C1-C4 units where the sRNA production rates are slightly decreased for IL but slightly increased for IS76, while other regions remain less affected. These slight alterations coincide with a pronounced increase in the production rates of viral mRNAs from all the three transcription units of IS76, while IL transcription units either remain unaffected (V2-V1) or slightly downregulated (C2-C3 and C1-C4) by IS76 at this early time-point. At 30 dpi, however, the presence of IL does not alter the sRNA production rates from any region of IS76, whereas IS76 has a substantial negative impact on the production rates of sRNAs from all the regions of the IL genome except for the part of IR with the rightward promoter (i.e., the recombination region) where the rates are very similar in single and mixed infection. This coincides with a dramatic decrease of the production rates of viral mRNAs from all the three transcription units of IL and a substantial decrease of its DNA accumulation, while the mRNA production rates and the DNA accumulation of IS76 are only slightly affected by IL. Notably, IS76 impairs DNA accumulation of IL and its mRNA production rates to comparable degrees (both ca. 3 times), whereas IL sRNA production rates are downregulated by IS76 much less pronouncedly (Fig. 4B vs 8B). In summary, while at earlier time-point of mixed infection of S plants, the negative impact

of IS76 on IL DNA accumulation coincides with only slightly increased production rates of both sRNAs and mRNAs from the leftward

Figure 12. Size profiles of viral sRNAs derived from the rightward transcription unit (V1-V2) in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

units of IL, accompanied with increased production rates of all mRNAs (but not sRNAs) from IS76, at the later-time point the production rates of all sRNAs and all mRNAs of IL are impaired by IS76, without any reciprocal effect of IL on IS76 sRNA or mRNA production rates. Thus, only the higher production rate of V2-V1 mRNA from IS76, owing to the recombination region, starting from the early time points of mixed infection can faithfully account for its competitive advantage over IL in S plants.

In mixed infection of R plants at 10 dpi, the production rates of viral sRNAs from all the regions and strands of the IL genome are substantially and almost proportionally (1.5-2 times) reduced, compared to single infection, while the production rates of viral sRNAs from any region or strand of the IS76 genome are not altered substantially. This coincides with a substantial decrease in both the production rates of viral mRNAs from all the three units of IL (each ca. 2 times) and its DNA load (ca. 8 times), accompanied by a slight increase in the production rates of all viral mRNAs from IS76 whose DNA load is also reduced, compared to single infection, albeit much less pronouncedly (ca. 2.5 times). By 30 dpi in confected R plants, when IS76 accumulates its DNA at a slightly lower level than in single infection (DNA load = 79 vs 85) and IL DNA is barely detectable (DNA load = 0.07 vs 20, i.e., 286 times lower than in single infection), the production rates of viral sRNAs from each region and strand of the IS76 genome are slightly increased, whereas those of the IL genome are increased strongly (3.9 to 7.6 times). A more pronounced increase is observed in the Pol II transcription units of IL, accompanied by slight alterations in viral sRNA forward-to-reverse strand biases in favour of the forward strand. The strong increase in IL sRNA production rates coincides with a substantial but less pronounced decrease in the production rates of all viral mRNAs of IL (each ca 1.6-1.7 times lower than in single infection), accompanied by a slight increase in the production rates of all mRNAs of IS76. Thus, in the presence of IS76, IL could evade RDRγ activity much less efficiently than in single infection and between 10 and 30 dpi its replication declined dramatically, concomitant with the strongly increased rate of viral sRNA production from its residual DNA. Note that despite the strongly increased production rates, the loads of viral sRNAs produced from IL in mixed infection at 30 dpi are still much lower than the loads of viral sRNAs produced from IS76 (488 vs 55,779 RPM) as they also are at 10 dpi (290 vs 4,612 RPM) (Fig 1C). Likewise, despite IL can still produce its mRNA at the substantial rate from its residual DNA in mixed infection, the loads of its mRNAs by 30 dpi become much lower than at 10 dpi (0.24 vs 3.0 RPM) and almost negligible compared to single infection of R plants at both 10 dpi (47 RPM) and 30 dpi (107 RPM). In contrast, the loads of IS76 mRNAs in mixed infection continue to increase between 10 dpi (121 RPM) and 30 dpi (418 RPM) and even reach the levels observed in single infection by 30 dpi (396 RPM) despite a substantial retardation at 10 dpi (121 vs 267 RPM in single infection) (Fig. 1B). Thus, the higher production rate of V2-V1 mRNA

observed for IS76 in coinfected R plants from the earlier time-point, compared to mixed infection of S plants, might account for its selective advantage over IL

Figure 13. Size profiles of viral sRNAs derived from the leftward transcription unit C2-C3 in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

evident already at 10 dpi. RDRγ-mediated enhancement of viral sRNA production rates from both viruses and the ability of IS76 to evade this action of RDRγ better than IL and even promote the RDRγ activity against IL can explain elimination of IL from mixed infection of R plants at the later time-points.

To further understand the role of RDRγ in viral sRNA biogenesis and the selective advantage of the recombinant IS76 over its parental IL we analysed size profiles of forward and reverse viral sRNAs produced from the whole viral genomes (Fig 9) as well as from each part of IR and each transcription unit in single and mixed infections of S and R plants at 10 and 30 dpi (Figs 10-14). Consistent with previous studies of TYLCV and other begomoviruses, the three major (and functional) size-classes of viral siRNAs (21, 22, and 24 nt) derived from both strands of the IL and IS76 genomes were observed in all conditions. Their relative abundance differed substantially between S and R plants (Fig. 10). In S plants, the 21 nt class was dominant for both viruses at both 10 and 30 dpi, followed by the second most abundant 22 nt class and much less abundant 24 nt class. In R plants in all conditions, the relative proportions of 22 and 24 nt sRNAs of both polarities were substantially higher than in S plants (Figure 10), indicating that RDRγ promotes the biogenesis of both 22-nt and 24-nt viral siRNAs from both viruses more pronouncedly than the biogenesis of 21-nt siRNAs. A similar difference in viral sRNA size profiles between susceptible and Ty-1 resistant plants infected with TYLCV-IL has been reported by Voorburg et al. (2021). In the *A. thaliana*-bipartite begomovirus system, the biogenesis of 21, 22 and 24 nt viral siRNAs is mediated by DCL4, DCL2 and DCL3, respectively, which all contribute to the transcriptional and post-transcriptional silencing of viral genes (Akbergenov et al., 2006; Aregger et al., 2012; Blevins et al., 2006). Thus, in addition to boosting the overall production of viral siRNAs by all the three tomato DCLs targeting both IL and IS76, RDRγ appears to specifically promote the activities of DCL2 and DCL3. As argued above, RDRγ might function indirectly by promoting Pol II-mediated bidirectional readthrough transcription of the entire virus genome generating sense and antisense strands of dsRNA precursors of viral siRNAs and/or directly by converting the viral RNAs produced by Pol II readthrough transcription or by other RNA polymerases (Pol IV or Pol V) into dsRNA precursors of viral siRNAs. In both cases, those dsRNA precursors might then be preferentially processed by DCL2 and DCL3 generating respectively 22 and 24 nt siRNAs and, less preferentially, by DCL4 generating 21 nt siRNAs. Consistent with this hypothesis, the size profile of viral sRNAs derived from the IR and the Pol II mRNA units in S plants infected with both viruses differ substantially in that both parts of the IR generate relatively higher proportions of 22 and 24 nt siRNAs (Figs 10 and 11) than the Pol II units (Figs 12-14). Thus, even in the absence of functional RDRγ, the dsRNA precursors produced from the IR, where only Pol II readthrough transcription and/or Pol V/Pol IV transcription might take place, are more preferentially processed by DCL2 and

DCL3 than those produced from the Pol II mRNA units. Compared to S plants, in R plants the proportion of 21 nt siRNAs derived the IR is strongly reduced in

Figure 14. Size profiles of viral sRNAs derived from the leftward transcription unit C1-C4 in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10 and 30 days post inoculation (dpi).

favour of 22 nt siRNAs and to a lesser extent 24 nt sRNAs, and as a result the 22 nt class becomes dominant. A similar alteration in the siRNA size profile is also observed in the Pol II mRNA units, although the proportion of 21-nt siRNAs is less strongly reduced. Thus, in addition to boosting bidirectional readthrough transcription, RDRγ might also convert the readthrough transcripts into dsRNAs that are preferentially processed by DLC2 and less preferentially DCL3. In the absence of RDRγ, the readthrough transcripts may only pair to the complementary transcripts to form dsRNAs and those events might be more frequent within the transcription units producing high-abundance mRNAs and less frequent within the IR. This hypothesis would explain why sRNA hotspots concentrate within the transcription units in S plants and spread to the IR in R plants where RDRγ would generate additional dsRNAs from unpaired readthrough transcripts.

Comparison of the viral siRNA size profiles of IL and IS76 revealed that the proportion of 24 nt siRNAs derived from all the regions of the viral genome is higher in IL-derived siRNAs compared to IS76-derived siRNAs in most conditions (Figs 9-13), except for coinfected R plans at 30 dpi where the proportions of 24 nt siRNAs produced from residual IL and highly abundant IS76 are comparable, Thus, IS76 evades DCL3 activity better than IL and at the same time attracts other two DCL activities better than IL (except nearly eliminated IL). Collectively, IS76 can be transcribed by Pol II more actively than IL owing to both the recombination region based cis-elements and the more efficient evasion of DCL3-mediated transcriptional silencing generating 24 nt siRNAs. More active transcription of viral mRNAs facilitates replication of its DNA but at the same time attracts the post-transcriptional silencing machinery including DCL2 and DCL4. These properties of IS-76 explain its selective advantage and competitiveness in mixed infection with IL both in the absence and the presence of functional RDRγ.

Concluding remarks

The recombination region-based cis-elements regulating both rightward-to-leftward transcription ratio and replication efficiency might be affected by cytosine methylation potentially directed by 24 nt viral siRNAs. In fact, a total number of cytosines on both strands of the recombination region is higher in IL. It remains to be investigated if those cytosines present in the recombination region of IL (but absent in IS76) are indeed methylated in any substantial fraction of viral circular dsDNA, thereby interfering with its transcription or replication, and whether or not RDRγ promotes cytosine methylation of viral dsDNA by boosting production of 24 nt siRNAs. Previously, cytosine methylation of TYLCV-IL DNA in susceptible tomato plants lacking functional RDRγ was studied using bisulfite sequencing and the results revealed substantial methylation at CG, CHG and CHH sites within the

entire IR as well as the V2 ORF and two parts of the C1 ORF flanking the C4 ORF, although no correlation was found between the siRNA hotspot and the methylation hotspot profiles (Piedra-Aguilera et al., 2019). It should be noted that the bisulfite sequencing approach used in that study could not distinguish between circular and linear forms of viral dsDNA both of which can potentially be methylated. The circular dsDNA serves as a template for (i) Pol II transcription producing viral mRNAs, (ii) rolling circle replication producing multiple copies of circular ssDNA (to be packaged in the virions) and (iii) recombination-dependent replication generating linear dsDNA of heterogeneous length. The population of viral linear dsDNA molecules including concatemers with more-than-one copies of the viral genome can potentially be transcribed by Pol II and/or by other polymerases (Pol V or Pol IV) in both directions to produce dsRNA precursors of viral siRNAs, and this population can also be targeted by 24 nt viral siRNAs for cytosine methylation as proposed earlier (Pooggin, 2013). Thus, linear viral dsDNA would serve as a decoy diverting the RNAi silencing machinery from the actively-transcribed circular viral dsDNA generating viral mRNAs. The proportion of heterogeneous linear dsDNA in total viral DNA we measured by qPCR might vary for both viruses depending on the time-point of infection or co-infection and the presence of functional RDRγ, which may also contribute to the discrepancy between the total viral DNA loads and the viral siRNA loads (and hence production rates).

Acknowledgements

The study was supported by ANR through the PRIMA project “Prevention and control of new and invasive geminiviruses infecting vegetables in the Mediterranean”. The PhD grant of Margaux Jammes was funded by the ANR project and CIRAD (https://www.cirad.fr/). We are thankful to Martine Granier and Sophie Le Blaye for technical assistance.

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Supplementary data

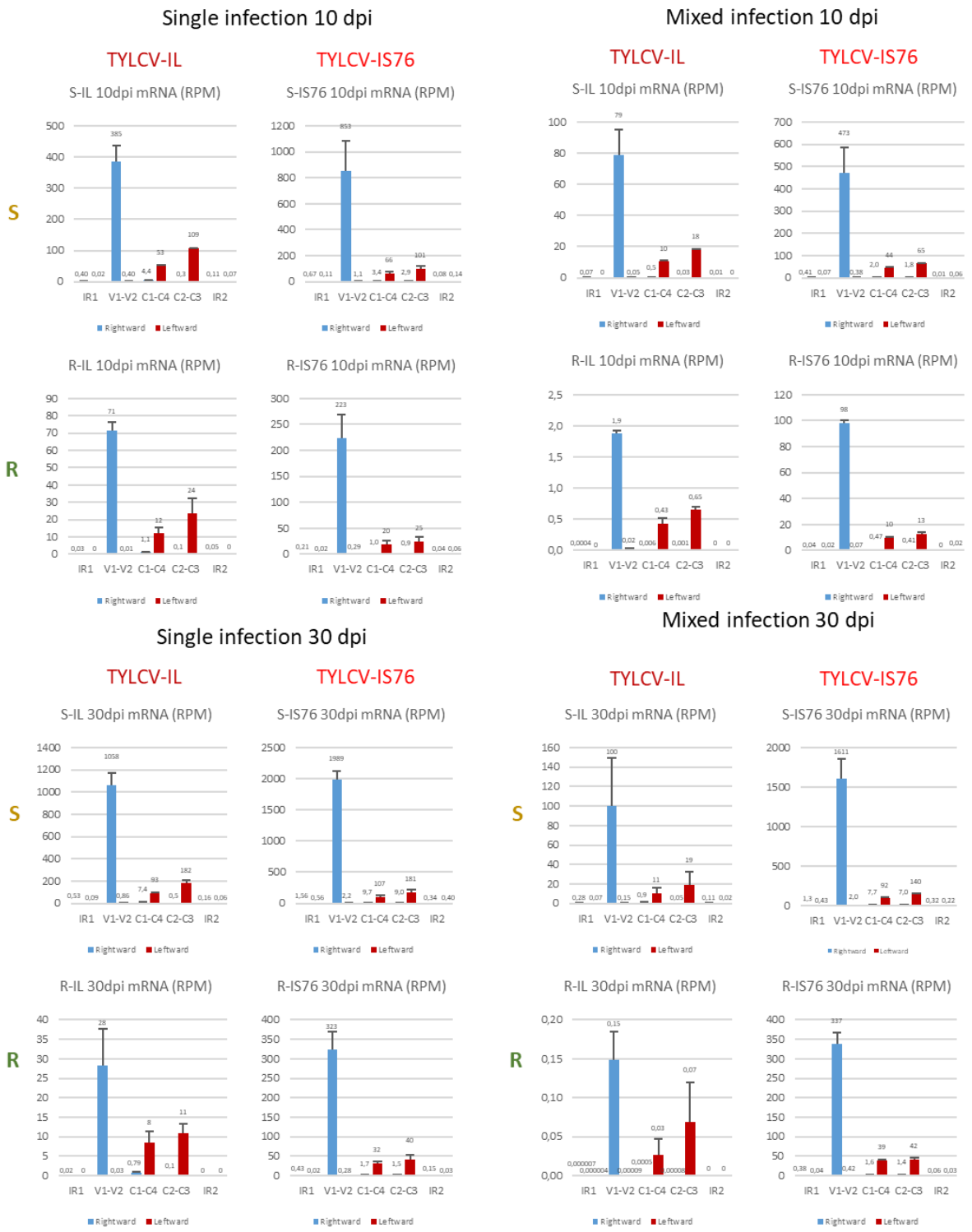

Figure S1. Counts of viral mRNA reads in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

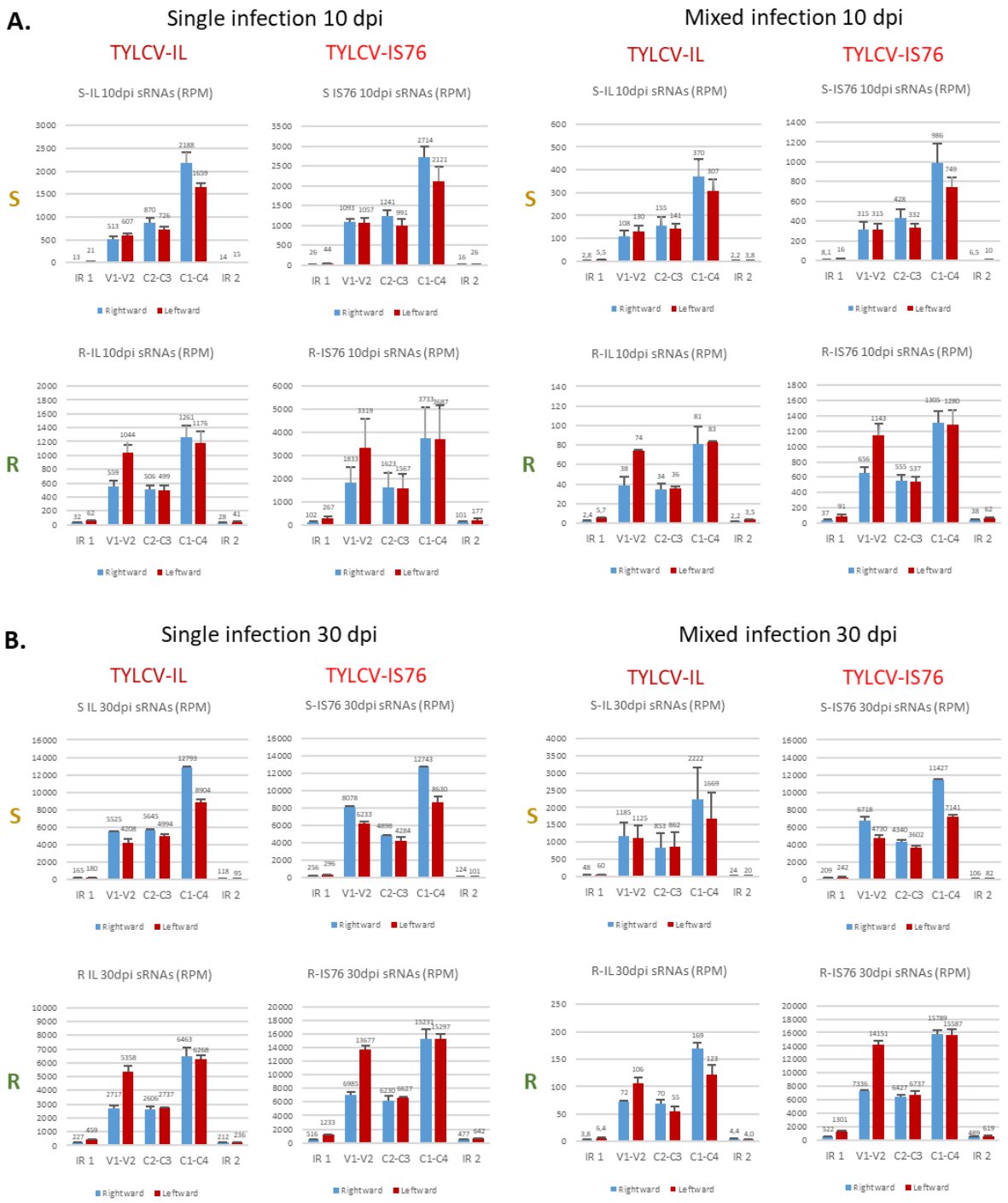

Figure S2. Counts of viral sRNA reads in susceptible (S) vs Ty-1 resistant (R) tomato plants infected with TYLCV-IL, its recombinant derivative TYLCV-IS76 or a combination thereof (IL+S76) at 10- and 30-days post inoculation (dpi).

CHAPITRE 4

Etude transcriptomique des gènes de plantes sensibles et résistantes infectées par TYLCV-IL et TYLCV-IS76 ou non infectées

1. Introduction

Le TYLCV-IS76 est un recombinant entre le TYLCV-IL et le TYLCSV apparu au Maroc et ayant presque complètement remplacé ses virus parentaux sur le terrain (Belabess et al., 2015). Il a été démontré que ce nouveau recombinant avait un avantage sélectif dans les plantes résistantes portant le gène Ty-1 et qu'il pouvait contourner la résistance. En effet, ce recombinant a été initialement révélé par la manifestation de symptômes sur des plantes résistantes Ty-1 et porteur de exclusivement de TYLCV-IS76 sans autre bégomovirus associé au tylc. Dans des plantes résistantes Ty-1, TYLCV-IS76 s'accumule davantage que les virus parentaux et, en situation de coïnfection il impacte lourdement l'accumulation virale de TYLCV-IL qui passe sous le seuil de détection 60 jours après inoculation (Belabess et al., 2016). De plus, selon d'autres expérimentations décrits dans les deux premiers chapitres de cette thèse, TYLCV-IS76 est capable de surinfecter des plantes préalablement infectées par ses virus parentaux (Jammes et al., 2023) et devenir majoritaire comme s'il avait été inoculé simultanément avec les parents. Cette dernière facette du phénotype de TYLCV-IS76 était finalement assez inattendue du fait que nous avons pu formellement démontrer que la prémunition est bien effective pour des variants de TYLCV. Toutes ces observations expliquent comment ce virus recombinant a pu envahir le Maroc et devenir majoritaire dans le pays, où les variétés de tomate résistantes portant le gène Ty-1 sont très répandues.

Le gène Ty-1 code pour une *RNA-dependent RNA polymerase* (RDR) de type gamma (Verlaan et al., 2013). Alors que les RDR de type alpha sont déjà connues pour jouer un rôle dans l'amplification du *gene silencing* les RDRϒ ont été beaucoup moins étudiés. Il a été montré cependant que le gène Ty-1 jouait également un rôle dans le renforcement du *gene silencing* dans des plantes infectées avec le TYLCV (Butterbach et al., 2014; Voorburg et al., 2021). Ce mécanisme permet de réprimer l'expression des gènes viraux par la production de petits ARNs complémentaires au génome du virus et qui vont pouvoir cibler ces ARNm pour dégradation (*gene silencing* post-transcriptionnel, PTGS) ou l'ADN viral directement pour méthylation et empêcher la transcription (*gene silencing* transcriptionnel, TGS). Dans le chapitre précédent, nous avons voulu comprendre comment le mécanisme du *gene silencing* pouvait donner un tel avantage au recombinant TYLCV-IS76 face à TYLCV-IL. Nous avons donc étudié le profil des ARN messagers (ARNm) et des petits ARN (siRNA) viraux dans des plantes sensibles ou résistantes infectées par TYLCV-IL et par TYLCV-IS76 en infection simple ou en infection mixte.

Cependant, le *gene silencing* n'est pas le seul mécanisme pouvant être impliqué dans l'avantage sélectif de TYLCV-IS76 dans les plantes résistantes Ty-1. Il est donc intéressant d'étudier plus largement les réactions se produisant dans les plantes après leur infection par TYLCV-IL ou TYLCV-

IS76 et de comparer ces réactions entre les plantes résistantes et les plantes sensibles. Dans ce chapitre, nous allons donc réaliser une étude transcriptomique afin de comparer les réactions entre plantes sensibles et plantes résistantes infectées par TYLCV-IL ou TYLCV-IS76 ou non infectées. Avec cette analyse nous souhaitons connaitre quel est l'impact de l'activation des mécanismes de résistance suite à la reconnaissance des virus sur la régulation des gènes de la tomate et si la résistance assurée par Ty-1 face aux virus s'explique par une différence dans l'expression de gènes spécifiques.

Nous avons vu dans le chapitre précédent que l'accumulation virale de TYLCV-IL et TYLCV-IS76, 10 jours après infection, était déjà diminuée dans les plantes résistantes par rapport aux plantes sensibles et que l'accumulation virale de TYLCV-IL était déjà inférieure à celle du TYLCV-IS76, dans les plantes résistantes, ce qui laisse penser que les mécanismes de résistance sont mis en place de manière précoce. 30 jours après inoculation, les accumulations virales ont atteint leurs niveaux maximaux, ceux des plantes résistantes restant inférieurs aux plantes sensibles. Contrairement à 10 jours après infection, les symptômes sur plantes sensibles sont bien développés alors que les plantes résistantes ne présentent pas de symptômes. Les analyses seront donc faites sur des plantes dès 10 jours après infection, afin d'étudier les mécanismes de résistance qui sont mis en place précocement dans les plantes Les analyses seront complétés avec des plantes échantillonnées 30 jours après infection, afin d'étudier les différences de réactions des plantes sensibles et résistantes associé à la différence drastique d'accumulation virale , d'un facteur 10 au minimum, à l'apparition ou non de symptômes, et aux phénomène de compétition conduisant à la disparition du parent TYLCV-IL dans les plantes résistantes.

L'analyse transcriptomique a donc été réalisée sur des plantes sensibles et des plantes résistantes portant le gène Ty-1, infectées soit par le TYLCV-IL, le TYLCV-IS76, ou non infectées, et échantillonnées à 10 jours ou 30 jours post inoculation.

2. Matériel et méthodes

Pour réaliser l'analyse du transcriptome des plantes, nous avons utilisés les extraits d'ARN totaux de plantes sensibles et résistantes non infectées et infectées par TYLCV-IL ou TYLCV-IS76 (en infection simple) collectées 10 jours ou 30 jours après inoculation. La préparation de ces extraits d'ARN a déjà été décrite dans le chapitre précédent car ces mêmes extraits ont servi aussi dans l'étude des mécanismes de gene silencing.

Les extraits d'ARN ont été divisé en deux fractions dont l'une a servi à la préparation des des cDNA pour l'analyse des petits ARNs (chapitre 3) et l'autre pour la préparation des cDNA pour l'analyse des ARNs messagers. La préparation des cDNA et le séquençage ont été réalisés par la société Fasteris. Pour l'analyse des ARNs messagers, la fraction a été enrichi en ARNs polyadenylés avant l'étape de synthèse de cDNA. Le séquençage des cDNA a été réalisé par la méthode Illumina comme décrit dans le chapitre précédent. L'*adapter trimming* des librairies est réalisé directement par la société Fasteris avec l'outil Trimmomatic. Les reads ont ensuite été alignés en utilisant le Burrow-Wheeler Aligner (BWA) sur le génome de référence de la tomate ITAG4.1 disponible sur *Sol Genomics Network* (www.solgenomics.net). Les comptes de reads ont été réalisés avec l'outil HTSeq count.

Les données ont ensuite été analysées avec le script DicoExpress (Lambert et al., 2020) développé par INRAe sur le logiciel R. Les gènes ont été filtrés, et seuls les gènes présentant un CPM (*Count per million*) supérieur ou égal à 5 pour la moitié au moins des échantillons sont conservés pour le reste de l'analyse. Les comptes de reads des gènes sélectionnés après filtration ont ensuite été normalisés grâce à la méthode TMM du package EdgeR. L'analyse différentielle a été réalisée par application d'un modèle GLM (*generalized linear model*) binomial négatif avec le package EdgeR. Un gène est considéré comme différentiellement exprimé si le FDR (*false discovery rate*) est inférieur ou égal à 0,05. L'analyse de coexpression des gènes différentiellement exprimés (DEGs) a été réalisée avec un modèle de mélange Gaussien (*Gaussian mixture model*) disponible dans le package coseq. Enfin, l'analyse d'enrichissement, faite sur les termes GO disponible dans le fichier d'annotation du génome de référence ITAG4.1 de *Sol Genomics Network*, a été réalisée grâce à un test hypergéométrique avec un seuil de significativité de 0,01.

3. Résultats

Le nombre total de gènes de la tomate connus et annotés sur la version du génome de référence que nous avons utilisé est de 34687. Après filtration de ces gènes sur la base d'un cpm seuil de 5 pour au moins la moitié de nos échantillons, 13939 gènes ont été conservés pour les plantes collectées 10 jours après infection, soit 40% du nombre total des gènes.

A 10 jours après infection, les gènes différentiellement exprimés (DEG) entre les plantes sensibles et les plantes résistantes sont peu nombreux, quel que soit leur profil d'infection. Ainsi, 98 DEGs ont été identifiés en absence d'infection (PC), 93 DEGs pour les plantes infectées par TYLCV-IL et 47 DEGs pour TYLCV-IS76 (Figure 1). Ce qui représente seulement 0,70%, 0,67% et 0,34% des gènes utilisés pour l'analyse, respectivement. Le faible nombre DEGs s'explique en partie par le fait que les deux cultivars sont isogéniques et diffèrent seulement au niveau de l'allèle Ty-1. Il est intéressant de noter

qu'il y a 2 fois moins de DEGs dans les plantes infectées par le TYLCV-IS76 que dans les plantes infectées par TYLCV-IL, ce qui suggère que les plantes réagissent moins à l'infection par le TYLCV-IS76 que par le TYLCV-IL. Alors que pour les plantes infectées, les gènes activés (up) sont plus nombreux que ceux qui sont réprimés (down), avec 68% de gènes activés pour TYLCV-IL et 70% pour TYLCV-IS76, pour les plantes non infectées, c'est le contraire avec seulement seulement 41 % de gènes activés (Figure 1).

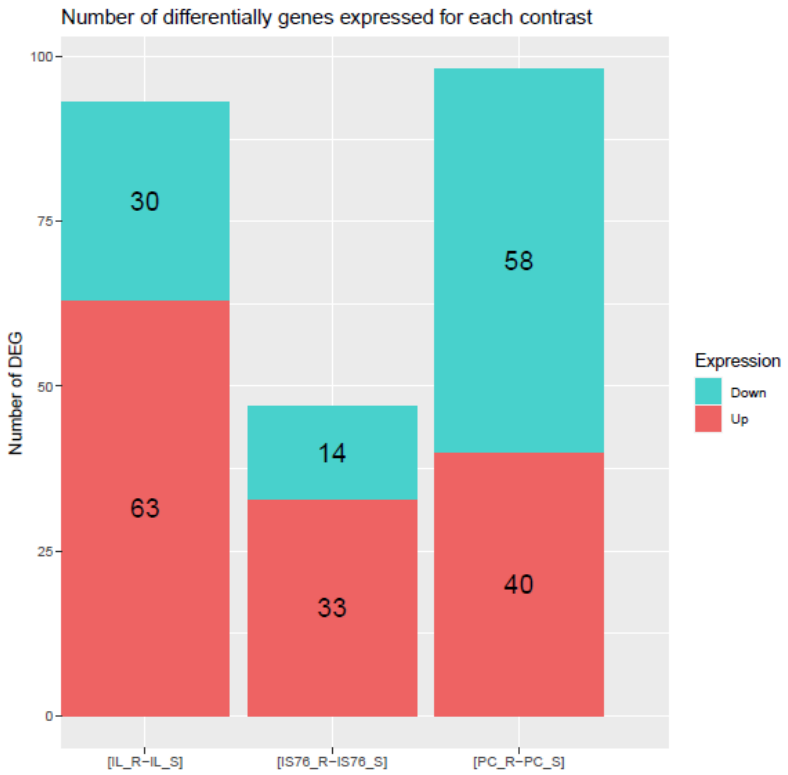

Figure 1 : Résultats de l'analyse d'expression différentielle des gènes à 10 jours après infection. Graphique en barres du nombre de gènes différentiellement exprimés entre les plantes résistantes et sensibles infectées par TYLCV-IL [IL_R-IL_S], par TYLCV-IS76 [IS76_R-IS76_S] ou non infectées [PC_R-PC_S]. Le nombre de gènes activés (up) ou réprimés (down) sont indiqués.

Parmi ces gènes différentiellement exprimés, pour les trois types d'infection, on retrouve le gène Ty-1 codant pour une RDR, et il fait partie des 10 gènes dont l'expression différentielle entre plantes sensibles et résistantes est la plus forte pour chaque profil d'infection. Ce gène est sur-exprimés dans les plantes résistantes par rapport aux plantes sensibles et légèrement plus exprimés lorsque les plantes sont infectées par TYLCV-IS76 par rapport aux autres types d'infection que ce soit dans les plantes sensibles ou les plantes résistantes (Figure 2). De plus, beaucoup de gènes différentiellement exprimés appartiennent au chromosome 6 pour chaque comparaison, 37 sur 93 soit 40% pour la comparaison entre plantes sensibles et résistantes infectées par TYLCV-IL, 29 sur 47, soit 62% pour les plantes infectées par TYLCV-IS76 et 31 sur 98 pour les plantes saines, soit 32% des gènes

différentiellement exprimés. Un test de Wilcoxon nous a permis de montrer que les gènes appartenant au chromosome 6 sont sur-représentés dans les gènes différentiellement exprimés entre les plantes sensibles et résistantes qu'elles soient infectées ou non infectées. Le gène Ty-1 est également situé sur le chromosome 6 donc ce phénomène est probablement dû au polymorphisme qui résulte de l'introgression du gènes Ty-1 dans les plantes résistantes et que les gènes différentiellement exprimés du chromosome 6 sont en déséquilibre de liaison avec le gène Ty-1.

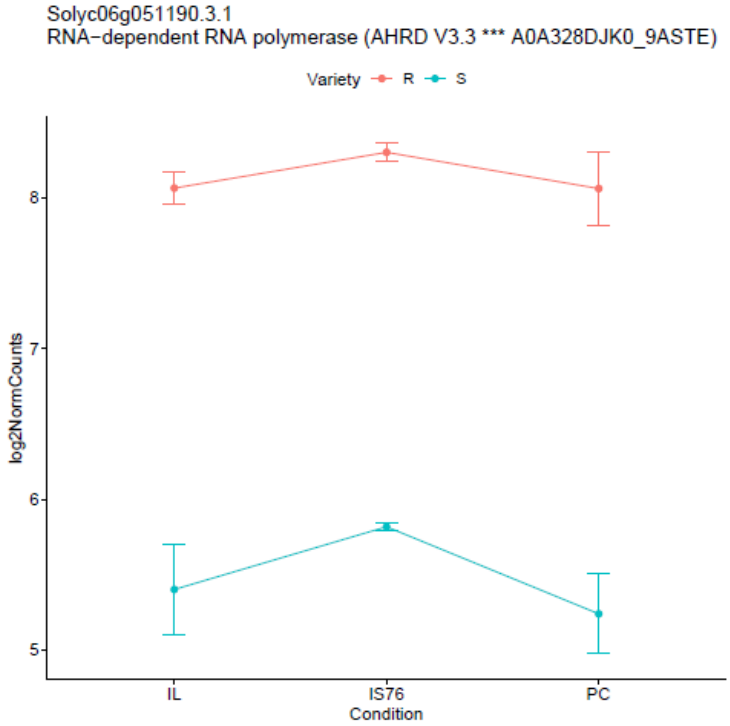

Figure 2 : Profils d'expression du gène Ty-1 dans les plantes sensibles (S, en bleue) et résistantes (R, en rouge), infectées soit par TYLCV-IL (IL) ou TYLCV-IS76 (IS76) ou non infectées (PC). L'expression du gène est exprimée en Log2 des comptes normalisés.

Les comparaisons entre plantes sensibles et résistantes, pour les trois types d'inoculation, partagent 21 DEGs communs (Figure 3). Outre le gène Ty-1, on trouve de nombreux gènes portés par le chromosome 6, vraisemblablement dû à l'introgression de Ty-1. En dehors du gène Ty-1 d'autres gènes pouvant être lié à la résistance des plantes à l'infection sont également identifiés parmi ces gènes communs, comme une protéine MLO, connues pour jouer un rôle dans la résistance contre l'oïdium. Cette résistance est une résistance récessive, la perte de fonction de la protéine MLO est associée à une perte de sensibilité au mildiou (Acevedo-Garcia et al., 2014; Büschges et al., 1997; Jørgensen, 1992). Ici, le gène de la protéine MLO est sous-exprimé dans les plantes résistantes. Les protéines MLO sont impliqués dans la réponse à différents stress biotiques et abiotiques. Elles peuvent agir comme des régulateurs de l'acide abscissique (Lim & Lee, 2014), modulent la réponse contre des bactéries et des oomycètes (Kim & Hwang, 2012) et la résistance liée à ces protéines repose sur des voies de signalisation commune à celle de l'immunité des plantes (Humphry et al.,

2006, 2010). Plusieurs études ont démontré une connexion entre les protéines MLO et des récepteurs aux protéines kinase (*receptor-like kinase* (RLK), (Humphry et al., 2010; Kessler et al., 2010) et il est intéressant de noter que dans les gènes communs aux trois comparaisons faites dans notre analyse transcriptomique, un gène codant pour un tel récepteur est sous-exprimés également dans les plantes résistantes.

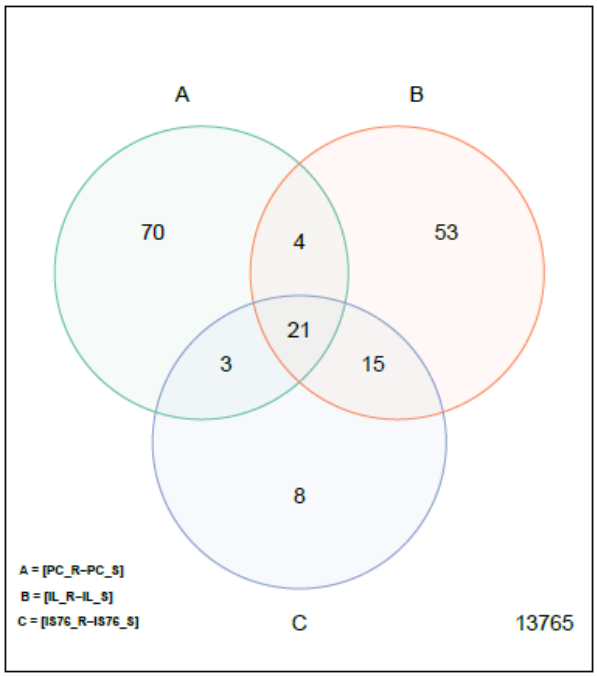

Figure 3 : Comparaison des gènes différentiellement exprimés entre plantes sensibles et résistantes non infectées ([PC_R-PC_S]) ou infectées soit par TYLCV-IL ([IL_R-IL_S]) soit par TYLCV-IS76 ([IS76_R-IS76_S]), à 10 jours après infection. Diagramme de Venn décrivant le nombre de gènes différentiellement exprimés pour les trois comparaisons [PC_R-PC_S], [IL_R-IL_S] et [IS76_R-IS76_S], et indiquant les gènes communs entre ces comparaisons.

La comparaison entre plantes sensibles et résistantes non infectées partagent seulement 4 gènes communs avec la comparaison des plantes infectées par le TYLCV-IL et 3 gènes communs avec la comparaison des plantes infectées par TYLCV-IS76. Les comparaisons entre plantes sensibles et résistantes infectées par le TYLCV-IL et par le TYLCV-IS76 ont 15 gènes communs dont un gène surexprimé dans les plantes résistantes, nommé Wound-induced protein 1 (WUN1), qui est impliqué dans les réponses aux blessures des plantes mais également lors de l'infection par certains pathogènes tels que les nématodes (Brodie, 1999) ou le champignon *Pseudomonas syringae* (Takemoto et al., 2003).

Alors qu'une minorité de DEGs des plantes infectées par TYLCV-IS76 lui sont spécifiques (17%), pour les plantes non infectées et les plantes infectées par le TYLCV-IL ils sont majoritaires avec 71% et 57% respectivement (Figure 3). Parmi les DEGs spécifiques, on trouve une protéine MLO et une protéine de la famille des TIR-NBS-LRR connu pour leur rôle dans la résistance des plantes aux pathogènes en permettant la détection des agents pathogènes et en activant des voies de signalisation pour la

défense des plantes contre ces pathogènes. Donc, d'autres mécanismes de résistance aux maladies se mettent en place lors de l'infection par le TYLCV-IL qui sont spécifiques à ce virus. Le TYLCV-IL est donc ciblé à la fois par la résistance Ty-1 mais également par d'autres gènes de résistance, ce qui peut expliquer son désavantage par rapport à TYLCV-IS76.

Les 174 DEGs identifiés 10 jours après infection entre les plantes résistantes et les plantes sensibles infectées et non infectées se regroupent en deux clusters distincts. Le premier cluster contient 19 gènes et regroupe des gènes dont l'expression est fortement supérieure dans les plantes résistantes par rapport à leur expression dans les plantes sensibles (Figure 4). Les annotations GO associées à ces gènes sur-exprimés dans les plantes résistantes sont listées dans le tableau 1. On y retrouve l'activité RDR du gène Ty-1 qui confirme que ce gène est bien lié à la résistance des plantes aux virus et également les termes *response to wounding* pouvant et *ubiquitin protein ligase binding* pouvant être impliqué dans des réponses de défenses de la plante. En effet, l'ubiquitination de protéines est une modification des protéines permettant la régulation de nombreux processus intracellulaires dont les voies de signalisation de défense aux pathogènes (Devoto et al., 2003). Le deuxième cluster regroupe 155 gènes pour lesquels l'expression moyenne est similaire entre les différentes conditions (Figure 20).

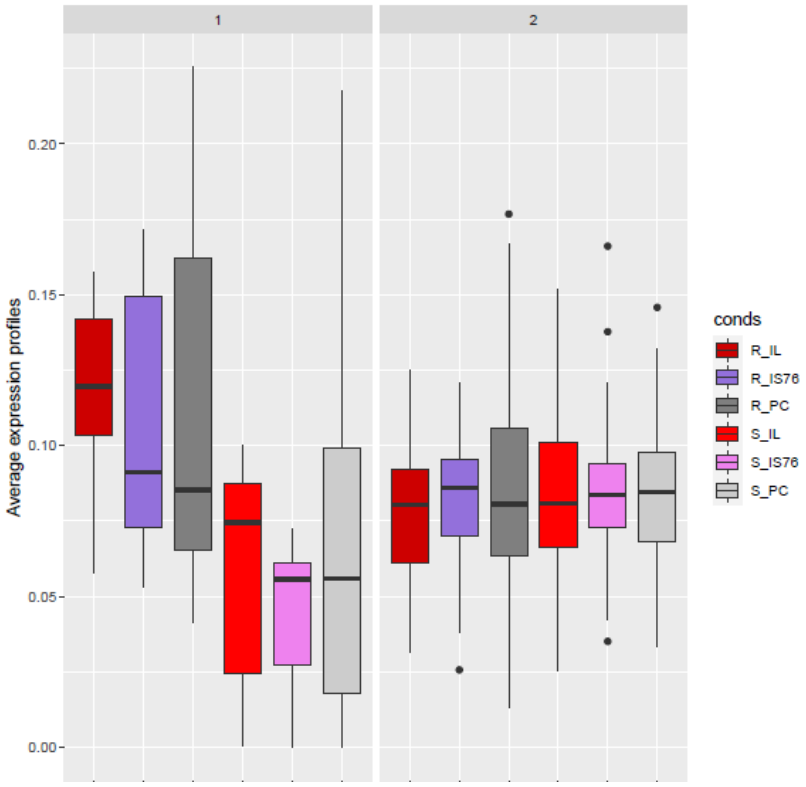

Figure 4 : Cluster de coexpression des gènes différentiellement exprimés entre plantes sensibles et résistantes non infectées (S_PC et R_PC) ou infectées soit par TYLCV-IL (S_IL et R_IL) soit par TYLCV-IS76 (S_IS76 et R_IS76) à 10 jours après infection. Profils d'expression moyenne des différents clusters calculés par l'analyse de coexpression des gènes différentiellement exprimés compris dans l'union des trois comparaisons [PC_R-PC_S], [IL_R-IL_S] et [IS76_R-IS76_S].

Tableau 1 : liste des termes GO pour les gènes du cluster 1 contenant 19 gènes.

GO term	Nombre de gènes
RNA-dependent RNA polymerase activity	1
response to wounding	1
hydrolase activity	2
ubiquitin protein ligase binding	1

L'analyse sur les plantes collectées 10 jours après l'inoculation a montré que très peu de gènes étaient différentiellement exprimés entre les plantes résistantes et les plantes sensibles, qu'elles soient saines ou infectées avec l'un ou l'autre des virus. A une date précoce d'infection, les réactions des plantes à l'infection sont donc très faibles. Cependant, on a observé qu'à cette date, le gène Ty-1 était déjà fortement exprimé dans les plantes résistantes comparées aux plantes sensibles. De plus, on observe cette différence d'expression dans les plantes infectées mais également dans les plantes non infectées, ce qui veut dire que ce gène est naturellement exprimé dans les plantes résistantes sans présence du virus. La protéine RDR codée par ce gène est donc déjà produite dans les plantes avant même l'infection, ce qui permet à la résistance induite par ce gène d'être efficace très tôt, dès le début de l'infection. En complément de l'activité du gène Ty-1, parmi les gènes surexprimés dans les plantes résistantes, on retrouve des gènes impliqués dans des fonctions de défense comme l'ubiquitination et la réponse à la blessure (tableau 1). D'autres gènes impliqués dans la résistance aux maladies ressortent différentiellement exprimés lorsque les plantes sont infectées avec TYLCV-IL tel que la protéine MLO (voir ci-dessus). Ces gènes pourraient donc intervenir également dans la résistance contre le TYLCV-IL spécifiquement et jouer un rôle dans la réduction de son accumulation virale dans les plantes résistantes.

Pour les plantes collectées 30 jours après infection, 14626 gènes ont été conservés pour l'analyse, soit 42% du nombre total de gènes compris dans notre génome de référence.

Dans les échantillons de plantes prélevées 30 jours après infection, les gènes différentiellement exprimés entre les plantes sensibles et résistantes infectées par le TYLCV-IL ou le TYLCV-IS76 sont beaucoup plus élevés que dans les plantes non infectées. Au total, 7594 gènes ont été identifiés pour la comparaison entre plantes sensibles et résistantes infectées par le TYLCV-IL ce qui représente 52% des gènes utilisés dans l'analyse, 6525 gènes ont été identifiés pour les plantes infectées par le TYLCV-IS76 soit 45% des gènes utilisés dans l'analyse alors que seulement 98 gènes ont été identifiés pour les plantes non infectées, représentant 0,68% des gènes de l'analyse. Chez les plantes infectées, la quantité de gènes réprimés (down) est équivalente à la quantité de gènes activés (up) avec 50,8%

de gènes réprimés pour les plantes infectées par TYLCV-IL et 50,4% pour les plantes infectées par TYLCV-IS76 (Figure 5).

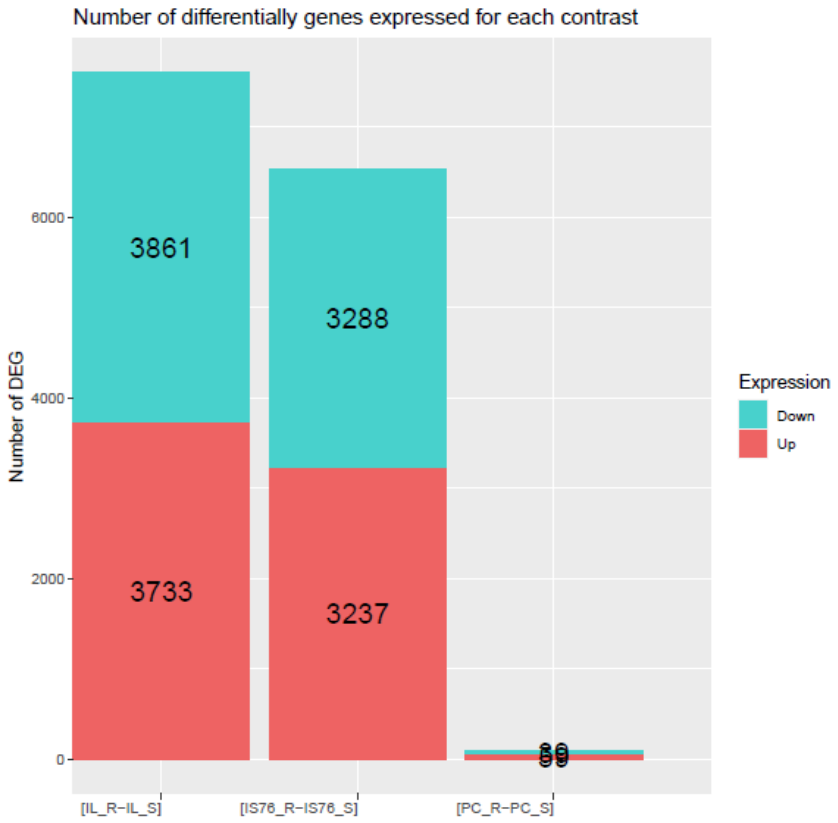

Figure 5 : Résultats de l'analyse d'expression différentielle des gènes 30 jours après infection. Graphique en barres du nombre de gènes différentiellement exprimés entre les plantes résistantes et sensibles infectées par TYLCV-IL [IL_R-IL_S], par TYLCV-IS76 [IS76_R-IS76_S] ou non infectées [PC_R-PC_S]. Le nombre de gènes activés (up) ou réprimés (down) sont indiqués.

Les gènes différentiellement exprimés dans les plantes non infectées sont équivalent au nombre de gènes identifiés 10 jours après infection alors que pour les plantes infectées, il y a 82 fois plus de gènes différentiellement exprimés à 30 jours qu'à 10 jours après infection pour les plantes infectées par TYLCV-IL et 139 fois plus pour les plantes infectées par TYLCV-IS76 (Figure 1 et 5).

Les trois comparaisons possèdent 80 gènes différentiellement exprimés communs, ce qui représente 82% des gènes identifiés pour la comparaison entre les plantes non infectées mais seulement 1% et 1,2% des gènes différentiellement exprimés pour l'infection par TYLCV-IL et TYLCV-IS76 respectivement (Figure 6). Parmi ces gènes communs, on retrouve toujours le gène Ty-1. La majorité des gènes différentiellement identifiés pour les plantes infectées sont communs entre les deux comparaisons, en effet, ces deux comparaisons partagent 5405 gènes soit 83% des gènes identifiés pour la comparaison des plantes infectées par TYLCV-IS76 et 71% des gènes identifiés pour la comparaison des plantes infectées par le TYLCV-IL (Figure 6). Parmi ces 5405 gènes communs, 17

sont des TIR-NBS-LRR et 2 CC-NBS-LRR, 20 gènes appelés « *Disease resistance protein* » et 3 sont des protéines d'amplification de la résistance (*Enhanced disease resistance 2*). 2103 gènes sont spécifiques à l'infection par TYLCV-IL dont 6 gènes codant pour des protéines de résistances aux maladies (2 TIR-NBS-LRR, 1 CC-NBS-LRR, 2 appelés *Disease resistance protein* et 1 appelé *Enhanced disease resistance 2*) et 1038 gènes sont spécifiques à l'infection par le TYLCV-IS76 dont seulement 2 gènes codant pour des protéines de résistance (1 TIR-NBS-LRR et 1 appelé *Disease resistance protein*).

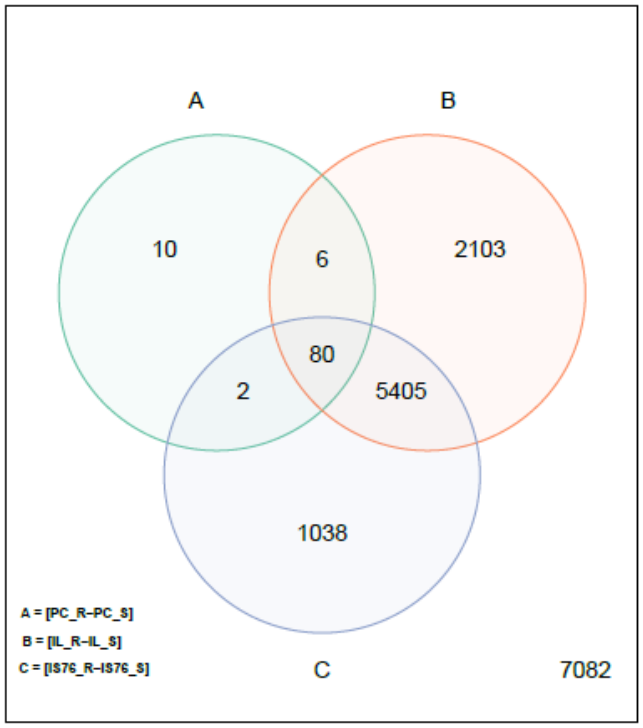

Figure 6 : Comparaison des gènes différentiellement exprimés entre plantes sensibles et résistantes non infectées ([PC_R-PC_S]) ou infectées soit par TYLCV-IL ([IL_R-IL_S]) soit par TYLCV-IS76 ([IS76_R-IS76_S]), à 30 jours après infection. Diagramme de Venn décrivant le nombre de gènes différentiellement exprimés pour les trois comparaisons [PC_R-PC_S], [IL_R-IL_S] et [IS76_R-IS76_S], et indiquant les gènes communs entre ces comparaisons.

Les nombreux gènes différentiellement exprimés entre plante sensibles et plantes résistantes collectées 30 jours après infection se regroupent en 22 clusters d'expression. Excepté dans le cluster 20 dans lequel les gènes identifiés sont surexprimés dans les plantes résistantes infectées et sous-exprimés dans les autres conditions, on remarque que dans tous les autres clusters, les plantes sensibles infectées par TYLCV-IL et TYLCV-IS76 ont des profils d'expression similaires et opposés aux autres traitements. Les plantes sensibles infectées présentent une expression des gènes identifiés supérieur à ceux des autres traitements dans les clusters 1, 3, 4, 5, 8, 10, 14, 15, 16, 18, 19 et 21 mais inférieur dans les clusters 2, 6, 7, 9, 11, 12, 13, 17 et 22 (Figure 7). Etant donné que la spécificité des plantes de ces deux traitements est l'expression de symptômes de tylc et une plus forte

accumulation virale, les DEGs identifiés sont probablement associés à des gènes mobilisés pour la réplication, la transcription et la traduction des génomes viraux et aux désordres physiologiques au sein de la plante provoqués par l'invasion.

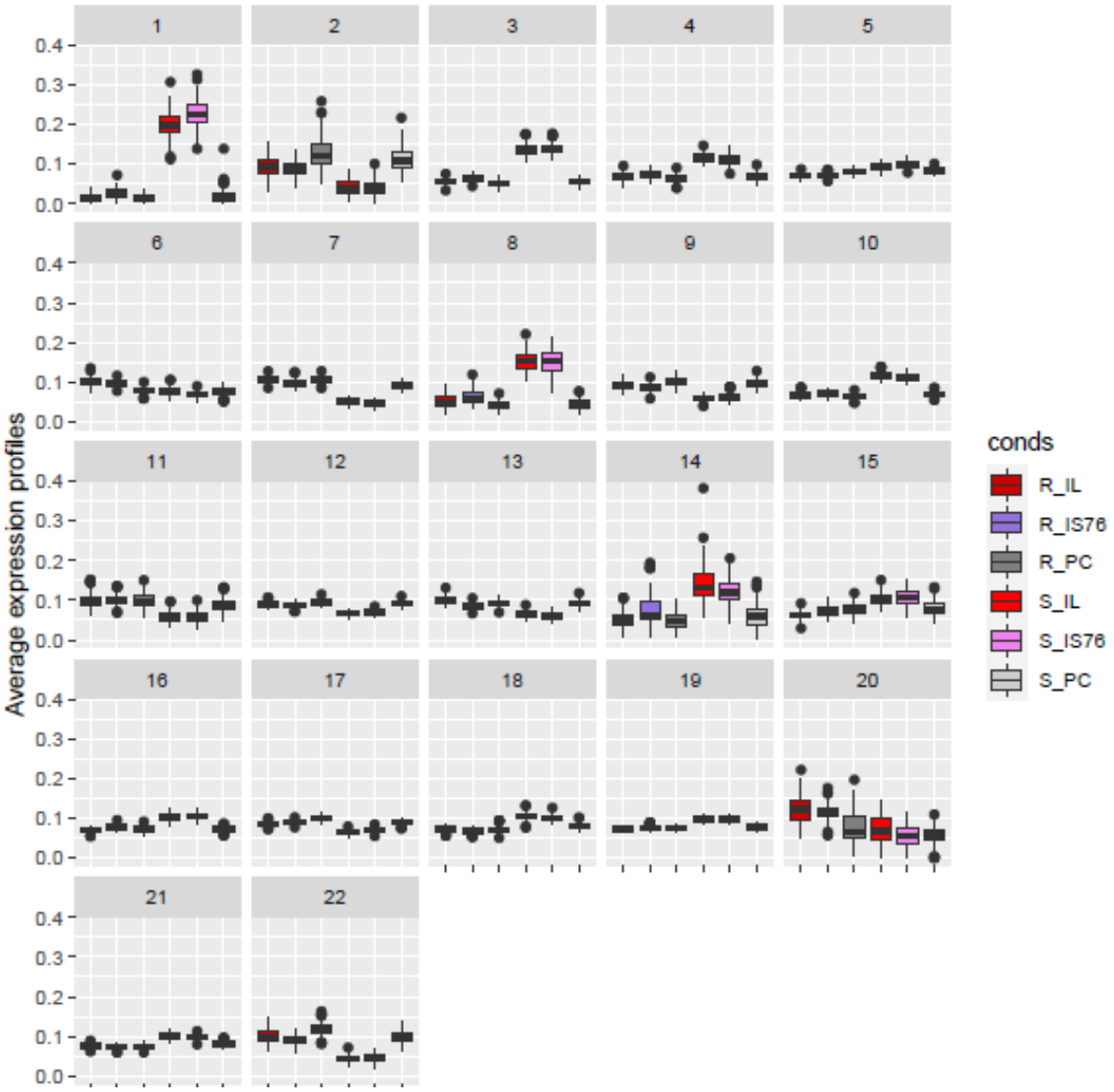

Figure 7 : Cluster de coexpression des gènes différentiellement exprimés entre plantes sensibles et résistantes non infectées (S_PC et R_PC) ou infectées soit par TYLCV-IL (S_IL et R_IL) soit par TYLCV-IS76 (S_IS76 et R_IS76) à 30 jours après infection. Profils d'expression moyenne des différents clusters calculés par l'analyse de coexpression des gènes différentiellement exprimés compris dans l'union des trois comparaisons [PC_R-PC_S], [IL_R-IL_S] et [IS76_R-IS76_S].

Les termes d'annotations GO confirment cela. En effet, on observe que dans les clusters où les gènes sont sous-exprimés dans les plantes sensibles infectées, de nombreux termes GO sont liés aux mécanismes de la photosynthèse. Ce qui est cohérent avec le fait que les symptômes de la maladie se caractérise notamment par un jaunissement des feuilles. De nombreux GO associés au métabolisme mitochondrial sont mis en évidence pour les gènes sous-exprimés dans les plantes

sensibles infectées, ce qui montre que les plantes sensibles infectées ont un métabolisme ralentit. Parmi les termes GO des gènes sous-exprimés dans les plantes sensibles infectées, on retrouve également des termes associés à la réplication, à la transcription et à la traduction des protéines (Tableau 3). Enfin deux termes GO (« defense response to bacterium » et « regulation of systemic acquired resistance ») indiquent que des gènes de résistance sont surexprimés dans les plantes sensibles infectées (Tableau 2). Donc l'absence du gène de résistance Ty-1 fait que d'autres mécanismes de résistance existant chez ces plantes se mettent en place pour tenter de contrer l'infection par les virus. Des gènes liés au transport cellulaire, à l'ubiquitinylation des protéines ou encore à la phosphorylation sont aussi surexprimés dans les plantes sensibles infectées (Tableau 2).

Tableau 2 : liste des termes GO pour les clusters 1, 3, 4, 5, 8, 10, 14, 15, 16, 18, 19 et 21. Dans ces clusters les gènes sont sur-exprimés dans les plantes sensibles infectées, quel que soit le virus. A l'inverse, les gènes sont sous-exprimés dans les plantes résistantes infectées et dans les plantes non infectées quel que soit le cultivar.

Termes GO	Nombre de gènes / cluster											
	C1	C3	C4	C5	C8	C10	C14	C15	C16	C18	C19	C21
nuclear-transcribed mRNA catabolic process	11	10	52	36		45				28	40	62
deadenylation-dependent decapping of nuclear-transcribed mRNA						3						
mRNA splicing											2	
nucleic acid binding										7	23	34
DNA binding						20				8		
chromatin binding												4
DNA-binding transcription factor activity	5	11			11		8					
RNA binding											8	11
DNA helicase activity						2						
helicase activity												3
histone acetyltransferase activity			2									
ribonuclease activity												3
guanyl-nucleotide exchange factor activity												2
double-strand break repair via nonhomologous end joining						2						
regulation of DNA-templated transcription	7	18			19	20	11					
RNA processing												5
mRNA processing												4
nucleotidyltransferase activity												4
U6 snRNA 3'-end processing		1										
ribosome biogenesis				2								
sequence-specific DNA binding	5	7		6		8	5					
1,3-beta-D-glucan synthase complex										2		
1,3-beta-D-glucan synthase activity										2		
(1->3)-beta-D-glucan biosynthetic process										2		
6-phosphofructokinase activity				2								
adenosine deaminase activity		1										
serine-type endopeptidase activity							3					
serine-type endopeptidase inhibitor activity							2					
homogentisate 1,2-dioxygenas activity		1										

inorganic diphosphate phosphatase activity									2			
phosphatidylinositol phospholipase C activity			2									
malate dehydrogenase (decarboxylating) (NAD+) activity			2									
chitinase activity	3											
poly(ADP-ribose)glycohydrolase activity					2							
protein kinase activity	10		36	24	35	36					43	37
protein phosphorylation	10		36	24	35	36					43	37
phosphate-containing compound metabolic process									2			
pyruvate kinase activity											2	
ubiquitin-protein transferase activity			4		3	6						
protein ubiquitination						4						
protein deubiquitination												5
cysteine-type deubiquitinase activity												5
endopeptidase inhibitor activity							2					
monoatomic ion channel activity	2		3									
voltage-gated chloride channel activity												3
monoatomic ion transport	2		3									
chloride transport												3
intracellular protein transport												6
endoplasmic reticulum to golgi vesicle mediated transport												3
retrograde transport, endosome to golgi												2
vacuolar transport									3			
metal ion transport		4										
Golgi vesicle transport											2	
transmembrane transport			21									
nitrogen compound transport			3									
iron ion binding					9							
calmodulin binding					4							
ATP binding			33		29	31				14	44	53
ADP binding			12		13							
GTP binding									9			
ATP hydrolysis activity					5					3		
spliceosomal complex											3	
sucrose metabolic process			3					2				
sucrose synthase activity			3					2				
trehalose biosynthetic process						3	2					
L-phenylalanine catabolic process		1										
tyrosine metabolic process		1										
phosphatidylserine biosynthetic process			2									
proteolysis	5											
autophagy						2						
enzyme activator activity						3						
cysteine-type peptidase activity	3											
zinc ion binding						13				6		
response to hormone										2		
regulation of systemic acquired resistance	1											

defense response to bacterium							1					
oxidoreductase activity	9		4		8		6					
hydrolase activity									9			
cell wall macromolecule catabolic process	3											
chitin catabolic process	3											
TBP-class protein binding											2	
nitronate monooxygenase activity		1										
heme binding					11							
COPII vesicle coat												3
retromer, cargo-selective complex												2
potassium ion binding											2	
Ino80 complex						2						
heat shock protein binding		2										
regulation of ARF protein signal transduction												2
intracellular signal transduction											2	
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase activity		1										
positive regulation of catalytic activity						3						
catalytic activity											15	
positive regulation of Notch signaling pathway		1										

Tableau 3 : liste des termes GO pour les clusters 2, 6, 7, 9, 11, 12, 13, 17 et 22. Dans ces clusters les gènes sont sous-exprimés dans les plantes sensibles infectées, quel que soit le virus. A l'inverse, les gènes sont sur-exprimés dans les plantes résistantes infectées et dans les plantes non infectées, quel que soit le cultivar.

Termes GO	Nombre de gènes / cluster								
	C2	C6	C7	C9	C11	C12	C13	C17	C22
nucleotide binding			4				7		
nucleic acid binding						22	11		
DNA binding									29
single-stranded DNA binding									2
RNA binding			7				7	13	
DNA replication				3		3			
DNA replication initiation							3		
DNA-directed DNA polymerase activity						2			
mismatch repair							2		
mismatched DNA binding							2		
transcription regulator complex				2					
nuclear-transcribed mRNA catabolic process	28	30	36	14	59				27
translation			22					70	10
translational termination						2			
translation elongation factor activity								3	
translation release factor activity						2			
positive regulation of translational elongation								2	
positive regulation of translational termination								2	
translational frameshifting								2	
tRNA aminoacylation for protein translation			4				5		

aminoacyl-tRNA ligase activity			4			4	4		
aminoacyl-tRNA hydrolase activity						2			
glycyl-tRNA aminoacylation							1		
aminoacyl-tRNA editing activity						2			
glycine-tRNA ligase activity							1		
ribosome			20					69	11
structural constituent of ribosome			23					70	10
ribosome biogenesis							2		2
ribosome binding								2	
rRNA processing						3			
rRNA binding			3						
5S rRNA binding								2	
chromosome							2		
chromosome organization							2		
histone acetyltransferase activity	2								
DNA topoisomerase type II							3		
purine nucleotide biosynthetic process						2			
DNA topological change							4		
nucleosome									26
nucleosome assembly						4			
protein folding			3					3	
protein transport						4			
protein peptidyl-prolyl isomerization			3						
peptidyl-prolyl cis-trans isomerase activity			3						
serine-type carboxypeptidase activity				6					
microtubule motor activity							7		
catalytic activity		19							
3-beta-hydroxy-delta5-steroid dehydrogenase activity					2				
3-hydroxyisobutyryl-CoA hydrolase activity					3				
GTPase activity						9		10	
cytochrome-c oxidase activity								2	
metalloendopeptidase activity			3				4		
fructose-bisphosphate aldolase activity		2							
inorganic diphosphate phosphatase activity							2		
methionine adenosyltransferase activity				2					
methylenetetrahydrofolate dehydrogenase (NADP+) activity									2
hydrolase activity						11			
phosphomannomutase activity						2			
prephenate dehydratase activity						2			
superoxide dismutase activity									2
uroporphyrinogen decarboxylase activity			2						
serine-type endopeptidase inhibitor activity	2								
voltage-gated chloride channel activity				2					
iron ion binding					18				
copper ion binding				5					
ATP binding						52	53		

GTP binding						11		9	
intracellular anatomical structure			11					45	
mitochondrial proton-transporting ATP synthase complex								3	
mitochondrion						3		2	
mitochondrial outer membrane									2
mitochondrial inner membrane								4	
mitochondrial respiratory chain complex I								2	
mitochondrial respiratory chain complex III								2	
mitochondrial electron transport								2	
mitochondrial pyruvate transmembrane transport								2	
carbohydrate metabolic process						16			
glycolytic process						4			
tricarboxylic acid cycle				2					
proteolysis	6						8		
amino acid metabolic process							3		
arginine biosynthetic process						2			
S-adenosylmethionine biosynthetic process				2					
lipid metabolic process		8							
fatty acid biosynthetic process		3			4	4			
steroid biosynthetic process					4			2	
porphyrin-containing compound biosynthetic process			3						
superoxide metabolic process			2						2
microtubule-based movement							7		
microtubule binding							7		
potassium ion transport							1		
phosphate ion transport							1		
inorganic phosphate transmembrane transporter activity							1		
chloride transport				2					
signal transduction		4							
methyltransferase activity						12			
O-methyltransferase activity					4				
RNA methyltransferase activity			2						
serine-type peptidase activity							3		
lipid binding		4							
lipid biosynthetic process									3
biosynthetic process						7			
L-phenylalanine biosynthetic process						2			
thiamine biosynthetic process	1								
riboflavin biosynthetic process			2						
glycolipid biosynthetic process						2			
GDP-mannose biosynthetic process						2			
photosynthesis			25						
photosystem I			13						
photosystem II			11						4
photosystem I reaction center			10						
photosystem II oxygen evolving complex			9						4

photosystem II assembly									2
chlorophyllide a oxygenase		2					2		
pyrophosphate hydrolysis-driven proton transmembrane transporter activity				2			2		
proton transmembrane transporter activity								6	
channel activity					4				
guanosine tetraphosphate metabolic process							2		
proton motive force-driven ATP synthesis			5					4	
proton transmembrane transport								4	
terpenoid biosynthetic process						2			
antioxidant activity			2						
oxidoreductase activity	7	2	6		17			3	
acyltransferase activity, transferring groups other than amino-acyl groups	7			8	8				
glycosyltransferase activity						8			
hexosyltransferase activity					7				9
cellulose synthase (UDP-forming) activity	3						4		
transferase activity, transferring alkyl or aryl groups			3						
hydrolase activity					13		2		
strictosidine synthase activity		2							
intramolecular lyase activity									2
outer membrane						2			
extrinsic component of membrane			6						4
protein kinase binding									2
heme binding					19				
cellulose biosynthetic process	3						4		
small-subunit processome						2			
tetrapyrrole biosynthetic process			2						
proton-transporting two-sector ATPase complex								3	
proton-transporting ATP synthase complex			2						
cell redox homeostasis			6					5	
phosphatidylinositol phosphate biosynthetic process			2						
proton-transporting ATP synthase			3						
NAD binding						5			
iron-sulfur cluster binding						5			
transmembrane transport	8				17				
NADPH binding							1		
uridylyltransferase activity						2			
proton transmembrane transport							2		
starch binding							2		
regulation of cyclin-dependent protein serine/threonine kinase activity									2
histidine biosynthetic process						4			
response to wounding	2								

Lorsque les plantes sont infectées depuis 30 jours, les mécanismes de résistance ont déjà été mis en place précocement, notamment la résistance apportée par le gène Ty-1, comme on a vu pour les plantes collectées 10 jours après l'infection. Ces résistances n'étant pas totales, les virus ont continué

à se répliquer jusqu'à atteindre leur niveau d'accumulation optimal. Dans les plantes sensibles, ce niveau d'accumulation est plus haut que dans les plantes résistantes où le gène Ty-1 limite la réplication des virus. Dans les plantes sensibles, ce niveau d'accumulation plus élevé des virus dans les plantes s'accompagne de la manifestation des symptômes typiques de la maladie. Les nombreux gènes différentiellement exprimés reflètent alors les réactions physiologiques de la plante dues à la maladie et à l'apparition des symptômes. Dans les plantes sensibles, on a pu observer une baisse de l'expression des gènes impliqués dans la photosynthèse, dans le métabolisme mitochondrial et dans les mécanismes de réplication, de transcription et de traduction principalement. En résumé, les plantes sensibles infectées semblent réagir à l'infection en mettant en place des réactions de défenses mais au détriment de leur métabolisme général, ce qui peut être la cause du jaunissement des feuilles ou encore du ralentissement de la croissance observées.

4. Discussion

Nous avons pu mettre en évidence dans ce chapitre que la résistance portée par le gène Ty-1 était une résistance mise en place de façon précoce puisque dans les plantes collectées 10 jours après infection, il est déjà fortement exprimé dans les plantes résistantes comparées aux plantes sensibles. Cette expression précoce de la résistance est une composante importante pour la défense de la plante à l'infection car plus une résistance se met en place rapidement, plus la défense de la plante sera efficace contre le pathogène. De plus, ce gène est différentiellement exprimé dans les plantes infectées, que ce soit par le TYLCV-IL ou le TYLCV-IS76, mais également dans les plantes non infectées. Ce qui veut dire que l'expression de ce gène n'est pas induite par la présence du virus mais qu'il est constamment exprimé dans les plantes résistantes ce qui permet à la plante d'être protégée dès l'entrée du virus et de ne pas souffrir du temps nécessaire à l'induction de la résistance. Le terme GO « response to wounding » identifié comme sur-représenté pour les gènes étant activé chez les plantes résistantes, correspond à une protéine appelée PPI3B2 associé à la réponse contre les lésions foliaires provoquées par des champignons phytopathogènes (Hermosa et al., 2006), mais n'est en réalité différentiellement exprimé que dans les plantes non infectées et n'a donc pas de lien avec la résistance à nos virus. On remarque également un gène dont le terme GO est lié à l'ubiquitination des protéines. L'ubiquitination est une modification des protéines qui peut être impliquée dans les voies de signalisation de défense de la plante (Marino et al., 2012) et il a été démontré que l'inhibition de l'ubiquitination par la protéine C2 des begomovirus diminuait les réponses des plantes à l'infection (Li et al., 2019). D'autres mécanismes de défense de la plante sont donc peut-être mis en place à ce temps précoce dans les plantes résistantes pour limiter l'infection par les virus.

Pour les plantes collectées à 30 jours après infection, peu de termes GO associés aux mécanismes de défense des plantes sont identifiés. Parmi ces termes, on trouve les termes suivants pour les gènes activés dans les plantes sensibles infectées : le terme « defense response to bacterium », qui correspond à un gène différentiellement exprimé lorsque les plantes sont infectées par TYLCV-IL seulement ; le terme « regulation of systemic acquired resistance » et le gène associé à ce terme est commun aux plantes infectées par TYLCV-IL et TYLCV-IS76 ; le terme « response to hormone » qui correspond à deux gènes associés à l'auxine, pouvant être impliqué dans les défenses de la plante et l'un de ces gènes est communs aux deux types d'infection et l'autre spécifique à l'infection par TYLCV-IS76 ; le terme « autophagy » connu dans les défenses des plantes aux pathogènes et notamment dans les résistances antivirales (Yang et al., 2020) et qui correspond à deux gènes communs à l'infection à TYLCV-IL et TYLCV-IS76 ; les termes GO liés à l'ubiquitination, aux protéines kinase, à la régulation de la transcription, la modification de la paroi cellulaire, aux métabolismes des sucres… Il est surprenant de retrouver autant de termes pouvant être liés à des défenses de la plantes pour des gènes surexprimés dans les plantes sensibles alors qu'on pourrait s'attendre au contraire. C'est d'ailleurs ce qui a été observé dans d'autres études de transcriptomique sur l'infection du TYLCV dans des plantes résistantes et sensibles. Par exemple, Chen et al (2013) ont montré que dans les plantes sensibles infectées par le TYLCV, il y avait une diminution d'expression des facteurs de transcription, des gènes de résistances et des protéines kinases. Mais dans une autre étude transcriptomique de plantes sensibles et résistantes infectées par le TYLCV, il a été montré qu'il y avait bien certains composés liés à la réponse au stress surexprimés dans les plantes sensibles tels que les anthocyanidine (Sade et al., 2015). Parmi les gènes différentiellement exprimés identifiés et qui sont réprimés dans les plantes sensibles infectées mais activés dans les plantes résistantes, de nombreux termes GO sont liés à la photosynthèse, à l'activité mitochondrial, à la transcription et à la traduction des gènes, à la réplication, au transport cellulaire… Cela montre une limitation des activités cellulaires dans les plantes sensibles contrairement aux plantes résistantes ou aux plantes non infectées et traduit les réactions physiologiques des plantes liées à une présence plus importante des virus dans les plantes sensibles et à l'apparition des symptômes. Cependant, peu de réaction de défense semblent ressortir comme étant activées dans les plantes résistantes ce qui est à nouveau surprenant, sachant que dans les précédentes études, de nombreuses réactions de défense ont pu être identifiées dans les plantes résistantes infectées par le TYLCV. Sade et al (2015) ont mis en évidence l'implication de la voie de signalisation des phenylpropanoïdes et la voie générale des phényls, impliqués dans la biosynthèse de l'acide salicylique et de la SAR, la voie de signalisation des flavonoïdes, des polyamines, de l'acide butyrique etc, qui sont toutes impliquées dans les mécanismes de défenses des plantes aux pathogènes. Chen et al (2013) ont également mis en évidence une activation importante des protéines kinases, de l'ubiquitination et de la réorganisation

des parois cellulaires liées également aux réponses de défense. Communément avec nos résultats, ces deux études ont aussi mis en évidence une activation de la transcription et une augmentation de la photosynthèse dans les plantes résistantes et leur diminution dans les plantes sensibles.

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Discussion Générale

TYLCV-IS76 est un recombinant entre le TYLCV-IL et le TYLCSV dont la valeur sélective dans les plantes résistantes portant le gène Ty-1 est supérieure à celle des virus parentaux. C'est par des symptômes inhabituels de tylc sur ces plantes résistantes que ce recombinant a été initialement détecté dans le Souss, au sud du Maroc. Il a envahi le pays et remplacé les virus parentaux. Nous avons montré qu'il était également capable d'infecter une plante Ty-1 préalablement infectée par le TYLCV-IL et le TYLCSV, et même de dépasser systématiquement l'accumulation de ces derniers, tout en manifestant son effet délétère typique sur son parent TYLCV-IL. L'étude que nous avons mené sur le potentiel de prémunition des plantes entre variants du TYLCV ne différant que de quelques SNP montre qu'une première infection avec un variant est capable de protéger la plante contre un autre variant, que ce soit dans les variétés sensibles ou résistantes Ty-1. Dans cette étude, il a été montré aussi que la prémunition était effective entre le TYLCV-IL et un variant du TYLCV-IL présentant le même profil de recombinaison que TYLCV-IS76 mais dont la séquence recombinante provient d'un bégomovirus asiatique. Ceci nous a permis de révéler une autre facette de la valeur sélective de TYLCV-IS76 qui est sa capacité de contourner la résistance induite par les mécanismes de prémunition des plantes, tout au moins dans les plantes résistantes Ty-1. Nous avons également montré que TYLCV-IS76 conserve son avantage sélectif dans les plantes Ty-1 peu importe l'âge de la plante infectée. Tous ces avantages sur plantes résistantes Ty-1 ne paient apparemment aucun coût sur l'infection de plantes sensibles ni sur son efficacité de transmission qui ne diffère pas de celle du TYLCV-IL le parent le plus efficacement transmis par *Bemisia tabaci*. Nous avons également montré que le gène Ty-1 codant pour une RDRγ était impliqué dans l'amplification du mécanisme antiviral du *gene silencing* par une plus grande production de petits ARN interférents (siRNA) viraux avec notamment une augmentation des siRNA de 22 et 24 nt et une augmentation de siRNA ciblant la région intergénique, conformément aux résultats de précédentes études (Butterbach et al., 2014; Voorburg et al., 2021). L'étude des ARN messagers et des siRNA viraux dans des plantes sensibles et résistantes Ty-1 infectées par TYLCV-IS76 et TYLCV-IL a montré que, pour TYLCV-IS76, les niveaux de réplication virale et de transcription des gènes de la capside (CP) et du suppresseur de *silencing* V1 étaient supérieurs à ceux du TYLCV-IL. De plus, le TYLCV-IL est préférentiellement ciblé par les siRNA, comparé au TYLCV-IS76, montrant que le TYLCV-IS76 a une meilleure capacité à échapper au *gene silencing*. Ces résultats expliquent pourquoi TYLCV-IS76 a un tel avantage sur le parent TYLCV-IL dans les plantes Ty-1 ; en effet, il se réplique mieux, produit plus de suppresseur de *silencing* et est moins ciblé par les siRNA que TYLCV-IL. Enfin, nous avons montré par une étude transcriptomique que la résistance induite par le gène Ty-1 était bien la principale source de défense impliquée lors de l'infection des plantes résistantes par le TYLCV-IL et le TYLCV-IS76. En effet, ce gène est fortement

surexprimé dans les plantes résistantes que les plantes soient infectées ou non par les virus. La protéine RDRγ est donc produite continuellement dans les plantes et n'est pas induite par la présence des virus ce qui permet une défense efficace contre l'infection car elle est déjà présente avant même l'entrée du virus dans la plante.

Le TYLCV-IS76 est un virus hybride issu de la recombinaison entre des représentants de deux espèces virales, dont la valeur sélective est supérieure à celle des virus parentaux. L'avantage apporté par l'hybridation est un phénomène bien connu dans le monde vivant, que ce soit chez les organismes eucaryotes, procaryotes ou chez les virus. Chez les plantes par exemple, la valeur sélective des hybrides est souvent supérieure à celle des lignées parentales, un résultat qui est largement exploité en sélection variétale pour une meilleure adaptation à l'environnement. Par exemple, l'espèce de tournesol *Helianthus paradoxus* est un hybride entre les lignées *Helianthus annuus* et *Helianthus petiolaris* et elle a une meilleure fitness que ses parents ; cela se traduit notamment par une meilleure adaptation à des environnements extrême comme la salinité (Donovan et al., 2009; Edelist et al., 2009; Karrenberg et al., 2006; Rieseberg et al., 2003). Chez les bactéries *E.coli*, des transferts de gènes horizontaux confèrent à différents isolats entérohémorragiques leur pathogénicité grâce au gain d'un gène codant pour une toxine spécifique (Ogura et al., 2009; Touchon et al., 2009). Le transfert horizontal d'éléments génétiques mobiles d'une souche non humaine à une souche humaine de *Staphylococcus aureus* a permis un saut d'espèces de l'homme au poulet grâce à une meilleure résistance de cette nouvelle souche au système immunitaire de l'animal (Lowder et al., 2009). Chez les virus, même si des génomes recombinants ou des réassortiments de virus multi-composants sont fréquemment décrits, ce n'est que dans quelques cas que leur avantage par rapport aux parents a été démontré. Outre le TYLCV-IS76 dont l'avantage de la recombinaison a été largement décrit (Belabess et al., 2015, 2016), on peut citer le cas du virus influenza A (H1N1) qui a provoqué une épidémie mondiale chez l'homme en 2009. Son avantage au niveau de la transmission et sa virulence accrue est le résultat d'un réassortiment de composants génomiques provenant de populations virales infectant l'homme, les oiseaux et les porcs (Smith et al., 2009).

Chez les organismes pathogènes, l'augmentation de virulence issue de génomes recombinants peut conduire au contournement de résistance obtenus par sélection variétale, tel celui de la résistance induite par le gène Ty-1 par TYLCV-IS76. Chez les animaux où les résistances sont apportées principalement par les vaccins, les cas de contournement sont malheureusement très fréquents. Ils sont surtout engendrés par des mutations ponctuelles ou des réassortiments mais peuvent également se produire par recombinaison comme décrit dans le cas d'un lagovirus recombinant au

sein de l'espèce rabbit haemorrhagic disease virus (RHDV, genre *Lagovirus*, Abrantes et al., 2020; Dalton et al., 2014; Le Gall-Reculé et al., 2011). Dans le cas du *Cotton leaf curl Burewala virus* (CLCuBuV) un virus dont le génome a été généré par recombinaison, la recombinaison aurait provoqué un contournement de résistance (Rajagopalan et al., 2012). Bien qu'aucune démonstration formelle n'en ait été faite, le bénéfice de la recombinaison a été suggéré par le fait que le recombinant s'est retrouvé dominant dans de nombreux champs au moment où des variétés précédemment résistantes ont présenté des symptômes de la maladie.

Garcia-Arenal et Mcdonald (2003) ont montré que pour les virus comme pour les autres pathogènes de plantes (McDonald & Linde, 2002), la durabilité d'une résistance était principalement déterminée par le potentiel évolutif du pathogène plutôt que par la nature de la résistance utilisée. Le potentiel évolutif des virus est défini par le calcul d'un risque prenant en compte trois dimensions évolutives, la taille effective de la population, le flux de gènes ou de génome, et la méthode de reproduction. Chez les virus, la taille effective de la population est très réduite comparée au nombre de virus produits dans une plante car il y a un goulot d'étranglement pendant le processus d'infection d'un nouvel hôte dû principalement au fait que l'insecte vecteur ne transmet qu'un petit nombre de particules virales (Pirone & Thornbury, 1988). La taille effective de la population des virus dépend également de la capacité du virus à survivre dans l'environnement en absence de culture, par leur capacité à infecter les graines, dans les débris de plantes, dans le sol, dans le vecteur, ou encore dans d'autres plantes hôtes. Le flux de gènes et de génomes dépend du potentiel de dispersion du virus. Des virus survivant dans le sol ou transmis par contact ont un potentiel de dispersion faible alors que des virus transmis par les graines ont, au contraire, un haut potentiel de dispersion. Pour ce qui est de la méthode de reproduction des virus, leur clonalité pourrait les priver de diversification génomique si elle n'était pas associée à des taux de mutation généralement élevé et à une forte capacité à recombiner pour beaucoup d'entre eux. En prenant en compte toutes ces conditions, le TYLCV est un virus à haut potentiel évolutif. Il a une large gamme d'hôte notamment des plantes adventices ou des plantes sauvages, lui permettant de survivre même en l'absence de culture de tomates. Son mode de transmission est persistant dans l'aleurode *Bemisia tabaci* et de plus cet aleurode vecteur est polyphage, très répandu dans le monde, et peut être déplacer sur de longues distances. Enfin, malgré son génome à ADN, il a un taux de mutation comparable à des virus ARN, et de plus a un haut potentiel de recombinaison. Garcia-Arenal et Mcdonald (2003) ont estimé le risque estimé du potentiel évolutif du TYLCV à 9, la note maximale pouvant être attribuée à un virus selon leur grille de notation. Le contournement de la résistance Ty-1 par TYLCV-IS76 n'est donc pas surprenant selon cette étude même si les auteurs ont montré que la durabilité des résistances contre

les virus était relativement élevée par rapport à celles contre des champignons ou des bactéries pathogènes.

Les virus présentant une virulence accrue dans les plantes conduisant à un contournement de résistance payent souvent un coût sur un autre aspect de leur fitness les empêchant de devenir prévalent sur le terrain. Par exemple, des isolats de Raspberry ringspot virus (RpRSV) pouvant contourner la résistance conférée par le gène Irr (Murant et al., 1968) n'ont pas été invasifs à cause de leur faible compétitivité en infection mixte et de leur faible transmission par les graines dans les plantes adventices (Hanada & Harrison, 1977; Jennings, 1964). Des isolats du Turnip mosaic virus (TuMV) capables de contourner la résistance du gène TuRB01 et de trois autres gènes de résistance présentaient une fitness diminuée dans les plantes sensibles comparée à celle d'un autre isolat qui ne contourne pas ces résistances (Jenner et al., 2002). A l'inverse, une diminution de la virulence du cowpea mild mottle virus (CPMMV) induite par des recombinaisons dans l'ORF1, était associé à une meilleure fitness du virus en plante et à une transmission plus efficace par vecteur (Zanardo et al., 2021). Par rapport à ces exemples, TYLCV-IS76 peut être classé dans les exceptions à la règle. En effet, aucun coût de fitness n'a été détecté par rapport à sa forte virulence dans les plantes résistantes. Il s'accumule aussi bien que les virus parentaux dans les plantes sensibles, sa compétitivité en coinfection dépasse celle des virus parentaux et aucune perte d'efficacité n'a pu être détectée pour sa transmission par vecteur. Ces caractéristiques expliquent pourquoi TYLCV-IS76 est devenu prévalent au Maroc.

Cependant, TYLCV-IS76 présente un désavantage. La génération et l'émergence de son génome est un phénomène extrêmement rare. En effet, sur la base des analyses phylogénique, l'évènement de recombinaison qui a conduit au génome de TYLCV-IS76 ne se serait produit qu'une seule fois (Belabess et al., 2015). Cette inférence basée sur des comparaisons de séquence est soutenu par des résultats de laboratoires sur des plantes de tomate coinoculées par des virus de type parentaux (Belabess et al., 2018). Lorsque des recombinants portant le point de recombinaison caractéristique du TYLCV-IS76 en position 76 sont détectés, ils représentent toujours un pourcentage extrêmement faible de la population virale intra-plante sans jamais augmenter en fréquence. Contrairement à TYLCV-IS76, les recombinants TYLCV/TYLCSV les plus courants dont le fragment génomique hérité de TYLCSV s'étend de l'origine de réplication en direction 3' jusqu'à la fin du gène V1 et au-delà, sont capables d'émerger plus facilement. En effet, ce type de recombinant est très fréquemment observé dans les plantes coinfectées par les virus parentaux en conditions contrôlées (Belabess et al., 2018; Davino et al., 2012; García-Andrés et al., 2007, 2009) et sur le terrain, dans plusieurs pays (Anfoka et al., 2016; Davino et al., 2009, 2012; Fiallo-Olivé et al., 2019; García-Andrés et al., 2006; Monci et al., 2002). Il est à noter qu'en dépit d'un profil de recombinaison très proche de TYLCV-IS76 et d'un

phénotype similaire, le recombinant TYLCV-IS141 a la capacité d'émerger bien plus fréquemment. En effet, alors que l'avantage sélectif de TYLCV-IS141 dans les plantes résistantes est similaire à celui de TYLCV-IS76 avec le typique effet délétère sur TYLCV-IL, il a émergé plusieurs fois en Italie et également en condition contrôlée (Belabess et al., 2015, 2018; Panno et al., 2018; Urbino et al., 2020). Cependant, comme il n'a jamais été identifié en dehors de l'Italie, ce recombinant semble lui aussi nécessiter des conditions d'émergence particulières, contrairement aux recombinants avec de longs fragments hérités du TYLCSV (> 1000 nts) qui sont détectés dès lors qu'une plante est coinfectée par TYLCV et TYLCSV. On ne peut exclure que la pré-émergence de TYLCV-IS76 ait eu lieu en dehors de la tomate, dans des plantes telle que le haricot ou des plantes sauvages. Mes premiers résultats sur haricot et sur *Solanum nigrum* n'ont pas permis de mettre en évidence une émergence de TYLCV-IS76 dans ces plantes coinoculés avec les virus parentaux. D'autres plantes sauvages connues pour être des hôtes du TYLCV devraient être ainsi testées pour continuer à explorer cette hypothèse.

Dans cette thèse, nous avons montré l'existence de la prémunition chez le TYLCV. L'implication de ce mécanisme de résistance dans la rareté de l'émergence de recombinants de type TYLCV-IS76 est une hypothèse captivante. Contrairement à l'inoculation du TYLCV-IS76 dans des plantes préalablement infectées par le TYLCV-IL et le TYLCSV où il est inoculé introduit vraisemblablement en plusieurs copies et parfois dans des cellules non infectées par le TYLCV-IL, la situation est probablement très différente pour un génome recombinant qui vient d'être généré au sein d'une cellule contenant déjà les virus parentaux. Selon le principe de prémunition qui a tendance à bloquer les virus les plus similaires, on peut imaginer que des virus de type TYLCV-IS76 seraient bloqués de par leur similitude avec le parent TYLCV alors que les autres recombinants avec des proportions de TYLCV et de TYLCSV plus équilibrées ne le seraient pas.

La plupart des variants décrits comme ayant un avantage sélectif permettant le contournement de résistance possèdent des mutations ou des recombinaisons dans des régions codantes avec des fonctions précises et parfois la cause de l'avantage sélectif induit est identifiée. Dans le cas de TYLCV-IS76, la modification génomique qui a apporté l'avantage sélectif, correspond à l'acquisition par recombinaison d'une courte séquence du TYLCSV dans le génome du TYLCV-IL. Cette courte séquence est située dans une région intergénique, dite non codante. Quoique non codante, cette région a des rôles importants de régulation de la réplication et de la transcription et donc des changements dans une telle région peut avoir des conséquences importantes. La région intergénique des virus du TYLCV est l'endroit de fixation de la protéine de réplication. Cette affinité de séquence est assurée par la présence, du côté 5' de la tige boucle, de séquences itérons spécifiques de la protéine de réplication sur lesquelles elle va venir se fixer pour entamer la réplication dont l'origine

est également dans la région intergénique. On a vu que TYLCV-IS76 avait une réplication plus rapide que TYLCV-IL et dans la région TYLCSV héritée par TYLCV-IS76, il a été identifié une séquence partielle inversée de l'itéron du TYLCV-IL qui pourrait permettre une plus grande affinité du génome de TYLCV-IS76 à la protéine de réplication. Nous avons également démontré que TYLCV-IS76 avait une meilleure transcription des gènes V1 et V2 traduisant un passage plus rapide de la transcription des gènes précoces codant principalement pour les protéines impliquées dans la réplication (C1 à C4) à la transcription des gènes tardifs (V1 et V2) codant pour la CP et le suppresseur de *silencing*. Cette transition de la transcription des gènes précoces aux gènes tardifs s'explique par la réplication plus rapide du génome du TYLCV-IS76. Sachant que la région recombinante est située en amont des gènes V1 et V2 dans la région promotrice de ces gènes, et qu'elle contient notamment la CAAT-box, un contexte génétique différent apporté par la recombinaison pourrait expliquer cet avantage dans la transcription des gènes V1 et V2. La transcription plus efficace des gènes V1 et V2 peut être due à une activation plus efficace du promoteur des gènes V1 et V2 et la protéine d'activation de la transcription (TrAP, C2). En effet chez le *Tomato golden mosaic virus* (TGMV) et le *Cabbage leaf curl virus* (CaLCuV), il a été démontré que la TrAP était requise pour l'activation de la transcription des protéines V1 et V2 (Lacatus & Sunter, 2008; Sunter & Bisaro, 1991, 1992) et que la séquence requise pour l'activation du promoteur de la CP chez le TGMV était situé entre les positions nucléotidiques 60 et 125 avant le site d'initiation de la transcription (Sunter & Bisaro, 2003). Si une telle séquence existe chez le TYLCV-IL, sa position pourrait donc être localisée dans la région recombinante. Selon cette hypothèse, l'évènement de recombinaison a pu apporter une séquence ayant une affinité plus forte avec la protéine TrAP. De plus, il a été montré qu'une séquence conservée appelé CLE (conserved late elements) situé dans le promoteur des gènes tardifs des begomovirus, était requis pour son activation (Ruiz-Medrano et al., 1999). Cet élément est présent dans le génome du TYLCV-IL et également dans la région recombinante du TYLCV-IS76 mais diffère d'un nucléotide. Cette mutation pourrait être à l'origine d'une meilleure transcription des gènes V1 et V2.

Etonnamment, le TYLCSV, qui possède la région recombinante de TYLCV-IS76, ne présente pas d'avantage sélectif vis-à-vis de TYLCV-IL. L'avantage sélectif apporté par la région recombinante est donc dépendant du fond génétique dans lequel elle est intégrée. Il a déjà été démontré que le fond génétique du PVY pouvait moduler le contournement de la résistance pvr2 par le PVY en changeant certaines régions du génome autre que le gène codant pour la VPg responsable du contournement de la résistance (Montarry et al., 2011). Le fond génétique a donc un impact important sur la capacité à contourner une résistance apportée par une région précise du génome et les effets de la recombinaison chez les virus sont donc difficilement prévisibles. Cet effet du fond génétique sur l'avantage sélectif peut être dû à de nouvelles interactions intragénomiques révélées par

l'évènement de recombinaison entre le génome du TYLCV-IL et la région recombinante. Martin et al (2011) a mis en évidence de possibles interactions intragénomiques non identifiées jusqu'à présent entre la région promotrice des gènes V1 et V2 et le gène V1. Sachant que les recombinants possédant une région héritée de TYLCSV longue n'ont jamais remplacé les virus parentaux et que leur augmentation en fréquence dans les plantes inoculées avec les virus parentaux n'affecte pas fortement l'accumulation du TYLCV-IL, on peut supposer qu'ils n'ont pas d'effet délétère sur le TYLCV-IL. Cette capacité serait donc spécifique au TYLCV-IS76 et TYLCV-IS141. Comme les autres recombinants ayant hérité d'un fragment TYLCSV long ont une protéine V1 correspondant à TYLCSV et non pas à TYLCV-IL, une possible intéraction entre V1 et son promoteur pourrait effectivement être impliquée dans l'effet délétère de TYLCV-IS76 et de TYLCV-IS141 sur TYLCV-IL. Il serait intéressant de tester la compétitivité de recombinant ayant des fragments hérités de TYLCSV de tailles différentes entre le début et la fin de l'ORF V1, vis-à-vis du parent TYLCV-IS76 pour identifier les régions précises interagissant entre elles. Enfin, TYLCV-IS76 a une meilleure capacité à échapper au *gene silencing* que TYLCV-IL et le gène *Ty-1* augmente fortement la quantité de siRNA viraux produits. Cela se traduit notamment par une augmentation des siRNA de 24nt, responsable du TGS par méthylation des cytosines de l'ADN viral, dans la région intergénique, où a eu lieu l'évènement de recombinaison. Or, dans cette région recombinante, TYLCV-IL possède trois cytosines de plus que TYLCV-IS76, il pourrait donc être plus méthylé que TYLCV-IS76 ce qui expliquerait la capacité de TYLCV-IS76 à échapper plus facilement au *gene silencing* que TYLCV-IL.

Des représentants de la souche TYLCV-IS76 ont été récemment détectés dans le sud de l'Espagne. Leur proximité génétique avec le TYLCV-IS76 marocain montre qu'ils ne proviennent probablement pas d'une nouvelle émergence de TYLCV-IS76 mais bien plutôt de la migration du virus depuis le Maroc. Tout comme au Maroc, le TYLCV-IS76 d'Espagne a été détecté sur des plantes résistantes Ty-1 qui présentaient des symptômes de la maladie (Torre et al., 2019). En Espagne et comme dans tous les pays du bassin méditerranéen, les variétés résistantes portant le gène Ty-1 sont très répandues. Il y a donc un risque important de propagation de ce virus dans le reste des régions productrices de tomate d'Espagne mais également de tous les autres pays proches. Il est donc urgent de développer de nouvelles variétés possédant une résistance au TYLCV-IS76.

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