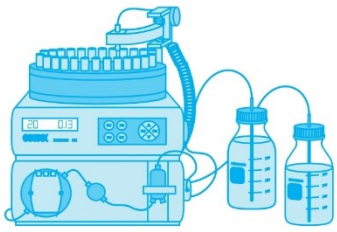
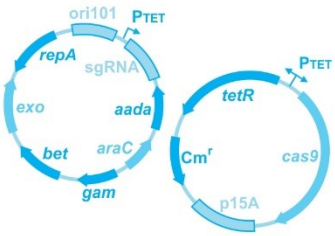
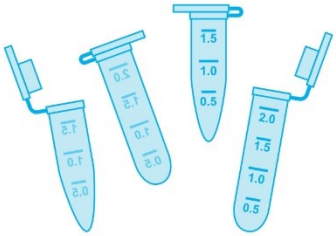
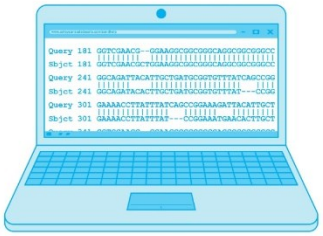
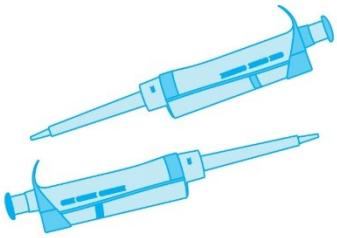
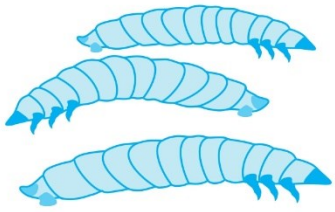
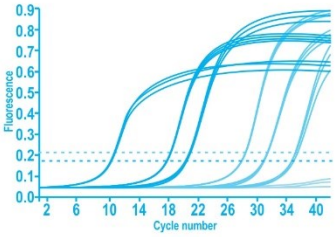



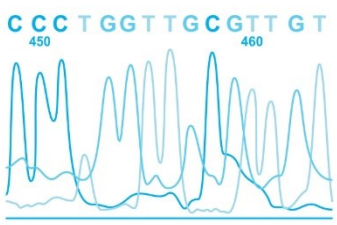




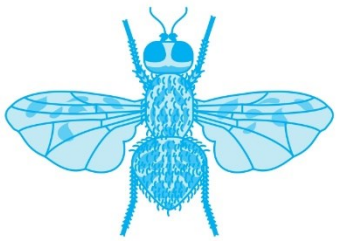



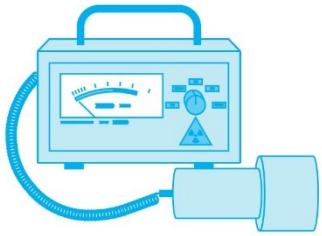
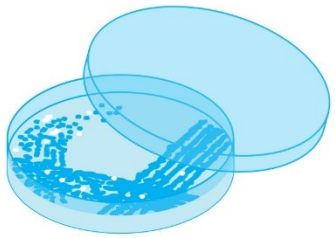
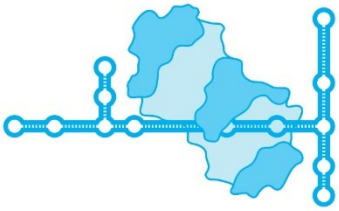
Heterologous expression of circular RNAs in *Escherichia coli* for analyzing the ligation process of chloroplastic viroids and producing double-stranded RNAs with insecticidal activity

Beltrán Ortolá Navarro

Doctoral Thesis





Supervisor: Dr. José Antonio Daròs Arnau

Valencia, December 2022

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Heterologous expression of circular RNAs in *Escherichia coli* for analyzing the ligation process of chloroplastic viroids and producing double-stranded RNAs with insecticidal activity

Beltrán Ortolá Navarro

Memory presented to qualify for the degree of Doctor in Biotechnology by the Universitat Politècnica de València

Supervisor: Dr. José Antonio Daròs Arnau

Valencia, December 2022

D. José Antonio Daròs Arnau, Doctor en Ciencias Biológicas por la Universitat de València y Profesor de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Biología Molecular y Celular de Plantas (IBMCP), centro mixto del CSIC y Universitat Politècnica de València,

CERTIFICA:

Que D. Beltrán Ortolá Navarro, Graduado en Biotecnología por la Universidad Católica de Valencia, ha realizado bajo su dirección el trabajo con título "Heterologous expression of circular RNAs in *Escherichia coli* for analyzing the ligation process of chloroplastic viroids and producing double-stranded RNAs with insecticidal activity" que presenta para optar al grado de Doctor en Biotecnología por la Universitat Politècnica de València.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia a 14 de Diciembre de 2022.

JOSE ANTONIO|DAROS|ARNAU

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D. José Antonio Daròs Arnau

A mis padres

But then science is nothing but a series of questions that lead to more questions, which is just as well, or it wouldn't be much of a career path, would it?

~ The Long Earth, Terry Pratchett and Stephen Baxter.

Table of contents

Index

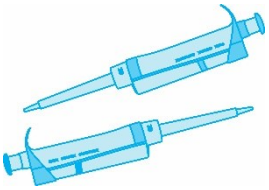
List of figures

General introduction

Chapter I

Chapter II

List of tables



Summary
Resumen
Resum

Summary

Viroids, with a minimal genome of highly structured, single-stranded, circular non-coding RNA, can parasitize plant cell components to replicate autonomously, establish systemic infections and usually cause diseases. Those of the family *Avsunviroidae* replicate and accumulate in chloroplasts through a symmetric rolling circle mechanism, in which a chloroplast RNA polymerase produces linear concatemers of complementary polarity that are reduced to monomeric intermediates by the activity of hammerhead ribozymes (HHR) present in the concatemer. The 5'-hydroxyl and 2',3'-cyclic phosphodiester ends generated are substrates in the formation of an intramolecular 5',3'-phosphodiester bond catalyzed by the chloroplastic isoform of tRNA ligase, generating circular molecules of complementary polarity that can enter another round of transcription, symmetrical to the first. In this Thesis the viroid sequences and structures that are essential for circularization have been analyzed, using as a model the eggplant latent viroid (ELVd), which induces asymptomatic infections in eggplant (*Solanum melongena* L.). To do this, we expressed in *Escherichia coli* linear ELVd (+) precursors flanked by two copies of their HHR. Its processing produces monomers with the appropriate ends for the ligation mediated by the chloroplastic isoform of the eggplant tRNA ligase, which is co-expressed. Point mutations and deletions at the wild-type ligation site suggest that only the HHR domain is essential in the circularization process. The apparent conservation between HHR sequence and structure with those of the enzyme's natural substrate (the tRNAs), lead us to propose that the HHR of

ELVd hijacks the ligase, mimicking the general characteristics of the anticodon loop of tRNAs.

The ELVd expression system has also been used to produce recombinant RNAs of interest, inserting them in a particular position of the ELVd (+) RNA. The chimeric molecules are processed by the flanking HHRs and their ends recognized and ligated by the tRNA ligase. The circular viroid scaffold, compact and possibly associated with the ligase, is responsible for increasing the half-life of the RNA of interest and its accumulation in the bacteria. In this Thesis we adapt this system to produce double-stranded RNA molecules (dsRNAs) capable of triggering the RNA interference (RNAi) response, a natural defense mechanism and transcriptional and post-transcriptional gene expression regulation in eukaryotic cells based on complementarity between RNAs. dsRNAs homologous to endogenous genes generate loss-of-function phenotypes by affecting the levels of cellular transcripts. In addition, insects and other organisms can take these dsRNAs from the environment, internalize them in their cells and distribute them systemically, which makes RNAi a promising strategy for pest control. For producing dsRNAs, we separated the inverted repeats of the target gene that generates the RNA hairpin with the cDNA of the group-I autocatalytic intron from the 26S rRNA of *Tetrahymena thermophila*, increasing the stability of the expression plasmids. After transcription, the intron is removed from the final product, a viroid molecule from which the dsRNA hairpin of interest protrudes. Furthermore, flanking the inverted repeats with an additional copy of the same intron in a permuted fashion allows the viroid molecule to be separated from the final product, a circular dsRNA molecule closed on both sides by small loops. Both types of molecules possess gen silencing activity: viroid-dsRNA chimeras with homology to the corn rootworm (*Diabrotica virgifera virgifera* LeConte; Coleoptera, Chrysomelidae) smooth septate junction 1 gene exhibit similar oral insecticidal activity against larvae to that of hairpins synthesized *in vitro*, whereas circular dsRNAs lacking the viroid scaffold

homologous to the vacuolar ATPase A subunit and ribosomal protein S13 genes repress their respective transcripts in adults of the Mediterranean fly (*Ceratitis capitata* Wiedemann; Diptera, Tephritidae) with high efficiency. The *C. capitata* case is of special relevance as it is the first demonstration of the RNAi strategy for the control of this devastating plague of agricultural crops.

In conclusion, ELVd is, despite its limited agricultural relevance, a very useful platform to investigate the molecular biology of the family *Avsunviroidae*, as well as a powerful biotechnological tool when used in combination with our *E. coli* expression system.

Resumen

Los viroides, con un genoma mínimo de RNA circular no codificante, monocatenario y altamente estructurado, parasitan componentes celulares de las plantas para replicarse autónomamente, establecer infecciones sistémicas y habitualmente causar enfermedades. Los de la familia *Avsunviroidae* se replican y acumulan en cloroplastos mediante un mecanismo de círculo rodante simétrico, en el que una RNA polimerasa cloroplástica produce concatémeros lineales de polaridad complementaria que son reducidos a intermedios monoméricos por la actividad de las ribozimas de cabeza de martillo (HHR) presentes en el concatémero. Los extremos 5'-hidroxilo y 2',3'-fosfodiéster cíclico generados son sustrato en la formación de un enlace intramolecular 5',3'-fosfodiéster catalizado por la isoforma cloroplástica de la tRNA ligasa, generando moléculas circulares de polaridad complementaria que pueden entrar otra ronda de transcripción, simétrica a la primera. En esta Tesis se han analizado las secuencias y estructuras viroidales esenciales para la circularización, empleando como modelo el viroide latente de berenjena (ELVd), que induce infecciones asintomáticas en berenjena (*Solanum melongena* L.). Para ello, expresamos en *Escherichia coli* precursores del ELVd (+) lineales flanqueados por dos copias de su HHR. Su procesamiento produce monómeros con los extremos apropiados para la ligación mediada por la tRNA ligasa de la berenjena, que es coexpresada. Mutaciones puntuales y deleciones en el sitio nativo de ligación sugieren que solo el dominio HHR es esencial en el proceso de circularización. La aparente conservación entre la secuencia y estructura de las HHR con aquellas del sustrato natural del enzima (los tRNAs), nos llevan a proponer que la

HHR del ELVd secuestra la ligasa mimetizando las características generales del bucle del anticodón de los tRNAs.

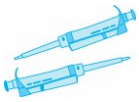
Este sistema de expresión se ha empleado también para producir RNAs recombinantes de interés, insertándolos en una posición particular del RNA del ELVd (+). Las moléculas quiméricas son procesadas por las HHR flanqueantes y sus extremos reconocidos y ligados por la tRNA ligasa. El andamiaje viroidal circular, compacto y posiblemente asociado a la ligasa, es responsable de aumentar la vida media del RNA de interés y su acumulación en la bacteria. En esta Tesis adaptamos este sistema para la producción de moléculas de RNAs de doble cadena (dsRNAs) capaces de desencadenar la respuesta de interferencia por RNA (RNAi), un mecanismo natural de defensa y regulación génica transcripcional y post-transcripcional en células eucariotas basado en la complementariedad de bases entre RNAs. dsRNAs complementarios a genes endógenos generan fenotipos de pérdida de función al afectar los niveles de transcritos celulares. Además, los insectos y otros organismos tienen capacidad de tomar estos dsRNAs del ambiente, internalizarlos en sus células y distribuirlos de manera sistémica, lo que hace al RNAi una estrategia prometedora para el control de plagas. Para la producción de los dsRNAs, separamos las repeticiones invertidas del gen diana que genera la horquilla dsRNA con el cDNA del intrón autocatalítico del grupo I del rRNA 26S de *Tetrahymena thermophila*, aumentando la estabilidad de los plásmidos de expresión. Tras la transcripción, el intrón es eliminado de la molécula final, una molécula viroidal de la que protruye una horquilla con el dsRNA de interés. Además, incorporar una copia adicional del mismo intrón en forma permutada flanqueando las repeticiones invertidas permite separar la molécula del viroide del producto final, una molécula de dsRNA circular cerrada en ambos lados por pequeños bucles. Ambos tipos de moléculas poseen actividad reguladora: las quimeras viroide-dsRNA con homología al gen de la unión septada suave 1 del gusano de la raíz del maíz (*Diabrotica virgifera virgifera* LeConte; Coleoptera, Chrysomelidae) exhiben una

actividad insecticida oral contra las larvas similar a la de horquillas sintetizadas *in vitro*, mientras que los dsRNAs circulares sin el andamiaje viroidal homólogos al gen de la subunidad A de la ATPasa vacuolar y la proteína ribosomal S13 silencian sus respectivos genes en adultos de la mosca del Mediterráneo (*Ceratitis capitata* Wiedemann; Diptera, Tephritidae) con elevada eficiencia. El segundo caso es de especial relevancia al ser la primera demostración de la estrategia de RNAi para el control de esta devastadora plaga de los cultivos agrícolas.

En conclusión, el ELVd es, a pesar de su limitada relevancia agrícola, una plataforma muy útil para investigar la biología molecular de la familia *Avsunviroidae*, así como una poderosa herramienta biotecnológica cuando se usa en combinación con nuestro sistema de expresión de *E. coli*.

Resum

Els viroides, amb un genoma mínim d'RNA circular no codificant, monocatenari i altament estructurat, parasiten components cel·lulars de les plantes per a replicar-se autònomament, establir infeccions sistèmiques i habitualment causar malalties. Els de la família *Avsunviroidae* es repliquen i acumulen en cloroplasts mitjançant un mecanisme de cercle rodant simètric, en el qual una RNA polimerasa cloroplàstica produeix concatèmers lineals de polaritat complementària que són reduïts a intermedis monomèrics per l'activitat dels ribozims de cap de martell (HHR) presents en el concatèmer. Els extrems 5'-hidroxil i 2',3'-fosfodièster cíclic generats són substrat en la formació d'un enllaç intramolecular 5',3'-fosfodièster catalitzat per la isoforma cloroplàstica de la tRNA lligasa, generant molècules circulars de polaritat complementària que poden entrar una nova ronda de transcripció, simètrica a la primera. En aquesta Tesi s'han analitzat les seqüències i estructures viroïdals essencials per a la circularització, emprant com a model el viroide latent d'albergínia (ELVd), que indueix infeccions asimptomàtiques en albergínia (*Solanum melongena L.*). Per aconseguir-ho, expressem en *Escherichia coli* precursors lineals de l'ELVd (+) flanquejats per dos còpies del seu HHR. El seu processament produeix monòmers amb els extrems apropiats per a la lligació mediada per la tRNA ligasa de l'albergínia, que és coexpressada. Mutacions puntuals i delecions al lloc nadiu de lligació suggereixen que només el domini HHR és essencial en el procés de circularització. L'aparent conservació entre la seqüència i estructura de les HHR amb aquelles del substrat

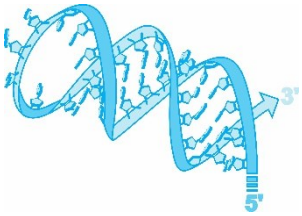


natural de l'enzim (els tRNAs), ens porten a proposar que la HHR de l'ELVd segresta la lligasa mimetitzant les característiques generals del bucle del anticodó dels tRNAs.

Aquest sistema d'expressió s'ha emprat també per a produir RNAs recombinants d'interés, inserint-los en una posició particular de l'RNA de l'ELVd (+). Les molècules quimèriques són processades pels HHR flanquejants i els seus extrems reconeguts i lligats per la tRNA lligasa. L'RNA viroïdal circular, compacte i possiblement associat a la ligasa, és responsable d'augmentar la vida mitjana de l'RNA d'interés i la seua acumulació en els bacteris. En aquesta Tesi adaptem aquest sistema per a la producció de molècules d'RNAs de doble cadena (dsRNAs) capaços de desencadenar la resposta d'interferència per RNA (RNAi), un mecanisme natural de defensa i regulació gènica transcripcional i post-transcripcional en cèl·lules eucariotes basat en la complementarietat de bases entre RNAs. dsRNAs complementaris a gens endògens generen fenotips de pèrdua de funció al afectar els nivells de transcrits cel·lulars. A més, els insectes i altres organismes tenen capacitat de captar aquests dsRNAs de l'ambient, internalitzar-los en les seues cèl·lules i distribuir-los de manera sistèmica, la qual cosa fa a l'RNAi una estratègia prometedora per al control de plagues. Per a la producció dels dsRNAs, separem les repeticions invertides del gen diana que genera la forqueta dsRNA amb el cDNA de l'intró autocatalític del grup I de l'rRNA 26S de *Tetrahymena thermophila*, augmentant l'estabilitat dels plasmidis d'expressió. Després de la transcripció, l'intró és eliminat de la molècula final, una molècula viroïdal de la qual protrueix una forqueta amb el dsRNA d'interés. A més, la incorporació de una còpia addicional del mateix intró en forma permutada flanquejant les repeticions invertides permet separar la molècula del viroide del producte final, una molècula de dsRNA circular tancada als dos costats per xicotets bucles. Els dos tipus de molècules posseeixen activitat reguladora: les quimeres viroide-dsRNA amb homologia al gen de la unió septada suau 1 del cuc de l'arrel de la dacsa (*Diabrotica virgifera virgifera* LeConte; Coleoptera, Chrysomelidae) exhibeixen una activitat insecticida oral contra

les larves similar a la de forquetes sintetitzades *in vitro*, mentre que els dsRNAs circulars sense l'RNA viroïdal homòlegs al gen de la subunitat A de la ATPasa vacuolar i la proteïna ribosomal S13 silencien els seus respectius gens en adults de la mosca del Mediterrani (*Ceratitis capitata* Wiedemann; Diptera, Tephritidae) amb elevada eficiència. El segon cas és d'especial rellevància perquè és la primera demostració de l'estratègia de l'RNAi per al control d'aquesta devastadora plaga dels cultius agrícoles.

En conclusió, l'ELVd és, malgrat la seua limitada rellevància agrícola, una plataforma molt útil per investigar la biologia molecular de la família *Avsunviroidae*, així com una poderosa ferramenta biotecnològica quan s'usa en combinació amb el nostre sistema d'expressió en *E. coli*.



General introduction

1. RNA interference as a tool for pest control

In a current scenario characterized by the need of increasing plant production to meet food needs of a world population in continuous growth and the rise of concerns about the environmental impact of human activity, the development of innovative solutions is required to optimize the crop yield, with improved nutritional properties and resistance to all kinds of stresses. In this sense, insect pests destroy around 20% of the worldwide annual agricultural production, with an estimated cost of around 470.000 million dollars (Culliney, 2014; Sharma et al., 2017), considering both the productive losses and the increase in costs due to pest control systems. In addition, most plant viruses are transmitted by insects and are benefiting from the emergence of new pests to increase their host range and geographic distribution. Thus, reinforcing the need of vector-resistant plants to reduce viral diseases (Escobar-Bravo et al., 2016; Monci et al., 2019; Fortes et al., 2020). In the same way, insects act as vectors of important human and animal diseases (with consequential risk of zoonoses) such as malaria, dengue or chikungunya disease (Chala and Hamde, 2021). Integrated pest management programs (IPMs) are currently being implemented, which along with good agricultural practices and pest monitoring, combine various control strategies such as baited traps with sexual pheromones or male lures, more eco-friendly new generation pesticides and releasing of sterile insects and pest predators, parasitoids and pathogens (Deguine et al., 2015). It is also promising the development of genetically edited plants capable of providing protection against insects by modifying mixtures of volatiles, increasing their

tolerance to herbicides or expressing *Bacillus thuringiensis* (*Bt*) entomotoxins against specific pests (Tyagi et al., 2020). In this sense, an alternative that is arousing great interest is the exploitation of the insects' natural mechanism of RNA interference (RNAi) for their control.

1.1. RNA interference overview

RNAi describes a series of mechanisms highly conserved in eukaryotes regulating gene expression and protecting against exogenous and endogenous genetic elements (such as viruses or transposons). RNAi is triggered by the presence in the cell of small RNAs (sRNA) with high sequence homology to the genetic element to be regulated or protected from. Silencing can occur at the transcriptional (TGS) and post-transcriptional (PTGS) level; the first involves epigenetic modifications in DNA and histones that repress the transcription process, and the second, mRNA degradation or translational repression.

1.2. RNAi discovery

The discovery of the RNAi mechanistic bases is attributed to the work of Fire et al., (1998) with the nematode and model organism *Caenorhabditis elegans*, which earned Andrew Z. Fire and Craig C. Mello the 2006 Nobel Prize in Physiology or Medicine. Their work established double-stranded RNA (dsRNA) as the main effector of RNAi silencing. They called this phenomenon "RNA interference" to distinguish it from previous gene silencing techniques using antisense RNAs (Izant and Weintraub, 1984). Their discovery served to explain previous phenomena of unexpected silencing in various organisms, such as the abovementioned nematode in which similar levels of silencing was achieved by using antisense and sense RNAs (the latter frequently used

as control in antisense strategies) (Fire et al., 1991; Guo and Kemphues, 1995), and in plants and fungi in which the use of transgenes for the overexpression of endogenous or exogenous proteins with high sequence homology sometimes resulted in the silencing of these genes (Matzke et al., 1989; Napoli et al., 1990; van der Krol et al., 1990; Romano and Macino, 1992; Cogoni et al., 1996). All these processes led to the cellular accumulation of dsRNAs, and consequently, the activation of the RNAi machinery. This seminal work also started a rapid race to identify this mechanism in other organisms, confirming its presence in other eukaryotes, such as plants and animals (including insects and mammals) but not in *Saccharomyces cerevisiae* (Kennerdell and Carthew, 1998; Hamilton and Baulcombe, 1999; Wianny and Zernicka-Goetz, 1999; Drinnenberg et al., 2009), as well as to unveil the basic cellular components that mediated the dsRNA-induced gene silencing. Ever since, RNAi has become a widely used tool in basic biological research and has many biotechnological applications.

1.3. Three different pathways of RNA-mediated silencing

Several types of sRNA are capable of triggering RNAi responses, which follow different processing pathways and silencing strategies, although the proteins involved are closely related: that mediated by small interfering RNAs (siRNAs), by micro RNAs (miRNAs) and by P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs). sRNAs can be exogenous (foreign genetic elements, such as viruses with dsRNA genomes or dsRNA intermediates, or experimentally introduced RNAs) and endogenous (genome-encoded and transcribed in the nucleus). An additional process regulating gene expression has also been described, known as RNA activation (RNAa), in which the machinery involved in RNAi have evolved to positively regulate the expression of target sequences at the transcriptional level in many eukaryotes (de Hayr et al., 2020; Li et al., 2006).

1.3.1. siRNAs

The first major pathway, mediated by siRNAs (**Figure 1**), is triggered by the presence of perfectly complementary dsRNAs or RNA hairpins in the cell cytoplasm. It is mainly involved in silencing exogenous RNAs (Galiana-Arnoux et al., 2006; Wang et al., 2006b), although multiple subtypes of siRNAs with endogenous origin (endo-siRNA or esiRNA) exist. esiRNAs can derive from transposable elements, genomic regions with inverted repeats and from mRNAs with overlapping sequences (natural antisense siRNA, natsiRNA) (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008a, 2008b). esiRNAs are involved in maintaining genome stability (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008), the response to several stresses (Lucchetta et al., 2009; Lim et al., 2011) and likely the regulation of gene expression in certain cellular processes, such as energy homeostasis (Lim et al., 2013).

dsRNAs are recognized and processed by multidomain enzymes belonging to Dicer and Dicer-like (DCL) family of proteins, dsRNA-specific bidentate endoribonucleases that generate shorter double-stranded molecules called siRNA (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001). In *Drosophila melanogaster* and presumably all insects, there are two different Dicer genes, being *Dicer-2* responsible for specifically producing siRNAs (Lee et al., 2004b). *Drosophila* Dicer-2 shares a common architecture with human Dicer, with six general domains: N-terminal helicase, central atypical dsRNA-binding domain (dsRBD), Platform/PAZ (Piwi, Argonaute, Zwille), two tandem RNase III (RNase IIIa and IIIb) and C-terminal dsRBD (Lau et al., 2012; Sinha et al., 2018). This enzyme can process dsRNAs according to their characteristics following two different mechanisms (Welker et al., 2011). In the first one, the helicase domain recognizes dsRNAs with blunt ends (that are characteristic of viral infection) and consumes ATP to unwind and translocate the dsRNA, placing its termini in the PAZ domain, processively producing siRNA from one end without dissociating the

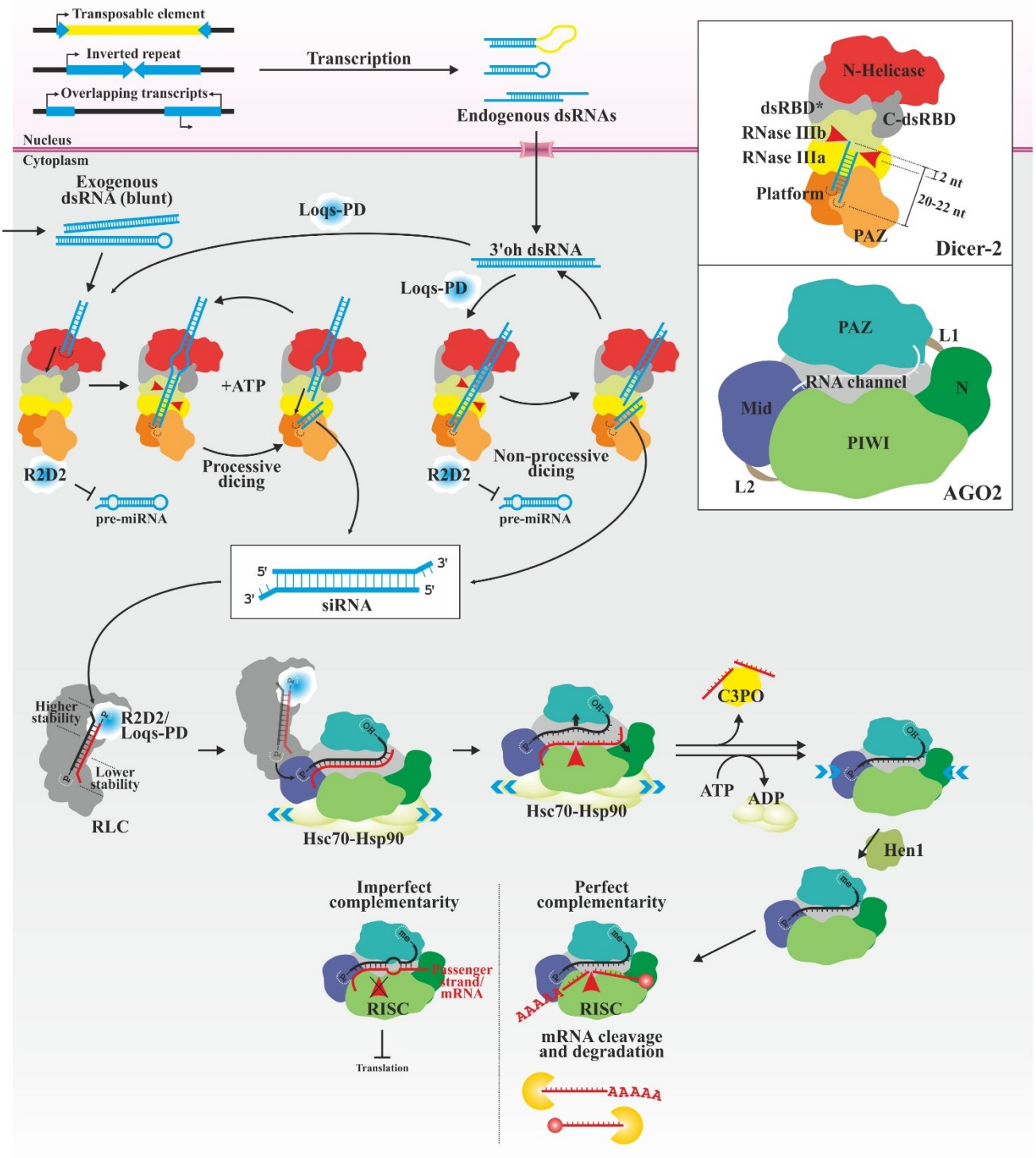


Figure 1. siRNA pathway in insects. Perfectly complementary long dsRNAs of exogenous or endogenous origin (depicted in blue) are differentially processed in the cytoplasm by Dicer-2 depending on the nature of their termini. Additional factors mediate the selection of suitable substrates in both processes. siRNAs of 20-22 bp are generated. Dicer-2 and R2D2/Loqs-PD select the guide strand by sensing the relative stability of both ends. They transfer both chains to AGO2 in an open state, which cleaves and removes the passenger strand (red). The guide (black) is methylated at the 3' end and AGO2 is closed. AGO2 uses the guide strand to cleave complementary mRNAs (red), marking them

for further degradation. The presence of mismatches with the substrate prevents its cleavage, thus repressing mRNA translation. Inserts in the right side show the domains of Dicer-2 and AGO2 (upper and lower, respectively). The characteristic cleavage of Dicer-2 is shown in its corresponding insert. 3'oh, 2 nt 3' overhang termini; -OH, 3'-hydroxyl termini; -P, 5'-phosphate termini; -me, 2'-O-methyl 3' termini; dsRBD*, atypical dsRBD dsRNA-binding domain.

dsRNA (Liu et al., 2003; Cenik et al., 2011; Welker et al., 2011; Sinha et al., 2015, 2018). In the second model, the PAZ/Platform domain interacts directly with dsRNAs with 2 nt 3' overhangs (that are characteristic of digestion by RNase III) independently of ATP. After each cleavage, the dsRNA dissociates, and its protruding ends can be recognized again (Welker et al., 2011; Sinha et al., 2018). Both mechanisms are typical, but not exclusive, for the described molecules (Naganuma et al., 2021).

The length of the produced RNAs is characteristic of the Dicer protein, since it depends on the separation between the PAZ domain and the active center of the RNase domains, acting as a sort of molecular rule (MacRae et al., 2006, 2007; Kandasamy and Fukunaga, 2016). Thus, typical insect siRNAs are 20-22 nt long (Santos et al., 2019). The presence of two RNase III domains and their characteristic positioning generate 2 nt overhanging 3' ends, with 5' phosphate and 3' hydroxyl ends (Zhang et al., 2004). The recognition and cleavage of dsRNAs may depend on the involvement of dsRNA binding proteins (dsRBPs). For example, it has been described that R2D2 (protein with two dsRBPs associated with Dicer-2) prevents Dicer-2 from processing miRNA precursors *in vitro*, increasing its affinity for long dsRNAs (Cenik et al., 2011), while its antagonist Loquacious-PD (Loqs-PD) is necessary for processing certain endo-siRNAs but not for viral siRNAs (Hartig et al., 2009; Zhou et al., 2009; Miyoshi et al., 2010a; Marques et al., 2013). It is possible that Loqs-PD promote the use of suboptimal dsRNAs by altering the Dicer-2 dependence of terminal structures (Sinha et al., 2015; Trettin et al., 2017; Jonely et al., 2021; Naganuma et al., 2021).

Only one strand of the siRNAs (called guide strand) becomes part of the RNA-induced silencing complex (RISC), in which it establishes the specificity of the silencing process by base complementarity with other RNAs (Martinez et al., 2002); the other strand (passenger strand) is usually degraded (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). Strand selection follows the thermodynamic asymmetry rule, whereby the strand with its 5' termini less stably paired with the complementary strand is preferentially selected (Khvorova et al., 2003b; Schwarz et al., 2003). The selection occurs in the so-called RISC loading complexes (RLC), consisting of Dicer-2, R2D2 and possibly additional factors (Liu et al., 2003, 2006; Tomari et al., 2004). R2D2 binds tightly to the 5' phosphate of the thermodynamically more stable passenger strand, restricting Dicer-2 to the opposite end of the siRNA duplex (Tomari et al., 2004). This function can be replaced by Loqs-PD in certain esiRNAs (Mirkovic-Hösle and Förstemann, 2014; Tants et al., 2017).

The cellular effector of the silencing is a protein of the Argonaute (AGO) family (Hammond et al., 2000, 2001; Martinez et al., 2002; Song et al., 2003), which forms RISC together with accessory proteins and the RNA. In insects, two proteins of the AGO subfamily have been described (Hammond et al., 2001; Kataoka et al., 2001; Rubio et al., 2018). They are multidomain proteins organized in two lobes: the N-terminal contains the variable domain N and the PAZ, connected by a linker (L1); a second linker (L2) connects it to the C-terminal lobe, which contains the middle (MID) and PIWI domains (Song et al., 2004; Schirle and MacRae, 2012). Both lobes surround a central channel that accommodates RNA. As with Dicer, AGO proteins have specialized functions, with AGO2 being involved in antiviral immunity and regulation mediated by esiRNAs (Keene et al., 2004; Okamura et al., 2004, 2008b; van Rij et al., 2006; Wang et al., 2006b; Ghildiyal et al., 2008). However, the selection of the AGO protein may occur according to the identity of the 5' terminal nucleotide of the guide strand and the presence and position of mismatches in the sRNA, and not according

to the Dicer that generates it. Thus, certain esiRNAs can be loaded in AGO1, and miRNAs passenger strands in AGO2 (Förstemann et al., 2007; Tomari et al., 2007; Czech et al., 2009; Kawamata et al., 2009; Okamura et al., 2009; Ghildiyal et al., 2010; Ameres et al., 2011; Nishida et al., 2013).

Loading requires Dicer-2/R2D2 and AGO2 (Liu et al., 2003, 2006; Tomari et al., 2004; Matranga et al., 2005; Iwasaki et al., 2015). Their interaction allows the MID domain to recognize and bind the 5' phosphate end of the guide strand, transferring the duplex (Ma et al., 2005; Parker et al., 2005). Weaker interactions are established between PAZ and the 3' overhang end (Lingel et al., 2003; Song et al., 2003). The duplex is then unwound, presumably by the N and PAZ domains (Okamura et al., 2004; Tomari et al., 2004; Gu et al., 2012; Kwak and Tomari, 2012; Park and Shin, 2015), and the PIWI domain cleaves the passenger strand (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005), which is rapidly removed by the C3PO endonuclease (RISC promoter component 3) (Liu et al., 2009). The 3' terminal nucleotide is methylated at the 2'-O position by RNA methyltransferase DmHen1 (Horwich et al., 2007), possibly to prevent its degradation. A complex of multiple Hsc70-Hsp90 chaperones (70 kDa heat shock analog protein and 90 kDa heat shock protein, respectively) also participates in the transfer (Iwasaki et al., 2010, 2015; Miyoshi et al., 2010b), maintaining AGO in an open state throughout the process; at the end of which it hydrolyzes ATP allowing AGO to acquire a closed, mature conformation.

The PIWI domain is responsible for the endonucleolytic cleavage of the target mRNA with base complementarity with the loaded sRNA. PIWI is similar in structure to RNase H, which typically catalyze the hydrolytic cleavage of RNA into RNA/DNA duplexes (Liu et al., 2004; Song et al., 2004). The cleavage generates 5′-phosphate and 3'-hydroxyl ends (Martinez and Tuschl, 2004), resulting in an RNA fragment without

a poly(A) tail and another without a 5' 7-methylguanosine cap, which are degraded by exonucleases of the RNA surveillance machinery (Lima et al., 2016). The AGO2-mediated cleavage, both in the passenger strand and in target mRNAs, do not occur if it is loaded with RNAs with central mismatches, explaining the differential loading of RNAs in both AGO isoforms (Matranga et al., 2005). In this case, the elimination of the passenger strand is slower, and silencing is established by blocking protein synthesis.

1.3.2. miRNAs

miRNAs (**Figure 2**) constitute the most abundant type of sRNAs in animals, regulating multiple biological processes, such as reproduction, development or immunity (Brennecke et al., 2003; Kang et al., 2018; Song et al., 2018; Ma et al., 2019). They are usually expressed as polycistronic RNAs from intergenic regions of the genome with their own promoters or from intragenic regions co-expressed with the gene they regulate (Lee et al., 2002; Qian et al., 2011; He et al., 2012). They are generally transcribed by RNA polymerase II as primary transcripts (pri-miRNA) with typical mRNA modifications (5' cap and 3' poly(A) tail) (Lee et al., 2002, 2004a; Cai et al., 2004) and a general hairpin structure with a long complementary double-stranded region flanked by a terminal loop and two unstructured single-stranded segments.

The pri-miRNAs are processed in the nucleus (Lee et al., 2002, 2004a; Cai et al., 2004) by the type-III ribonuclease Drosha (Lee et al., 2003), with the help of Pasha (Denli et al., 2004; Landthaler et al., 2004), a dsRBPs with which it forms the Microprocessor complex. Two Pasha (Herbert et al., 2016) proteins recognize and bind the terminal loop of the pri-miRNA, and their dsRBD domains interact with part of the dsRNA region. Drosha's dsRBD binds the other half of the dsRNA, and additional interactions are established between the enzyme and the other ssRNA-dsRNA region.

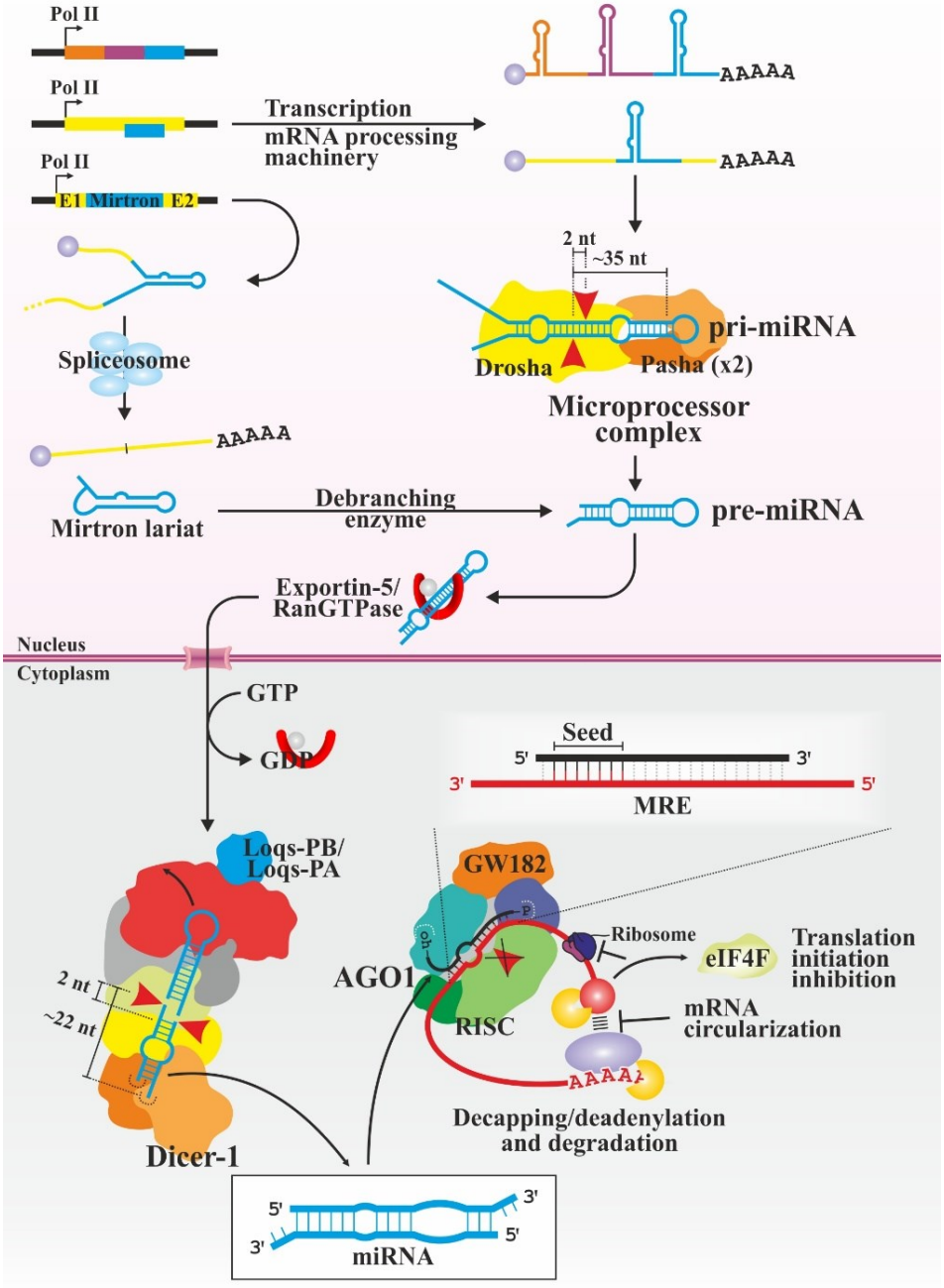


Figure 2. miRNA pathway in insects. miRNA-containing genomic loci are transcribed by RNA polymerase II generating long, partially dsRNA primary precursors (depicted in blue) that are trimmed into shorter hairpins by the Microprocessor complex Drosha/Pasha in the nucleus. This step is not necessary in the case of mirtrons, whose processing depends on spliceosomal and debranching machinery. The precursors are then exported into the cytoplasm where Dicer-1, aided by Loqs isoforms, eliminates the loop resulting in ~22 bp miRNA. The characteristic cleavage of the microprocessor and Dicer-1 are shown. miRNA guide strand (black) is loaded into AGO1 that induces cleavage-independent mRNA (red) degradation and translation suppression of the partially complementary mRNAs. E1/E2, exon 1 and 2, respectively; -OH, 3'-hydroxyl termini; -P, 5-phosphate termini.

Both Drosha and Pasha contacts ensure the binding of suitable substrates, acting together as a molecular rule and accepting RNAs with two ssRNA regions separated by a dsRNA of about 35 nt (Ma et al., 2013; Nguyen et al., 2015; Kwon et al., 2016; Jin et al., 2020; Partin et al., 2020). Additional motifs may affect processing efficiency (Fang and Bartel, 2015). The two RNAse III domains of Drosha eliminate the basal single-stranded segments and part of the dsRNA, generating miRNA precursors (pre-miRNAs), hairpins of about 70 nt with one of the characteristic 2 nt protruding 3' ends of miRNAs. One subtype of miRNAs is derived from introns; thus, they are known as mirtrons. For their maturation they depend on the splicing machinery and debranching enzymes but not on Microprocessor, directly entering the pathway as pre-miRNAs with a hairpin structure (Okamura et al., 2007; Ruby et al., 2007).

The next step in its processing occurs in the cytoplasm (Lee et al., 2002, 2004a; Cai et al., 2004). Exportin-5 (Exp-5) mediates pre-miRNAs translocation to the cytoplasm through the nuclear pores in combination with GTPase Ran (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004). The efficient transport of precursors by Exp-5 seems to depend largely on the presence of 2 nt 3' overhangs and to a lesser extent on the characteristics of the apical loop (Zeng and Cullen, 2004; Okada et al., 2009; Zhang et al., 2021b). Release of pre-miRNA into the cytosol requires hydrolysis of GTP by GTPase Ran.

There they are processed by Dicer-1 (Lee et al., 2004b; Förstemann et al., 2007). As with Dicer-2, the PAZ domain recognizes and binds to the 3' overhang generated by Drosha while the helicase domain, which is not functional in this isoform (Lee et al., 2004b), binds to the loop region (Tsutsumi et al., 2011). The helicase senses the loop size while PAZ measures the distance from the loop to the 3' overhang. Thus, tandem RNase III domains only cleave the substrate if they are within adequate distance from the 3' end (Tsutsumi et al., 2011). The cleavage removes the loop and

generates the second 3' overhang, resulting in ~22 nt RNA. The PB isoform (and to a lesser extent PA) of Loqs (Förstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005) is involved in the processing of most but not all (Förstemann et al., 2005; Liu et al., 2007; Lim et al., 2016) miRNAs, possibly stabilizing RNAs with unstable structures at the cleavage site (Lim et al., 2016).

Next, one of the miRNA strands is loaded onto RISC containing AGO1 (Okamura et al., 2004), following the same strand selection rule as siRNAs. Unlike R2D2/Loqs-PD and AGO2, Loqs-PB seems to be dispensable in AGO1 loading (Liu et al., 2007), although it is unknown whether Dicer-1 by itself is sensitive to the asymmetry or requires additional factors. The loading is dependent on ATP, possibly to keep AGO1 in an open state. The duplex unwinds in a similar way as in AGO2 and the passenger strand is removed without endonucleolytic cleavage, facilitated by miRNAs characteristic mismatches (Kawamata et al., 2009; Park and Shin, 2015). It is worth mentioning that miRNAs and their non-mature forms can be edited, adding or modifying nucleotides critical for their maturation, regulation and functionality (Kawahara et al., 2007; Chawla and Sokol, 2014; Reimão-Pinto et al., 2015).

In animals, the recognition elements of miRNAs (MREs) are usually found in the 3' untranslated region (3' UTR) of mRNAs. Their interaction is usually imperfect but with a characteristic pattern: nucleotides 2-7 of the miRNA 5' end (called seed region) present perfect complementarity with the mRNA and is sufficient for its function, although additional pairing may participate in the process (Lai, 2002; Stark et al., 2003; Bartel, 2009). This short sequence allows a miRNA to regulate several mRNAs and different miRNAs can act on a single mRNA (John et al., 2004). In *D. melanogaster*, both AGOs have cleavage activity; however, the catalytic rate of AGO1 is limited by the inefficient dissociation of the reaction product (Förstemann et al., 2007). Thus, AGO1 induce silencing in several ways in which GW182 proteins generally

participate recruiting cell factors and serving as scaffolding. It can inhibit translation by preventing interactions between the poly(A) tail and the 5' cap that pseudocircularize mRNA during translation, as well as preventing ribosomal recruitment by dissociating the eukaryotic initiation factor eIF4F from the cap (Zdanowicz et al., 2009; Zekri et al., 2009; Fukaya et al., 2014). It can recruit deadenylases and decapping enzymes (Behm-Ansmant et al., 2006; Eulalio et al., 2007; Zekri et al., 2009; Braun et al., 2011; Nishihara et al., 2013), making the mRNA sensitive to the action of the exoribonuclease 5′-3′ XRN1 (Rehwinkel et al., 2005).

1.3.3. piRNAs

The piRNA pathway (**Figure 3**) is mediated by proteins of the PIWI subfamily (Piwi, Aubergine (Aub) and AGO3). It occurs mainly in the germ line and is associated with RNAs originating from genomic repetitive intergenic regions and transposons (piRNA clusters). Its main function is to maintain genomic integrity (Vagin et al., 2006; Brennecke et al., 2007). piRNAs also participate in both cell lines of processes such as fertility, maintenance and differentiation of stem cells or defense against some viruses (Gonzalez et al., 2015; Klein et al., 2016; Dietrich et al., 2017; Katsuma et al., 2018; Kotov et al., 2019).

Unlike siRNA and miRNA pathways, piRNAs are processed from single-stranded precursors that are transcribed by RNA pol II and processed and exported as typical mRNAs (Goriaux et al., 2014; Dennis et al., 2016). In germline, bidirectional clusters are common (Brennecke et al., 2007; Malone et al., 2009; Mohn et al., 2014), involving the same polymerase and specialized cell factors. Rhino recognizes these clusters, generally with characteristic modified histones (H3K9me3), and uses Deadlock as a scaffold to recruit the Moonshiner transcription factor, Cutoff (which prevents transcript splicing and modification) and nuclear export factor 3 (Nxf3 -Nxt1, that

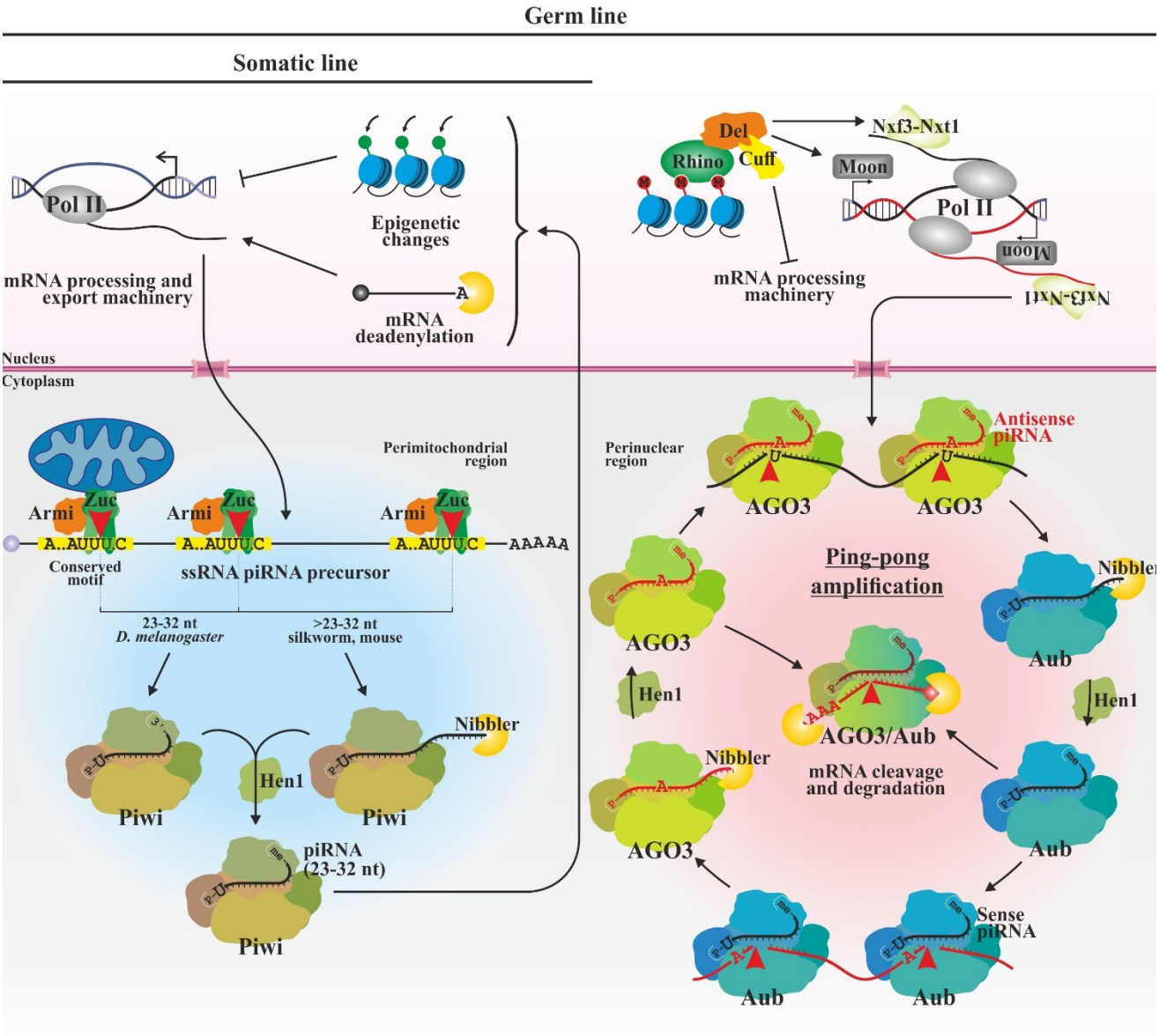


Figure 3. piRNA pathway in both, insects' somatic and germline cells. In somatic cells, piRNA precursors are transcribed from unidirectional piRNA clusters. Linear precursors are exported to cytoplasmic perimitochondrial regions where Zucchini and in some cases additional exonucleases generate single-stranded 23-32nt piRNAs that are loaded on Piwi proteins and methylated at their 3' end. In germline, the presence of bidirectional clusters and specialized transcription machinery also allows the generation of piRNAs of both senses that participate in the generation of more piRNAs of the complementary sense (ping-pong cycle) in perinuclear regions; Aub/AGO3 differentially load RNAs of both polarities that are also terminally methylated (sense in black, and antisense in red, respectively). Piwi-bound piRNAs enter the nucleus where they transcriptionally regulate gene expression while Aub/AGO3 remain in the cytoplasm and induce degradation of complementary mRNAs. Pol II, RNA polymerase II; Armi, Armitage; -P, 5'-phosphate termini; -me, 2'-O-methyl 3' termini; M, H3K9me3; Del, Deadlock; Moon, Moonshiner transcription factor; Cuff, Cutoff protein.

mediates export of precursors to the cytoplasm) (Klattenhoff et al., 2009; Mohn et al., 2014; Zhang et al., 2014b; Chen et al., 2016; Andersen et al., 2017; ElMaghraby et al., 2019; Kneuss et al., 2019).

piRNA precursors accumulate together with proteins that intervene in their biogenesis in perinuclear and perimitochondrial electron-dense regions in the germline but only in perimitochondrial regions in somatic cells (Ai and Kai, 2007; Qi et al., 2011). Argonaute proteins tightly bind the 5' phosphate end of RNAs. In germline cells, this end is generated by AGO3 proteins containing antisense guide RNAs that recognize and cleave sense precursors (Han et al., 2015; Mohn et al., 2015; Gainetdinov et al., 2018; Nishida et al., 2018; Izumi et al., 2020), or by the Zucchini (Zuc) endonuclease, possibly with the help of additional factors such as Armitage, which recognizes conserved motifs with a moderate preference to those that generate uridines at the 5′ end (Izumi et al., 2020). Only the second pathway occurs in somatic line since the machinery required for generating complementary piRNAs is not expressed. The 5'-P intermediate generated in both pathways is loaded onto a Piwi protein (somatic cells) or Piwi and Aub (germline) (Brennecke et al., 2007). The 3' cleavage of piRNA is mediated by additional downstream action of Zuc or AGO3 proteins. In several species the 3' ends are trimmed by a 3'-5' exonuclease to generate optimal RNAs to be fully accommodated by PIWI subfamily proteins (Kawaoka et al., 2011; Izumi et al., 2016; Nishimura et al., 2018). In *D. melanogaster* it has been suggested that the Nibbler exonuclease only is needed if the 3' cleavage is generated by Aub/Ago3, while Zuc would directly generate the appropriate end of the piRNA (Hayashi et al., 2016). The maturation of the piRNAs concludes with the 2'-O-methylation of its 3' end by the Hen1 methyltransferase (Horwich et al., 2007; Saito et al., 2007), which is required for correct interaction with the PAZ domain. Mature piRNAs are slightly longer than miRNAs and siRNAs (23-32 nt).

In germline, the silencing signal is amplified with the generation of secondary piRNAs in the so-called "ping-pong mechanism", in which sense precursors loaded to Aub serve as a guide for the cleavage of homologous antisense transcripts (Brennecke et al., 2007; Gunawardane et al., 2007). The new molecule is recognized, loaded and processed in AGO3. Due to the cleavage pattern, the molecules loaded in AGO3 have 10 nt homology to the primary piRNA, and most of them have an adenine in the tenth position. piRNA-AGO3 in turn recognizes and cuts transcripts from the piRNA cluster itself, generating new piRNAs with the same sequence as primary piRNAs. In addition, the Zuc activity on the precursors generates new intermediates that can be processed and loaded by other PIWIs, generating phased piRNAs that increase the sequence diversity of piRNAs (Han et al., 2015; Mohn et al., 2015; Gainetdinov et al., 2018; Izumi et al., 2020).

Aub and AGO3 remain in the cytoplasm where they mediate PTGS by cleaving target RNAs (Brennecke et al., 2007; Reuter et al., 2011; Zhang et al., 2015b), repressing translation and promoting mRNA degradation (Grivna et al., 2006; Ai et al., 2009; Rouget et al., 2010; Gou et al., 2015; Rojas-Ríos et al., 2017). Piwi develops its function mainly in the nucleus, inducing epigenetic changes (Klenov et al., 2011; Sienski et al., 2012; Shpiz et al., 2014) and deadenylating mRNAs in sites of active transcription of transposable elements (Kordyukova et al., 2020).

1.4. dsRNA cell uptake and systemic distribution of the silencing signal

The mechanisms described in the previous section are known as cell-autonomous RNAi, while non-cell autonomous RNAi encompasses the processes that occur before and after it. These are the uptake of dsRNA from the extracellular medium (environmental RNAi) and the transport of the silencing signal to other cells (systemic RNAi)

(Whangbo and Hunter, 2008) (**Figure 4**). Both processes require additional cellular machinery that seems highly variable between organisms; thus, less information is known about them. The following sections will focus on those aspects that are relevant for the oral delivery of dsRNAs to insects.

Most of the information about non-cell autonomous RNAi comes from *C. elegans*, in which is mediated by the endocytosis pathways and systemic RNA interference deficiency (SID) membrane proteins. SID-2 is only expressed in the apical membrane of intestinal cells and mediates the endocytic uptake of dsRNA from the intestinal lumen into the cells, not being required for systemic RNAi (Hunter et al., 2006; Winston et al., 2007). SID-1 is a ubiquitously expressed (except in neurons) dsRNA-specific transmembrane channel that mediates endosomal dsRNA release into the cytoplasm during environmental and systemic RNAi (Winston et al., 2002; Feinberg and Hunter, 2003). Two endosomal vesicle-associated proteins, SID-5 (Hinas et al., 2012) and SID-3 (Jose et al., 2012; Gao et al., 2020), are also required in both dsRNA uptake and systemic distribution by mediating the import and export of vesicles containing such dsRNAs.

Other than *C. elegans*, SID-2 homologs have only been found within the genus *Caenorhabditis*, in species resistant to environmental RNAi (Winston et al., 2007). SID-1 homologous genes have been identified in most insects, except for those of the superorder Antliophora (Diptera, Mecoptera and Siphonaptera) (Dowling et al., 2016). However, these genes may have more homology with tag-130/CHUP-1, a SID-1 paralogue that does not participate of RNAi in *C. elegans* but is involved in cholesterol internalization (Tomoyasu et al., 2008; Pinheiro et al., 2018). This protein has been indirectly related to dsRNA movement by influencing the composition of the plasma membrane. The effect on the uptake efficiency of altering the membrane fatty acids composition has been demonstrated as an immunological mechanism to protect insects

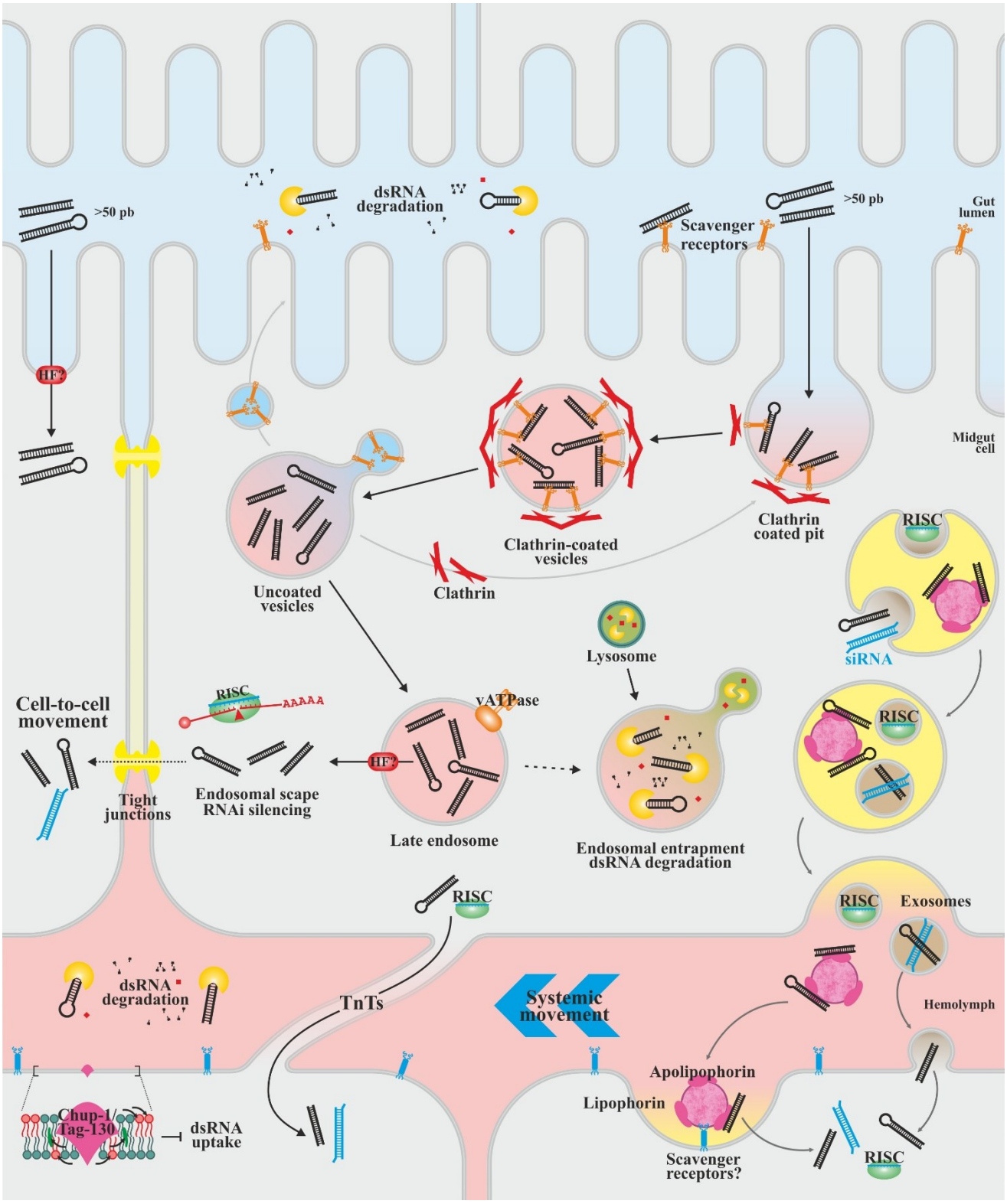


Figure 4. Proposed mechanisms of exogenous dsRNA cell-uptake, cell-to-cell and systemic movement in insects. Scavenger receptor-mediated clathrin-dependent endocytosis is the main pathway for cellular uptake of dsRNAs in midgut cells, although additional unknown factors may be involved in the process. The uptake is dependent on the dsRNA size, and nucleases in the digestive system may compromise the dsRNA stability. Unknown cellular factors mediate dsRNA egress from the late endosome; inefficient endosomal escape induces dsRNA degradation after endosome-lysosome

fusion. In the cytoplasm, the dsRNAs mediate the silencing of endogenous host genes. dsRNAs, RNAi machinery and RNA intermediates can be transported to neighboring cells through cytoplasmic projections (TnTs) and possibly through tight junctions. Systemic movement to distant cells through hemolymph depends on exosomal encapsulation and it has been proposed that may also be mediated by dsRNA binding to apolipophorins. The interaction with these cellular components prevents their degradation by the hemolymph nucleases. Alterations in plasma membrane composition may be consequential in the uptake and distribution of the silencing signal. vATPase, Vacuolar-type ATPase; HF?, unknown host factor; Chup-1/Tag-130, cholesterol uptake protein 1.

from subsequent exposures to environmental dsRNA (Dong et al., 2017). This is consistent with the elusive role of this proteins in insects, since there is no straightforward association between absence, presence and number of Sid-1 genes with the uptake and systemic distribution of dsRNAs (Tomoyasu et al., 2008; Cappelle et al., 2016; Yoon et al., 2016). Therefore, other mechanisms must facilitate these non-cell autonomous RNAi in insects.

Studies with *D. melanogaster* S2 cells showed the role of clathrin-mediated endocytosis in the uptake of dsRNAs (Saleh et al., 2006; Ulvila et al., 2006). In addition, chemical blockade of pattern recognition receptors disrupts uptake (Saleh et al., 2006), and two scavenger receptors, SR-CI and Eater, have been implicated as the main mediators of the process (Ulvila et al., 2006). A similar role for these receptors and clathrin-mediated endocytosis has been demonstrated in several insect species (Aung et al., 2011; Wynant et al., 2014b; Li et al., 2015b; Xiao et al., 2015; Cappelle et al., 2016; Meng et al., 2018; Pinheiro et al., 2018; Abbasi et al., 2020), and could also play a role in the uptake of dsRNAs encapsulated in bacteria (Rocha et al., 2011). However, several of these works shown that silencing or blocking these receptors does not completely interrupt uptake. Therefore, it is likely that dsRNAs can be recognized by different receptors that could differ between species. Similarly, the involvement of other clathrin-independent pathways in the process cannot be ruled out, as has been seen to occur with some dsRNA structures (Abbasi et al., 2020).

Interestingly, the recognition of naked dsRNAs by the insect uptake machinery is length dependent. It has been shown to be efficient for long molecules (greater than ~50 bp) but not for short molecules such as siRNAs (Saleh et al., 2006; Bolognesi et al., 2012; Miller et al., 2012), which can be a disadvantage in many dsRNA delivery methodologies. Another factor that limits the development of this type of strategies is the differential capacity to degrade exogenous dsRNAs of the RNases within the digestive system of the various insect orders (Wynant et al., 2014c; Wang et al., 2016a; Singh et al., 2017). Similarly, efficient endosomal escape of dsRNAs is required for developing the RNAi response. For example, lepidopteran species have low sensitivity to RNAi, at least in part, due to dsRNA entrapment in endosomes and degradation after fusing with lysosomes (Shukla et al., 2016; Yoon et al., 2017). Blocking the interaction of late endosomes with lysosomes enhances si- and miRNA-mediated silencing in *D. melanogaster*, while blocking late endosome formation limits silencing (Lee et al., 2009), thus restricting dsRNA escape between late endosome formation and lysosomal fusion (Saleh et al., 2006). The activity of the vacuolar H^+-ATPase has been related to dsRNA cell entry in several species (Saleh et al., 2006; Xiao et al., 2015; Shi et al., 2022); however, the mechanism mediating the endosomal escape is not exactly known.

In some insects, dsRNA delivery can result in the generation of a systemic response by short- and long-distance RNAi signal movement. It is likely that the signal is transmitted by direct intercellular contact through membranous protrusions called tunneling nanotubes (TnTs) that allow the connection between cells. Viral infection in *D. melanogaster* cell cultures induces the formation of these structures that transport dsRNA and components of the RNAi machinery (Karlikow et al., 2016). In addition, movement of the RNAi signal through tight junctions, as occurs in mammals, has been proposed, although not yet demonstrated (Valiunas et al., 2005).

Over long distances, dsRNA movement appears to occur through hemolymph. In certain insects, hemolymph nucleases efficiently degrade dsRNA (Garbutt et al., 2013; Ren et al., 2014; Li et al., 2022; Ma et al., 2022c), hampering the systemic response and effectiveness of RNAi. dsRNA transport in the hemolymph is mediated by carrier molecules, thus protecting it from degradation. The dsRNA-binding ability of apolipophorins purified from the hemolymph of *Bombyx mori* (Sakashita et al., 2009) and *Schistocerca gregaria* (and probably in species of the orders Orthoptera, Blattodea and Diptera) (Wynant et al., 2014a) has been demonstrated, strongly suggesting a conserved mechanism in insects. Apolipoforins are the protein components of lipophorin, hemolymphal lipoprotein complexes which function in lipid transport and are also part of the insect antiviral defense. Lipophorins are scavenger receptor ligands, and in ticks these receptors have also been implicated in systemic RNAi (Aung et al., 2011). RNAs are also carried by extracellular vesicles. miRNA-containing vesicles have been identified in *D. melanogaster* cell cultures (van den Brande et al., 2018) and their occurrence *in vivo* has been proposed (Dhahbi et al., 2016). Furthermore, during viral infection in this species, hemocytes capture dsRNAs from infected cells and generate viral siRNAs that are packaged in vesicles that circulate through hemolymph, systemically diffusing the RNAi signal (Tassetto et al., 2017). As for exogenously supplied RNAs, the encapsulation of long dsRNAs and derived siRNAs has been shown in extracellular vesicles of *Tribolium castaneum* and *Leptinotarsa decemlineata* cell cultures (Mingels et al., 2020; Yoon et al., 2020). In the latter, some of the factors related with endosomal generation and recycling pathways participating in the process were detailed. The full extent of these mechanisms, as well as the possible involvement of additional factors, have yet to be fully resolved.

1.5. Sources of dsRNAs with insecticidal effect

The first strategy used to assess pest control by means of RNAi was to develop transgenic plants expressing specific dsRNAs for silencing (Baum et al., 2007; Mao et al., 2007) (**Figure 5**). Since then, countless reports of plant-produced, dsRNAs-mediated gene silencing in insects have been published, with the first commercial variety approved by competent Canadian, USA and Chinese administrations (2016, 2017 and 2021, respectively). It is SMARTSTAX PRO corn (Bayer), that produces dsRNA against the Snf7 gene of *Diabrotica virgifera virgifera* (Head et al., 2017). Its commercialization is scheduled to start in 2022. However, multiple limitations hold back the development of this kind of technologies. On the one hand, GMOs generation is laborious, expensive due to the rigid commercialization regulations (AgbioInvestor, 2022), and currently have scant public acceptance (Sikora and Rzymski, 2021). On the other hand, plant RNAi machinery recognizes the produced dsRNAs, generating siRNAs (Gordon and Waterhouse, 2007; Mao et al., 2007), which can negatively affect their uptake by the insect. It is possible to circumvent this problem by expressing dsRNAs in chloroplasts or other compartments lacking RNAi machinery (Jin et al., 2015; Zhang et al., 2015a; Bally et al., 2016; Thagun et al., 2022), although their accumulation is very size-sensitive (Burke et al., 2019; He et al., 2020) and their usefulness with sap feeding Hemiptera is limited (Kaplanoglu et al., 2022). It has also been proposed the use of RNAs with structures resistant to the plant RNAi machinery, such as artificial pre-miRNAs (Bally et al., 2020; Flynt, 2021).

Other approaches not requiring plant modification have been developed, such as RNA uptake by roots and petiole or trunk injection (Hunter et al., 2012; Li et al., 2015a; Dalakouras et al., 2018; Pampolini et al., 2020). In these strategies, the dsRNA enters the plant but it is retained in the xylem and apoplast, thus not being processed by the plant RNAi machinery. High-pressure dsRNA spraying has been shown to induce systemic silencing of plant genes and confer resistance to fungi (Dalakouras et

al., 2016b; Koch et al., 2016; Biedenkopf et al., 2020; Dalakouras and Ganopoulos, 2021), in a process in which dsRNAs have been also detected in the phloem.

Plants infected with modified plant viruses have been widely used in functional genetics with insects (Kumar et al., 2012; Wuriyanghan and Falk, 2013; Hajeri et al., 2014; Khan et al., 2018), and similar viruses may also confer protection to plants against fungi and nematodes (Valentine et al., 2007; Dubreuil et al., 2009). The virus acts as a dsRNA factory during its replication in the plant (Fang et al., 2017). The wide variety of vectors commonly used to produce molecules of interest with minimal damage to plants makes them an interesting alternative. Similarly, insect-specific viruses have been used to silence endogenous pest genes (Hajós et al., 1999; Uhlirova et al., 2003; Huang et al., 2007; Gu et al., 2011; Kontogiannatos et al., 2013; Taning et al., 2018). Replicating engineered viruses can be useful in those cases in which the insect is resistant to environmental and systemic RNAi, given its ability to transfer its genome into the cells, multiplying and establishing systemic infections. Additionally, it would provide another layer of specificity as viruses can be highly host-specific (Gu et al., 2011; Taning et al., 2018). An interaction between both types of viruses is found in the Flock House virus, which replicates in insects and plants (Selling et al., 1990; Dasgupta et al., 2001; Zhou et al., 2015). Although viruses represent an interesting insecticidal strategy, the cross-kingdom status of the silencing suppressors encoded in both virus types must be considered (Li et al., 2002; Sinha et al., 2021). In addition, the environmental release of transgenic viruses can pose biosafety problems. An alternative is the use of virus-like particles (VLPs) synthesized *in vitro* or in modified microorganisms and plants, expressing the dsRNA of interest and viral capsid proteins that self-assemble into virus-like structures enclosing the nucleic acids (Aalto et al., 2007; Fang et al., 2017; Niehl et al., 2018; Le and Müller, 2021). VLPs have been used in plants to induce resistance against viruses (Niehl et al., 2018) and to produce recombinant proteins. Although they lack replicative capacity, they confer protection

to dsRNA and retain the potential to transfer dsRNAs to the cytoplasm, in addition to some host specificity. Also, they lack silencing suppressors. With the perspective of continuous production in the insect and host specificity, the use of bacteria and fungi able to parasitize or symbiotize pest insects and modified for producing dsRNA has been studied (Chen et al., 2015; Taracena et al., 2015; Hu and Wu, 2016; Murphy et al., 2016; Whitten et al., 2016).

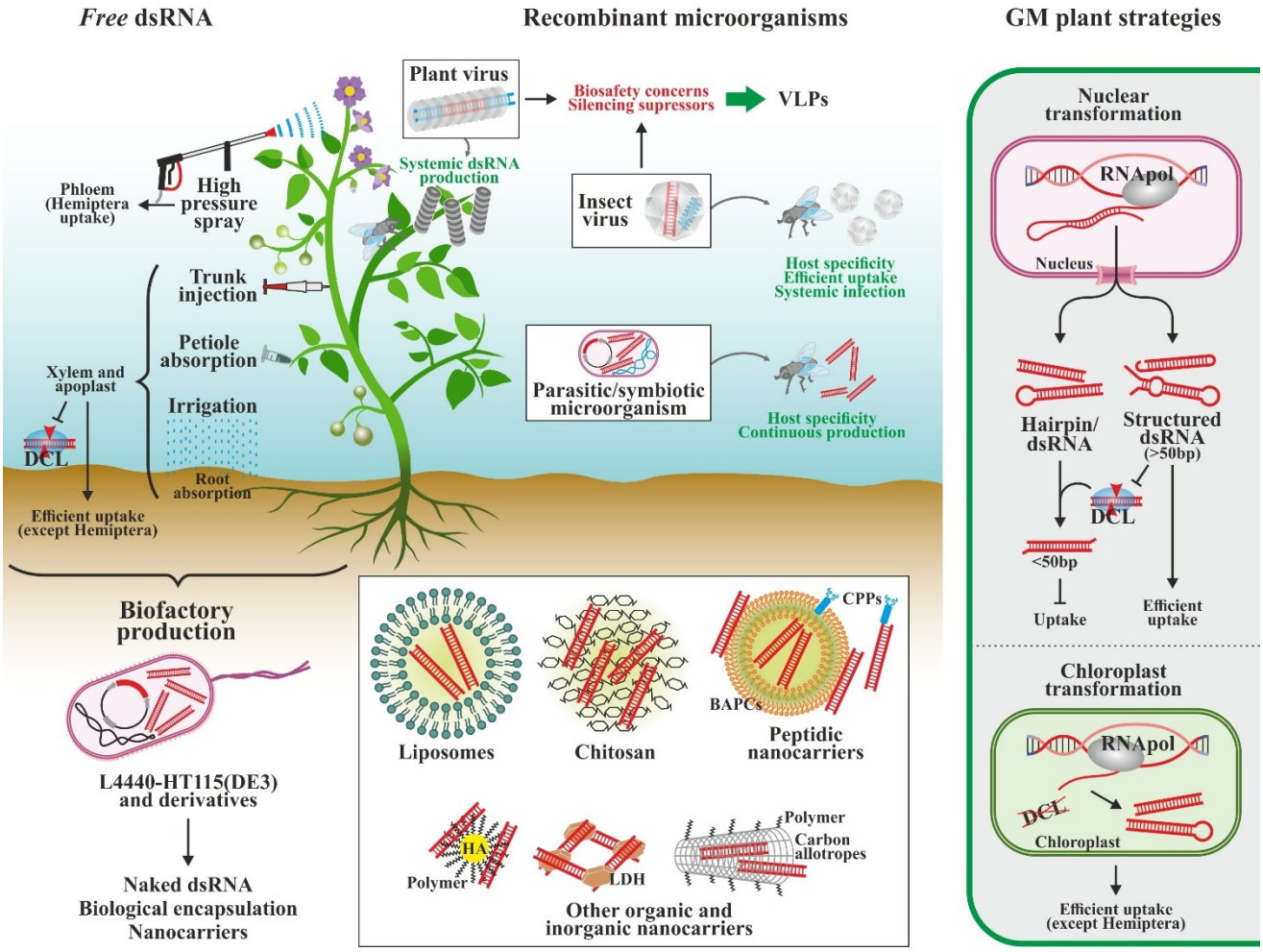


Figure 5. Proposed strategies for supplying dsRNAs to insects for RNAi-mediated pest control in the field. dsRNAs can be produced in biofactories and used, alone or in combination with nanomaterials (insert), with different application systems. The use of plant and insect viruses, VLPs derived from them, as well as other insect pathogens or symbionts, taking advantage of its intrinsic characteristics, has been also studied. The development of nuclear and chloroplast transformants allows the continuous production of dsRNAs in genetically modified plants. RNApol, RNA polymerase; HA, hydroxyapatite.

The exogenous application of dsRNAs as a non-transformative alternative for plant protection was pioneered with work against viral diseases (Tenllado et al., 2003), while the use of dsRNAs pulverized as a conventional pesticide was proposed by San Miguel and Scott (2016). These strategies (and some of the previously mentioned) require the production of dsRNAs in heterologous systems, as well as their purification and encapsulation. mRNAs and dsRNAs for vaccines are produced by *in vitro* systems as they are more quickly developed and entail fewer concerns regarding their production in microorganisms. Similar systems are unfeasible for intensive use in the field given the enormous quantities required and their high production cost (Das and Sherif, 2020; Taning et al., 2020), which would increase crop production prices. *In vivo* production methods, using microorganisms as a biofactory, are more attractive. The microbiological variety, easy handling, fast growth and heterologous production capacity make them economically viable. The most widely used procedure is the L4440-HT115(DE3) system (Timmons and Fire, 1998; Timmons et al., 2001) developed for the initial RNAi experiments in *C. elegans*. It is based on the transformation of plasmid L4440, which contains two opposing T7 promoters flanking the cDNA of the gene to be silenced, into a modified *Escherichia coli* bacterial strain that lacks the dsRNA-specific endonuclease RNase III but has the bacteriophage T7 RNA polymerase under the control of the inducible lac operon. Bidirectional transcription results in two complementary ssRNA strands that hybridize; both, the whole bacteria or further dsRNA purification are feasible for RNAi strategies. Multiple advances have been made on this type of system in order to increase performance, such as the use of different strains and microorganisms (including their genetic modification), the development of new expression vectors, the improvement of fermentation and extraction methods, etc. (Posiri et al., 2013; Thammasorn et al., 2015; Papić et al., 2018; Ahn et al., 2019; Ma et al., 2020; Park et al., 2020; Hashiro et al., 2021; Islam et al., 2021). To put these advances in perspective, Tenllado et al. (2003) reported the production of 4 μg of

dsRNA per ml of culture with the L4440-HT115(DE3) system, while Hashiro et al. (2021) achieved amounts greater than 1 mg/ml using the modified Gram-positive bacterium *Corynebacterium glutamicum* lacking the rnc gene by co-expressing a high copy number plasmid with convergent T7 promoters and an additional plasmid encoding the polymerase under lac control. As a result of these improvements and the advent of novel systems, the price per gram of dsRNA produced has fallen from over $12,000 in 2008 to about $1 today, and up to half of that in cell-free systems (Taning et al., 2020).

Naked dsRNA molecules are susceptible to degradation by several biotic and abiotic stresses when used as pesticides. Furthermore, in some insects they have a limited ability to be efficiently uptaken and systemically distributed. Therefore, they are usually formulated in combination with carrier molecules of different natures that increase dsRNA bioavailability in cells. Most of these strategies are based on the advances made in human therapies, and as a common feature, they have cationic surfaces that allow the interaction and encapsulation of the negatively charged phosphate backbone of nucleic acids, as well as the interaction with the negatively charged cell membrane (Han et al., 2020). One common strategy is to encapsulate dsRNA in liposomes, lipid bilayer spherical structures (Whyard et al., 2009; Taning et al., 2016; Castellanos et al., 2019; Gurusamy et al., 2020b; Wang et al., 2020). Multiple commercial transfection reagents, with different lipid compositions, may be useful to improve species-dependent recalcitrances; however, they are usually expensive and, in many cases, potentially cytotoxic, and may affect beneficial insect species. Another widespread strategy is the use of the natural polysaccharide chitosan, due to its abundance, low cost, biocompatibility and degradability (Zhang et al., 2010; Das et al., 2015; Gurusamy et al., 2020a; Wang et al., 2020). Extensive modifications to these natural polymers have been made to improve their stability and cell delivery in pest control and other applications (Dhandapani et al., 2019; Dou et al., 2019; Huang et al.,

2020). Several organic and inorganic nanoparticles can also be used, such as hydroxyapatite, silica, phosphate calcium, carbon allotropes or quantum dots (Das et al., 2015; Edwards et al., 2020; Elhaj Baddar et al., 2020; Wang et al., 2020; Henry and Lacarriere, 2021). They have low toxicity and a high surface/volume ratio that allows efficient loading of RNAs. Usually, they are functionalized or associated with polymers of synthetic origin, whose chemical variety allow highly versatile particle designs, thus modulating cytotoxicity, dsRNA stability in specific insect environments, cell uptake, etc. (He et al., 2013; Christiaens et al., 2018). An inorganic nanoparticle that does not need functionalization or association with other polymers is layered double hydroxide (LDH), used in plants to provide resistance to virus and fungi, and with potential for pest control applications (Mitter et al., 2017; Worrall et al., 2019; Sundaresha et al., 2022). There are also two interesting protein alternatives. On the one hand, branched amphipathic peptide capsules (BAPCs), bilayer structures very similar to liposomes but made up of peptides. Protein nanostructures such BAPCs have been described as potentially more biocompatible and biodegradable than synthetic polymers, and more stable than those composed of lipids and polysaccharides (Gudlur et al., 2012). They have been used to enhance the RNAi response in insects such as *T. castaneum*, *Acyrthosiphon pisum* (Avila et al., 2018), and *Spodoptera frugiperda* (McGraw et al., 2022). In the latter case, clathrin-mediated endocytosis and macropinocytosis uptake has been described, along with high endosomal escape and an increase of dsRNA transcytosis to hemolymph, improving the systemic response. On the other hand, peptides can be used as uptake mediators. Membrane penetrating peptides (CPPs), both derived from natural proteins or engineered, are rich in basic amino acids that can establish complexes with dsRNA or coat other nanostructures. Their variety allows dsRNA cell internalization in several ways, thus overpassing recalcitrancies to any specific entry pathway, as reviewed extensively (Falato et al., 2021). This strategy has been successfully used in insects, using a CPP fused with a

dsRBD to improve the silencing effect in *A. grandis* (Gillet et al., 2017). A similar improvement is obtained with the fusion of a dsRBD with agglutinin in *Spodoptera exigua* (Martinez et al., 2021).

2. Viroids

Viroids constitute a group of intracellular parasites exclusive of higher plants, composed of a small RNA (246 to 430 nt) covalently closed and single-stranded, but highly structured given its high self-complementarity. Its short sequence does not code for any protein in either the viroid RNA or the complementary strand. They lack a protective protein coat and depend on sequences and structures in their RNAs to parasitize host plant cell structures in order to replicate autonomously and complete their infectious cycle. Among viroids host species are several crop and ornamental plants; since the infective process leads to host diseases in many cases, viroids are economically relevant as well as interesting from a biological point of view.

2.1. Discovery and possible origin of viroids

The process that led to the discovery of viroids began in the late 1960s with studies that focused on elucidating the causative agent of potato spindle tuber disease (Martin, 1922), which was suspected of viral etiology (Schultz and Folsom, 1923). Diener and Raymer (1967) and Singh and Bagnall (1968) isolated from diseased plants a low molecular weight free RNA, with characteristics of dsRNA, capable of producing the infection. Later it was proposed that the causal agent of this disease must depend entirely on the host enzymes to its replication, as it is composed by a genome smaller than that of known viruses, too small to encode the genetic information necessary for

its replication, but do not need an auxiliary virus to do so (Diener, 1971b). Therefore, the existence of a new type of pathogens, similar but different from viruses and RNA satellites, was proposed. T.O. Diener coined the term "viroid" (virus-like) to describe these RNAs. Although this proposal was not initially well accepted by many scientists, similar causal agents were described for citrus exocortis (CEVd) and chrysanthemum dwarf (CSVd), which contributed to its consolidation (Semancik and Weathers, 1968; Diener and Lawson, 1973). Later works refined the molecular and mechanistic knowledge of these pathogens.

Its origin is an enigma. Several hypotheses have been considered, such as viroids originating from or being the origin of primitive RNA viruses, as well as deriving from introns, transposable elements or other cell RNAs (Diener, 1981; Kiefer et al., 1983; Catalán et al., 2019; Jain et al., 2020). Nowadays, a widespread hypothesis is that viroids and other current catalytic RNAs are remnants of the "RNA world" (Diener, 1989; Moelling and Broecker, 2021). In this hypothesis RNA was the basis of life, given its ability to store information and catalyze its own synthesis, before the unfolding of these functions in DNA and proteins. Existing viroids can no longer replicate on their own, possibly having lost that function when they became strict plant parasites. Currently, viroids are classified, together with satellite RNAs, defective interfering particles and prions, as subviral agents, and are considered the smallest infectious agents described to date. It has been proposed that viroids and viroid-like satellite RNAs (previously known as virusoids) have a monophyletic origin, with the family *Avsunviroidae* acting as an evolutionary link between them (Diener, 1989; Elena et al., 1991, 2014), although this proposal is controversial.

2.2. General structure and phylogenetic classification of viroids

Currently, 33 different viroids and several sequence variants have been biologically and molecularly characterized. Based on structural characteristics, and their impact on the biological properties, viroids have been classified by the International Committee on Virus Taxonomy (ICTV) into two families (**Figure 6**).

The family *Pospiviroidae* (named after its type species, potato spindle tuber viroid (PSTVd) (Gross et al., 1978), to which most viroids belong, adopt rod-shaped structures containing conserved sequences and structural motifs: the central conserved region (CCR) and the terminal conserved region (TCR) or the terminal conserved hairpin (TCH) (**Figure 7A**). The sequence of the CCR and the presence or absence of TCR and TCH allow the members of this family to be classified into five genera (**Figure 6**). Five distinct domains have been mapped in these viroids (Keese and Symons, 1985): the central domain (C), containing the CCR and flanked by the pathogenic (P) and variable (V) domains to its left and right, respectively, and two terminal domains, right (TR) and left (TL), the latter containing the TCR or TCH (**Figure 7A**). Although they are named by specific functions, there is a more complex correlation between different parts of the viroid genome and the biological functions they perform (Sano et al., 1992; Zhong et al., 2008; Wu et al., 2020). These viroids replicate and accumulate in the nucleus (Diener, 1971a; Sänger, 1972; Takahashi et al., 1982; Bonfiglioli et al., 1994; Qi and Ding, 2003) by an asymmetric rolling circle mechanism.

The family *Avsunviroidae* on the other side is much smaller. Named after its type species, avocado sunblotch viroid (ASBV) (Symons, 1981), they do not contain CCR region or other conserved motifs typical of the former family, but they contain functional hammerhead ribozymes (HHR) in the RNA of both polarities (**Figure 7B**). These viroids replicate and accumulate in chloroplasts (Bonfiglioli et al., 1994; Lima et

al., 1994; Bussière et al., 1999; Navarro et al., 1999) by a symmetric rolling circle mechanism. Three of its members, peach latent mosaic viroid (PLMVd),

***Pospiviroidae* family**		
Genus *Pospiviroid*	PSTVd	Potato spindle tuber viroid
	CEVd	Citrus exocortis viroid
	CSVd	Chrysanthemum stunt viroid
	CLVd	Columnea latent viroid
	IrVd 1	Iresine viroid 1
	PCFVd	Pepper chat fruit viroid
	TASVd	Tomato apical stunt viroid
	TCDVd	Tomato chlorotic dwarf viroid
	TPMVd	Tomato planta macho viroid
Genus *Hostuviroid*	HSVd	Hop stunt viroid
	DLVd	Dahlia latent virus
Genus *Apscaviroid*	ASSVd	Apple scar skin viroid
	ADFVd	Apple dimple fruit viroid
	AGVd	Australian grapevine viroid
	CBLVd	Citrus bent leaf viroid
	CDVd	Citrus dwarfing viroid
	CVd-V	Citrus viroid V
	CVd-VI	Citrus viroid VI
	GYSVd 1	Grapevine yellow speckle viroid 1
	GYSVd 2	Grapevine yellow speckle viroid 2
	PBCVd	Pear blister canker viroid
Genus *Cocadviroid*	CCCVd	Coconut cadang-cadang viroid
	CTiVd	Coconut tinangaja viroid
	CBCVd	Citrus bark cracking viroid
	HLVd	Hop latent viroid
Genus *Coleviroid*	CbVd 1	Coleus blumei viroid 1
	CbVd 2	Coleus blumei viroid 2
	CbVd 3	Coleus blumei viroid 3

***Avsunviroidae* family**		
Genus *Avsunviroid*	ASBVd	Avocado sunblotch viroid
Genus *Pelamoviroid*	PLMVd	Peach latent mosaic viroid
	CChMVd	Chrysanthemum chlorotic mottle viroid
	AHVd	Apple hammerhead viroid
Genus *Elaviroid*	ELVd	Eggplant latent viroid

Figure 6. ICTV taxonomic classification of viroids (2020). The 33 viroids are grouped into two families: *Pospiviroidae*, with five genera, and *Avsunviroidae*, with three genera. The type species of each genus is highlighted on a gray background. For each species, the abbreviation of its name is indicated.

chrysanthemum chlorotic mosaic viroid (CChMVd), and apple hammerhead viroid (AHVd) (Hernández and Flores, 1992; Navarro and Flores, 1997; Zhang et al., 2014a), have a branched conformation stabilized by kissing-loops and pseudoknots, and are classified in a single genus (*Pelamoviroid*), while ASBVd and the eggplant latent viroid (ELVd) adopt quasi-rod-like conformations (Symons, 1981; Fadda et al., 2003). These form genera with a single member currently described: *Avsunviroid*, characterized by a high content (62%) in A+U, distinctive among the other viroids (Flores et al., 2014), and *Elaviroid*, with intermediate properties between the previous families (Fadda et al., 2003) (**Figures 6 and 7B**).

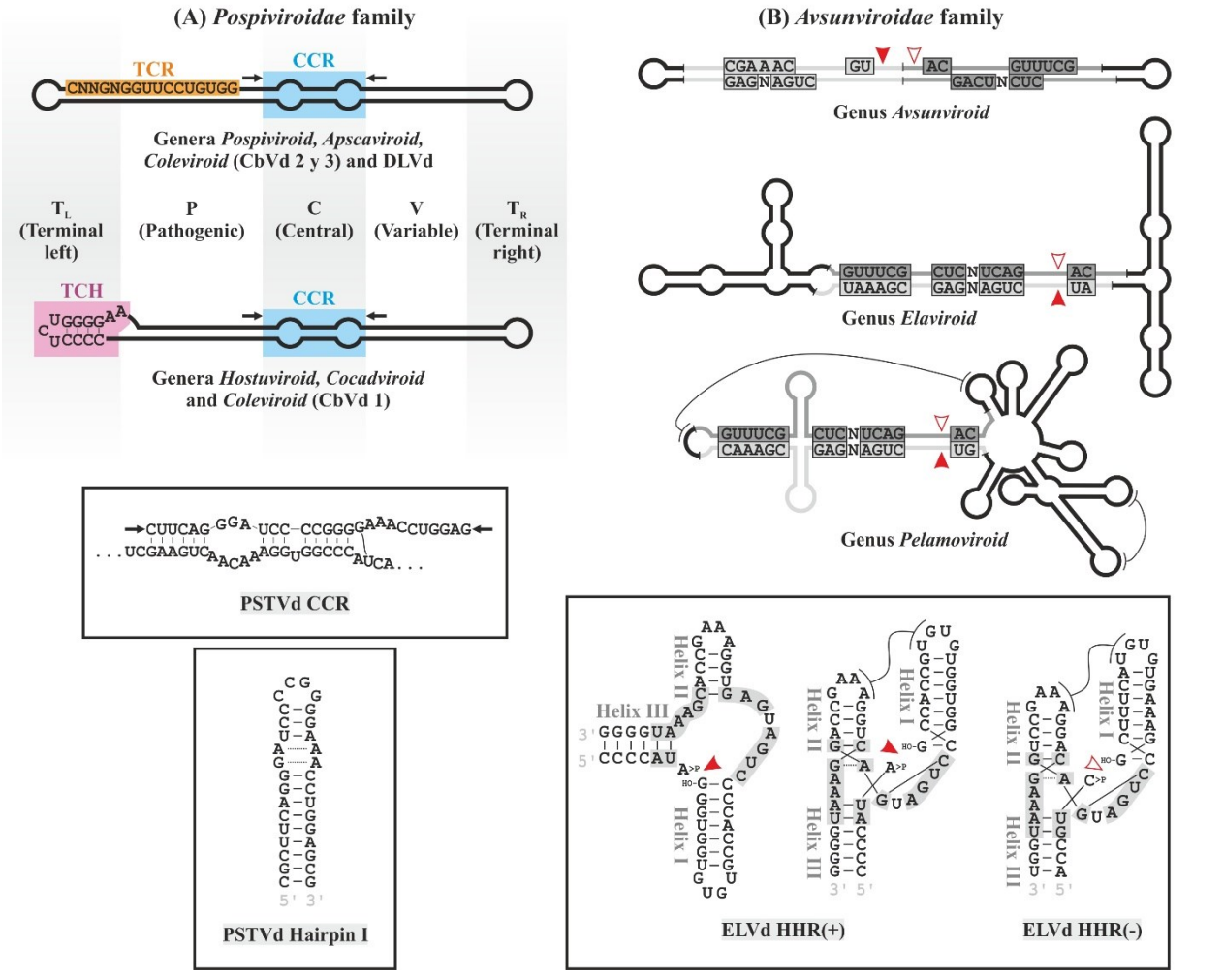


Figure 7. Structural characteristics of the viroids in the families *Pospiviroidae* and *Avsunviroidae*. (A) Members of the family *Pospiviroidae* adopt a rod-shaped secondary structure that has been functionally separated in five domains (TL, P, C, V and TR; differentially shaded). They

contain conserved motifs: the features of the CCR (blue box) and the presence of TCR or TCH (orange and pink boxes, respectively) define the characteristics of each genus, as indicated. Together with the conserved sequence of the upper strand of the CCR, the flanking variable nucleotides (indicated by arrows) form an imperfect hairpin (hairpin I). Both, the characteristic CCR sequence of PSTVd and the hairpin that forms are shown in the upper and lower inserts, respectively. (B) *Avsunviroidae* viroids adopt rod-shaped, branched or semibranched secondary structures (genus *Avsunviroid, Pelamoviroid* and *Elaviroid*, respectively). They contain conserved sequences of hammerhead ribozymes that are functional in positive and negative strands (light and dark gray boxes, respectively, with the self-cleavage sites indicated by solid or empty arrowheads, respectively). In PLMVd, 'kissing-loops' tertiary interactions are indicated by lines. The insert includes the sequence of the HHR of ELVd with the classic representation that gives name to these ribozymes (left) next to the same HHR in both polarities according to the data of X-ray crystallography and NMR. Tertiary interactions between loops 1 and 2 are shown with lines. N, any nucleotide; -OH and >P, 5′-hydroxyl and 2′,3′-phosphodiester groups, respectively.

2.3. Viroid replication mechanisms

A rolling circle replication mechanism was proposed, with differences between both families (Branch et al., 1981; Branch and Robertson, 1984). This proposal was based on (i) the non-detection of homologous DNAs in infected tissues (Branch and Dickson, 1980; Zaitlin et al., 1980; Rohde and Sänger, 1981), (ii) the circular nature of viroids (Sanger et al., 1976; Owens et al., 1977) and (iii) the presence of longer-than-unit RNAs, apparently with tandem repeats of both polarities (by convention, the + polarity is assigned to the most abundant circular RNA), in dsRNA complexes (Grill and Semancik, 1978; Branch et al., 1981; Bruening et al., 1982; Hadidi et al., 1982; Owens and Diener, 1982; Branch and Robertson, 1984).

PSTVd do not accumulate circular forms of - polarity (Branch et al., 1988; Feldstein et al., 1998), so the replication is restricted in this viroid and the other members of its family to the asymmetric variant of the rolling circle mechanism (**Figure 8A**). Circular RNAs (+) are repeatedly transcribed producing a linear concatemer of RNAs of complementary (-) polarity. This RNA enters directly into a new replication cycle, generating an oligomer of + polarity, which is processed (cleaved

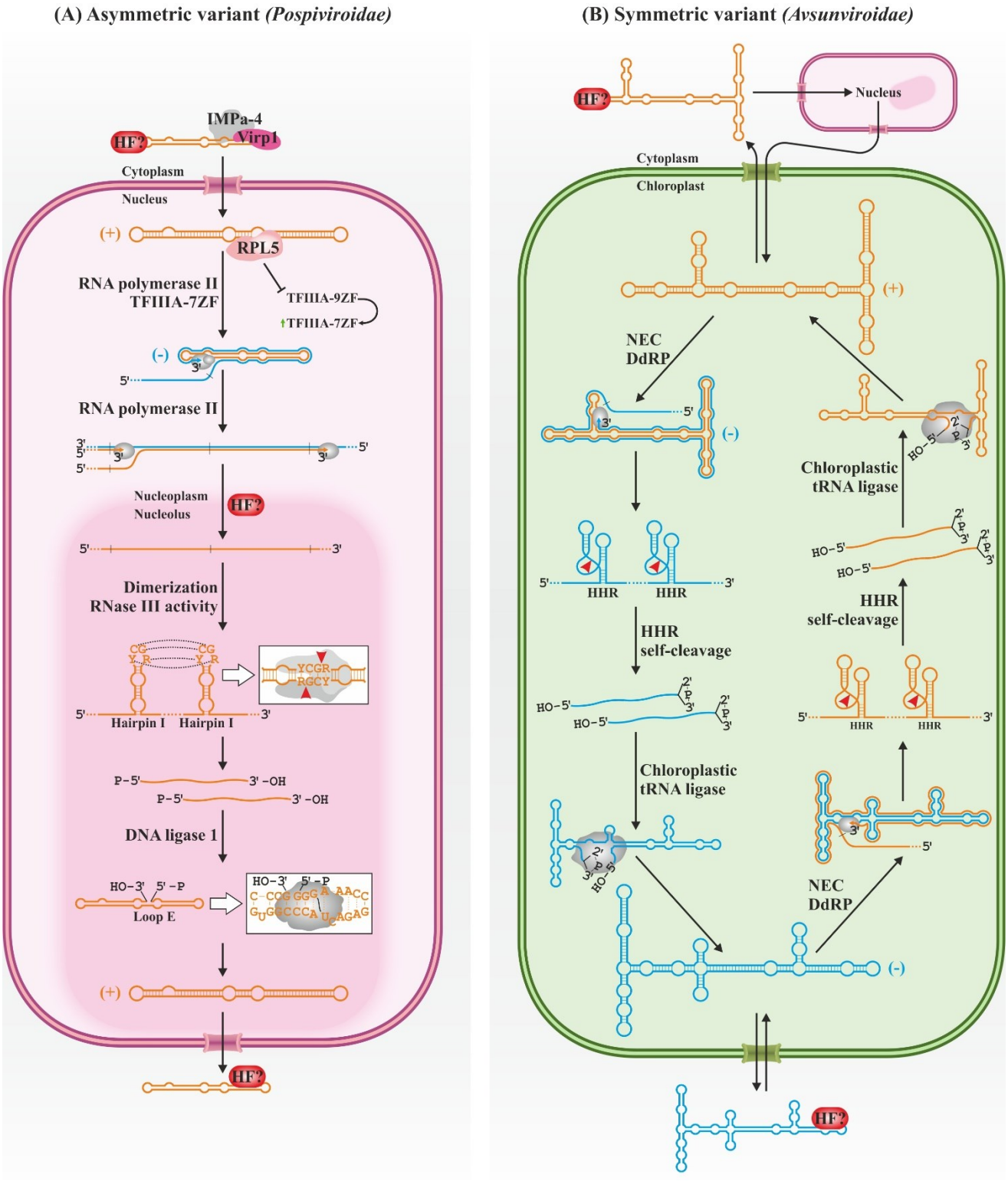


Figure 8. Rolling-circle mechanism in its (A) asymmetric and (B) symmetric variant proposed for the replication of viroids of the families *Pospiviroidae* in the nucleus and *Avsunviroidae* in chloroplasts, respectively. In both cases, the positive and negative viroid RNA polarity are represented in orange and blue, respectively. Host proteins and viroid RNA motifs involved in the replicative cycle are indicated. Arrowheads indicate RNA cleavage sites. NF?, unknown host factor; NEC DdRP, nuclear-encoded chloroplastic DNA-dependent RNA polymerase; -P, -OH and >P, 5′-phosphate, 5′-hydroxyl and 2′,3′-phosphodiester groups, respectively.

and ligated) by host enzymes to generate circular (+) monomers of the viroid. On the other hand, *Avsunviroidae* members follow the symmetric pathway (**Figure 8B**). Circular monomers (+) are repeatedly transcribed producing a viroid concatemer of - polarity. The self-cleavage activity of the HHRs in the concatemer generates monomeric units that are circularized by host factors, resulting in circular viroids of - polarity which can enter a new round of replication to generate more circular (+) viroids.

The first step in the replication of the *Pospiviroidae* family members is their entry into the nucleus, which appears to be dependent on interaction with host factors (Seo et al., 2020). The participation of Virp1 (bromodomain-containing protein 1) in the process has recently been demonstrated in CEVd (Seo et al., 2021). Virp1 can also bind efficiently to PSTVd (Gozmanova et al., 2003; Maniataki et al., 2003; Martínez de Alba et al., 2003; Kalantidis et al., 2007), interacting with a C-loop conserved in nuclear-replicating viroids (Ma et al., 2022a). This protein of unknown function contains a nuclear localization signal, and it locates in such organelle (Martínez de Alba et al., 2003; Kalantidis et al., 2007), being also able to mediate the nuclear import of the satellite RNA of cucumber mosaic virus (Chaturvedi et al., 2014), also containing a C-loop (Ma et al., 2022a). These latter authors also showed the likely involvement of importin alpha-4 (IMPa-4) in the viroid trafficking process. However, CEVd can be also imported independently of Virp1, while additional nuclear localization domains have been described in PSTVd, in the upper strand of the CCR and/or hairpin I (Abraitiene et al., 2008). Thus, additional cell factors and viroid signals may mediate the import process. For instance, HSVd contains open reading frames encoding short peptides with nuclear localization signals and is associated with polysomes, raising a possible novel viroid trafficking strategy (Márquez-Molins et al., 2021).

Replication takes place specifically in the nucleoplasm (Qi and Ding, 2003). Viroids of this family hijack the host DNA-dependent RNA polymerase II (Pol II)

(Mühlbach and Sänger, 1979; Flores and Semancik, 1982; Yoshikawa and Takahashi, 1986), an ability apparently shared with the complementary (-) strand (Warrilow and Symons, 1999). The Pol II involved in this process, however, has a remodeled architecture with reduced number of components in contrast to the polymerase complex on DNA templates (Dissanayaka Mudiyanselage et al., 2022). In the case of PSTVd, the transcription factor TFIIIA-7ZF is also required (Wang et al., 2016b), being essential for the polymerase to use RNA as a template, while other canonical factors of general transcription do not participate, suggesting a different transcription machinery (Dissanayaka Mudiyanselage et al., 2022). The binding sites of Pol II and TFIIIA-7ZF are found in the left terminal region of the PSTVd (+), where the transcription start site is consequently found (nucleotide U359 or C1) (Kolonko et al., 2006). An study (Bojić et al., 2012) suggests that the polymerase recognizes the general rod structure between loops 1 to 5, rather than its specific sequence, while TFIIIA-7ZF has been mapped to the lower strand, between nucleotides 331-347 (loops 3 to 5) (Wang et al., 2016b). The CCR region is also essential for PSTVd replication. Loop 15 can adopt a Loop E structure, characterized by 5-6 consecutive non-Watson-Crick base pairs. This structure is also present in the 5S rRNA, mediating its binding to cellular proteins, such as TFIIIA or the ribosomal protein L5 (RPL5). PSTVd loop E interacts with RPL5 (Eiras et al., 2011), affecting its ability to regulate TFIIIA splicing and favoring the production of the 7ZF variant over the 9ZF, and thus benefiting its own transcription (Jiang et al., 2018). It remains to be resolved whether the processes described with PSTVd are general to other members of its family.

The multimeric strands of both polarities produced during replication have different sublocations. Those of - polarity remain anchored in the nucleoplasm, giving rise to more (+) multimers; those of + polarity are selectively transported to the nucleolus (Qi and Ding, 2003), an organelle in which several cell RNAs are processed. Therefore, the existence of some transport mechanism capable of discerning between

both polarities is expected. In this sense, it has been proposed that Loop E, which only occurs in PSTVd (+), is involved in this transport through its interaction with the RPL5 protein, that is related to the movement of ribosomal RNAs (Eiras et al., 2011).

The (+) oligomers are then cleaved to monomeric units. The cleavage takes place between the nucleotides G96 and G97 in the upper strand of the CCR in PSTVd, and in equivalent sites in other viroid species, always between two G. In the currently accepted model, the sequence of the upper strand of the CCR, together with a short flanking inverted repeat, form a stem-loop structure with a central CG-rich region and a terminal YCGR tetraloop (hairpin I). Two consecutive hairpins in (+) oligomers interact via kissing loops to form a quasi-double-stranded structure that is recognized and processed by a type III RNase, which cleaves at the hairpin loops (now a dsRNA region) of the two units at once, releasing a monomeric linear unit of the concatemer. Although the enzyme responsible for this cleavage is formally unknown, current evidence points to the involvement of a host RNase III, since these act on dsRNA and the viroid cleavage generates RNA termini expected for these enzymes: 2 nt overhang 3' ends with 5′-phosphomonoester and 3′-hydroxyl termini groups (Gas et al., 2007, 2008). Upon cleavage, the monomers likely rearrange into rod-like structure, stabilizing the new 3' and 5' ends by base pairing with the lower strand, while loop 15 acquires the abovementioned E-loop structure (Gas et al., 2007). The host DNA ligase 1, whose usual substrate is DNA and consumes ATP, recognizes and ligates the 5'-phosphomonoester and 3'-hydroxyl ends of the linear replicative intermediate, both *in vitro* with recombinant enzyme produced in *E. coli*, and *in vivo*, as suggested by silencing assays (Nohales et al., 2012a). However, the details that mediate the recognition of the replicative intermediate by the enzyme are currently unknown.

On the other hand, the viroids of the family *Avsunviroidae* are the only infectious agents able to enter the chloroplast, where they replicate in the thylakoid membrane

(Bonfiglioli et al., 1994; Daròs et al., 2006), although the specific trafficking mechanism of these pathogenic RNAs to the chloroplast is unknown. Viroid import seems to be mediated by a viroid localization signal, either sequences or specific structural motifs, which in ELVd have been mapped in the left terminal region (nucleotides 52 to 150) (Gómez and Pallás, 2010). Nuclear-expressed transcripts containing these sequences are efficiently transported to chloroplasts, leading these authors to hypothesize an initial step of the viroid infection in which the ELVd is transported from the cytoplasm to the nucleus prior to being exported to the chloroplast. It has been shown that a region of ELVd (nt 16-182) can effectively mediate transcript import into the nucleus; interestingly this region is partially overlapping with that required for its import into the chloroplast (Gómez and Pallás, 2012). As for nuclear-replicating viroids, the translation of small peptides containing subcellular localization signals has been also proposed for the subcellular trafficking of the ELVd and other members of its family (Márquez-Molins et al., 2021). The cellular factors involved in the intracellular movements of the *Avsunviroidae* are however unknown.

It has been proposed that the nuclear-encoded chloroplastic RNA polymerase is the main host factor involved in the replication of these viroids (Navarro et al., 2000; Rodio et al., 2007). Less known are the mechanisms by which the enzyme is recruited, since the transcription start sites are not conserved between species. In ASBVd, this site is located at U121 and U119 in the (+) and (-) RNA, respectively, in the AU-rich right terminal loop of the predicted quasi-rod-like structure of both polarities (Navarro et al., 2000). In PLMVd, positions A50/C51 and A284/A286 have been determined as the transcriptional start for the (+) and (-) strands, respectively (Delgado et al., 2005; Motard et al., 2008), both located in short stems within conserved HHR motifs. Although it is speculated that in both, ASBVd and PLMVd, specific promoter sequences are necessary for polymerase recognition, it cannot be ruled out the involvement of structural motifs. Such is the case of the ELVd, in which these sites

(U138 and A48 for the (+) and (-) chains, respectively) are not related at the sequence level. Thus, it has been proposed that the polymerase (and/or accessory factors) hijack it is dependent on some common but unknown structural feature (López-Carrasco et al., 2016).

The linear concatemers of both polarities are processed by HHRs present in their sequences, generating viroid linear monomeric units without the need for host enzymes (Hutchins et al., 1986; Hernández and Flores, 1992; Navarro and Flores, 1997; Fadda et al., 2003; Zhang et al., 2014a). HHRs are small RNA domains with autocatalytic activity. First discovered in satellite RNA (Prody et al., 1986) and shortly thereafter in viroids (Hutchins et al., 1986), they are more widely distributed than initially anticipated, having been identified in all domains of life (Webb et al., 2009; de La Peña and García-Robles, 2010a, 2010b). Structurally, HHRs are composed of three stems (named Helix I, II and III), which may or may not be capped by terminal loops, all surrounding a set of 15 highly conserved nucleotides that mediate catalysis. The ribozymes of all family *Avsunviroidae* viroids are type III, as this stem houses the 5' and 3' ends of the HHR. Despite their name, derived from the original two-dimensional representations (Hutchins et al., 1986; Forster and Symons, 1987), ribozymes fold into a γ-shaped structure in which stems I and II establish with each other essential interactions for an efficient catalysis under physiological conditions (Pley et al., 1994; de la Peña et al., 2003; Khvorova et al., 2003a). These interactions are stabilized by divalent metal ions, usually Mg^{2+}; its additional involvement in catalysis is currently discussed (Murray et al., 1998; O'Rear et al., 2001; Roychowdhury-Saha and Burke, 2007; Lee et al., 2008; Mir and Golden, 2016). In any case, HHRs induce cleavage in RNA through a transesterification reaction that converts a 5',3'-phosphodiester bond into a cyclic 2',3'-phosphodiester, also generating a 5'-hydroxyl end. The process is potentially reversible, although the efficiency of the reverse reaction in viroid ribozymes is highly variable and generally low (Canny et al., 2007; Przybilski and Hammann,

2007). Furthermore, the ASBVd (+) RNA interacts with two chloroplast RNA-binding proteins, PARBP33 and PARBP35, usually involved in stabilization, maturation and editing of chloroplast transcripts (Daròs and Flores, 2002). PARBP33 acts as an RNA chaperone for the viroid, facilitating self-cleavage of viroid oligomers *in vitro* and possibly *in vivo*.

After the cleavage, the monomers are efficiently circularized by the chloroplastic isoform of the host tRNA ligase, at least in the ELVd, as has been shown both *in vitro* and *in vivo* (Nohales et al., 2012b). The main function of this enzyme is to ligate the 5'-hydroxyl and 2',3'-cyclic phosphodiester ends generated with the excision of introns in pre-tRNAs during the tRNA maturation process (Englert et al., 2007). Its function in viroid processing can be replaced by an enzymatic activity of the unicellular green algae *Chlamydomonas reinhardtii* (Molina-Serrano et al., 2007; Martínez et al., 2009), while eggplant tRNA ligase can process the other members of the family *in vitro* (Nohales et al., 2012b). Thus, suggesting both the involvement of this kind of enzymes in the processing of all the *Avsunviroidae* and a conserved mechanism of enzymatic recruitment and processing among them. The exact nature of the viroid-enzyme interaction is unknown, although the quasi-rod-like structure in the central part of the ELVd (containing the ligation site in an internal loop) appears to be necessary for ligation (Martínez et al., 2009; Nohales et al., 2012b). Other domains outside this region, however, appear to be not necessary for the circularization (Daròs et al., 2014, 2018).

2.4. Movement of viroids within the plant

The viroid progeny must leave the organelle where replication occurs to colonize the rest of the plant, developing a systemic infection. Viroid spread occurs proximally between cells symplastically connected by plasmodesmata (Ding et al., 1997) and

through the phloem in long-distance transport (Palukaitis, 1987; Zhu et al., 2001) (**Figure 9**). However, exceptions to this have been described, such as some citrus viroids in which movement through phloem is restricted (Bani-Hashemian et al., 2015). Together with the existence of mutations that specifically affect systemic infection (Zhong et al., 2007, 2008), this suggests that the movement depends on interactions with host's cell components.

Several host factors have been proposed to be involved in viroid movement, such as the chaperone-type cucumber phloem protein 2 (CsPP2), which is the most abundant component of cucumber phloem exudate. It forms a ribonucleoprotein complex with the hop stunt viroid (HSVd) *in vitro* (Gómez and Pallás, 2001), and spread the infection through intergeneric grafts, suggesting its contribution to long-distance phloem trafficking of HSVd (Gómez and Pallás, 2004). The same group described two additional phloem proteins translocatable through intergeneric grafting (a phloem-specific lectin and an unidentified 14 kDa protein) able to bind ASBVd, suggesting that similar mechanisms could govern the expansion of chloroplast viroids (Gómez et al., 2005). On the other hand, silencing an *Arabidopsis thaliana* phloem protein of unknown function (At-4/1) seems to enhance PSTVd transport to young developing leaves (Solovyev et al., 2013; Morozov et al., 2014) evidencing its possible role in the vascular movement of the viroid, although how it does so is unknown. Adkar-Purushothama et al. (2015a) proposed the role of small RNAs derived from loops 7 and 8 of PSTVd in movement regulation by silencing CalS11 and CalS12, callose synthases that regulate plasmodesmata function by reducing the transit space through callose deposition. Callose-mediated plasmodesmata size exclusion limit has already been related to viral expansion (Iglesias and Meins, 2000; Bucher et al., 2001; Li et al., 2012). Whether movement through plasmodesmata occurs as free RNAs or associated with plant proteins remains a mystery.

In addition to these host factors, several PSTVd RNA motifs have been related to its movement, being common for specific motifs to mediate transport from or to specific areas of the plant, possibly by interacting with different factors (Qi et al., 2004; Zhong et al., 2007, 2008; Takeda et al., 2011; Wu et al., 2020; Ma and Wang, 2022).

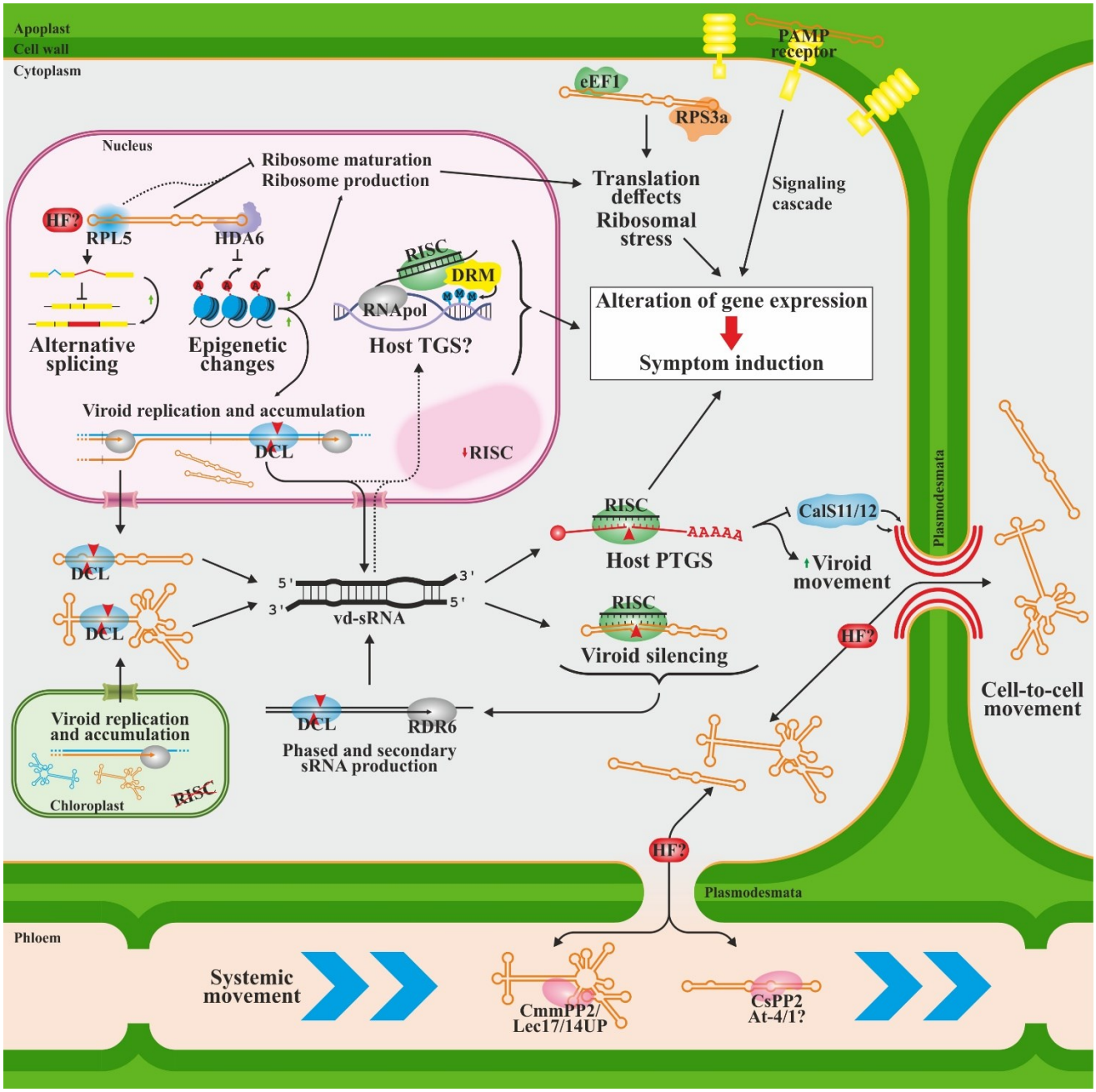


Figure 9. Proposed mechanisms of host defense responses, viroid pathogenesis and intercellular movement. Plant RNAi response is responsible of much of the viroid symptoms. dsRNA replicative intermediates and the cytoplasmic passage of viroids triggers the production of vd-sRNAs in plant cells. vd-sRNA-loaded RISC targets viroids and inhibit the expression of host genes

containing complementary sequences, post-transcriptionally by mRNA degradation and translation inhibition, and possibly transcriptionally via RNA-directed DNA methylation. RDRs may transform sRNA fragments into additional DCL and RISC substrates. Viroid may also be recognized by cell membrane PAMP receptors and stimulates plant innate immunity, resulting in the alteration of host gene expression. Additional interactions with proteins and host factors are responsible of global epigenetic changes, alternative splicing and interference with translational machinery, thus are also involved in the development of symptoms. Viroids use plasmodesmata for proximal movement and phloem for systemical transport, likely interacting with specific (and in some cases unknown) host factors. RNAi silencing of plant pathogen infection response genes can increase intercellular movement. PAMP, pathogen-associated molecular pattern; DRM, domains rearranged methylase; RNApol, RNA polymerase; Cmm14UP, *Cucumis melo* uncharacterized protein of 14 kDa; NF?, unknown host factor.

2.5. Host defense and pathogenesis

During their infectious cycle, viroids must be able to interact with various host factors while overcoming the plant defensive strategies to stop pathogenic infection (**Figure 9**). The dependence of viroids on cellular factors to complete its biological cycle make likely that hijack of host resources is direct and main cause of the phenotypic effects of the infection. However, additional causes for symptom development may explain the lack of linearity between titer and symptoms (especially considering the existence of latent viroids that despite being asymptomatic reach significant concentrations in the infected tissue) as well as the effect of specific nucleotide changes able to transform mild strains into aggressive strains.

The almost dsRNA structure of viroids of both polarities, and potentially its dsRNA replication intermediates, make them ideal for the generation of RNAi responses. Several studies have detected viroid-derived small RNAs (vd-sRNAs) of both polarities in infected plants, first in PSTVd (Papaefthimiou et al., 2001; Itaya et al., 2007a) and later in multiple viroid species of both families (Martínez de Alba et al., 2002; Markarian et al., 2004; Navarro et al., 2009; Martinez et al., 2010). Viroids are not only origin of vd-sRNAs but also seem to be substrates for degradation via host RNAi defense. The viroid titer is reduced, and the onset of infection symptoms delayed

by overexpression of AGO proteins (Minoia et al., 2014) or by the experimental introduction of vd-sRNA (Carbonell et al., 2008; Schwind et al., 2009; Adkar-Purushothama et al., 2015b; Carbonell and Daròs, 2017). Viroid overaccumulation is achieved by silencing RNA-dependent RNA polymerases (RDR) 1 and 6, responsible for generating secondary siRNAs (Gómez and Pallás, 2007; di Serio et al., 2010; Li et al., 2021), as well as in co-infections with viruses that express silencing suppressors or through the ectopic expression of these suppressors (Serra et al., 2014). Similarly, salicylic and gentisic acids appear to enhance the resistance against CEVd in tomato plants by inducing factors that mediate RNAi silencing (Campos et al., 2014). Despite these evidences, a moderate resistance of mature viroids against RNAi-mediated degradation has also been described. Unlike plant viruses, viroids do not express silencing suppressor. This resistance thus must reside in the viroid compact secondary structure, its association with proteins that prevent their recognition by the RNAi machinery and/or the fact that viroids of both families replicate in organelles where RISC is not as active (Wang et al., 2004; Gómez and Pallás, 2007; Itaya et al., 2007b; Dalakouras et al., 2015), particularly in the chloroplast, in which the RNAi machinery has not been detected (Bally et al., 2018). It is assumed that chloroplastic viroids produce vd-sRNAs during transit through the cytoplasm before reaching the organelle (Flores et al., 2005).

An important part of viroid pathogenicity derives from the generated vd-sRNAs that can be directed against host mRNAs and trigger the induction of disease symptoms. This hypothesis, which was raised on a theoretical framework by the group of Dr. Peter Waterhouse (Wang et al., 2004), was initially demonstrated with the cucumber mosaic virus Y RNA satellite (Shimura et al., 2011), and later in a PLMVd variant that induces extreme leaf chlorosis or peach calico (PC) (Navarro et al., 2012). This conspicuous symptom only occurs if the viroid sequence variant contains the insertion of a specific 12-13 nt hairpin (Malfitano et al., 2003). Two vd-sRNAs derived

from the PC-associated insert are homologous to the mRNA encoding the chloroplastic heat shock protein 90 (cHSP90). Thus, vd-sRNAs induce mRNA degradation and promote chloroplasts destabilization, leading to PC symptoms (Navarro et al., 2012). Similar observations of vd-sRNA involvement in downregulation of host genes have been reported in various viroids (Adkar-Purushothama et al., 2017, 2018; Aviña-Padilla et al., 2018; Thibaut and Claude, 2018; Bao et al., 2019). Secondary, trans-acting, phased vd-sRNAs has also been proposed, thus expanding the repertoire of silencing targets (Gómez et al., 2009). Interestingly, the distribution of vd-sRNAs is not uniform throughout the viroid RNA, but rather vd-sRNAs are concentrated in specific regions of the RNA molecules of both families, many of which had been previously described as pathogenicity determinants (Itaya et al., 2007a; di Serio et al., 2009; Navarro et al., 2009; Martinez et al., 2010; Adkar-Purushothama et al., 2015b; Jiang et al., 2019). It is likely that the secondary structures of these regions are more susceptible to being processed by the RNAi machinery (Martínez de Alba et al., 2002; Adkar-Purushothama et al., 2015b; Jiang et al., 2019).

PSTVd and CEVd also induce the expression of genes of the RNA-dependent DNA methylation (RdDM) pathway in tomato (Torchetti et al., 2016; Thibaut and Claude, 2018), and members of both families can induce transcriptional silencing (TSG) by methylating its own transgene (Wassenegger et al., 1994; Vogt et al., 2004; Dalakouras et al., 2015, 2016a; Wassenegger and Dalakouras, 2021). In addition, trans-methylation of the partial sequence of PSTVd has been achieved experimentally after infection with TASVd, with which it shares some sequence homology (Dalakouras et al., 2016a), and methylation of some promoters of endogenous genes has been described after PSTVd infection (Lv et al., 2016; Thibaut and Claude, 2018). However, the molecular basis of host gene methylation and the functional impact for both the plant and the viroid need to be clarified (Gómez et al., 2022). Direct interactions have been described between HSVd and histone deacetylase 6 (HDA6), reducing its activity and

promoting epigenetic alterations (Castellano et al., 2016b). It has been hypothesized that this interaction favors the spurious recognition of the viroid as RNA to replicate and has been related to the hypomethylation of the 5S rRNA gene and transposable elements, increasing its transcription (Martinez et al., 2014; Castellano et al., 2015, 2016a). In this sense, transcriptomic studies have shown extensive changes in gene expression as a result of nuclear (Zheng et al., 2017; Thibaut and Claude, 2018; Štajner et al., 2019) and, to a lesser extent, chloroplastic viroid infection (Herranz et al., 2013). Zheng et al. (2017) also observed other global effects with PSTVd infection, such as deregulation of lncRNAs, alteration of miRNA and phasiRNA function, and changes in the splicing pattern of coding transcripts. In this regard, the PSTVd interacts with at least one splicing factor, RPL5, interfering with its function (Eiras et al., 2011; Jiang et al., 2018). However, it is unknown whether this interaction can induce the described effects or whether interactions with other regulators are required instead.

On the other hand, affecting the translational machinery seems to be an important mechanism of viroid infection. In addition to the transcriptional reactivation of rRNA genes and the PSTVd-RPL5 interaction, it has been described that members of the *Pospiviroid* family and/or derived RNAs interfere with the activity of the eukaryotic elongation factor 1 (Lisón et al., 2013), the maturation of the 18S subunit (Cottilli et al., 2019), repress the ribosomal protein S3a (Adkar-Purushothama et al., 2017) and induce the ribosomal stress response (Prol et al., 2021).

Finally, it is speculated that, as occurs with viral dsRNAs (Niehl et al., 2016), the almost dsRNA structure of viroids or its replicative intermediates are recognized as pathogenic molecular patterns by the plant immune system. The induction of several proteins related to this process during viroid infection has been described (Thibaut and Claude, 2018; Prol et al., 2021) and thus, immune response could be partly responsible of viroid symptomatology. In this sense, it has been proposed as for viral genomes that

post-transcriptional modifications on viroid RNA might prevent its detection by host immunity mechanisms (Márquez-Molins et al., 2022b). Given the dynamic complexity of the host-viroid interaction during the infection process, a recent study by Márquez-Molins et al. (2022c) provides an overall vision that gives temporal relativity to many of the abovementioned host changes.

2.6. Host range and symptoms

Viroids infect dicotyledon plants, except CCCVd and CTiVd which infect monocots. Some viroids, such as HSVd and PSTVd, have a wide host range, while others, such as CbVd1-6 and those of the family *Avsunviroidae*, are mainly restricted to their natural hosts (Flores et al., 2020). Generally, members of the family *Pospiviroidae* produce late, nonspecific, systemic symptoms. Those attributed to PLMVd and other members of the family *Avsunviroidae* are, on the contrary, earlier, specific and local (Flores et al., 2020). Pathogenicity depends on the genomes of both the viroid and the host plant, as well as the environmental conditions. Viroids cover a wide range of symptoms (**Figure 10**), from asymptomatic infections to those that induce plant death, and generally are similar to those induced by viruses. At the macroscopic level, they induce epinasty and chlorosis of the leaves, deformation in flowers, fruits and reserve organs, stem and bark cracking, growth retardation, dwarfism, etc. At the subcellular level, they induce malformations of cell walls and chloroplasts, formation of plasmalemmasomes and electron-dense deposits in the cytoplasm and chloroplasts (Flores et al., 2005).

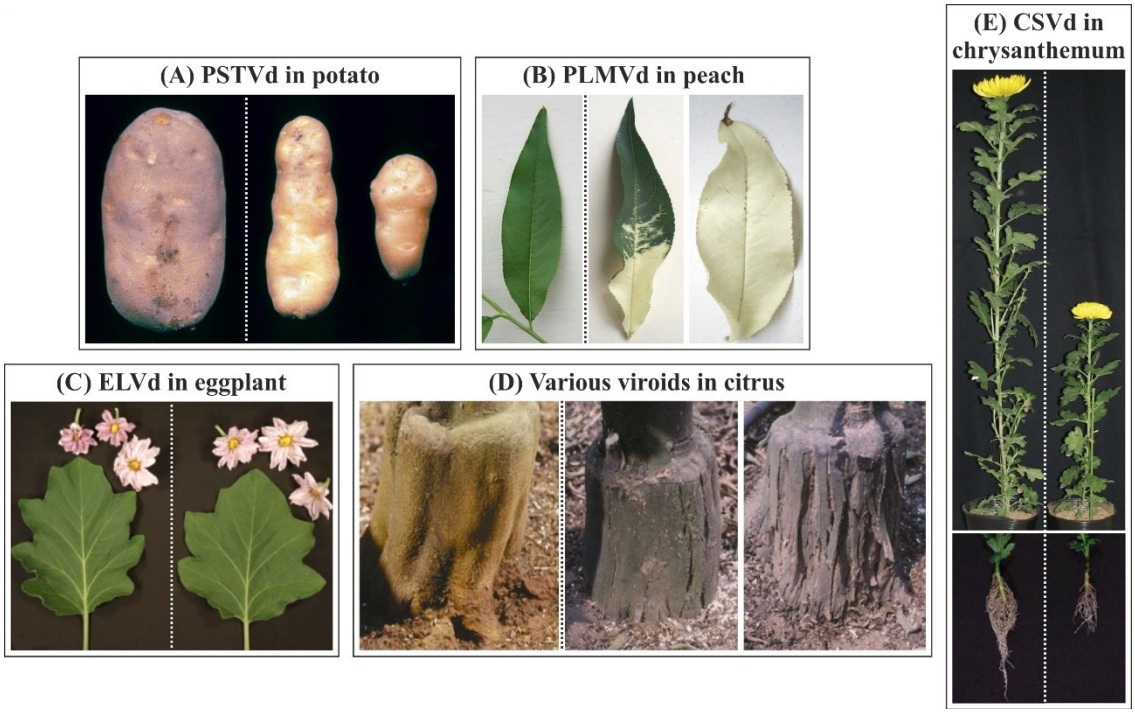


Figure 10. Typical symptoms of viroid infection in various crops. In all cases, mock-inoculated or symptomless plants are in the left and viroid-infected plants are in the center and/or right. (A) PSTVd induces potato tuber malformations. (B) Variants of PLMVd induce partial and complete peach calico (center and right, respectively) in peach leaves. (C) Symptomless infections induced by ELVd in eggplant (cv. Black Beauty). (D) Co-inoculation of citrus trees with CEVd and CVd-IV induces severe bark scaling (center), while co-inoculation with CEVd and CVd-IV induces bark cracking (right). (E) CSVd induces reduction of plant height and flower size in chrysanthemum; the root development of both healthy and inoculated plants is shown in the bottom panels. Adapted from Daròs, 2016, Navarro et al. 2012, Matsushita, 2013, Vernière et al. 2006.

2.7. Transmission between plants and control strategies

Several strategies are used by viroids for its dissemination, some of which are facilitated by modern agricultural practices (**Figure 11**). The most effective strategy is the vegetative propagation of viroids through bulbs, tubers, rhizomes, grafts, etc. (Ito et al., 1993; Kim et al., 2006), followed by mechanical transmission, especially during manipulations that involve mechanical damage to the plants, such as pruning and harvesting, and allow direct transmission by plant-to-plant contact or the use of contaminated agricultural machinery (Ito et al., 1993; Fadda et al., 2003; Kim et al., 2006). Much lower efficiency has been described for seed transmission of several viroids

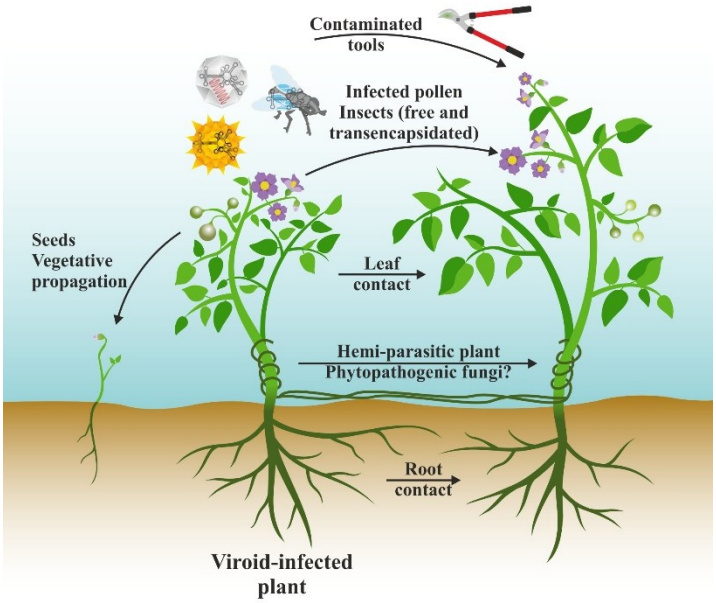


Figure 11. Proposed mechanisms of viroid transmission between plants.

(Kryczyński et al., 1988; Fadda et al., 2003; Kim et al., 2006; Antignus et al., 2007), as well as for infected pollen (Desjardins et al., 1979; Kryczyński et al., 1988). Spatial analyses of infection spread suggest viroid transmission through roots, which has been proven under experimental conditions (Schnell et al., 2011; Fukuta et al., 2012; Mehle et al., 2014). The spread of viroid-like RNAs through parasitic plants and phytopathogenic fungi has also been proposed (Vachev et al., 2010; Wei et al., 2019; Leichtfried et al., 2020). Insects are potential vectors of transmission, possibly by spreading infected pollen (Antignus et al., 2007) , and certain insect species could mediate the direct transmission of viroids of both families between plants (Schumann et al., 1980; Desvignes, 1986; Leichtfried et al., 2020), although the efficiency of these transmissions seems to be very low and unimportant from an epidemiological point of view. It has been described that the efficiency improves with the transencapsidation of the viroid RNA with an insect-transmitted plant virus, probably given the adaptation of the virus to its vector and its ability to efficiently infect plant cells (Syller et al., 1997).

Effective commercial methods for the control of viroid infections are currently lacking, relying only on good agronomic practices to prevent, detect and eradicate the infection. Additionally, several strategies have been proposed for the control of viroids, such as genetic improvement of resistant varieties (Matsushita et al., 2012; Naoi and Hataya, 2021), cross-protection with latent viroids (Niblett et al., 1978; de La Peña and Flores, 2002), or the generation of resistant transgenic plants, including RNAi-based strategies (Carbonell et al., 2008; Carbonell and Daròs, 2017; Carbonell, 2022).

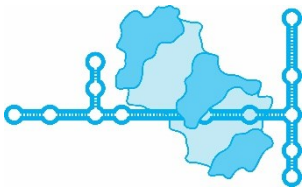
Objectives

In the current and near future context influenced by the need of considerably increasing agricultural production with methods that involve minimal impact on health and environment, biotechnology-based strategies are essential to address problems such as the effect on agricultural yield caused by pathogens and plant pests. On the one hand, it is still essential to deepen into the mechanisms of the viroid-plant interactions related with replication, transport, immune response evasion, symptomatology and transmission, in order to develop effective control strategies for these small pathogens. On the other hand, the proven efficiency of RNAi as a pesticidal strategy makes it necessary to develop efficient production systems of double-stranded RNAs (dsRNAs) that trigger pest gene silencing, among which stand out bacterial biofactories. In this Thesis, an eggplant latent viroid (ELVd)-based expression system in *Escherichia coli* has been used to address these two aspects. The specific objectives defined have been:

1. To study the effect of modifications in the native ligation site of ELVd of + polarity on the circularization activity catalyzed by the eggplant tRNA ligase to better understand the structural and sequence requirements that mediate the recognition between the enzyme and the viroids of the family *Avsunviroidae*.

2. To develop a system for the overproduction of recombinant dsRNAs in *E. coli* using ELVd as a scaffold, as well as making use of the intrinsic properties of an autocatalytic intron of *Tetrahymena thermophila*, both in its complete and permuted form.

3. To analyze the insecticidal activity of the dsRNAs produced against two economically important pests of crops, *Diabrotica virguifera virguifera* and *Ceratitis capitata*, by means of feeding assays with larvae or adults of these species.



Chapter I

Mutational analysis of *Eggplant latent viroid* RNA circularization by the eggplant tRNA ligase in *Escherichia coli*

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Author Contributions:

J-AD conceived the work and designed the experiments in close collaboration with TC and BO. TC and BO performed the experiments. TC, BO, and J-AD analyzed the data. J-AD wrote the manuscript with inputs from TC and BO. All authors read and approved the final manuscript.

Abstract

Eggplant latent viroid (ELVd) is a relatively small non-coding circular RNA that induces asymptomatic infections in eggplants (*Solanum melongena* L.). Like other viroid species that belong to the family *Avsunviroidae*, ELVd contains hammerhead ribozymes in the strands of both polarities that self-cleave RNAs producing terminal 5′-hydroxyl and 2′,3′-cyclic phosphodiester groups. Available experimental data indicate that ELVd replicates in the chloroplasts of infected cells through a symmetric rolling-circle mechanism, in which RNA circularization is catalyzed by the chloroplastic isoform of the tRNA ligase. In this work, a mutational analysis was performed to gain insight into the sequence and structural requirements of the tRNA ligase-mediated circularization of ELVd RNAs. In the predicted minimum free energy conformation of the monomeric linear ELVd RNA intermediate of plus (+) polarity, the ligation site is located in the lower part of an opened internal loop, which is present in a *quasi*-rod-like structure that occupies the center of the molecule. The mutations analyzed herein consisted of punctual nucleotide substitutions and deletions surrounding the ligation site on the upper and lower strands of the ELVd *quasi*-double-stranded structure. Computational predictions of the mutated ELVd conformations indicated different degrees of distortions compared to the minimum free energy conformation of the wild-type ELVd linear monomer of + polarity. When these mutant RNAs were expressed in *Escherichia coli*, they were all circularized by the eggplant tRNA ligase with approximately the same efficiency as the wild-type ELVd, except for those that directly affected the ribozyme domain. These results suggest that the viroid ribozyme domains, in addition to self-cleavage, are also involved in the tRNA ligase-mediated circularization of the monomeric linear replication intermediates.

Keywords

Eggplant latent viroid, *Avsunviroidae*, tRNA ligase, RNA ligation, circular RNA, hammerhead ribozyme.

Introduction

Viroids are a very unique type of plant infectious agents as they are exclusively constituted by a relatively small (246–401 nt) non-coding circular RNA. Despite the small size and with no help from self-encoded proteins, when these RNAs manage to enter compatible host cells they are able to move to appropriate cellular structures and to recruit the right host proteins to start their replication. Then the viroid progeny moves cell-to-cell through the plasmodesmata, the channels that traverse the cell walls of plant cells, and long-distance through the phloem to establish a systemic infection in the host plant frequently inducing a disease. The more than 30 viroid species known to date are classified into two families, *Pospiviroidae* and *Avsunviroidae*, depending on the presence of particular domains in the viroid molecule; more specifically a central conserved region (CCR) that is present in all members of the family *Pospiviroidae*, but is missing in those of the *Avsunviroidae*, and hammerhead ribozymes that are exclusive of this last family (Flores et al., 2015; Daròs, 2016b; Steger and Perreault, 2016; Brass et al., 2017; Giguère and Perreault, 2017).

Viroids replicate through an RNA-based rolling circle mechanism in which viroid circular RNAs serve as templates for reiterative transcription to produce multimeric RNAs of opposite polarity. Since viroids do not code for proteins, plus (+) polarity is arbitrarily assigned to the circular RNA strand that most abundantly accumulates in the host plant. Analyses of the replication mechanisms in different viroid species have indicated that members of both families follow two different variants of the rolling circle, termed asymmetric and symmetric (Branch and Robertson, 1984; Flores et al., 2008). *Potato spindle tuber viroid* (PSTVd), and possibly all members of the family *Pospiviroidae*, follow the asymmetric variant in which the minus (-) multimeric RNAs directly serve as templates for the transcription of multimeric + RNAs, which are then cleaved into monomeric linear viroid RNAs, and are finally circularized acquiring their mature form (Branch et al., 1988). In contrast, *Avocado sunblotch viroid* (ASBVd) and

members of the family *Avsunviroidae* follow the symmetric variant in which the multimeric - RNAs self-cleave after the transcription through embedded hammerhead ribozymes, and the resulting monomers are ligated into monomeric circular RNAs. Next these viroid molecules, which are distinctive of this family, serve as templates in a second (symmetric) rolling circle for the transcription of multimeric + RNAs, which once again, are processed into monomeric circular RNAs of + polarity (Hutchins et al., 1985; Daròs et al., 1994).

Potato spindle tuber viroid, and apparently its family members, replicate in the nucleus of infected cells (Diener, 1971; Spiesmacher et al., 1983), while ASBVd and the other members of its family replicate in chloroplasts (Bonfiglioli et al., 1994; Navarro et al., 1999). However, things could be more intricate as *Eggplant latent viroid* (ELVd; family *Avsunviroidae*) is able to traffic from the cytoplasm to the nucleus and from there to the chloroplasts (Gómez and Pallás, 2012). Viroid RNA transcription is mediated by host DNA-dependent RNA polymerase II and chloroplastic nuclear-encoded polymerase (NEP), respectively, in viroids of the families *Pospiviroidae* and *Avsunviroidae* (Mühlbach and Sänger, 1979; Navarro et al., 2000). While multimeric replication intermediates of both polarities self-cleave through hammerhead ribozymes in viroids of the family *Avsunviroidae* (Flores et al., 2001), a host type-III RNase has been proposed to cleave the multimeric + transcripts of members of the *Pospiviroidae* by acting on transient double-stranded structures (Gas et al., 2007, 2008). Finally, viroid RNA circularization is catalyzed by host DNA ligase 1 and the chloroplastic isoform of the tRNA ligase in members of the families *Pospiviroidae* and *Avsunviroidae*, respectively (Nohales et al., 2012a, b).

Eggplant latent viroid, the only species in the genus *Elaviroid* (family *Avsunviroidae*) (Fadda et al., 2003; Daròs, 2017), induces asymptomatic infections in eggplants (*Solanum melongena* L.) and has been recently suggested to be a friendly

experimental system to research many aspects of the *Avsunviroidae* molecular biology (Daròs, 2016a). Experiments done with this viroid have provided notable findings about hammerhead ribozyme activity (Carbonell et al., 2006), viroid replication (Nohales et al., 2012b; López-Carrasco et al., 2016), viroid movement (Gómez and Pallás, 2010, 2012) and viroid structure (Giguère et al., 2014; López-Carrasco et al., 2016). Regarding viroid circularization, a pioneering work showed that, despite not replicating in these cells, the dimeric ELVd transcripts expressed in the chloroplast of the unicellular green alga *Chlamydomonas reinhardtii* (phylum *Chlorophyta*) are efficiently processed to monomers and circularized (Molina-Serrano et al., 2007). Further work in *C. reinhardtii* chloroplasts showed that efficient ligation requires a *quasi*-double-stranded structure present in the central part of the molecule, which contains the ligation site in an internal loop (Martínez et al., 2009). A combination of *in vitro* and *in vivo* experiments indicated that the chloroplastic isoform of tRNA ligase (Englert et al., 2007) is the host enzyme involved in the circularization of ELVd and, most probably, of all the viroids in the family (Nohales et al., 2012b). *In vitro* experiments using a recombinant version of eggplant tRNA ligase produced in *Escherichia coli* have shown that, among several monomeric linear + ELVd RNAs, only those opened at the hammerhead ribozyme cleavage site are efficiently circularized (Nohales et al., 2012b). A circularization analysis in *E. coli* - ELVd does not replicate in these cells either - in which longer than unit ELVd + transcripts were co-expressed along with the eggplant tRNA ligase further supported that the domains of the viroid molecule outside the central *quasi*-double-stranded structure are dispensable for ligation (Daròs et al., 2014, 2018).

To gain further insight into the sequence and structural requirements of the tRNA ligase-mediated circularization of ELVd, we herein used the *E. coli* co-expression system to analyze the effect of the mutations surrounding the ligation site in this reaction. Surprisingly, all the assayed ELVd mutants were efficiently circularized by

the eggplant tRNA ligase in *E. coli*, provided they did not directly affect the ribozyme domain, which supports the notion that this RNA domain, in addition to viroid RNA cleavage, is also involved in the circularization step that is mediated by the tRNA ligase.

Materials and methods

Construction of series of plasmids to express ELVd mutants in *E. coli*

Plasmid pLELVd contains a longer-than-unit ELVd cDNA (from C327 to G46, GenBank accession number AJ536613; note that ELVd is circular and A333 is followed by G1), which included the repetition of the + -strand hammerhead ribozyme domain under the control of the *E. coli* murein lipoprotein promoter and the 5S rRNA (rrnC) terminator (Darós et al., 2014, 2018). To induce a series of nucleotide substitutions and deletions in ELVd cDNA, this plasmid was employed as a template in polymerase chain reactions (PCR), for which the Phusion High-Fidelity DNA polymerase (Thermo Scientific) and different pairs of divergent primers were used (**Table 1**). Most of these pairs of primers, which harbored the desired mutations, contained a recognition site for type-IIS restriction enzyme *Bpi*I at the 5′ ends. The PCR products of full plasmid size were eluted after separation by electrophoresis in 1% agarose gels. Next they were digested with *Bpi*I (Thermo Scientific) and subjected to ligation with T4 DNA ligase (Thermo Scientific). In those cases in which primers did not harbor *Bpi*I sites (**Table 1**), deletions simply resulted from ligation of phosphorylated (T4 polynucleotide kinase, Thermo Scientific) blunt end PCR products. In both cases, *E. coli* DH5α were electroporated with the products of the ligation reaction and the recombinant clones selected on plates with Luria-Bertani (LB) medium that included ampicillin. The plasmids that contained the desired mutations were selected after sequencing (3130xl Genetic Analyzer, Life Technologies).

Table 1. Primers used to mutagenize the ELVd cDNA.

Mutant	Sequence*
C197A	5′-GGCGGAAGACGCTTTC***A***GACGGTGGGTTCGTCGAC-3′
	5′-CCGCGAAGACCGGAAAGTGTGTACTTTCCCTG-3′
G198U	5′-GGCGGAAGACGCTTTCC***T***ACGGTGGGTTCGTCGAC-3′
	5′-CCGCGAAGACCGGAAAGTGTGTACTTTCCCTG-3′
C200G	5′-GGCGGAAGACGCTTTCCGA***G***GGTGGGTTCGTCGAC-3′
	5′-CCGCGAAGACCGGAAAGTGTGTACTTTCCCTG-3′
G201A	5′-GGCGGAAGACGCTTTCCGAC***A***GTGGGTTCGTCGAC-3′
	5′-CCGCGAAGACCGGAAAGTGTGTACTTTCCCTG-3′
ΔU194-G204	5′-GGCGGAAGACGCCACTGGTTCGTCGACACCTCTCCC-3′
	5′-CCGCGAAGACCGAGTGTGTACTTTCCCTGATG-3′
ΔC197-G201	5′-GGCGGAAGACGCTTTCGTGGGTTCGTCGACACCTC-3′
	5′-CCGCGAAGACCGGAAAGTGTGTACTTTCCCTG-3′
ΔG1-U7	5′-TATGGGGCAGCGTTACAAGT-3′
	5′-GTGTGCCACCCCTGATGAGAC-3′
ΔG38-U42	5′-GACCTTTCGGTCTCATCAGG-3′
	5′-GGGGTTTCGCCATGGGTCGG-3′
ΔU47-G56	5′-CCCCATTTCGACCTTTCGGTC-3′
	5′-GGTCGGGACTTTAAATTCGG-3′
ΔG317-U326	5′-CTCTATCTCTCCTGGAAGGC-3′
	5′-CCCCATAGGGTGGTGTGTGC-3′

*BpiI recognition and cleavage sites are on a gray background and underlined, respectively. The punctual nucleotide substitutions are in italics and bold.

ELVd and tRNA ligase co-expression in *E. coli*

Escherichia coli DH5α were co-electroporated with pLELVd (or derivatives with the different mutations) and p15LtRnlSm, a plasmid with a compatible p15A replication origin to express a recombinant version of the chloroplastic isoform of the eggplant tRNA ligase (GenBank accession no. JX0225157) under the control of the *E. coli* murein lipoprotein promoter and the rrnC terminator (Daròs et al., 2014, 2018). The recombinant clones that harbored both plasmids were selected on LB plates that contained ampicillin and chloramphenicol. To co-express the ELVd RNA and the tRNA ligase in *E. coli*, the colonies from these plates were grown in 2 ml of liquid LB medium that contained both antibiotics for 24 h at 37°C with vigorous shaking (225 revolutions per min). The K219A mutant of eggplant tRNA ligase was obtained by mutagenic

PCR on template p15LtRnlSm using primers 5′-CAAGTGTGACCTCGACTATAG-3′ and 5′-CGCATTCTGGATCTCTTTTTATG-3′, as previously described for ELVd mutants.

RNA purification from *E. coli*

Cells in the 2-ml cultures were harvested by centrifugation and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). To break cells, one volume (50 µl) of a 1:1 mixture of buffer saturated phenol (1 M Tris-HCl, pH 8.0) and chloroform was added and the mix was vigorously vortexed. The aqueous phase that contained the *E. coli* RNA was recovered after centrifugation and was frozen.

RNA electrophoresis

Aliquots of 10 µl of the RNA preparations were mixed with one volume of loading buffer that contained 98% formamide, and was denatured by heating for 1.5 min at 95°C, followed by snap cooling on ice. After denaturation, RNA was separated by electrophoresis in 5% polyacrylamide gels (37.5:1 acrylamyde:*N,N'*-methylenebisacrylamide) that contained 8 M urea in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). In some experiments, in order to further separate circular molecules, RNA preparations were subjected to a two-dimension electrophoresis. After separating the RNA as explained above, the whole lane was transversally laid on top of a second urea gel at a lower buffer concentration (0.25 × TBE) and electrophoresis continued.

Northern blot hybridization analysis

After electrophoresis, RNAs were electroblotted to positively charged nylon membranes (Nytran SPC, Whatman) and were cross-linked by irradiation with 1.2 J/cm^2 UV light

(254 nm, Vilber Lourmat). Hybridization was performed overnight at 70°C in 50% formamide, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 100 ng/ml salmon sperm DNA, 1% sodium dodecyl sulfate (SDS), 0.75 M NaCl, 75 mM sodium citrate, pH 7.0, with approximately 1 million counts per minute of a ^{32}P-labeled ELVd probe of - polarity. The hybridized membranes were washed three times for 10 min with 2 × SSC, 0.1% SDS at room temperature and once for 15 min at 55°C with 0.1 × SSC, 0.1% SDS (the SSC buffer is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Hybridization signals were recorded by autoradiography using X-ray films (Fujifilm). The radioactive RNA probe consisted of a full-length ELVd RNA monomer of complementary polarity. This probe was obtained by *in vitro* transcription of a linearized plasmid with 20 U of T3 bacteriophage RNA polymerase (Roche) in 20-µl reactions that contained 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 20 mM DTT, 2 mM spermidine, 0.5 mM each of ATP, CTP and GTP, and 50 µCi of [α-^{32}P]UTP (800 Ci/mmol), 20 U RNase inhibitor (RiboLock, Thermo Scientific) and 0.1 U yeast inorganic pyrophosphatase (Thermo Scientific). Reactions were incubated for 1 h at 37°C. After transcription, the DNA template was digested with 20 U DNase I (Thermo Scientific) for 10 min at 37°C. The probe was purified by chromatography in a Sephadex G-50 column (mini Quick Spin DNA Columns, Roche).

Prediction of RNA secondary structures

The Mfold algorithm (Zuker, 2003) was used to predict the minimum free energy conformation of the different monomeric linear ELVd RNAs using the default parameters[1].

[1]http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form

Results

In the predicted conformation of minimum free energy of the monomeric linear + ELVd RNA (sequence variant AJ536613), the ligation site (positions 333-1) lays on the border of an internal opened loop in a long *quasi*-double-stranded structure that is present in the central part of the molecule (**Figure 1**). In this region, this predicted conformation mostly agrees on those of two independent experimental determinations of circular ELVd + RNAs obtained by high-throughput selective 2′-hydroxyl acylation analyzed by primer extension (hSHAPE) (Giguère et al., 2014; López-Carrasco et al., 2016). To study in-depth the sequence and structural requirements of the ELVd RNA circularization by the eggplant tRNA ligase, we used an *E. coli*-based co-expression system to analyze the effect of mutations on this loop. To avoid interference with the hammerhead ribozyme-mediated cleavage of the expressed ELVd longer-than-unit transcripts (a processing step prior to circularization), the first set of mutations were placed on the upper strand of the loop.

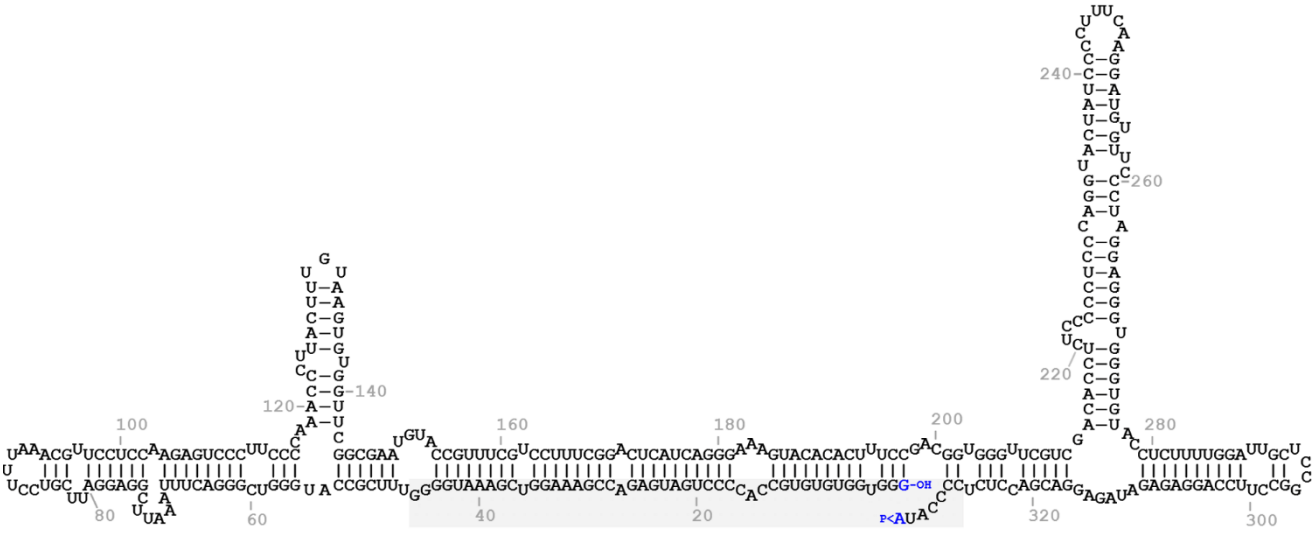

Figure 1. Predicted minimum free energy conformation of the monomeric linear ELVd replication intermediate of + polarity. The terminal nucleotides are highlighted in blue. The 5′-hydroxyl and 2′,3′-phosphodiester groups of the terminal nucleotides are indicated by -OH and >P, respectively. The domain of the + hammerhead ribozyme is on a gray background.

Effect on circularization of punctual substitutions that change the conformation of the loop that contains the ELVd ligation site

By means of PCR with mutagenic primers, we created four different nucleotide substitutions on the upper strand of the loop where the ligation site is located on the ELVd RNA of + polarity. These four mutations were designed to alter the secondary structure around the ligation site. According to the Mfold prediction of minimum free energy conformations, substitution C197A opens the loop on the left-hand side, while G198U does not substantially change the structure (**Figure 2**). Substitution C200G closes the loop on the right-hand side, whereas G201A causes a strong restructuring of the whole region (**Figure 2**). The longer-than-unit ELVd transcripts that contained these mutations where co-expressed in *E. coli* along with the eggplant tRNA ligase.

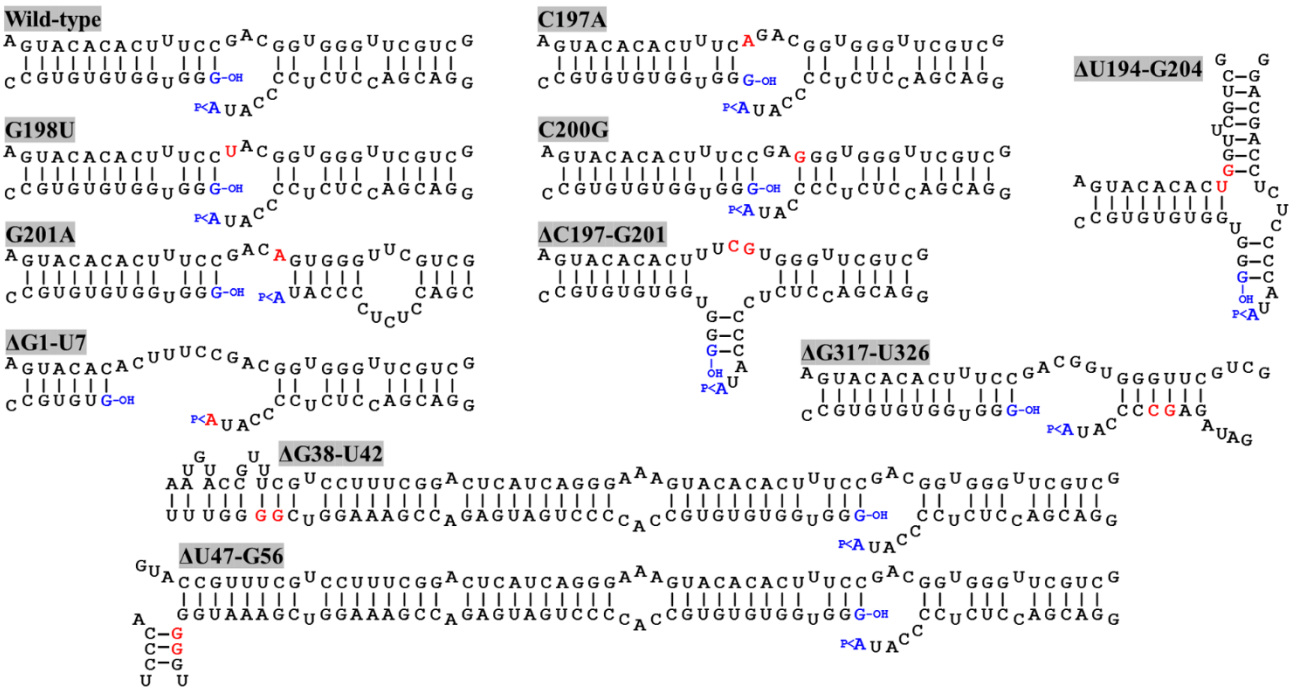

Figure 2. Predicted minimum free energy conformations of the regions around the ligation site of wild-type ELVd and different mutants. C197A, G198U, C200G, and G201A are punctual nucleotide substitution mutants, and ΔU194-G204, ΔC197-G201, ΔG1-U7, ΔG38-U42, ΔU47-G56, and ΔG317-U326 are deletions mutants. Nucleotide positions refer to ELVd sequence variant AJ536613. Mutated nucleotides and nucleotides on the border of deletions are highlighted in red. The terminal nucleotides ligated during circularization are in blue. The 5′-hydroxyl and 2′,3′-phosphodiester groups of the terminal nucleotides are indicated by –OH and >P, respectively.

Total RNA was extracted from *E. coli* cells and separated by denaturing PAGE. Finally, ELVd + strands were detected by Northern blot hybridization using a complementary radioactive probe. Two prominent bands that apparently corresponded to the monomeric linear and monomeric circular ELVd RNAs of + polarity were detected in the lanes that corresponded to all four mutants (**Figure 3A**, lanes 1–4). The intensity of these bands did not differ from those in the wild-type ELVd control (**Figure 3A**, lane 6). The controls of this experiment also included the single expressions of the wild-type ELVd or the tRNA ligase. In these controls, *E. coli* cells were co-transformed with the corresponding empty expression plasmids. Only faint bands were detected when the tRNA ligase was not expressed (**Figure 3A**, lane 5),

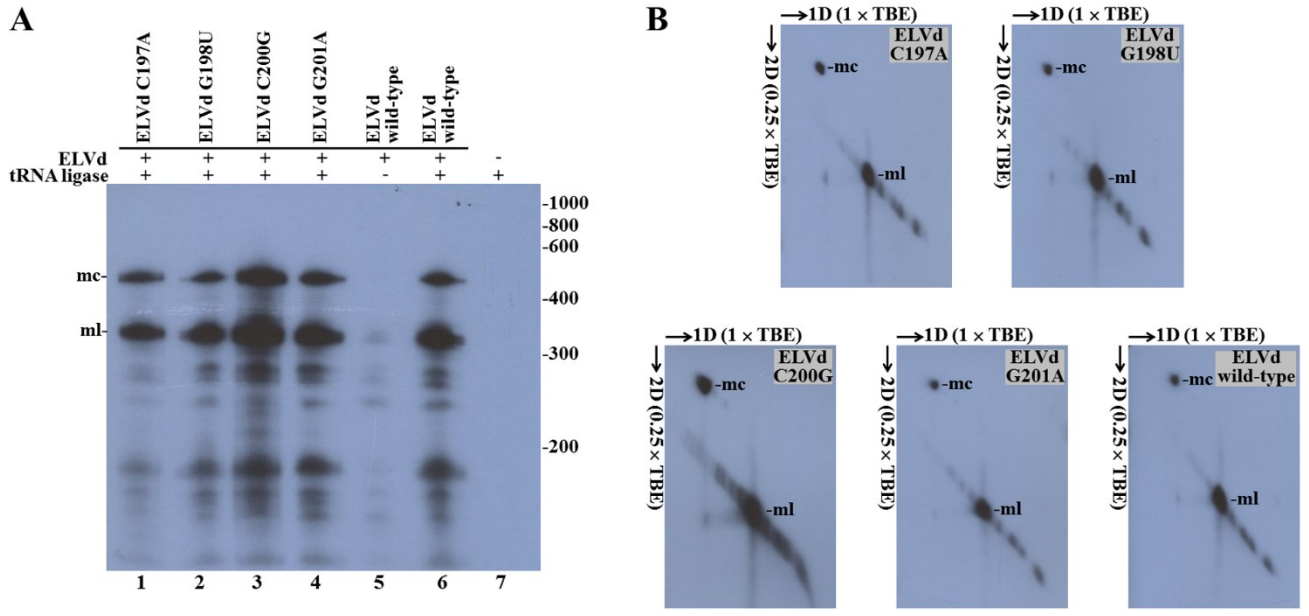

Figure 3. Eggplant tRNA ligase-mediated circularization of ELVd punctual substitution mutants in *Escherichia coli*. Total RNA from *E. coli* clones, in which longer-than-unit ELVd transcripts and tRNA ligase were co-expressed, were separated by (A) single denaturing PAGE in an 8 M urea, (×1) TBE gel or (B) two-dimension PAGE, first in an 8 M urea, (×1) TBE gel and second in an 8 M urea, (×0.25) TBE gel. ELVd + strands were detected by Northern blot analysis. (A) Lanes 1 to 4, E. coli clones in which ELVd mutants C197A, G198U, C200G and G201A were co-expressed with eggplant tRNA ligase; lanes 5 and 6, control clones in which wild-type ELVd was expressed alone or co-expressed with tRNA ligase, respectively; lane 7, control clone in which only tRNA ligase was expressed. The positions and sizes (in nt) of RNA markers are indicated on the right (B) The migration directions of RNA in both dimensions are indicated by arrows. (A,B) The positions of the monomeric circular (mc) and linear (ml) forms of ELVd are indicated.

and no band was observed when ELVd RNA was not expressed (**Figure 3A**, lane 7). As previously shown, ELVd RNAs only accumulate efficiently in *E. coli* cells in the presence of eggplant tRNA ligase (Darós et al., 2018). The same results were obtained in two additional replicates done of the whole experiment, in which independent *E. coli* clones were analyzed.

Of the two prominent bands, the lower (ml in **Figure 3A**) very precisely matches the expected position of the ELVd linear monomers of 333 nt, while the upper one (mc in **Figure 3A**) corresponds to a species that displays the electrophoretic behavior expected for the ELVd circular monomers, which must be delayed from the linear counterparts under denaturing conditions. To ensure that the upper bands corresponded to the ELVd circular monomers, equivalent aliquots of the above samples were separated by two-dimension denaturing PAGE at two different ionic strengths and the ELVd + strands were detected by Northern blot hybridization. As expected for circular RNA molecules, the slow migrating species further delayed in the second dimension at a low ionic strength, and deviated from the diagonal of the linear RNAs (**Figure 3B**). Taken together, these results indicate that the four assayed punctual nucleotide substitutions, which induced different alterations in the secondary structure of the loop on which the + ELVd ligation site is located have no effect on the eggplant tRNA ligase-mediated circularization.

Effect on the circularization of deletions on the upper strand of the *quasi*-double-stranded domain that contains the ELVd ligation site

We reasoned that the assayed punctual nucleotide substitutions may not have sufficiently altered the secondary structure of the loop that contains the ligation site to affect circularization. To test this hypothesis, two additional ELVd mutants were created, which consisted in the deletion of five (from position C197 to G201) or eleven

(from position U194 to G204) nucleotides of the upper RNA strand. The Mfold prediction of the minimum free energy conformation of these deleted monomeric linear ELVd + RNA forms showed a profound alteration of the secondary structures around the ligation site in these two cases (**Figure 2**). The ELVd longer-than-unit transcripts that contained these deletions were co-expressed with eggplant tRNA ligase in *E. coli* and total RNA was extracted. RNA was separated by denaturing PAGE and the + ELVd strands were revealed by Northern blot hybridization. Two prominent bands, whose position was consistent with the migration of the monomeric linear and circular ELVd RNAs, were once again detected (**Figure 4A**, lanes 1 and 2). Like the above experiment, the intensity of these bands did not substantially differ from those in the control in which the wild-type ELVd was expressed (**Figure 4A**, lane 4). The same results were observed in the analysis of two additional independent *E. coli* clones of each mutant. These results indicate that the two assayed deletions, despite the strong impact on the secondary structure of the ligation domain, have no effect on the efficiency of the tRNA ligase-mediated circularization of ELVd.

Effect on the circularization of deletions on the lower strand of the *quasi*-double-stranded domain that contains the ELVd ligation site

Since punctual mutations and deletions on the upper strand of the *quasi*-double-stranded domain that contains the ligation site did not affect ELVd circularization, we assayed four deletions on the lower strand. Note that this strand contains the viroid + hammerhead ribozyme domain (**Figure 1**) and some of these deletions were expected to have a deleterious effect on the self-cleavage of the ELVd + RNA precursor. The two deletions that directly affected the ribozyme domain were the 7-nt deletion from G1 to U7 that completely eliminated the upper strand of the hammerhead helix I and the 5-nt deletion from G38 to U42 that eliminated a key hammerhead conserved motif. The 10-nt deletions from U47 to G56 and from G317 to U326 were created in both

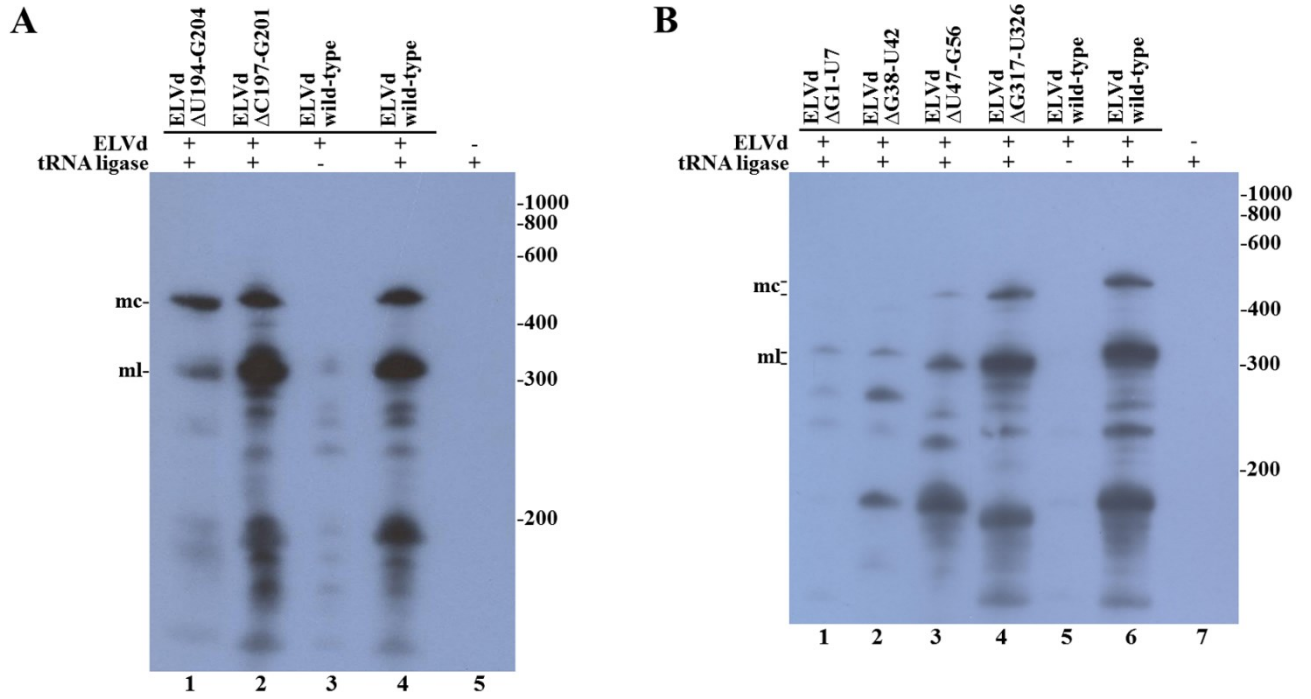

Figure 4. Circularization of ELVd deletions mutants by eggplant tRNA ligase in *E. coli*. Total RNA from *E. coli* clones, in which longer-than-unit ELVd transcripts and tRNA ligase were co-expressed, were separated by denaturing PAGE and transferred to a membrane for Northern blot hybridization of ELVd + strands. (A) Lanes 1 and 2, *E. coli* clones in which ELVd deletion mutants ΔU194-G204 and ΔC197-G201 were co-expressed with eggplant tRNA ligase; lanes 3 to 5, control *E. coli* clones in which wild-type ELVd, but not tRNA ligase, wild-type ELVd and tRNA ligase, and tRNA ligase, but not ELVd RNA, were, respectively, expressed as indicated. (B) Lanes 1 to 4, *E. coli* clones in which ELVd deletion mutants ΔG1-U7, ΔG38-U42, ΔU47-G56, and ΔG317-U326 were co-expressed with eggplant tRNA ligase; lanes 5 to 7, control *E. coli* clones in which wild-type ELVd alone, wild-type ELVd and tRNA ligase, and tRNA ligase alone were, respectively, expressed as indicated. (A,B) The positions and sizes (in nt) of RNA markers are indicated on the right, and the positions of the monomeric circular (mc) and linear (ml) forms of ELVd are indicated on the left of both panels.

borders of the ribozyme domain (**Figure 2**). We co-expressed longer-than-unit transcripts containing these deletions with the eggplant tRNA ligase in *E. coli* and analyzed the ELVd + RNAs that accumulated in the bacteria by Northern blot hybridization. Bands corresponding to the monomeric linear ELVd + RNA were detected for all four mutants, but bands corresponding to monomeric circular ELVd + RNAs were only observed for deletion mutants not directly affecting the ribozyme

domain (**Figure 4B**, compare lanes 1 and 2 with lanes 3 and 4). Same results were obtained in the analysis of two additional independent *E. coli* clones of each mutant.

In view of these last results, we decided to rule out the possibility that, in our *E. coli* experimental system, the hammerhead ribozyme in addition to ELVd RNA cleavage was also catalyzing the RNA circularization. To do that, we compared the circularization of the wild-type ELVd transcript in the presence of the eggplant wild-type tRNA ligase and the K219A catalytic mutant. This mutation in a nucleotidyltransferase key lysine completely prevents the binding of an ATP molecule, which is critical for the function of different ATP-dependent ligases

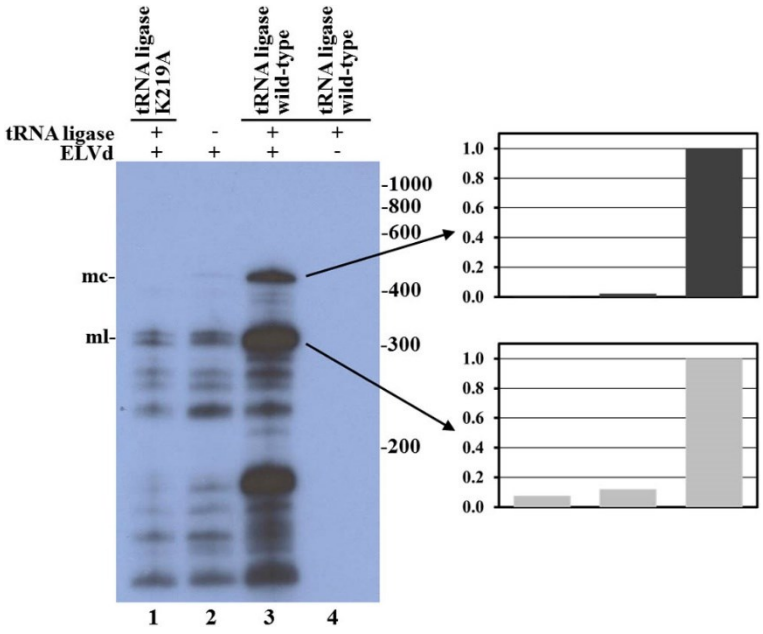

Figure 5. Analysis of eggplant tRNA ligase role in ELVd RNA circularization in *E. coli*. Northern blot hybridization analysis of total RNA from *E. coli* clones in which longer-than-unit ELVd transcripts were co-expressed with eggplant tRNA ligase. RNAs were separated by denaturing PAGE and transferred to a membrane. ELVd + strands were detected with a complementary ^{32}P-labeled RNA probe. Lanes 1, 2, and 3, *E. coli* clones in which the wild-type ELVd was co-expressed with the K219A catalytic mutant of eggplant tRNA ligase (lane 1), no tRNA ligase (lane 2) or the wild-type tRNA ligase (lane 3). Lane 4, control clone in which the wild-type tRNA ligase was expressed alone. The positions and sizes (in nt) of RNA markers are indicated on the right of the panel, and the positions of the monomeric circular (mc) and linear (ml) forms of ELVd are indicated on the left. Normalized histograms of band intensities corresponding to monomeric circular and linear ELVd forms are presented on the right.

(Sawaya et al., 2003). By Northern blot hybridization analysis, we were unable to detect substantial amounts of ELVd circular monomers in bacteria that expressed the inactive tRNA ligase, contrary to what happens in bacteria that expressed the wild-type ligase (**Figure 5**, compare lanes 1 and 3). This result supports the notion that, despite the observed importance of the viroid hammerhead ribozyme domain, the catalytic activity that circularizes the ELVd RNA resides in the co-expressed eggplant tRNA ligase.

Discussion

Viroids are non-coding RNAs capable of replicating and moving in the plants that they manage to infect. To do so, they must recruit host factors, such as RNA polymerases, RNases, nucleic acid ligases, RNA chaperones or RNA transporters, and take advantage of cellular structures to mediate the different steps of the infectious process. To design durable resistance strategies against viroid infection, we need to improve our knowledge about the molecular details that rule the interactions between viroid molecules and these host factors. One such host factor is the tRNA ligase, a conserved eukaryotic enzyme involved in the maturation of nuclear tRNAs. Intron-containing pre-tRNAs are cleaved by a tRNA splicing endonuclease that produce tRNA halves with 2′,3′-cyclic phosphodiester and 5′-hydroxyl ends, which are joined by the tRNA ligase using ATP (Englert and Beier, 2005; Phizicky and Hopper, 2015). Apart from the nucleus and cytoplasm, in plants this enzyme is located in chloroplasts (Englert et al., 2007), this being the replication site of the viroids that belong to the family *Avsunviroidae*, like ELVd (Flores et al., 2000). This enzyme ligates RNA molecules that contain 5′-hydroxyl and 2′,3′-cyclic phosphodiester ends, which are precisely the terminal groups produced by hammerhead ribozymes during the self-cleavage of the multimeric replication intermediates of these viroids. Indeed, a combination of *in vitro*

experiments, in which a recombinant version of the chloroplastic isoform of the eggplant tRNA ligase was used, and *in vivo* experiments, in which the endogenous tRNA ligase from *Nicotiana benthamiana* was silenced, supported the notion that this is the enzyme involved in the circularization of the viroids that belong to the family *Avsunviroidae* during replication (Nohales et al., 2012b).

In infectious agents like viroids, where genetic information is so densely packed, an analysis of a particular step of the infection process by site-directed mutagenesis normally requires the use of an appropriate experimental system since mutations will most probably have side effects on other steps. These experimental systems aim to dissect the step of interest from the whole infectious process. In the past, we set up one of these systems to analyze the processing (cleavage and ligation) of the RNAs of the family *Avsunviroidae*. This system consisted of transplastomic clones of the unicellular green alga *C. reinhardtii* in which longer-than-unit viroid RNAs were expressed in the algal chloroplast (Molina-Serrano et al., 2007). With the viroids of the family *Avsunviroidae*, these RNAs self-cleaved and circularized rather efficiently in the algal chloroplast, but efficiency depended on particular viroid species (Molina-Serrano et al., 2007). In this system, in which viroids do not replicate, circularization is most probably mediated by the *C. reinhardtii* homolog of plant tRNA ligase. This system served to perform a mutational analysis of ELVd RNA processing, which supported that the hammerhead ribozyme domain is necessary and sufficient to mediate transcript cleavage. However, this analysis also indicated that during RNA circularization, other viroid parts were involved, most probably a *quasi*-double-stranded structure present in the central part of the molecule that contains the ligation site in an internal loop (Martínez et al., 2009). This result is consistent with a subsequent finding obtained with an *in vitro* circularization analysis of different ELVd monomeric forms using a recombinant version of the chloroplastic isoform of the eggplant tRNA ligase. Under those particular *in vitro* reaction conditions, this enzyme only circularized the genuine

monomeric linear ELVd replication intermediate of + polarity, opened at the site which corresponded to the native hammerhead ribozyme. In contrast, five other monomeric linear ELVd RNAs opened at different sites along the molecule were not circularized, despite containing the same 5′-hydroxyl and 2′,3′-phosphodiester terminal groups (Nohales et al., 2012b).

Even though, the chloroplastic *C. reinhardtii* system having many interesting properties to study viroid processing, it also has some limitations. First, *C. reinhardtii* chloroplast transformation is labor-intensive and time-consuming. Second, and possibly most importantly, viroid ligation is catalyzed by an undefined enzyme in *C. reinhardtii* chloroplasts. To overcome these limitations, and once the host enzyme involved in viroid circularization was known, we set up a new experimental system that consisted of *E. coli* recombinant clones in which ELVd longer-than-unit transcripts and the chloroplastic isoform of the eggplant tRNA ligase were co-expressed. In these cells, ELVd transcripts self-cleaved to monomers, which were recognized by the eggplant tRNA ligase and efficiently circularized. However, no viroid RNA-to-RNA replication was detected (Daròs et al., 2014, 2018).

In the present work, we used this *E. coli*-based experimental system to further study the sequence and structural requirements of ELVd circularization by the eggplant tRNA ligase. We more specifically analyzed the effect of mutations on both strands of the central *quasi*-double-stranded structure that contains the ligation site in an internal loop in the predicted conformation of minimum free energy of the monomeric linear ELVd intermediate of + polarity (**Figure 1**). In the upper strand, we assayed four punctual nucleotide substitutions that have different effects on the secondary structure of this domain, as well as two deletions with a strong effect. In the lower strand, we assayed two deletions that directly affected the viroid ribozyme and only one of them had a strong effect on the secondary structure of the ligation domain, as well as two

deletions outside the ribozyme domain, again with only one having a strong effect on the secondary structure of the ligation domain (**Figure 2**). Interestingly, both ELVd deletion mutants directly affected in the ribozyme domain were still able to self-cleave in *E. coli*, although with a low efficiency, most likely through the *trans*-complementation activity of the second wild-type ribozyme. Note that the + ELVd longer-than-unit transcripts expressed in *E. coli* consisted of viroid monomers flanked by two complete ribozymes. In these transcripts, the full-length viroid monomers (from G1 to A333) were preceded by ELVd nucleotides C327 to A333 and followed by ELVd nucleotides G1 to G46 to form two complete ribozymes that release the full-length monomeric linear intermediates.

Despite having more or less affected the secondary structure of the ligation domain, most of the ELVd mutants analyzed in this work (C197A, G198U, C200G, G201A, ΔU194-G204, ΔC197-G201, ΔU47-G56, and ΔG317-U326) were efficiently circularized by the eggplant tRNA ligase in *E. coli*, except for those (ΔG1-U7 and ΔG38-U42) that directly affected the ribozyme domain (**Figures 3**, **4**). With the only concern that the precursors of these two mutants self-cleave in *E. coli* with low efficiency, due to mutations in one of the ribozymes, lack of circularization of the corresponding monomeric linear intermediates suggests the hypothesis that the eggplant tRNA ligase may not recognize the conformation of minimum free energy, rather a hypothetical transient structure formed by the lower strand of ELVd. This hypothesis would reconcile previous *in vitro* results in which + ELVd monomers opened at sites different to that which corresponded to the hammerhead ribozyme were not circularized by a purified eggplant tRNA ligase (Nohales et al., 2012b). The hammerhead ribozyme fold is just one of the many potential transient structures that the lower strand of the *quasi*-double-stranded structure can adopt. However, if this would finally be the physiological ligation fold, the experimental results presented herein clearly indicated that viroid circularization is not the result of a backward ribozyme reaction (Nelson et al., 2005;

Canny et al., 2007) because no substantial circularization was detected when the eggplant tRNA ligase was not expressed in *E. coli* (**Figures 3**, **4**) or when a ligase catalytic mutant was expressed (**Figure 5**). A deeper analysis with additional mutants in the ribozyme domain will be necessary to shed some more light about the contribution of the viroid hammerhead ribozyme domain in ELVd circularization.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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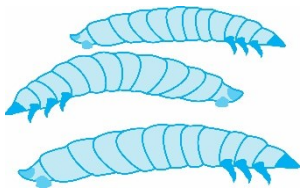

Chapter II

Intron-assisted, viroid-based production of insecticidal circular double-stranded RNA in *Escherichia coli*

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Author contributions:

J-AD conceived the work and designed the experiments in close collaboration with TC and BO. TC, BO and XH performed the experiments. All authors carried out data analysis and interpretation of the results. BO and JAD wrote the manuscript with input from TC and XH. All authors read and approved the final manuscript.

Abstract

RNA interference (RNAi) is a natural mechanism for protecting against harmful genetic elements and regulating gene expression, which can be artificially triggered by the delivery of homologous double-stranded RNA (dsRNA). This mechanism can be exploited as a highly specific and environmentally friendly pest control strategy. To this aim, systems for producing large amounts of recombinant dsRNA are necessary. We describe a system to efficiently produce large amounts of circular dsRNA in *Escherichia coli* and demonstrate the efficient insecticidal activity of these molecules against Western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte), a highly damaging pest of corn crops. In our system, the two strands of the dsRNA are expressed in *E. coli* embedded within the very stable scaffold of *Eggplant latent viroid* (ELVd), a small circular non-coding RNA. Stability in *E. coli* of the corresponding plasmids with long inverted repeats was achieved by using a cDNA coding for a group-I autocatalytic intron from *Tetrahymena thermophila* as a spacer. RNA circularization and large-scale accumulation in *E. coli* cells was facilitated by co-expression of eggplant tRNA ligase, the enzyme that ligates ELVd during replication in the host plant. The inserted intron efficiently self-spliced from the RNA product during transcription. Circular RNAs containing a dsRNA moiety homologous to *smooth septate junction 1* (*DvSSJ1*) gene exhibited excellent insecticide activity against WCR larvae. Finally, we show that the viroid scaffold can be separated from the final circular dsRNA product using a second *T. thermophila* self-splicing intron in a permuted form.

Keywords

RNA interference; double-stranded RNA; *eggplant latent viroid*; group-I self-splicing intron; intron-exon permutation; *Escherichia coli*; *Diabrotica virgifera.*

Introduction

RNA silencing, also known as RNA interference (RNAi), is a eukaryotic natural defence mechanism against exogenous RNA and transposon mobilization that has evolved to also regulate gene expression. RNAi is induced by the presence of highly structured or double-stranded RNA (dsRNA) and typically results in the silencing of homologous genes (Fire et al., 1998). Since efficient silencing can be equally induced by endogenously transcribed or exogenously delivered RNA, RNAi-mediated gene knockdown is frequently used in many organisms for basic research to study gene function, as well as for biotechnological applications, from therapeutics (Setten et al., 2019) to plant breeding (Guo et al., 2016). More specifically, in recent years, remarkable progress has been made in the use of exogenously supplied dsRNA as a highly specific and environmentally friendly anti-pest and anti-pathogen agent in agriculture (Das and Sherif, 2020; Fletcher et al., 2020; Liu et al., 2020). The ingestion of long dsRNAs by nematodes, insects, or other arthropods induces silencing of endogenous homologous genes, which may cause pest death or, at least, affect development, feeding, mobility, or progeny production, reducing crop damage in any case (Cagliari et al., 2019).

The dsRNA molecules required for RNAi applications can be obtained via chemical synthesis or bi-directional *in vitro* transcription. Both strategies generate two complementary RNAs that must be subsequently hybridized. These strategies are time-consuming, expensive, and particularly difficult to scale up to produce the large amounts of dsRNAs required, for example, in pest control. A more feasible strategy is *in vivo* production using a biofactory system, such as the bacteria *Escherichia coli* (Ponchon and Dardel, 2007). In this approach, the dsRNA can be expressed from a single transcriptional unit, which results in a hairpin RNA consisting of two complementary strands of the target sequence separated by a single-stranded loop (Saksmerprome et al., 2009; Posiri et al., 2013; Thammasorn et al., 2015; Zhong et al., 2019). However, the presence of inverted repeats in plasmid vectors significantly

damages stability (Leach, 1994; Lai et al., 2016). Alternatively, the two complementary RNA strands are usually synthesized *in vivo* from two promoters in inverted orientations (Timmons et al., 2001; Israni and Rajam, 2017; Papić et al., 2018). Again, this strategy requires hybridization of both complementary strands, thereby lowering efficiency and rendering the whole process prone to degradation.

We have recently developed a system to produce large amounts of recombinant RNA in *E. coli* based on elements of viroid biology (Cordero et al., 2018a; Daròs et al., 2018). Viroids are a unique class of plant infectious agents that are exclusively composed of a relatively small (246–434 nt) circular non-coding RNA molecule (Flores et al., 2005; Darós, 2016; Adkar-Purushothama and Perreault, 2020). Our RNA production system is based on co-expression in *E. coli* of an *Eggplant latent viroid* (ELVd) (Daròs, 2016) scaffold, in which the RNA of interest is grafted, along with the eggplant (*Solanum melongena* L.) tRNA ligase, the host enzyme involved in viroid circularization in the infected plant (**Figure 1**) (Nohales et al., 2012a, 2012b). Although there is no ELVd replication in *E. coli*, the viroid-derived RNA can be efficiently transcribed in these bacteria and it undergoes processing through the embedded hammerhead ribozymes. The resulting monomers that contain the RNA of interest are recognized by the tRNA ligase and circularized (**Figure 1**). The expression product likely remains bound to the tRNA ligase, forming a ribonucleoprotein complex that reaches high concentration in *E. coli* cells. Using this system, tens of milligrams of RNAs of interest, such as RNA aptamers, can be easily obtained per litre of *E. coli* culture under regular laboratory conditions (Daròs et al., 2018; Yu et al., 2020). We aim to apply this system for producing the large amounts of dsRNAs required to fight Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera: Chrysomelidae), using RNAi strategies. WCR is considered one of the most harmful insect pests of cultivated corn in the USA and it has received increasing attention globally because of repeated invasion events outside this country (Aragón et

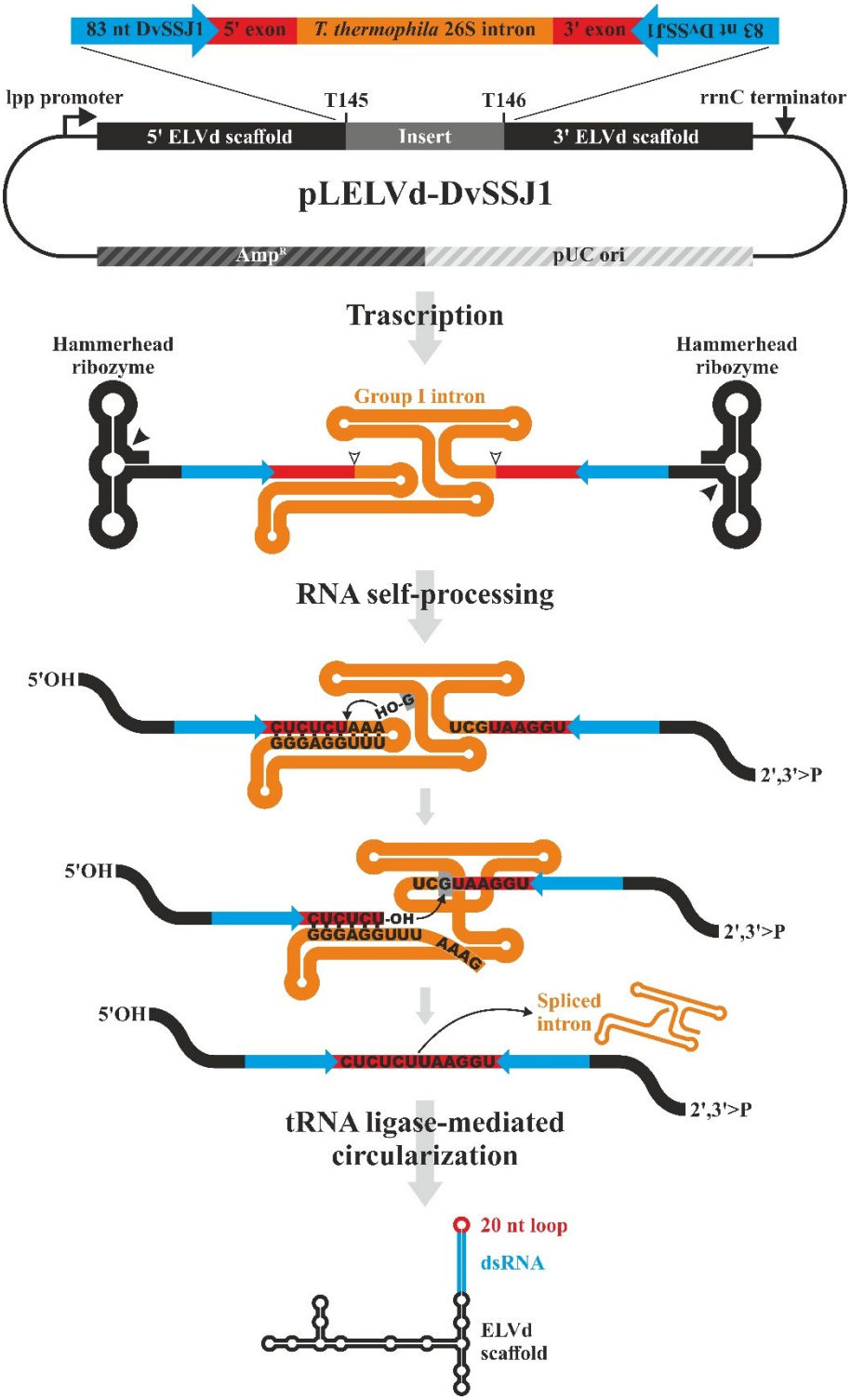

Figure 1. Schematic representation of the pLELVd-DvSSJ1 plasmid and the process for producing dsRNA in *E. coli*. In the primary transcript (not at scale), the inverted repeats homologous to *DvSSJ1* are separated by the *T. thermophila* 26S rRNA intron and the 10-nt native flanking exons. Spacing the inverted repeats in the expression plasmid is critical to stabilizing the constructs. After transcription, the intron self-splices very efficiently through two sequential transesterification reactions. First, the 3′-OH of an exogenous guanosine nucleoside docked in the intron structure attacks the phosphodiester bond between the first exon-intron boundary, generating a 3′-OH

group at the end of the exon and leaving the G residue attached to the 5′ end of the intron. Next, the intron terminal G is docked in the same place of the intron structure and the 3′-OH group of the first exon attacks the phosphodiester bond between the second exon-intron boundary, resulting in the ligation of both exons and the release of the catalytic intron in a linear form. The intron-processing facilitates the hybridization of the inverted repeat sequences to form a hairpin composed of a dsRNA capped by a 20-nt loop arising from the two flanking exons. Concomitantly, the self-splicing activity of the two ELVd hammerhead ribozymes present in the precursor yields the 5′-hydroxyl and 2′,3′-phosphodiester termini that are recognized and ligated by the co-expressed eggplant tRNA ligase, generating a circular chimera.

al., 2010). However, despite our initial success in producing recombinant hairpin RNAs of small length, we experienced major difficulties in building the expression plasmids that contain long inverted repeats. Thus, we first aimed to adapt the viroid-based system for the large-scale production in *E. coli* of hairpin RNAs with long double-stranded regions, which are required in anti-pest RNAi approaches. Second, we sought to use these recombinant RNAs to fight WCR. Third, we refined the *in vivo* production system to automatically remove the viroid scaffold from the dsRNA product.

We show that plasmids with long inverted repeats become completely stable in *E. coli* when the corresponding sequences are separated by a sequence coding for a group-I self-splicing intron (**Figure 1**). Interestingly, this intron self-splices with extremely high efficiency after transcription *in vivo*, facilitating the formation of an RNA product that contains the long hairpin RNA and that accumulates to high concentration in *E. coli*. We also demonstrate that the resulting RNA product, which consists of a structured viroid-derived scaffold from which the dsRNA protrudes, shows a potent insecticide activity against WCR larvae. Finally, we show that the dsRNA of interest can be efficiently excised from the viroid scaffold through the addition of a permuted version of the same intron flanking the inverted repeats (**Figure 2**); this yields a highly-stable and compact circular molecule consisting of a perfect dsRNA locked at both ends with small terminal single-stranded loops.

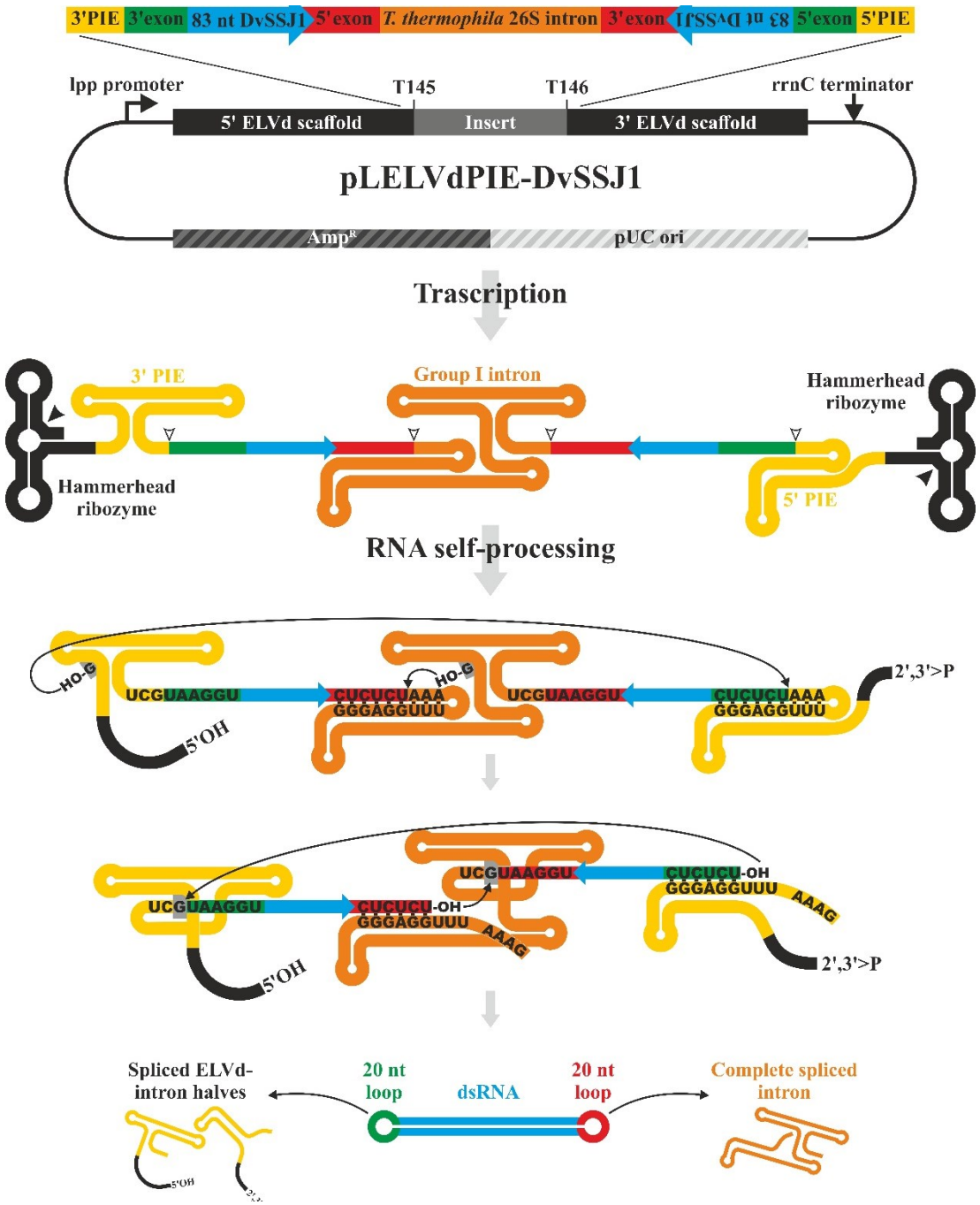

Figure 2. Schematic representation of the double-intron strategy to produce recombinant circular dsRNA in which the ELVd scaffold is removed. A permuted additional copy of the autocatalytic intron is added to the features of the pLELVd-DvSSJ1 flanking the inverted repeats to generate pLELVdPIE-DvSSJ1 (not at scale). Two self-splicing reactions are carried out in parallel (following the same two sequential transesterification reactions detailed in Figure 1). As a result, the complete intron is released, in addition to the two halves of the permuted intron covalently linked to the 3′ or 5′ ELVd scaffold; a circular dsRNA molecule, consisting of a 83-bp *DvSSJ1* dsRNA capped at both ends by the exon fragments is produced. Concomitantly, the self-splicing activity of the two ELVd hammerhead ribozymes present in the precursor yields the 5′-hydroxyl and 2′,3′-phosphodiester termini that are recognized and ligated by the co-expressed eggplant tRNA ligase, generating a circular chimera.

Results

Plasmids with long inverted repeats are stabilized in *E. coli* when these sequences are separated by a cDNA corresponding to a self-splicing group-I intron

D. virgifera smooth septate junction 1 (*DvSSJ1*) gene encodes a membrane protein associated with smooth septate junctions, which are required for intestinal barrier function. Ingestion by WCR larvae of dsRNA homologous to *DvSSJ1* induces mRNA suppression and larval growth inhibition and mortality (Hu et al., 2016, 2019, 2020). Using the viroid-derived system to produce recombinant RNA in *E. coli*, we attempted to produce the large amounts of various dsRNAs homologous to *DvSSJ1* to analyse their anti-WCR activity via oral feeding of insect larvae. However, we were unable to obtain the corresponding expression plasmids with long inverted repeat sequences, although we tried multiple cloning strategies, *E. coli* strains, and growth conditions. We reasoned that plasmid instability would revert if inverted repeats were separated by a sufficiently long spacer sequence. Inspired by previous work to produce hairpin RNAs in plants – in which inverted repeats were separated by a cDNA corresponding to a plant intron that efficiently spliced when the RNA was transcribed in the plant cells (Smith et al., 2000) – we searched for introns potentially able to self-splice in *E. coli*. We selected the group-I *Tetrahymena thermophila* 26S rRNA intron (Zaug and Cech, 1986).

In contrast to previous fruitless results, we easily obtained a plasmid in which 83-nt inverted repeats homologous to *DvSSJ1* were separated by a 433-bp cDNA that corresponded to the *T. thermophila* 26S rRNA intron (GenBank accession number V01416.1), plus both 10-nt native flanking exons. To build this plasmid, we electroporated the product of a Gibson assembly reaction in *E. coli* and selected transformed clones in plates containing ampicillin. Electrophoretic analysis of recombinant plasmids from 12 independent *E. coli* colonies showed that all were the

same size and exhibited a migration delay consistent with the inserted cDNA (**Supplemental Figure S1**, compare lane 1 with lanes 2 to 13). The expected sequence was confirmed in one of these plasmids, hereafter named pLELVd-DvSSJ1 (**Supplemental Dataset S1**).

Remarkable amounts of the dsRNA of interest, inserted into the ELVd molecule, accumulate in *E. coli* when co-expressed with tRNA ligase

We used pLELVd-DvSSJ1 to co-electroporate the RNase III-deficient strain of *E. coli* HT115(DE3), along with plasmid p15LtRnlSm (**Supplemental Dataset S1**), from which eggplant tRNA ligase is constitutively expressed. As controls, p15LtRnlSm was also co-electroporated with the empty expression plasmid (pLPP) or the plasmid to express empty ELVd (pLELVd), with no RNA of interest inserted (**Supplemental Dataset S1**). Three independent colonies were selected from plates containing ampicillin and chloramphenicol and grown for 24 h in Terrific Broth (TB). We extracted total RNA from the cells and analysed it by polyacrylamide gel electrophoresis (PAGE) in denaturing conditions (8 M urea). Two prominent bands above the 600-nt and slightly below the 400-nt RNA markers were observed in the lanes containing RNA from bacteria transformed with pLELVd-DvSSJ1 (**Figure 3A**, lanes 7 to 9, orange and black arrows, respectively). Note that these bands exhibited a fluorescence signal higher than those corresponding to endogenous *E. coli* rRNAs (**Figure 3A**, upper part of the gel), indicating a large accumulation *in vivo*. RNA extracts from the empty ELVd controls exhibited a single prominent band above the 400-nt marker that, according to our previous analyses (Fadda et al., 2003; Cordero et al., 2018b), corresponds to the 333-nt circular ELVd RNA (**Figure 3A**, lanes 4 to 6, white arrow). In denaturing conditions, circular RNAs migrate more slowly than the linear counterparts of the same size. Finally, RNA extracts from the empty plasmid control did not exhibit any particular prominent band (**Figure 3A**, lanes 1 to 3). To

confirm the identity of the expressed RNA species, we also analysed the RNA preparations by northern blot hybridization, using radioactive RNA probes complementary to ELVd and the sense strand of the *DvSSJ1*-derived dsRNA. While the ELVd probe hybridized with the prominent RNA band in the empty ELVd controls and the slowly migrating band in the ELVd-DvSSJ1 samples (**Figure 3B**, lanes 4 to

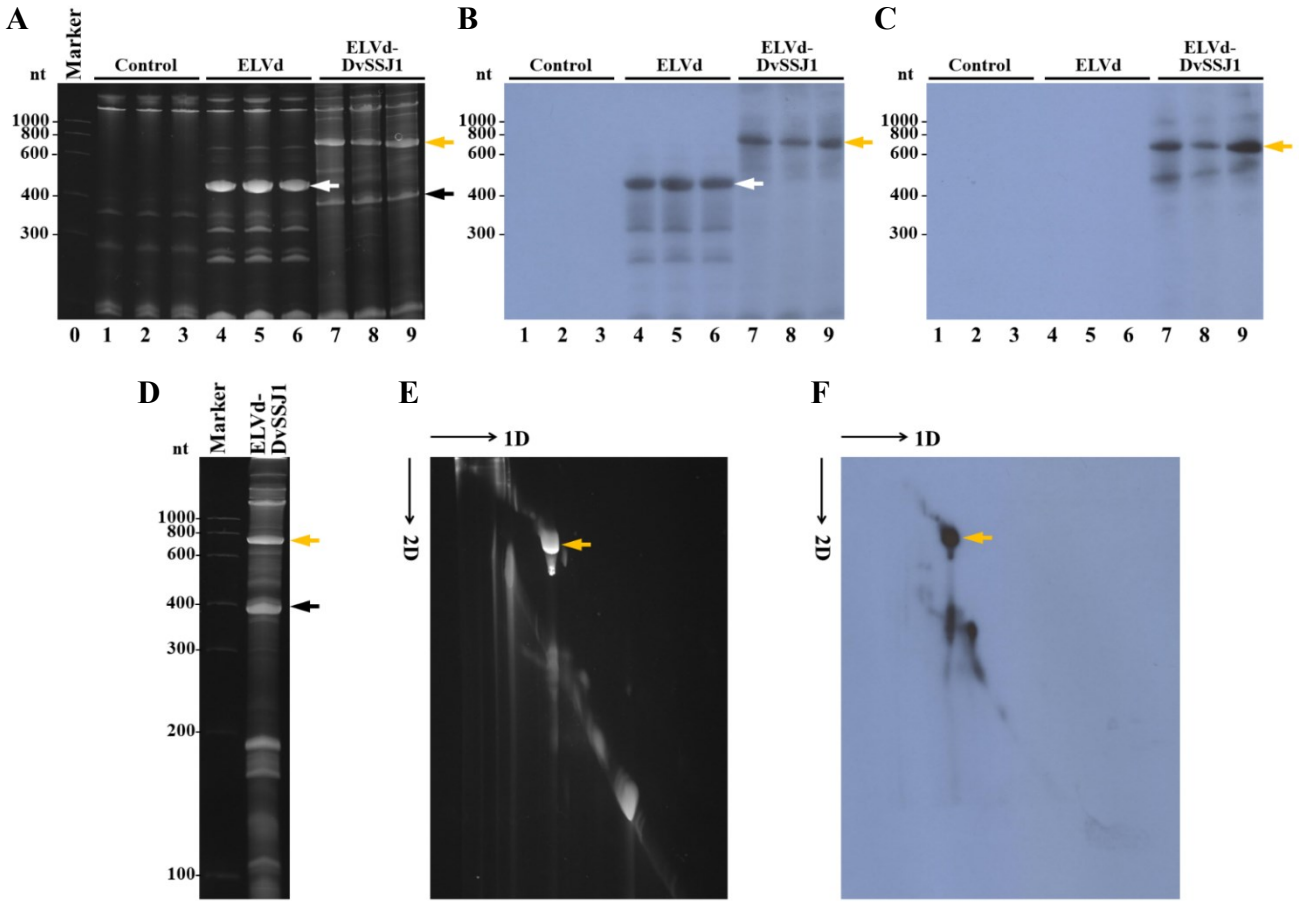

Figure 3. Analysis of *DvSSJ1*-derived dsRNA produced in *E. coli*. (A to C) Bacterial RNA was extracted from three independent cultures of *E. coli* co-transformed with p15LtRnlSm and pLPP (lanes 1 to 3), pLELVd (lanes 4 to 6) or pLELVd-DvSSJ1 (lanes 7 to 9), separated by denaturing PAGE, and stained with ethidium bromide (A) or transferred to membranes for hybridization with ELVd (B) or *DvSSJ1* (C) probes. (A) Lane 0, RNA marker with sizes in nt on the left. (D to F) An RNA preparation from *E. coli* co-transformed with p15LtRnlSm and pLELVd-DvSSJ1 was separated by denaturing 2D PAGE. The first dimension was under high ionic strength, and the RNAs were stained with ethidium bromide (D). The second dimension was under low ionic strength; the RNAs were first stained with ethidium bromide (E) and then transferred to a membrane and hybridized with a *DvSSJ1* probe (F). (E and F) Slim black arrows indicate the direction of RNA migration in both dimensions of 2D PAGE. Thick white, orange, and black arrows point to ELVd, ELVd-DvSSJ1, and spliced intron, respectively.

9, white and orange arrows, respectively), the DvSSJ1 probe only hybridized with the slowly migrating band of the ELVd-DvSSJ1 samples (**Figure 3C**, lanes 7 to 9, orange arrow). These results indicate that this slowly migrating band corresponds to a composite RNA species consisting of ELVd and *DvSSJ1* moieties. To determine whether this RNA species was linear or circular, we separated an RNA preparation from bacteria co-transformed with p15LtRnlSm and pLELVd-DvSSJ1 using denaturing 2-dimension (2D) PAGE, under conditions of high ionic strength (**Figure 3D**) and then low ionic strength (**Figure 3E** and **3F**). In this electrophoretic separation, circular RNAs are selectively delayed when conditions change from high to low ionic strength; they deviate from the diagonal of the linear RNAs. We observed the electrophoretic behaviour of a circular RNA for the prominent slowly migrating species when, after the second run, the gel was either stained with ethidium bromide (**Figure 3E**, orange arrow) or hybridized with the *DvSSJ1* probe (**Figure 3F**, orange arrow). Hybridization spots in the diagonal of linear molecules must correspond to linear counterparts of the ELVd-DvSSJ1 RNA circular form (**Figure 3F**).

We also sought to determine the nature of the rapidly migrating prominent band in the ELVd-DvSSJ1 samples (**Figure 3A**, lanes 7 to 9, black arrow). An RNA preparation from *E. coli* co-transformed with pLtRnlSm and pLELVd-DvSSJ1 was separated via denaturing PAGE (**Figure 4A**) and hybridized with a probe complementary to the *T. thermophila* 26S rRNA group-I intron. The probe specifically recognized this rapidly migrating species in the ELVd-DvSSJ1 RNA preparation (**Figure 4B**, black arrow), indicating that this band corresponds to the spliced intron that also accumulates to a high concentration in *E. coli*. The electrophoretic mobility of this species (close to the 400-nt RNA marker) suggests a linear form. The full size of the spliced *T. thermophila* 26S rRNA intron is 413 nt. We were surprised that we did not obtain hybridization bands corresponding to unspliced forms of the intron. This suggests an extremely efficient self-splicing reaction in *E. coli* cells. To investigate the

efficiency of intron splicing *in vivo*, we sampled a liquid *E. coli* culture at several time points and analysed bacterial RNA by electrophoretic separation and northern blot hybridization with the *DvSSJ1* and the intron probes (**Figure 4C**). Time-course analysis definitively supported a highly efficient self-splicing reaction of the *T. thermophila* intron in *E. coli*. No substantial amounts of processing intermediates were detected at any time point. A deletion analysis of the flanking 10-nt exon fragments suggested that their size could also be reduced with no substantial effect on self-cleavage (**Supplemental Figure S2**).

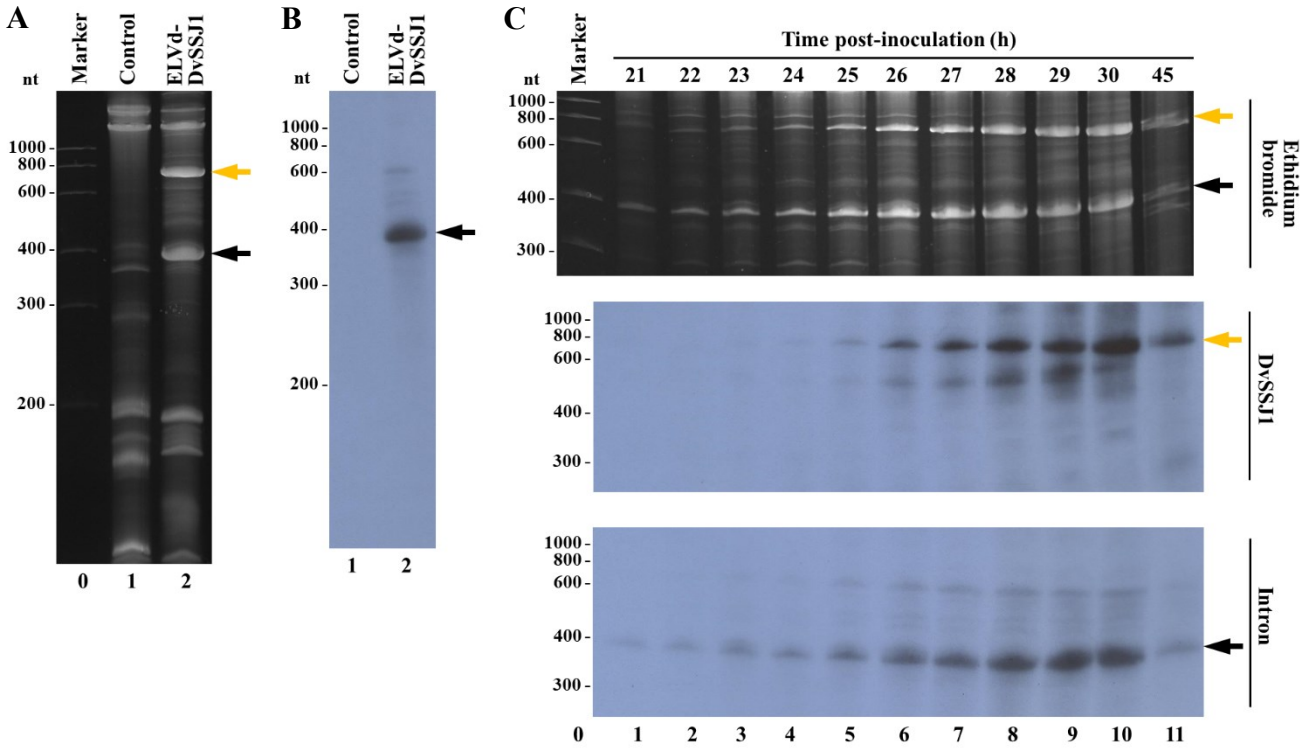

Figure 4. Analysis of the 26S rRNA intron processing in *E. coli*. (A and B) RNA was extracted from *E. coli* transformed with p15LtRnlSm and either pLPP (lane 1) or pLELVd-DvSSJ1 (lane 2), separated by denaturing PAGE, stained with ethidium bromide (A), and then transferred to a membrane and hybridized with an intron-specific probe (B). (C) RNA was extracted from aliquots of a liquid culture of *E. coli* co-transformed with p15LtRnlSm and pLELVd-DvSSJ1 at different time points (as indicated). Lanes 1 to 11, RNAs were separated by denaturing PAGE and stained with ethidium bromide (top) or transferred to a membrane and hybridized with a *DvSSJ1* (middle) or intron-specific (bottom) probe. Orange and black arrows point to ELVd-DvSSJ1 RNA and the spliced intron, respectively. (A and C) Lane 0, RNA markers with sizes in nt on the left.

These results indicate that, while the *T. thermophila* cDNA serves to stabilize the inverted repeats in the *E. coli* expression plasmids, the intron very efficiently self-splices from the primary transcript in bacterial cells, facilitating the accumulation of a circular RNA product that consists of an ELVd scaffold from which the *DvSSJ1*-derived 83-bp hairpin RNA is presented. The entire process is schematized in **Figure 1**.

The ELVd-DvSSJ1 RNA produced in *E. coli* possesses insecticide activity against WCR larvae

To test whether the chimeric ELVd-DvSSJ1 RNA displays anti-WCR activity, we performed a bioassay with WCR larvae. First, we grew 250-ml cultures of *E. coli* co-transformed with p15LtRnlSm and either pLELVd or pLELVd-DvSSJ1. The cells were harvested at 24 h and total bacterial RNA was purified. Our electrophoresis dilution analysis, along with a comparison to standards of known concentration, allowed us to quantify the concentration of empty ELVd and ELVd-DvSSJ1 in both RNA preparations (**Supplemental Figure S3**). As a control for this assay, we also produced the same *DvSSJ1*-derived 83-bp dsRNA that is contained in ELVd-DvSSJ1, using conventional *in vitro* transcription and hybridization (Hu et al., 2016). Next, equivalent amounts of the three RNA preparations were mixed with the artificial rootworm diet. While empty ELVd had no effect on larval growth at the top dose of 35 ng/µl, ELVd-DvSSJ1 and *in vitro*-transcribed DvSSJ1 induced similar larval growth inhibition (50% inhibition concentration, IC_{50} = 0.159 vs. 0.215 ng/µl) and mortality (50% lethal concentration, LC_{50} = 0.642 vs. 0.665 ng/µl) (**Table 1**).

Production of a circular version of the dsRNA of interest without the viroid scaffold using a permuted intron

Because carrying a sequence derived from an infectious agent may not be desirable for the commercial use of recombinant RNAs, we next aimed to automatically remove, *in*

Table 1. Insecticidal activity of conventional *DvSSJ1 in vitro*-transcribed dsRNA (IVT DvSSJ1) and ELVd-DvSSJ1 dsRNA against WCR.

WCR	LC_{50}/IC_{50}*	RNA (ng/µl)	Lower 95% CL*	Upper 95% CL	n
IVT DvSSJ1	LC_{50}	0.665	0.426	0.928	188
	IC_{50}	0.215	0.096	0.301	157
ELVd-DvSSJ1	LC_{50}	0.642	0.384	0.925	188
	IC_{50}	0.159	0.028	0.277	124
Empty ELVd	LC_{50}	>35 ppm			
	IC_{50}	>35 ppm			

*LC50, 50% lethal concentration; IC50, 50% inhibition concentration; CL, confidence limit.

vivo, the viroid scaffold moiety from the dsRNA product. For this purpose, we inserted into our construct an additional copy of the *T. thermophila* group-I autocatalytic intron, albeit with permuted intron-exon (PIE) sequences (Puttaraju and Been, 1992). More specifically, we incorporated cDNAs corresponding to the 3′ half of the *T. thermophila* intron (from position C236 in the intron to C10 of exon 2) just between the end of the 5′ ELVd moiety and the sense copy of the inverted repeat; between the antisense copy of the inverted repeat and the start of the 3′ ELVd moiety, we inserted the 5′ half of the *T. thermophila* intron (from −10A of exon 1 up to intron U235), as depicted in **Figure 2**. The two intron halves were still able to recognize the intron-exon boundaries and undergo the two transesterification reactions. Because the 3′ end of the second exon is covalently linked to the 5′ end of the first exon, both exons and the sequence between them are released as a circular RNA molecule (Puttaraju and Been, 1992).

The two intron halves were amplified by PCR and inserted into the right places by the Gibson assembly reaction to build plasmid pLELVdPIE-DvSSJ1 (**Supplemental Dataset S1**). We co-electroporated the *E. coli* RNase III-deficient strain containing this new plasmid together with p15LtRnlSm, the plasmid to express

eggplant tRNA ligase. Following plate selection of transformed clones, four independent colonies were grown in TB media for 24 h. Total bacterial RNA was extracted using phenol:chloroform and analysed using denaturing PAGE. The controls included bacteria co-electroporated with p15LtRnlSm and either the empty ELVd plasmid (pLELVd) or pLELVd-DvSSJ1. Electrophoretic analysis of RNA preparations from bacteria transformed with pLELVdPIE-DvSSJ1 showed a new prominent band between the 100-nt and 200-nt RNA markers, which was absent in both controls (**Figure 5A**, compare lanes 9 to 12, see blue arrow, with lanes 1 to 8). Surprisingly, the RNA molecule producing this band exhibited a differential migration depending on the position in the gel, creating an inverted smile pattern (**Figure 5A**, lanes 9 to 12,

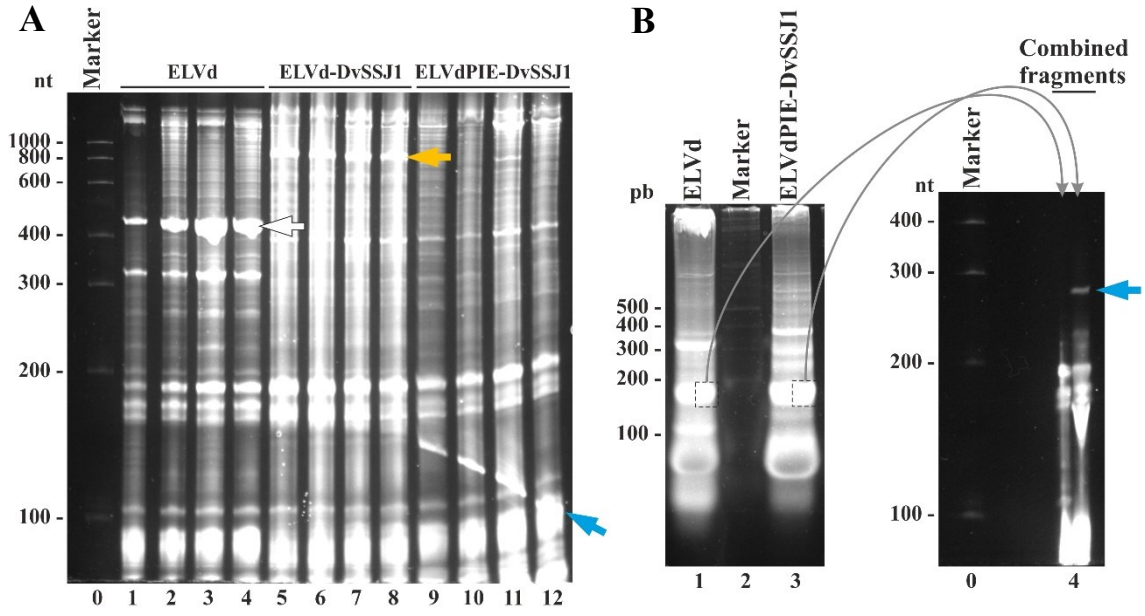

Figure 5. Recombinant circular dsRNA production in *E. coli* with the two-intron strategy. (A) *E. coli* HT115(DE3) was co-electroporated with p15LtRnlSm along with pLELVd (lanes 1 to 4), pLELVd-DvSSJ1 (lanes 5 to 8), or pLELVdPIE-DvSSJ1 (lanes 9 to 12). Bacterial RNAs were extracted from culture aliquots after 24 h and separated by denaturing PAGE. The gel was stained with ethidium bromide. (B) Equivalent aliquots of the total RNAs from *E. coli* co-transformed with p15LtRnlSm and either pLELVd (lane 1) or pLELVdPIE-DvSSJ1 (lane 3) were subjected to 2D PAGE separation. The first dimension (left) was conducted under non-denaturing conditions. Lane 2, DNA marker with sizes in bp on the left. The indicated bands were recovered from the first gel and placed together in a single well of a second denaturing gel (right; lane 4). The second dimension was done under denaturing conditions. (A and B) Lanes 0, RNA marker with sizes in nt on the left. Gels were stained with ethidium bromide. White, orange, and blue arrows point to ELVd, ELVd-DvSSJ1, and the circular dsRNA, respectively.

blue arrow). This anomalous electrophoretic behaviour is expected for a very compact circular dsRNA molecule, whose denaturation degree, and consequent electrophoretic mobility, changes with temperature. In this kind of electrophoresis, the temperature in the centre of the gel is higher than at the sides, as is the degree of denaturation. Consequently, a compact circular dsRNA migrates at the side of the gel (less denaturing) more rapidly than it does at the centre (more denaturing) (**Supplemental Figure S4**).

To further confirm the circularity of the recombinant dsRNA product, we subjected equivalent aliquots of RNA preparations from bacteria transformed with pLELVd and pLELVdPIE-DvSSJ1 to 2D PAGE separation. First, we separated the RNA using PAGE under non-denaturing conditions. We detected a prominent band close to the 200-bp DNA marker in the pLELVdPIE-DvSSJ1 sample; this was also present in the pLELVd control (**Figure 5B**, compare lanes 1 and lane 3). However, when we split both bands, directly loaded them side-by-side on top of a second denaturing polyacrylamide gel (containing 8 M urea), and continued electrophoresis, we observed a differential band corresponding to a species with a delayed electrophoretic mobility. This arose exclusively in the half lane corresponding to ELVdPIE-DvSSJ1 (**Figure 5B**, lane 4, blue arrow). These results indicate that, in bacteria transformed with pLELVdPIE-DvSSJ1, both introns (the regular and the permuted) self-splice efficiently to form a circular dsRNA product in which the ELVd scaffold has been removed, as depicted in the scheme in **Figure 2**.

Circular dsRNA is also produced in *E. coli* in the absence of eggplant tRNA ligase

Since the viroid scaffold is very effectively removed *in vivo* by the self-catalytic reactions of both introns, we examined whether the eggplant tRNA ligase added any benefit to the process. We grew cultures of bacteria co-transformed with pLELVdPIE-

DvSSJ1 and either p15LtRnlSm or the corresponding empty plasmid (p15CAT; **Supplemental Dataset S1**). Electrophoretic analysis of the RNA preparations showed that the recombinant circular dsRNA was produced in the presence or absence of the eggplant tRNA ligase (**Figure 6A**, see blue arrow and compare lanes 1 to 3 with lanes 4 to 6).

Since this result demonstrated that the tRNA ligase was no longer required to produce the recombinant circular dsRNA, we wondered whether the ELVd scaffold itself was required. To investigate this, we constructed a new plasmid in which the two ELVd moieties were deleted (pLPIE-DvSSJ1; **Supplemental Dataset S1**). The RNase-III-deficient *E. coli* strain was transformed with this plasmid alone and the RNA was purified from bacteria growing in a liquid culture. Electrophoretic analysis showed that, under these new conditions with the two introns, deletion of the ELVd

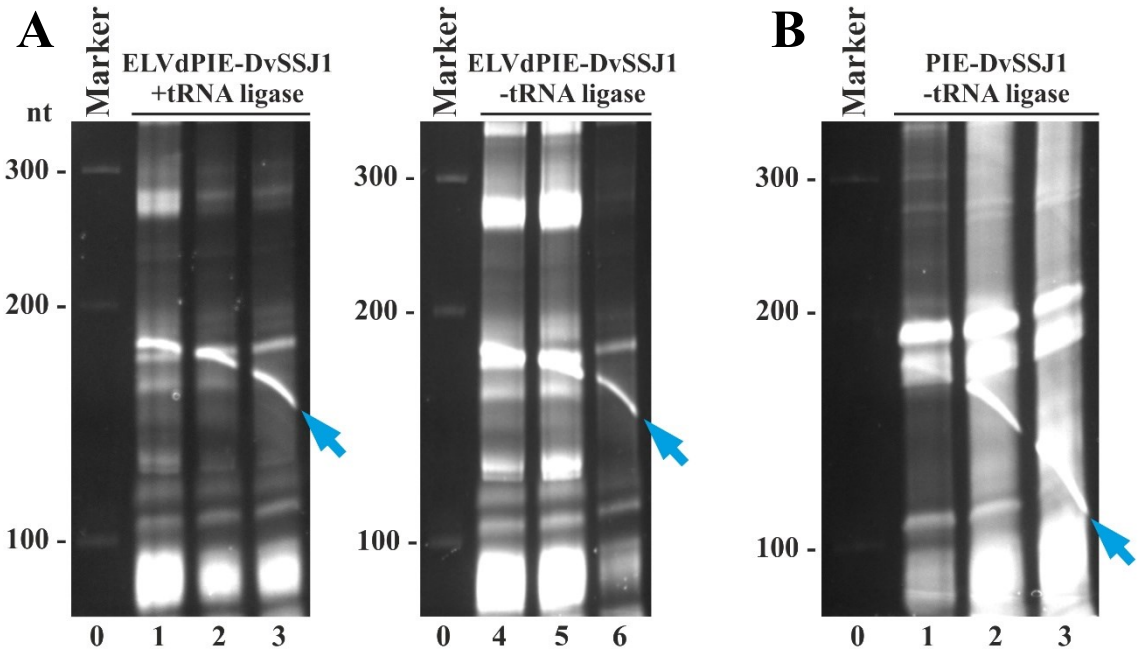

Figure 6. Recombinant circular dsRNA production in *E. coli* without tRNA ligase and the ELVd scaffold. RNA preparations from (A) bacteria transformed with pLELVdPIE-DvSSJ1 and p15LtRnlSm (lanes 1 to 3) or the corresponding empty plasmid p15CAT (lanes 4 to 6), and (B) bacteria transformed with pLPIE-DvSSJ1 and p15CAT (lanes 1 to 3) were separated by denaturing PAGE and the gels were stained with ethidium bromide. Lanes 0, RNA marker with sizes in nt on the left. In all cases, RNA was extracted from bacteria growing in liquid cultures after 24 h. The circular dsRNA is indicated with blue arrows.

scaffold had only a minor effect on circular dsRNA production (**Figure 6B**, blue arrow; and **Supplemental Figure S5**). Note that, although the recombinant circular dsRNA in this type of separation co-migrates with *E. coli* RNAs or degradation products thereof, it can be easily distinguished in the gels as a crooked band resulting from temperature-dependent migration (**Figure 6**).

Discussion

RNAi can be driven by endogenous or exogenous RNA molecules that are processed in eukaryotic cells by RNase-III-type enzymes (Dicer), resulting in small double-stranded molecules from 21 to 25 bp with two protruding nucleotides at each 3′ end. One of the strands is loaded by an Argonaute protein to form the RNA-induced silencing complex (RISC), which serves as a guide for searching RNA targets based on base complementary. Target RNAs can be processed in various ways, but the whole mechanism typically results in reduced gene expression (Baulcombe, 2004). Due to its mechanistic simplicity, RNAi has become a powerful tool for basic research and biotechnological applications. While transgenic technologies may be ideal for inducing RNAi, stringent legislation regarding genetically modified organisms makes it necessary to consider exogenously supplied RNA molecules in many instances. However, possibly due to its intrinsically low half-life, there is no easy way to produce the large amounts of recombinant RNAs that are required for many practical applications, although substantial advances in RNA expression have been made using stable RNA scaffolds in *E. coli* (Ponchon and Dardel, 2007; Liu et al., 2010; Ponchon et al., 2013). We have recently contributed to this effort with a system that expresses the RNA of interest in *E. coli* in a circular form that is grafted into an extremely stable scaffold consisting of a viroid RNA backbone (Daròs et al., 2018). Viroids are plant infectious agents constituted by a relatively small naked circular RNA able to survive and replicate in

the hostile environment of the host cell. The circular, highly base-paired viroid structure – likely bound to the co-expressed tRNA ligase – provides the recombinant RNA with high stability and exonuclease resistance, contributing to the vast accumulation (tens of milligrams per litre of *E. coli* culture in laboratory conditions) of the RNA of interest in this system. However, this system proved to be inefficient for producing the long dsRNAs that are preferred for inducing RNAi in many applications (Daròs et al., 2018).

In this work, we aimed to adapt the viroid-based system for producing recombinant RNA in *E. coli* to generate the dsRNAs that are used for RNAi-based pest control. As a target, we chose *D. virgifera*, a relevant corn pest for which RNAi-based control has been recently demonstrated by targeting the *DvSSJ1* gene (Hu et al., 2016, 2019, 2020). To express a dsRNA homologous to a fragment of the *DvSSJ1* gene in the viroid-based system, we had to create a long inverted repeat in the sequence of the vector. This resulted in the longstanding and well-known problem of plasmid instability (Lai et al., 2016). The use of asymmetric inverted repeats (Saksmerprome et al., 2009; Thammasorn et al., 2015), or the insertion of long spacers between the repeats (Posiri et al., 2013; Zhong et al., 2019), has been proposed to avoid plasmid instability in bacteria. However, in other systems such as plants or *Drosophila melanogaster*, the repeats have been successfully separated with intron cDNAs from genes (of the same or related organisms) containing short fragments of both flanking exons. In this way, once transcribed, the introns are recognized and processed by the splicing machinery, eliminating them from the final product: a hairpin consisting of a long dsRNA capped by a short loop resulting from the flanking exon fragments (Smith et al., 2000; Lee and Carthew, 2003; Bao and Cagan, 2006; Eamens and Waterhouse, 2011). Inspired by these studies, we separated the inverted repeats with the sequence corresponding to a self-splicing group-I intron from *T. thermophila*. Group I introns are found in genes encoding proteins, rRNA, and tRNA in algae, fungi, lichens, some

lower eukaryotes, and especially in bacteria. These introns are ribozymes, capable of catalysing their own splicing from primary transcripts without the involvement of any protein or additional factor, other than Mg^{2+} and guanosine (Raghavan and Minnick, 2009). In addition to the full-length cDNA of the *T. thermophila* 26S rRNA intron (433 nt), we added each 10-nt flanking exon to ensure optimal recognition of intron-exon boundaries. As expected, the separation of the inverted repeats with the intron plus flanking exons was key to achieving plasmid stability in *E. coli* (**Supplemental Figure S1**). The expression of the corresponding precursor transcript in an *E. coli* RNase III-deficient strain, along with that of the eggplant tRNA ligase, led to the efficient accumulation of chimeric molecules of circular RNA consisting of the ELVd scaffold containing the 83-nt *DvSSJ1* hairpin (**Figure 3**). Interestingly, the 433-nt processed intron also accumulated in *E. coli*, while unprocessed intermediates were not detected, indicating very efficient *T. thermophila* group-I intron self-cleavage in bacteria (**Figure 4**) (Guo and Cech, 2002). The amount of the chimeric RNA containing the dsRNA of interest increased with time up to 24 h in the *E. coli* culture, in contrast to the usual 12-h accumulation peak of single-stranded RNA aptamers, as in Spinach (Daròs et al., 2018). The amount decreased after that point, with the band of circular RNA at 40 h being almost negligible (**Figure 4**). In addition, the 50-fold upscaling of production (maintaining the expression conditions) does not seem to affect the large-scale accumulation of the dsRNA of interest. Thus, we suggest that this approach is an efficient way to produce large amounts of recombinant dsRNAs, due to the fact that both the scaffolding and the hairpin loop are constructed to avoid degradation by the bacterial RNases.

The produced dsRNA maintains a potent anti-WCR activity, as we observed in the WCR larvae feeding bioassay (**Table 1**). As previously shown, the inserted RNA is fully functional, probably due to the position in which the recombinant RNA is inserted – at the end of the right upper arm, thus allowing both scaffold and

recombinant RNA to form two independent domains (Daròs et al., 2018). Furthermore, it should be noted that the silencing obtained with the ELVd-dsRNA chimera is slightly better than that obtained using the same dsRNA molecules produced by *in vitro* transcription (**Table 1**). We speculate that the presence of the protective scaffold may help increase the half-life of dsRNA during its production and application, both in the field and in the intestines and cells of insects, thereby making it available for processing by the RNAi machinery in greater quantities than the same dsRNA produced *in vitro*.

Various releasing methods have been described to remove the protective scaffold after expression of recombinant RNA. *In vitro* strategies such as the use of RNases (Ponchon et al., 2012; Posiri et al., 2013) or sequence-specific DNAzymes (Liu et al., 2010), although they remove single-stranded RNA, would add an additional layer to the production system. Furthermore, the resulting RNA would not be circular. Another feasible approach is to flank the recombinant RNA ends with hammerhead ribozymes, which leads to *in vivo* efficient cleavage of the RNA of interest and its release as a linear (Nelissen et al., 2012) or even a circular molecule (Litke and Jaffrey, 2019). In this work, we explore the use of an additional copy of the same group-I autocatalytic intron, although in a permuted fashion in which the 3′ half of the intron is placed upstream of the 5′ half (**Figure 2**). As reported, both intron halves undergo the two transesterification reactions (Puttaraju and Been, 1992), even inserting between both halves very long sequences (Wesselhoeft et al., 2018) such as, in this case, the long inverted repeats plus the full-length separating intron. The resulting product is a circular molecule composed of the dsRNA locked at both sides by loops derived from the exon fragments (**Figure 2**). This strategy allows us to remove the viroid scaffolding without losing, in the final molecule, two key characteristics in recombinant RNA accumulation – circularity and compaction – that make the molecules highly resistant to degradation, as seen from the fact that they accumulate in large quantities in the

bacteria (**Figure 5**). Interestingly, although there are two complete intron sequences, they are both removed correctly without mutual interference. Due to the efficient self-splicing of both introns, the circular dsRNA can be efficiently obtained in *E. coli*, even if neither the eggplant tRNA ligase nor the viroid scaffold are present (**Figure 6**). Note that we did not assay the insecticidal activity of the released circular dsRNA, although taking into consideration that the chimeric ELVd-DvSSJ1 and the empty ELVd scaffold displayed, respectively, strong and negligible anti-WCR activities (**Table 1**), the free circular DvSSJ1 dsRNA is likely to also possess insecticidal activity.

Purified dsRNA or even inactivated complete bacterial extracts have been used to induce RNAi effectively (Timmons et al., 2001; Israni and Rajam, 2017). For many applications, it is preferable, however, to completely isolate the dsRNA from other bacterial RNAs. Thus, both recombinant dsRNAs produced here could be purified by affinity chromatography, using recombinant-specific dsRNA-binding proteins or antibodies (Atsumi et al., 2015). The circularity of the produced molecules and the absence of such structures in *E. coli* may also be exploited, as the circular molecules can be purified to homogeneity by 2D-PAGE, both under denaturing conditions (with reduction of the ionic strength in the second separation) and subsequent elution from the gel.

In conclusion, we have developed a strategy to adapt our viroid-based expression system to overproduce in *E. coli* RNA hairpins with extended double-stranded regions able to induce RNAi in insects, as we demonstrate in the case of WCR. Further, we used a second self-splicing group-I intron (permuted) to produce very compact circular dsRNAs in which the viroid scaffold is completely removed *in vivo*. Both strategies are based on the activity of autocatalytic introns. We assert that they are fast, high-output, cost-effective, and scalable alternatives for industrially producing the large amounts of large dsRNAs that are required in pest control approaches. Because of their robustness

and flexibility, these strategies could be used in any other RNAi biotechnological applications.

Materials and methods

Plasmid construction

To build pLELVd-DvSSJ1, we first produced an 83-bp cDNA molecule homologous to a fragment of *DvSSJ1* (from positions 50 through 132 of GenBank KU562965.1) via the polymerase chain reaction (PCR), using the Phusion high-fidelity DNA polymerase (Thermo Scientific) and primers D2623 and D2624. All primers used in this work are in **Supplemental Table S1**. Next, we amplified three cDNAs corresponding to this *DvSSJ1* fragment in two opposite orientations, using primers D2625-D2626 and D2629-D2630, as well as the *T. thermophila* 26S rRNA intron, which includes 10 nt of the flanking exons (from positions 43 through 475 of V01416.1) with primers D2627 and D2628. Finally, we assembled (Gibson et al., 2009) these three cDNAs into pLELVd-BZB (**Supplemental Dataset S1**), digested with *Bpi*I (Thermo Scientific). The sequence of the resulting plasmid, pLELVd-DvSSJ1 (**Supplemental Dataset S1**), was experimentally confirmed (3130xl Genetic Analyzer, Life Technologies). From this plasmid, we built pLELVdPIE-DvSSJ1 by adding, through two consecutive Gibson assembly reactions, two halves of the autocatalytic intron: 3′ (opening the plasmid with the D2936 and D2937 primers) and 5′ (opening the plasmid with the primers D2940 and D2941). We also sequentially removed the 5′ and 3′ viroid moieties from this latter plasmid via PCR with the phosphorylated primers (T4 polynucleotide kinase, Thermo Scientific) D3606 and D3285, and D3607 and D3608, followed by self-ligation of the products (T4 DNA ligase, Thermo Scientific). Finally, we obtained plasmid pLPIE-DvSSJ1 (**Supplemental Dataset S1**).

Escherichia coli culture

The strain HT115(DE3) (Timmons et al., 2001) of *E. coli* was co-electroporated (Eporator, Eppendorf) with p15LtRnlSm along with pLPP, pLELVd, pLELVd-DvSSJ1, pLELVdPIE-DvSSJ1, or pLPIE-DvSSJ1. In some experiments, bacteria were co-electroporated only with pLELVdPIE-DvSSJ1 or pLPIE-DvSSJ1, and p15CAT. Transformed clones were selected and grown as previously described (Daròs et al., 2018).

RNA extraction

At the desired time, 2-ml aliquots of the liquid cultures were harvested; the cells were sedimented by centrifugation at 13,000 rpm for 2 min. Cells were resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). One volume (50 µl) of a 1:1 (v/v) mix of phenol (saturated with water and equilibrated at pH 8.0 with Tris-HCl, pH 8.0) and chloroform was added; the cells were broken by vigorous vortexing. The mix was centrifuged for 5 min at 13,000 rpm; the aqueous phase, containing total bacterial RNA, was recovered. For large-scale preparation of ELVd and ELVd-DvSSJ1 RNA, *E. coli* were grown in a 2-l Erlenmeyer flask with 250 ml of TB medium at 37°C and 225 rpm for 24 h. Cells were sedimented by centrifugation for 15 min at 8000 rpm, resuspended in H_2O, and sedimented again under the same conditions. Cells were resuspended in 10 ml buffer 50 mM Tris-HCl, pH 6.5, 0.15 M NaCl, and 0.2 mM EDTA. One volume of a 1:1 mix of phenol and chloroform was added; the mix was intensively vortexed. The phases were separated by centrifugation; the aqueous phase, once recovered, was re-extracted with one volume of chloroform. Finally, RNAs were precipitated from the aqueous phase adding sodium acetate pH 5.5 to 0.3 M and 2.5 volumes of ethanol. RNAs were resuspended in H_2O and re-precipitated with one volume of isopropanol.

RNA electrophoresis

Aliquots of the RNA preparations (20 µl; corresponding to 0.8 ml of the original *E. coli* culture) were separated by denaturing PAGE as previously described (Daròs et al., 2018). The Riboruler low range RNA ladder (Thermo Scientific) was used as a standard. Gels were run for 2 h at 200 V in 5% polyacrylamide gels (37.5:1 acrylamyde:N,N'-methylenebisacrylamide) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) that included 8 M urea. In one experiment, an RNA preparation was separated by denaturing electrophoresis in a second dimension at a lower (0.25× TBE) ionic strength. After the first dimension, an entire lane from a 5% polyacrylamide, 8 M urea, TBE gel was cut and laid transversely on top of a 5% polyacrylamide gel of the same dimensions in 0.25× TBE buffer containing 8 M urea; it was run for 2.5 h at 25 mA. In another experiment, the RNA was separated first in a non-denaturing 5% PAGE gel in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) without urea. The gel was run for 1.5 h at 75 mA. The bands of interest were cut after the electrophoresis separation and placed on top of a 5% PAGE gel in 0.25× TBE buffer containing 8 M urea; they were run as previously explained.

Northern blot hybridization analysis of RNA

After electrophoretic separation, RNAs were electroblotted to positively charged nylon membranes (Nytran SPC, Whatman) and cross-linked by irradiation via 1.2 J/cm^2 UV light (254 nm, Vilber Lourmat). Hybridization was performed overnight at 70°C in the presence of 50% formamide as previously described (Daròs et al., 2018). Radioactive RNA probes complementary to ELVd, the *DvSSJ1* fragment, and the *T. thermophila* intron were obtained by *in vitro* transcription of the corresponding linearized plasmids with T3 bacteriophage RNA polymerase (Roche) (Daròs et al.,

2018). After transcription, the DNA template was digested with DNase I (Thermo Scientific) and the probe was purified by chromatography using a Sephadex G-50 column (mini Quick Spin DNA Columns, Roche).

Double-stranded RNA production by *in vitro* transcription

The target-specific primers containing T7 RNA polymerase sites at the 5′ end of each primer were used to generate the PCR product; this served as the template for dsRNA synthesis by *in vitro* transcription using a MEGAscript kit (Life Technologies). The dsRNAs were purified using the Megaclear kit (Life Technologies) and examined by 12-well E-gel electrophoresis (Life Technologies) to ensure dsRNA integrity. They were quantified using Phoretix 1D (Cleaver Scientific) or a NanoDrop 8000 Spectrophotometer (Thermo Scientific).

WCR bioassays

We prepared the WCR diet according to the manufacturer's guideline for a *D. virgifera* diet (Frontier, Newark, DE), with modifications (Zhao et al., 2016). The dsRNA samples (5 µl) were incorporated into 25 µl of WCR diet in a 96-well microtiter plate and shaken on an orbital shaker for 1 min until the diet solidified. For each RNA sample, nine doses (35, 17.5, 8.8, 4.4, 2.2, 1.09, 0.55, 0.27, and 0.14 ng/µl) were evaluated, for a total of 32 observations per dose or water control. We transferred two one-day-old larvae into each well. The plates were incubated at 27°C and 65% relative humidity. Seven days after exposure, the larvae were scored for growth inhibition (severely stunted larvae with >60% reduction in size) and mortality. We analysed the data using PROC Probit analysis in SAS (Finney, 1971) to determine LC_{50}. The total numbers of dead and severely stunted larvae were used to analyse the IC_{50}.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supporting information

Supplemental Dataset S1. Nucleotide sequences and elements of plasmids pLELVd, pLELVd-BZB, pLPP, pLELVd-DvSSJ1, pLELVdPIE-DvSSJ1, pLPIE-DvSSJ1, pLtRnlSm and p15CAT.

>pLELVd (2050 bp)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTG**CCCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGG**TTTCGCCATGGGTCG
GGACTTTAAATTCGGAGGATTCGTCCTTTAAACGTTCCTCCAAGAGTCCCTTCCCCAAACCCTTACTTTGTAAGTGTGGTT
CGGCGAATGTACCGTTTCGTCCTTTCGGACTCATCAGGGAAAGTACACACTTTCCGACGGTGGGTTCGTCGACACCTCTCC
CCCTCCCAGGTACTATCCCCTTTCAAGGATGTGTTCCCTAGGAGGGTGGGTGTACCTCTTTTGGATTGCTCCGGCCTTCCA
GGAGAGATAGAGGACGACCTCTCCCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGGGAAATC
ATCCTTAGCGAAAGCTAAGGATTTTTTTTATCTGAAATGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGC
ATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCT
CCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTT
CTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTC
AGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAG
CCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACA
CTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA
AACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATC
CTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCG
TCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGG
CTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCC
AGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAG
GCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCC
CCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCA
TGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCA
AGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCA
GAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT
CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAA
GGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA
GCATTTATCAGGGTTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In **bold**, ELVd cDNA (C327 to G46 of AJ536613), with the repeated hammerhead ribozyme domain **highlighted in yellow**, and their self-cleavage sites **underlined**. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>pLELVd-BZB (2574 bp)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTG**CCCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGG**TTTCGCCATGGGTCG
GGACTTTAAATTCGGAGGATTCGTCCTTTAAACGTTCCTCCAAGAGTCCCTTCCCCAAACCCTTACTTTGTAAGTGTGGTT
CGGCGAATGTACCGTTTCGTCCTTTCGGACTCATCAGGGAAAGTACACACTTTCCGACGGTGGGTTCGTCGACACCTCTCC
CCCTCCCAGGTACTATCCCCTTGCGTCTTCGCGGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTC
ATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG
GAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCCCCC

TCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCG
CGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA
AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC
GATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGGGCGGAAGACGCTCAAGGATGTGTTCCCTAGGA
GGGTGGGTGTACCTCTTTTGGATTGCTCCGGCCTTCCAGGAGAGATAGAGGACGACCTCTCCCCATAGGGTGGTGTGTGCC
ACCCCTGATGAGACCGAAAGGTCGAAATGGGGGAAATCATCCTTAGCGAAAGCTAAGGATTTTTTTTATCTGAAATGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC
AGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATA
CCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGT
TCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTC
CAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGC
TACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT
TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCA
GCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGAT
CTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC
CAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA
GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCC
AGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAG
CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGAT
CGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATC
CGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCG
AAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATG
TTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In **bold**, (C327 to G46 of AJ536613), with the repeated hammerhead ribozyme domain **highlighted in yellow**, and their self-cleavage sites **underlined**. In blue cDNA coding for LacZ gene inserted between T245 and T246 of ELVd cDNA. Highlighted in dark blue, recognition sites for type IIS restriction enzyme BpiI, with the cleavage sites in **underlined bold**. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>pLPP (1916 bp)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTGGGAGACCGCGG*CAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGG*
AACAAAAGCTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGAT
CCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCC
*GTCGTTTTACA*GGCGGGTCTCGGAAATCATCCTTAGCGAAAGCTAAGGATTTTTTTTATCTGAAATGCGTTGCTGGCGTTT
TTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAA
AGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG
CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTA
AGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC
TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA
AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACG
CGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT
CGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCA
ATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGT
GGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGT
TTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCC
CAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGA
AGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGC
TTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCA
ATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCA

AGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACC
AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTC
ATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In **bold blue**, polylinker with double recognition sites for the enzyme IIS type Eco31I (highlighted in yellow, with the cleavage sites underlined) separating the polylinker from the plasmid pBSIIKS + (in reverse, in *blue italics*). In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>pLELVd-DvSSJ1 (2649 bp)
CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTG**CCCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGG**TTTCGCCATGGGTCG
GGACTTTAAATTCGGAGGATTCGTCCTTTAAACGTTCCTCCAAGAGTCCCTTCCCCAAACCCTTACTTTGTAAGTGTGGTT
CGGCGAATGTACCGTTTCGTCCTTTCGGACTCATCAGGGAAAGTACACACTTTCCGACGGTGGGTTCGTCGACACCTCTCC
CCCTCCCAGGTACTATCCCCTTACCATTGTCCTGAAATTGCTGAAGTTGGTGATCAATTTGATATGTCTCATCTTGTACCG
AACCGGATATCAAGGCTACTTCTTATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACC
TGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCG
TTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCA
CGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTAT
TCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACT
AATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCAAGAAGTAGCCTTGATATCCGGTTCGGTAC
AAGATGAGACATATCAAATTGATCACCAACTTCAGCAATTTCAGGACAATGGT**TCAAGGATGTGTTCCCTAGGAGGGTGGG**
TGTACCTCTTTTGGATTGCTCCGGCCTTCCAGGAGAGATAGAGGACGACCTCTCCCCATAGGGTGGTGTGTGCCACCCCTG
ATGAGACCGAAAGGTCGAAATGGGGGAAATCATCCTTAGCGAAAGCTAAGGATTTTTTTTATCTGAAATGCGTTGCTGGCG
TTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTA
TAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC
GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCG
GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC
GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATT
ACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTA
TTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT
GCAATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA
AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAAT
AGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGT
TCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTC
AGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCG
TCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTC
TCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC
ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATA
CTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In **bold**, (C327 to G46 of AJ536613), with the repeated hammerhead ribozyme domain **highlighted in yellow**, and their self-cleavage sites **underlined**. Highlighted in green, inverted repeat of an 83 nts fragment of the *DvSSJ1* gene inserted between T245 and T246 of ELVd cDNA. In green, between the two copies of the *DvSSJ1* gene, the cDNA of the group-I *Tetrahymena termophila* 26S rRNA intron, with the 10 nts of both flanking exons underlined. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>pLELVdPIE-DvSSJ1 (3080 bp)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTG**CCCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGGTTTCGCCATGGGTCG**
GGACTTTAAATTCGGAGGATTCGTCCTTTAAACGTTCCTCCAAGAGTCCCTTCCCCAAACCCTTACTTTGTAAGTGTGGTT
CGGCGAATGTACCGTTTCGTCCTTTCGGACTCATCAGGGAAAGTACACACTTTCCGACGGTGGGTTCGTCGACACCTCTCC
CCCTCCCAGGTACTATCCCCTTCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTT
CTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATT
TGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCACCATTGTCCTGAAATTGCTGAAGTTGGTGATCA
ATTTGATATGTCTCATCTTGTACCGAACCGGATATCAAGGCTACTTCTTATGACTCTCTAAATAGCAATATTTACCTTTGG
AGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAA
ATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAAT
AAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGAC
TAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGA
TGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCAAGAA
GTAGCCTTGATATCCGGTTCGGTACAAGATGAGACATATCAAATTGATCACCAACTTCAGCAATTTCAGGACAATGGTATG
ACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGAT
TGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTT
TGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGAT**T**
CAAGGATGTGTTCCCTAGGAGGGTGGGTGTACCTCTTTTGGATTGCTCCGGCCTTCCAGGAGAGATAGAGGACGACCTCTC
CCCATAGGGTGGTGTGTGCCACCCCTGATGAGACCGAAAGGTCGAAATGGGGGAAATCATCCTTAGCGAAAGCTAAGGATT
TTTTTTATCTGAAATGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGT
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCG
ACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTAT
CTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC
GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA
GCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATC
TGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATGCTTAATCAGT
GAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGG
GAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATCAGCAATAAAC
CAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCG
TTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT
AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT
TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATG
CGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATT
GGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCC
AACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGA
ATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTC
AT

In red, *E. coli* murein lipoprotein promoter. In **bold**, (C327 to G46 of AJ536613), with the repeated hammerhead ribozyme domain **highlighted in yellow**, and their self-cleavage sites **underlined**. In orange, inserted between T245 and T246 of ELVd cDNA, group-I *Tetrahymena termophila* 26S rRNA intron with intron-exon permutation between T235 and C236; 10 nts of both flanking exons underlined. Highlighted in green, inverted repeat of an 83 nts fragment of the *DvSSJ1* gene. In green, non-permutated sequence of the same intron, with the 10 nts of both flanking exons underlined. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>pLPIE-DvSSJ1 (2694 bp)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTGCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGA
TATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGA
AAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCACCATTGTCCTGAAATTGCTGAAGTTGGTGATCAATTTGATAT

GTCTCATCTTGTACCGAACCGGATATCAAGGCTACTTCTTATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAG
TTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGA
AAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACG
GACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCG
GTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACT
GGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCAAGAAGTAGCCTTG
ATATCCGGTTCGGTACAAGATGAGACATATCAAATTGATCACCAACTTCAGCAATTTCAGGACAATGGTATGACTCTCTAA
ATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGT
TTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGC
CTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATGAAATCATCC
TTAGCGAAAGCTAAGGATTTTTTTTATCTGAAATGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCA
CAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCT
CGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCA
TAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCC
CGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCAC
TGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAG
AAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA
AACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTT
TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGT
GTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGGCTCC
AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC
TATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCAT
CGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCAT
GTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGT
TATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTC
ATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAAC
TTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGAT
GTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCA
AAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCAT
TTATCAGGGTTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In orange, group-I *Tetrahymena termophila* 26S rRNA intron with intron-exon permutation between T235 and C236; 10 nts of both flanking exons underlined. Highlighted in green, inverted repeat of an 83 nts fragment of the *DvSSJ1* gene. In green, non-permutated sequence of the same intron, with the 10 nts of both flanking exons underlined. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>p15LtRnlSm (5415 bp)

CCCGGGGGCGGCGGCCGCCCGCGAAATCGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATA
TTCTCAACATAAAAAACTTTGTGTAATACTTGTAACGCTGGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTA
ACTTTAAGAAGGAGATATACCATGTCGGTTTCGCATAGGGTCATTTACTCTTTCACTCATTACAAACTCTATAATCTCTCT
TCTTCTTTATCATCTTTGCCTTCTAGAATCTTCTTCCCTTTTCAATCTCCTTCCTTTCACACGTTCTCTTCACTCATGCCC
AACAATCAGGAAAGGGGTGGTTATGAAGGAAAAAAATGGCAAGTGAGGCCAAGTTCCAATAGGGTACCAGGCTCGTCTTCA
AATGTGGAACCTGTATCTGCTGCAACTGCTGAAGCCATTACCGACCGTCTAAAGTCCGTGGATATTACTGAAAGTGGTGCA
CAGTCTAGTGTTCCAGTCACATCTCTTCAGTTTGGCAGCGTTGGATTAGCACCCCAGTCACCTGTGCAACATCAAAAAGTA
ATCTGGAAACCCAAATCATATGGAACAGTGTCTGGAGCCCCAGTGGTTGAAGCTGGAAAAACACCAGTTGAACAAAAAAGT
GCTCTTTTAAGTAAATTATTCAAGGGTAATTTATTGGAAAATTTTACTGTAGATAACTCAACATTCTCGAGAGCCCAAGTA
AGGGCCACTTTCTACCCAAAATTTGAGAATGAGAAATCAGATCAGGAGATCAGGACAAGGATGATAGAGATGGTCTCCAAA
GGCTTGGCTATAGTCGAGGTCACACTTAAGCATTCTGGATCTCTTTTTATGTATGCTGGGCATGAAGGTGGAGCATATGCC
AAGAATAGCTTCGGGAATATCTATACTGCCGTTGGCGTCTTTGTTCTTGGACGGATGTTTCGTGAGGCATGGGGAACTAAA
GCAAGCAAGAAGCAAGCAGAGTTCAATGAGTTTCTTGAGCGCAATCGTATGTGCATATCAATGGAGTTGGTCACGGCAGTG
TTGGGGGACCACGGACAACGCCCACGAGATGATTATGCGGTTGTGACTGCAGTCACGGAGTTGGGAAATGGAAAACCAACT
TTCTATTCAACTCCCGATGTAATTGCTTTTTGCAGGGAATGGCGATTACCAACAAATCATGTATGGCTGTTCTCAACAAGG
AAATCAGTGACTTCCTTCTTTGCTGCGTATGATGCACTTTGCGAGGAAGGTACAGCAACCACCGTTTGCGAGGCTCTCAGC
GAAGTTGCTGATATTTCTGTACCTGGATCAAAAGACCATATAAAAGTGCAGGGTGAAATTTTGGAGGGTCTCGTGGCCCGC

ATCGTAAAACGTGAGAGCTCAGAGCATATGGAGCGGGTTCTGAGAGATTTTCCTCCTCCGCCATCAGAGGGTGAGGGTTTG
GACCTGGGACCTACGTTACGTGAAATTTGTGCTGCAAACAGATCAGAAAAGCAGCAAATAAAGGCACTTCTTCAGAGTGCT
GGCACGGCTTTCTGCCCGAATTATTTGGACTGGTTTGGAGATGAAAACTCTGGTTCACATTCAAGAAATGCTGATCGATCT
GTTGTCTCAAAGTTCTTACAATCACATCCTGCTGATCTTTATACAGGAAAAATACAGGAAATGGTTCGCTTGATGAGGGAA
AAGCGCTTTCCTGCTGCTTTCAAGTGTCATTATAACTTACATAAAATTAATGATGTATCGAGTAACAACCTGCCTTTCAAA
ATGGTGATCCATGTATATAGTGATTCAGGCTTCCGCCGGTACCAGAAAGAGATGAGGCACAAACCAGGACTATGGCCTTTG
TATCGAGGCTTTTTTGTTGACCTGGATTTATTCAAGGTCAATGAGAAGAAAACTGCTGAAATGGCAGGAAGCAACAATCAA
ATGGTAAAAAATGTGGAAGAGGACAACAGTTTAGCTGATGAAGATGCAAATCTGATGGTCAAGATGAAATTTCTTACTTAC
AAGTTGAGAACTTTTTTGATCCGTAATGGCTTGTCGACTCTTTTCAAAGAAGGACCTTCTGCGTATAAGTCTTATTACCTG
AGGCAAATGAAAATTTGGAATACTTCAGCAGCCAAGCAACGAGAACTCAGCAAGATGCTTGATGAATGGGCAGTATATATA
CGCAGAAAATATGGGAACAAACCATTGTCATCATCCACATACCTAAGTGAAGCTGAGCCTTTCCTTGAACAATATGCAAAG
CGTAGTCCACAAAATCATGCTTTGATAGGATCTGCTGGAAATTTTGTCAAAGTTGAAGATTTCATGGCTATTGTTGAAGGA
GAAGATGAAGAGGGTGATCTCGAGCCTGCGAAAGATATTGCTCCTTCAAGCCCTAGTATTTCCACCAGAGACATGGTGGCA
AAGAATGAGGGTCTCATTATTTTCTTTCCAGGAATACCAGGTTGTGCTAAATCTGCACTTTGTAAGGAAATACTGAATGCT
CCAGGAGGGCTTGGAGATGATCGACCAGTTAACAGTTTAATGGGTGATCTTATTAAAGGTAGATATTGGCAAAAAGTTGCT
GATGAACGTCGAAGAAAACCTTACTCGATCATGCTTGCTGACAAGAATGCACCAAATGAGGAAGTATGGAAACAAATTGAG
AACATGTGCCTAAGCACCGGAGCATCTGCTATTCCAGTTATACCTGATTCAGAAGGAACTGAAACTAATCCATTCTCTATT
GATGCACTTGCGGTTTTTATATTCCGAGTACTTCACCGTGTCAATCATCCGGGAAATCTTGACAAGTCATCTCCAAATGCT
GGATATGTGATGCTTATGTTTTATCACCTTTATGATGGAAAGAGCCGTCAGGAGTTCGAGAGTGAGCTTATTGAACGTTTT
GGATCGCTTGTCAGAATTCCTGTACTGAAACCTGAGAGGTCTCCTCTTCCGGATTCTGTGAGGTCTATTATCGAGGAGGGA
CTCAGTCTGTACAGACTTCATACAACGAAACATGGAAGATTGGAGTCTACAAAAGGGACATATGTACAAGAGTGGGTTAAA
TGGGAGAAGCAATTGAGAGATATTCTACTTGGAAATGCAGACTATCTCAATTCAATACAGGTTCCATTTGAATTTGCCGTT
AAAGAAGTCCTTGAACAACTGAAAGTTATTGCGAGGGGCGAATATGCAGTGCCTGCTGAGAAGAGGAAGCTAGGATCCATT
GTATTCGCCGCTATCAGCCTGCCAGTTCCAGAAATTCTAGGTCTTCTAAATGATCTAGCAAAGAAAGATCCAAAGGTTGGC
GATTTCATTAAGGACAAGAGCATGGAGAGCAGCATTCAGAAGGCCCATCTTACCCTGGCTCACAAGAGAAGTCACGGTGTC
ACTGCAGTTGCCAATTACGGTTCCTTTCTTCATCAAAAGGTGCCAGTAGACGTGGCTGCTTTGTTGTTCTCCGATAAATTG
GCTGCACTAGAAGCTGAGCCTGGCTCTGTTGAAGGTGAAAAGATCAATTCTAAAAACTCATGGCCCCATATCACATTATGG
TCTGGTGCAGGAGTTGCCGCAAAAGATGCCAATACACTACCACAGTTACTTTCCCAAGGGAAGGCTACCCGCATTGATATA
AATCCACCGGTCACTATAACTGGCACTCTCGAATTCTTTCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCC
CGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
GGTTTTTTGCTGAAAGGAGATCTGGCGGGGCCCGCGCTAGCGGAGTGTATACTGGCTTACTATGTTGGCACTGATGAGGGT
GTCAGTGAAGTGCTTCATGTGGCAGGAGAAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATTCCG
CTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTCGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCC
TGGAAGATGCCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATAGGCTCCGCCCCCCTGA
CAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGG
CGGCTCCCTCGTGCGCTCTCCTGTTCCTGCCTTTCGGTTTACCGGTGTCATTCCGCTGTTATGGCCGCGTTTGTCTCATTC
CACGCCTGACACTCAGTTCCGGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCCGTTCAGTCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACATGCAAAAGCACCACTGGCAGCAGCCACTGGTAATT
GATTTAGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGCTCCTCCA
AGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTCA
GAGCAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAATTACGCCCCGCCCTGCCACTCATCGCAG
TACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGGCATGATGAACCTGAATCGCCAGCGGCATC
AGCACCTTGTCGCCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAA
TCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGAAATAGGCCAGG
TTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGAT
GAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATT
GCCATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTTTC
TTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCA
AAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCT
CCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAA

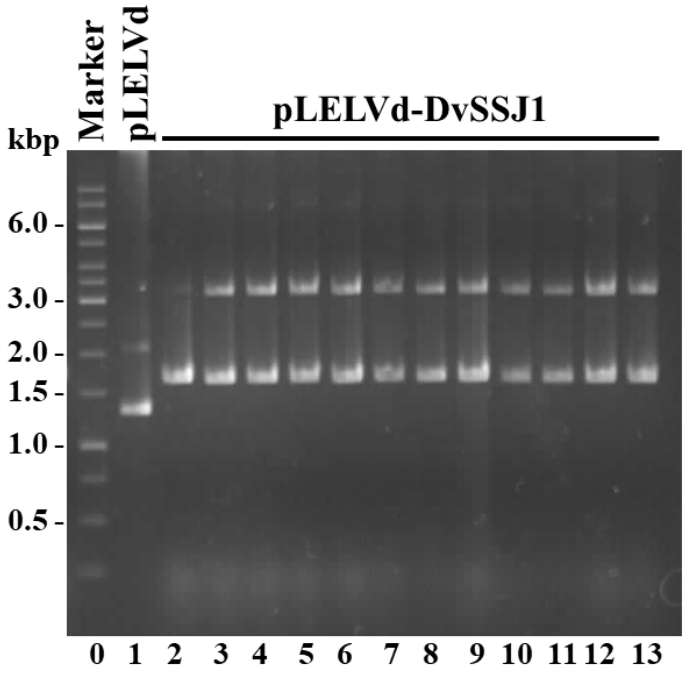
In red, *E. coli* murein lipoprotein promoter. In **bold**, chloroplastic isoform of the eggplant tRNA ligase (JX0225157), with the theoretical amino-terminal transit peptide **highlighted in green**, the carboxyterminal hexahistidine tag in blue and the Start and Stop codon **underlined**. In fucsia, T7 bacteriophage terminator. In gray, p15A replication origin. Highlighted in light grey, chloramphenicol resistance gene (in inverse orientation), with the promoter highlighted in dark gray).

>p15CAT (1634 bp)

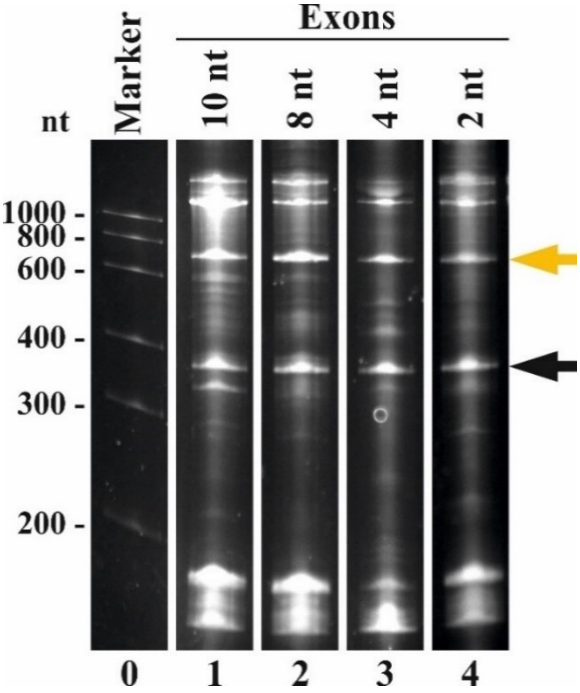
CCCGGGGGCGGCGGCCGCGGCGGACGTCGGCGCCTAAGGGGCGAGATCTGGCGGGGCCCGCGCTAGCGGAGTGTATACTGG
CTTACTATGTTGGCACTGATGAGGGTGTCAGTGAAGTGCTTCATGTGGCAGGAGAAAAAAGGCTGCACCGGTGCGTCAGCA
GAATATGTGATACAGGATATATTCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTCGACTGCGGCGAGCGGAAAT
GGCTTACGAACGGGGCGGAGATTTCCTGGAAGATGCCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAAGCCG
TTTTTCCATAGGCTCCGCCCCCCTGACAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTA
TAAAGATACCAGGCGTTTCCCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCCTGCCTTTCGGTTTACCGGTGTCATTCCG
CTGTTATGGCCGCGTTTGTCTCATTCCACGCCTGACACTCAGTTCCGGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCA
CGAACCCCCCGTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACATGCAAAAGC
ACCACTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGA
CAAGTTTTGGTGACTGCGCTCCTCCAAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGC
CCTGCAAGGCGGTTTTTTCGTTTTCAGAGCAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAATT
ACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGGCAT
GATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGA
AGTTGTCCATATTGGCCACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAA
TAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGAAAT
CGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCC
ATATCACCAGCTCACCGTCTTTCATTGCCATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGG
CCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTAC
ATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTT
TTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTCATTAT
GGTGAAAGTTGGAA

In gray, p15A replication origin. Highlighted in light grey, chloramphenicol resistance gene (in inverse orientation), with the promoter highlighted in dark gray). Highlighted in yellow, polylinker with recognition sites for NotI (underlined) and BglII (underlined).

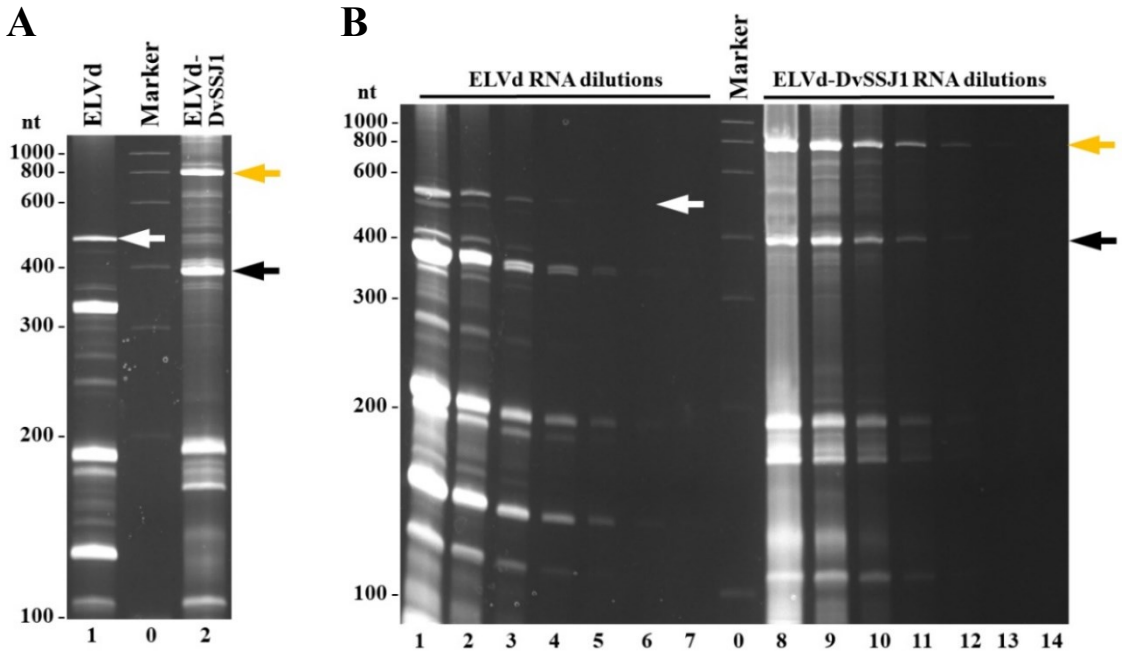
Supplemental Figures S1-S5



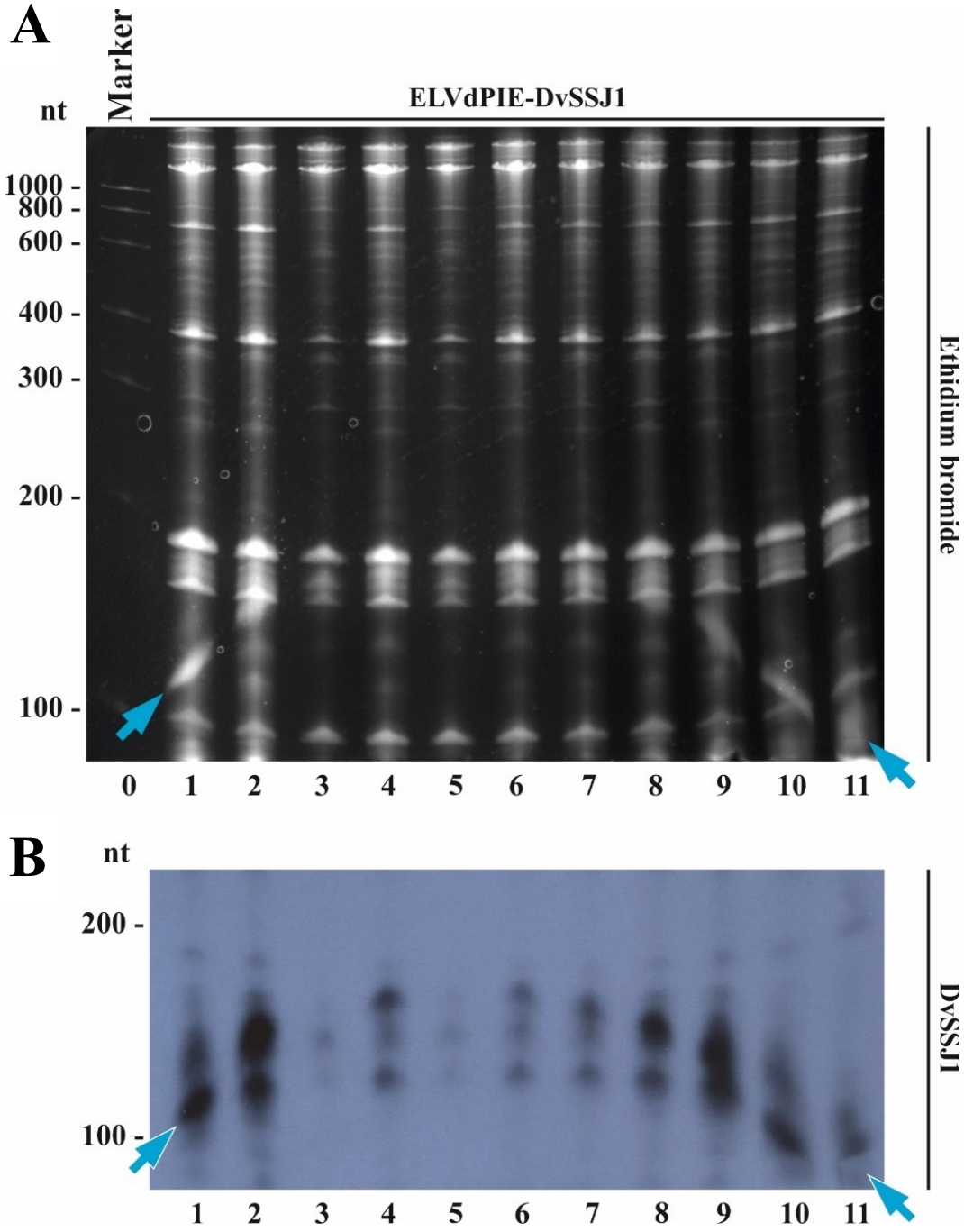
Supplemental Figure S1. Construction of expression plasmids to produce *DvSSJ1*-derived dsRNA in *E. coli*. Plasmids purified from independent *E. coli* clones were separated by electrophoresis through an agarose gel, which was stained with ethidium bromide. Lane 0, DNA marker ladder with some of the sizes in bp on the left; lane 1, control plasmid pLELVd expressing an empty ELVd; lanes 2 to 13, plasmids pLELVd-DvSSJ1 to express the *DvSSJ1*-derived dsRNA on an ELVd scaffold obtained from 12 independent *E. coli* colonies.



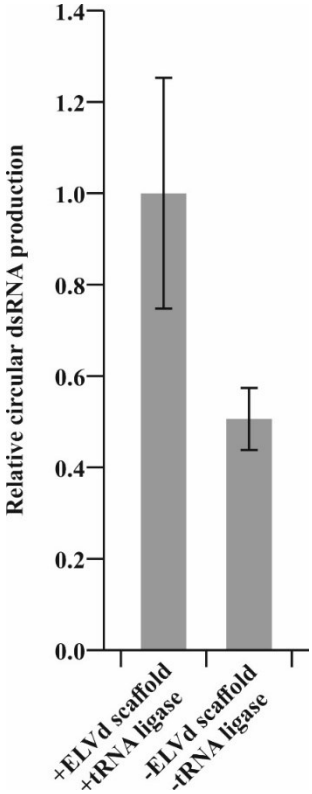
Supplemental Figure S2. Effect of exon size in *T. thermophila* intron processing in the ELVd-based system to produce dsRNA in *E. coli*. Aliquots of RNA preparations from *E. coli* clones cotransformed with pLtRnlSm and a series of pLELVd derivatives to produce a 100-bp dsRNA, in which the exons that flank the *T. thermophila* intron are increasingly shorter, as indicated, were separated by denaturing PAGE. The gel was stained with ethidium bromide. Lane 0, RNA marker with sizes (in nt) on the left; lanes 1 to 4, RNAs from constructs with 10, 8, 4 and 2-nt exons, respectively. Orange and black arrows point the positions of the recombinant ELVd-dsRNA and the spliced introns, respectively.



Supplemental Figure S3. Large scale RNA preparations produced in *E. coli* by means of the viroid-based system and used in the WCR bioassay. RNAs were separated by denaturing PAGE and the gels stained with ethidium bromide. (a and b) Lane 0, RNA marker ladder with sizes in nt on the left. (a) Lanes 1 and 2, large-scale RNA preparations from *E. coli* transformed with pLtRnlSm and pLELVd or pLELVd-DvSSJ1, respectively. (b) Dilution analysis of the ELVd (lanes 1 to 7) and the ELVd-DvSSJ1 (lanes 8 to 14) RNA preparations. White, orange and black arrows point to ELVd, ELVd-DvSSJ1 and spliced-intron RNAs, respectively.



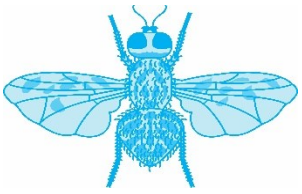
Supplemental Figure S4. Analysis of the recombinant circular dsRNA. RNApreparations from different *E. coli* clones (lanes 1 to 11) co-transformed with pLtRnlSm and pLELVdPIE-DvSSJ1 were separated by denaturing PAGE. The gel was (a) stained with ethidium bromide and (b) the RNA transferred to a membrane and hybridized with a ^{32}P-labelled probe to detect DvSSJ1 RNA. Lane 0, RNA marker with sizes in nt on the left. Blue arrows point to the recombinant circular dsRNA that exhibits an inverted smile migration across the gel.



Supplemental Figure S5. Effect of the the ELVd scaffold and the tRNA ligase on accumulation of a recombinant circular dsRNA. RNA preparations from *E. coli* clones cotransformed with pLtRnlSm and a pLELVdPIE-derivative to produce a 100-bp dsRNA or the empty ligase plasmid (p15CAT) and a pLPIE-derivative (no ELVd scaffold) to produce the same 100 bp dsRNA were separated by denaturing PAGE. After staining the gels with ethidium bromide, recombinant circular dsRNA accumulation was quantified (in fluorescence arbitrary units) using an image analyzer. Normalized average fluorescence is plotted. Error bars represent standard deviation (n = 5).

Supplemental Table S1. Primers used in the PCR amplifications to build expression plasmids pLELVd-DvSSJ1, pLELVdPIE-DvSSJ1 and pLPIE-DvSSJ1, and to generate the DNA template for *in vitro* transcription.

Name	Sequence (5' to 3')
D2623	ACCATTGTCCTGAAATTGCTGAAGTTGGTGATCAATTTGATATGTCTCA
D2624	AAGAAGTAGCCTTGATATCCGGTTCGGTACAAGATGAGACATATCAAAT
D2625	CCTCTCCCCCTCCCAGGTACTATCCCCTTACCATTGTCCTGAAATTG
D2626	TTTAGAGAGTCATAAGAAGTAGCCTTGATATCCG
D2627	AAGGCTACTTCTTATGACTCTCTAAATAGCAATATTTAC
D2628	AAGGCTACTTCTTGGCTACCTTACGAGTACTCC
D2629	TCGTAAGGTAGCCAAGAAGTAGCCTTGATATCCG
D2630	ACCCACCCTCCTAGGGAACACATCCTTGAACCATTGTCCTGAAATTG
D2936	AAGGGGATAGTACCTGGGAG
D2937	ACCATTGTCCTGAAATTGCTG
D2940	ACCATTGTCCTGAAATTG
D2941	TCAAGGATGTGTTCCCTAG
D3606	CTTCTGTTGATATGGATG
D3285	CAGCGTTACAAGTATTACAC
D3607	GAAATCATCCTTAGCGAAAGC
D3608	ATCTGTTGACTTAGGACTTGGC



Chapter III

RNAi-mediated silencing of Mediterranean fruit fly *(Ceratitis capitata)* endogenous genes using orally-supplied circular double-stranded RNAs produced in *Escherichia coli*[1]

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Author Contributions:

All authors participated in work conception and design. BO, ME and MPH performed the experiments. All authors carried out data analysis and interpretation of the results. BO wrote a first draft and all authors participated in manuscript writing. All authors read and approved the final manuscript.

[1]To be submitted.

Abstract

The Mediterranean fruit fly (medfly), *Ceratitis capitata* Wiedemann, is a major pest affecting fruit and vegetable production worldwide, whose control is mainly based on insecticides. Double-stranded RNA (dsRNA) able to down-regulate endogenous genes, thus affecting essential vital functions via RNA interference (RNAi) in pests and pathogens, is envisioned as a more specific and environmentally-friendly alternative to traditional insecticides. However, this strategy has not been explored in medfly yet. Here, we screened seven candidate target genes by injecting in adult medflies gene-specific dsRNA hairpins transcribed *in vitro*. Several genes were significantly down-regulated, resulting in increased insect mortality when compared to flies treated with a control dsRNA homologous to the green fluorescent protein (GFP). Three of the dsRNAs, homologous to the beta subunit of ATP synthase *(ATPsynbeta)*, a vacuolar ATPase *(V-ATPase)*, and the ribosomal protein S13 *(RPS13)*, were able to halve the probability of survival in only 48 h after injection. We then produced new versions of these three dsRNAs and that of the GFP control as circular molecules in *Escherichia coli* using a two-self-splicing-intron-based expression system and tested them as orally-delivered insecticidal compounds against medfly adults. We observed a significant down-regulation of *V-ATPase* and *RPS13* mRNAs (approximately 30 and 90%, respectively) compared with the control medflies after three days of treatment, although no significant mortality was recorded. In sum, we report the potential of dsRNA molecules as oral insecticide in medfly.

Keywords

Medfly, Tephritidae, pest control, bait treatment, RNA interference, double-stranded RNA, circular RNA, self-splicing intron, vacuolar ATPase, ribosomal protein S13, RPS13, *Escherichia coli*.

Key message

Orally delivered recombinant circular dsRNAs produced in *Escherichia coli* induce strong silencing of targeted genes in *Ceratitis capitata*. This result opens the possibility to use RNA interference-based crop protection strategies against this insect pest.

Introduction

The Mediterranean fruit fly (medfly) *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is a devastating agricultural pest with a wide range of unrelated host species, including hundreds of fruit and vegetables for human consumption, and an amazing capacity to disperse, adapt and invade new ecological niches in tropical and temperate areas (Dias et al., 2022). The medfly damage is associated with egg laying inside ripe fruits and vegetables and the subsequent larval feeding, causing annual losses in the order of billions of dollars, which result from reduced production, cost of control measures, and lost markets (Shelly et al., 2014). Strict quarantine regulations have been established to limit the spread between regions (Urbaneja et al., 2020). Medfly control in crops has been traditionally based on chemical pesticides (Urbaneja et al., 2009). However, some species-specific and eco-friendly alternatives are also being used or developed as part of integrated pest management (IPM) programs (Monzó et al., 2012; Juan-Blasco et al., 2014; Llopis-Giménez et al., 2017).

The induction of RNA interference (RNAi) in pests and pathogens is currently considered a promising strategy for more specific and environmentally-friendly crop protection. RNAi is a highly conserved mechanism in eukaryotic organisms for gene regulation and protection against harmful exogenous and endogenous genetic elements. It is efficiently triggered by different types of RNAs, including double-stranded RNA (dsRNA), highly homologous to the regulated genetic factors. Intake of dsRNAs

homologous to endogenous genes in pests and pathogens may induce downregulation of these genes, potentially affecting essential physiological processes in those organisms. In some, such as nematodes, insects, and other arthropods, cuticle and midgut cells can intake exogenously supplied dsRNA molecules present in the environment and ingested food (Araujo et al., 2006; Turner et al., 2006; Wang et al., 2011; El-Shesheny et al., 2013; Killiny et al., 2014). Once in the cell, the dsRNAs are diced by the RNase-III-type enzyme Dicer-2 to generate small interfering RNAs (siRNAs) that guide the slice of complementary RNAs through the activity of the RNA-induced silencing complex (RISC) that contains the endoribonuclease Argonaute (Ago)-2, a process that likely results in substantial degradation of the endogenous RNA. In arthropods, the RNAi effect may spread from the initial cells to the rest of the organism producing a systemic effect (Tomoyasu and Denell, 2004; Li et al., 2011; Bolognesi et al., 2012), although unlike in nematodes, fungi, or plants, the RNAi response is not amplified by endogenous RNA-dependent RNA polymerases (RdRp) (Roignant et al., 2003; Li et al., 2018; Pinzón et al., 2019).

Several lines of evidence suggest the potential susceptibility of medfly to RNAi-mediated control. dsRNA-mediated silencing has been extensively reported in species of Tephritidae through diverse delivery systems, developmental stages, and target genes (Maktura et al., 2021). The transcriptomic analysis of *C. capitata* revealed that key genes involved in the RNAi pathways, such as Dicer and Ago, are actively expressed (Salvemini et al., 2014; Papanicolaou et al., 2016). In addition, the functionality of the RNAi pathway induced by exogenous RNAs has been experimentally demonstrated as sterile insects of both sexes have been obtained after knocking down sex determination genes by injecting specific dsRNA directly into embryos (Pane et al., 2002; Salvemini et al., 2009; Gabrieli and Scolari, 2016; Gabrieli et al., 2016). Due to the sheltered oviposition, larval development inside the fruit, and the pupation in the soil, in-field control of species from Tephritidae relies almost

exclusively on adults. These strategies may be likely based on supplementing food lures in artificial baits with target-specific dsRNAs (Guo et al., 2021). Thus, two essential aspects for using RNAi as a pesticide in *C. capitata* remain undetermined: the susceptibility of adult flies to RNAi-mediated post-transcriptional knockdown and whether orally supplied dsRNAs can achieve RNAi. This is especially relevant as RNAi efficiency is highly variable not only between insect orders and even close-related species but also between juvenile and adult stages (Araujo et al., 2006; Griebler et al., 2008; Guo et al., 2015; Pereira et al., 2016; Powell et al., 2017) –larvae are usually more sensitive when subjected to equal dsRNA treatments– and delivery methods, with several reports of effective silencing by injection coupled with complete oral insensitivity (Luo et al., 2013; Wynant et al., 2014; Nishide et al., 2021). The variation is related to insect intrinsic factors, such as nuclease-mediated dsRNA degradation in saliva, gut, and hemolymph (Garbutt et al., 2013; Ren et al., 2014; Wynant et al., 2014; Wang et al., 2016; Sharma et al., 2021; Li et al., 2022), variable cellular uptake and intercellular transport activities, and even differences in expression of RNAi machinery (Dowling et al., 2016; Cooper et al., 2019; Arraes et al., 2021).

An additional challenge for implementing RNAi strategies is still the cost-effective production and formulation of target-specific dsRNAs. Recent strategies involve the production of dsRNAs in microbiological factories and its encapsulation in nanoparticles to overcome the low stability of RNA against biotic and abiotic stresses and to improve their absorption (Whyard et al., 2009; Zhang et al., 2010; Das et al., 2015; Taning et al., 2016). In this regard, we recently developed a strategy to overproduce dsRNAs in *Escherichia coli* by emulating the structural characteristics of plant pathogenic RNAs, namely viroids (Navarro et al., 2021), whose high compaction and circular structure make them resistant to degradation and prone to bacterial accumulation (Ortolá et al., 2021). The system also takes advantage of the autocatalytic properties of group-I introns (Zaug and Cech, 1986), which allows plasmid

stabilization and circularization of dsRNA molecules without incorporating viroid-derived sequences in the resulting molecules. We have speculated that the circularity and compaction of the resulting circular dsRNA molecules may also be beneficial to improving the amount of RNA that reaches cells in field application.

Here, we used the circular dsRNA production system to analyze the effectiveness of RNAi control strategies in medfly. Screening of seven target genes for *C. capitata* adults showed high susceptibility to three microinjected *in vitro*-produced hairpins homologous to *ATPsynbeta*, a component of the channel that produces ATP using the energy of proton gradient; *V-ATPase*, a vacuolar proton pump that produces a proton gradient with ATP consumption; and *RPS13*, encoding a ribosomal protein that is part of the 40S subunit. The latter two genes were also significantly and specifically repressed when evaluated via orally delivered circular dsRNA produced in *E. coli*. Thus, we have established a starting point for a novel medfly control strategy based on orally-delivered dsRNAs that, combined with other IPM strategies, may provide more sustainable crop protection against this devastating pest.

Materials and methods

Insect rearing and maintenance

Medfly adults used in the experiments described below came from a laboratory colony maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada (Valencia, Spain). The medfly populations' maintenance and diet followed the methodology previously described (Jacas et al., 2008). The environmental chamber conditions were 25 ± 2°C, 60–70% relative humidity with a photoperiod of 14:10 (L:D) h. This colony is annually reinforced with regular additions of flies emerging from pupae collected on naturally infested fruit in the field. For each experiment, to obtain a cohort

of adults, approximately 1,000 24-h-old pupae were collected and kept in transparent polymethylmethacrylate cages (20 x 20 x 20 cm) until adult emergence. Unfed 24-hour-old adults were used in all experiments.

Construction of plasmids to express dsRNA against *C. capitata*

We constructed a series of plasmids for the *in vitro* transcription of dsRNAs homologous to seven *C. capitata* genes and the enhanced green fluorescent protein (GFP) cDNA as a negative control. Total RNA from *C. capitata* adult flies, purified using silica-gel columns (Zymo Research), was used as a template in reverse transcription (RT) reactions (RevertAid reverse transcriptase, Thermo Scientific) using an oligo(dT) primer. The resulting cDNAs were used in polymerase chain reactions (PCR) with specific primers (**Supplemental Table S1**) and the Phusion high-fidelity DNA polymerase (Thermo Scientific) to amplify each target sequence (400-500 bp) in sense and antisense orientations. A region of similar size of the GFP cDNA in both orientations was also obtained. We also amplified the cDNA corresponding to the *Tetrahymena thermophila* 26S rRNA type-I intron, including 10 nt of the flanking exons (positions 43 to 475 of GenBank V01416.1). Each inverted repeat and the autocatalytic intron were inserted into the *Bpi*I-digested plasmid pMT7 (**Supplemental Dataset S1**) by the Gibson assembly reaction (Gibson et al., 2009), using the NEBuilder HiFi assembly master mix (New England Biolabs). The assembled products were electroporated in *E. coli* DH5α. Recombinant resistant clones were selected in plates of lysogeny broth (LB) medium containing ampicillin (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 1.5% agar, and 50 µg/ml ampicillin), and grown in liquid LB media (as above minus 1.5% agar), at 37°C with vigorous shaking (225 revolutions per minute, rpm). Plasmids were purified (NucleoSpin Plasmid, Macherey-Nagel), and sequences were experimentally confirmed by gel electrophoresis and sequencing (3130xl Genetic Analyzer, Life Technologies). We additionally built

plasmids to express circular dsRNAs in *E. coli*. For this purpose, we sequentially digested the plasmid pLPIE-LacZ (**Supplemental Dataset S1**) with *Esp*3I and *Bpi*I and added in two consecutive Gibson assembly reactions the target gene or the GFP sequences in each orientation. With the same methodology, we also constructed plasmids for expressing circular RNA molecules under the control of the inducible T7 promoter in bacteria. The complete sequences of all resulting plasmids are detailed in **Supplemental Dataset S1**.

In vitro production of dsRNA

For the *in vitro* synthesis of dsRNA hairpin molecules, pMT7 derivatives were linearized with *BsaI* (BsaI-HFv2; New England Biolabs), purified in silica columns, and used as templates for *in vitro* transcription with bacteriophage T7 RNA polymerase (Roche Life Science). The quality and concentration of the produced dsRNA were electrophoretically assessed by non-denaturing polyacrylamide gel electrophoresis (PAGE) in 5% polyacrylamide gels (37.5:1 acrylamide:N,N'-methylenebisacrylamide) in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) for 1.5 h at 75 mA. Gels were stained by shaking in 200 ml of 1 µg/ml ethidium bromide for 15 min and, after washing three times with water, documented using a UV transilluminator (UVIdoc-HD2/20MX, UVITEC). Total RNAs from the *in vitro* transcription reactions were extracted by mixing with one volume of a 1:1 (v/v) mix of phenol (saturated with water and equilibrated at pH 8.0 with Tris-HCl, pH 8.0) and chloroform, and then re-extracted with chloroform. The aqueous phases were then precipitated overnight at -20°C with 0.1 volume of sodium acetate and 2.5 volumes of 96% ethanol. After centrifugation (15 min, 13,000 rpm at 4ºC), the RNA pellet was washed with cold 70% ethanol, dried, and resuspended in 20 µl of H_2O. Serial dilutions of a purified and spectrophotometrically quantified *E. coli* 5S rRNA were used as concentration standards.

Production of circular dsRNA in *E. coli*

An RNase III-deficient *E. coli* BL21(DE3) derivative [BL21(DE3)Δrnc], generated according as previously described (Reisch and Prather, 2015), was electroporated (Eporator, Eppendorf) with pLPIE-derivatives and transformed clones were selected in plates with ampicillin. Individual recombinant *E. coli* clones were grown in 50-ml tubes containing 5 ml of Terrific Broth (TB) medium (12 g/l tryptone, 24 g/l yeast extract, 0.4% glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4) containing ampicillin (50 µg/ml), at 37ºC with vigorous shaking (225 rpm). In some cases, induction of dsRNA expression under the T7 promoter was carried out by growing *E. coli* cells to an optical density at 600 nm (OD_{600}) of 0.6 and then adding isopropyl thiogalactopyranoside (IPTG) to 0.4 mM. At the required time (24 h), bacteria in 2 ml of the cultures were sedimented by centrifugation, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 50 µl of a 1:1 (v/v) phenol:chloroform was added and vigorously mixed by vortexing to break cells. After centrifugation, the aqueous phase containing total bacterial RNA was recovered and frozen. Aliquots of the RNA preparations were analyzed by PAGE as previously explained. For large-scale production of circular dsRNA, 2-l baffled Erlenmeyer flasks with 250 ml of TB buffer containing ampicillin were used. After 24-h culture, cells were sedimented (8000 rpm for 15 min) and resuspended in 50 ml of H_2O. After the second sedimentation under the same conditions, cells were resuspended in 10 ml buffer 50 mM Tris-HCl, pH 6.5, 0.15 M NaCl, and 0.2 mM EDTA. The cells were mixed with one volume of a 1:1 (v/v) phenol:chloroform and broken by vigorous vortexing. Total bacterial RNA contained in the aqueous phase was recovered after centrifugation (8000 rpm for 15 min) and re-extracted with one volume of chloroform. The RNA was then precipitated, adding 0.1 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of ethanol, and resuspended in H_2O. The RNA was re-precipitated with one volume of isopropanol. Aliquots of the RNA preparations were analyzed by electrophoresis, as previously explained.

Injection of dsRNAs and feeding assays in *C. capitata* adults

For the study of the effect of dsRNA by injection, in each knockdown experiment, medfly adults less than 24-h-old were cold anesthetized and injected intrathoracically with 0.5 µg of dsRNA using a Drummond Nanoject II microinjector and a micromanipulator (Drummond Scientific Company, Broomall, PA) as described previously (Pérez-Hedo et al., 2013). To manipulate adults, groups of about 10 individuals, either males or females, were placed in Petri dishes of 140 mm in diameter and were cold anesthetized by placing them 1 min in a -15°C freezer. Once removed from the freezer and until use, they were kept anesthetized by placing the Petri dish on ice. This process was repeated until the necessary number of repetitions was achieved. Each dsRNA was injected into 30 males and 30 females. Both males and females were grouped into three biological replicates of 10 individuals each and placed in a plastic cage (15 x 7 x 10 cm), with a hermetic lid having a mesh area of 12 x 8 cm for ventilation (Urbaneja et al., 2009). Medfly adults were fed with sugar placed on a 1.5-ml microcentrifuge tube lid fixed to the bottom of the cage with plasticine. In all experiments, mortality of medfly adults was evaluated daily until the 30th day after adults' introduction. Additionally, 72 hours after the adult injection, 3 live individuals were removed from each cage, placed in 1.5 tubes and immediately frozen by immersion in liquid nitrogen. On these adults, expression levels of the seven targeted genes at 72 h post injection were determined by reverse transcription (RT)-quantitative PCR (qPCR) relative to alpha tubulin.

Following microinjection assays, the three dsRNAs that resulted in higher mortality (ATPsynbeta, V-ATPase, and RPS13 genes) were selected and administered orally to adult medflies. Equal amounts of circular dsRNAs with homology to the three endogenous genes or GFP, as a control, produced in *E. coli,* were dissolved in 30% sucrose and used as a sole nutritional source for adult medflies. Following the similar methodology described above, each dsRNA was orally offered to 30 males and 30

females. Both males and females were grouped into three biological replicates of 10 individuals each, as indicated above. Adult medflies were fed for three days with the 30% sucrose solutions containing circular dsRNAs at 1 µg/µl. With the help of a micropipette, a 10 µl droplet was placed on a 1.5-ml microcentrifuge tube lid fixed to the bottom of the cage with plasticine. Diet solutions were replaced daily. Transcript levels 72 h post-treatment were analyzed in six randomly picked flies of both sexes as previously detailed. In all experiments, the mortality of medfly adults was evaluated daily until the 30th day after adults' introduction.

Analysis of gene silencing by RT-qPCR

Total RNA from *C. capitata* adults treated with gene-specific or control dsRNA was purified from frozen flies 72 h post-injection using silica-gel columns (Zymo Research). Aliquots (500 ng) of each purified RNA were used to remove genomic DNA contamination and first-strand cDNA synthesis (iScript gDNA Clear cDNA Synthesis, Bio-Rad). cDNAs were used in qPCR amplification with target-specific primers (D4077 to D4090) in a 96-well plate using a CFX96 thermal cycler (BioRad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). All primers used for these analyses are in **Supplemental Table S2**. PCR amplifications were performed with the following cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 60°C for 15 s. In all cases, a melting analysis was performed to confirm the specificity of the qPCR amplicons (from 65°C to 95°C in 0.5°C increments with a hold time of 5 s for each read). Alpha-tubulin (amplified with specific primers D3007 and D3008) was used as a reference gene for data normalization (Sagri et al., 2017). The qPCR analysis was based on three biological replicates (individual flies) for each treatment and three technical replicates for each biological replicate. The data were analyzed with the Bio-Rad CFX manager software using the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of target genes compared to the control. An unpaired one-tailed t-test was used to determine

statistical significance. The amplification efficiency for each gene was evaluated with a standard curve.

Data analysis

The survival of the adult medflies in both RNAi experiments (microinjection and oral feeding) was evaluated using Kaplan-Meier survival analyses. Adult survival curves of the time to death were plotted to reflect the mortality due to the corresponding RNAi treatment. Comparisons of the survival curves of each gene and control treatment were tested for statistical significance (Gehan-Breslow-Wilcoxon tests; $P<0.05$). Data of gene expression quantification were analyzed using a one-tailed Student's t-test ($P<0.05$). Statistical analyses were performed using GraphPad Prism Software (San Diego, CA, USA). All data are reported as means ± SE.

Results

Selection of *C. capitata* candidate target genes and *in vitro* transcription of homologous RNA hairpins

Since there were no previous studies available on *C. capitata*, we first selected seven candidate genes aiming to identify suitable targets for robust RNAi-mediated control of this insect. The selection was based on previous successful reports of increased mortality in other dipteran and insect species: *Snf7* (Das et al., 2015; Dhandapani et al., 2020), *RyR* (Majidiani et al., 2019; Gurusamy et al., 2021), *Nep4* (Sitnik et al., 2014; Ohsako et al., 2021), *IAP2* (Gurusamy et al., 2021), *ATPsynbeta* (Prentice et al., 2017; Castellanos et al., 2019), *V-ATPase* (Baum et al., 2007; Cagliari et al., 2020; Rahmani and Bandani, 2021), and *RPS13* (Taning et al., 2016, 2018; Castellanos et al., 2019). Target regions between 400 and 500 nt in the coding sequences of these

genes were selected, taking into account their essentiality and specificity. In addition, we selected a GFP cDNA fragment of similar size as a negative control.

By *in vitro* transcription, we produced hairpin RNAs that included dsRNAs regions homologous to the selected sequences in the abovementioned target genes and the control. Based on our previous work on the production of dsRNA in *E. coli* (Ortolá et al., 2021), we constructed a series of plasmids in which a single bacteriophage T7 promoter controls the transcription of an inverted repeat of each target gene. In these plasmids, the inverted repeats were separated by a cDNA corresponding to the self-splicing type-I *Tetrahymena thermophila* 26S rRNA intron, plus 10 nt of both native flanking exons. This intron was observed to self-splice efficiently out of the primary transcript during the *in vitro* transcription reaction. While the intron cDNA separates the long inverted repeats in the plasmids to increase stability, efficient self-splicing produces a hairpin RNA composed of two perfect complementary strands separated by a 20-nt loop derived from exon fragments (**Figure 1A**). Aliquots of the *in vitro* transcripts were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Analysis showed prominent bands corresponding to RNA species that migrated between the 500 and 600-bp DNA markers and that likely correspond to the properly processed RNA hairpins (**Figure 2**).

Injection of long dsRNAs in *C. capitata* adults triggers knockdown of target genes and increased mortality

Aliquots (0.5 µg) of the purified hairpin RNAs were injected into the hemolymph of adult medflies of both sexes. Three live flies of each sex were randomly selected and frozen 72 h after injection to analyze the RNAi response to the injected dsRNAs by RT-qPCR (**Figure 3A**). Pooled data (flies per treatment and sex) were analyzed with an unpaired one-tailed t-test. Five of the seven tested dsRNAs significantly

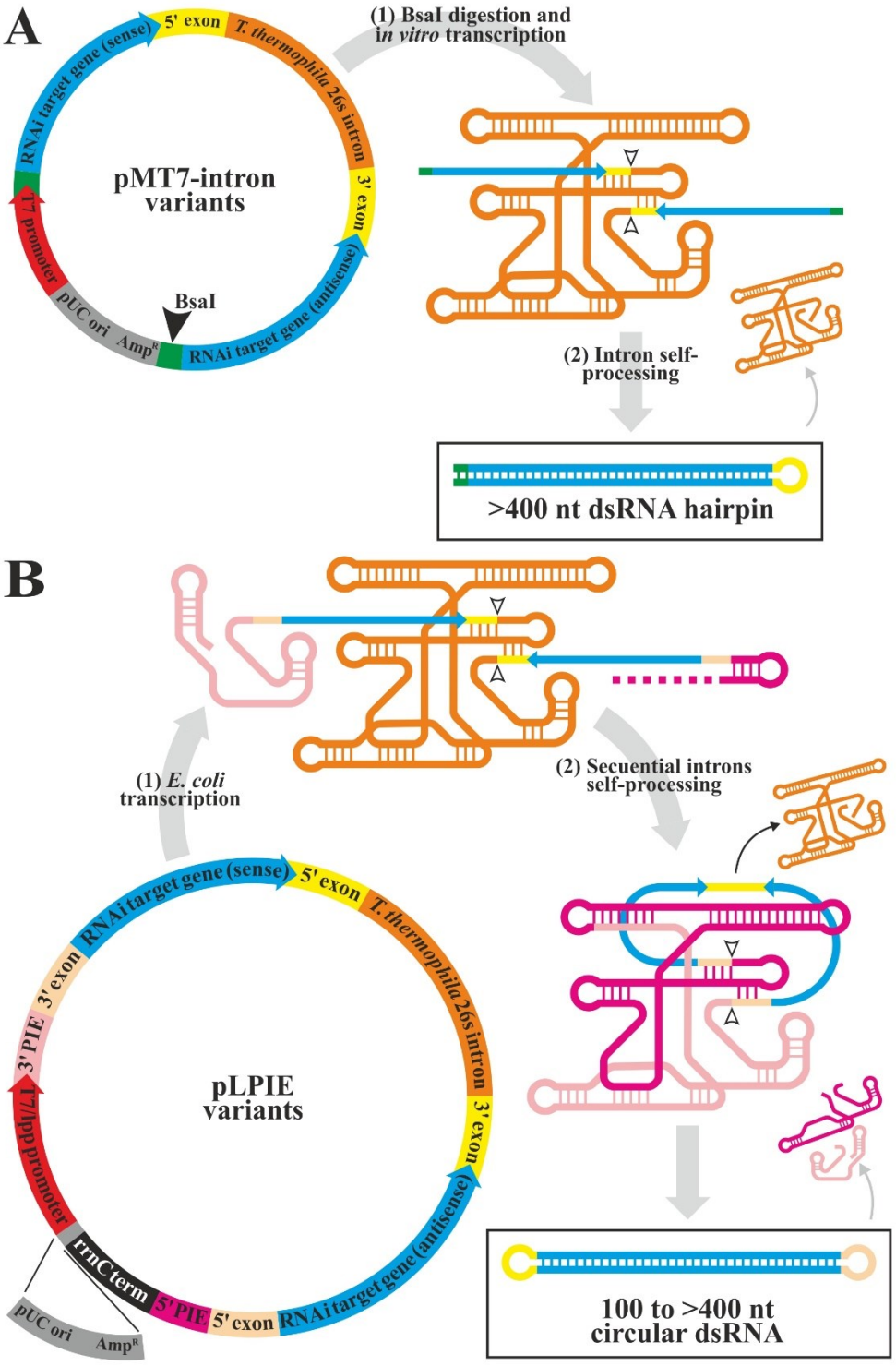


Figure 1. Schematic representation of the intron-mediated strategies for the (A) *in vitro* production hairpin dsRNAs or (B) circular dsRNAs in *E. coli* against *C. capitata* genes. (A and B) A *Tetrahymena thermophila* autocatalytic intron (plus 10-nt flanking exons) stabilizes the plasmids containing inverted repeats; the intron is entirely removed from the final transcript, generating a hairpin with a short 20-nt loop containing both exon sequences. (B) For circular dsRNA generation, the intron-separated inverted repeats are placed between two halves of a second autocatalytic intron with intron-exon permutation. The central regular intron is likely processed prior to the permuted one, possibly while the antisense direction of the inverted repeat is still being

transcribed, hence bringing both halves of the introns closer to each other. Then, the second intron is self-spliced, releasing the dsRNA as a circular molecule, capped at both ends by the exon fragments, as the 3' end of the second exon is covalently linked with the 5' end of the first exon. (A and B) Schemes are not at scale.

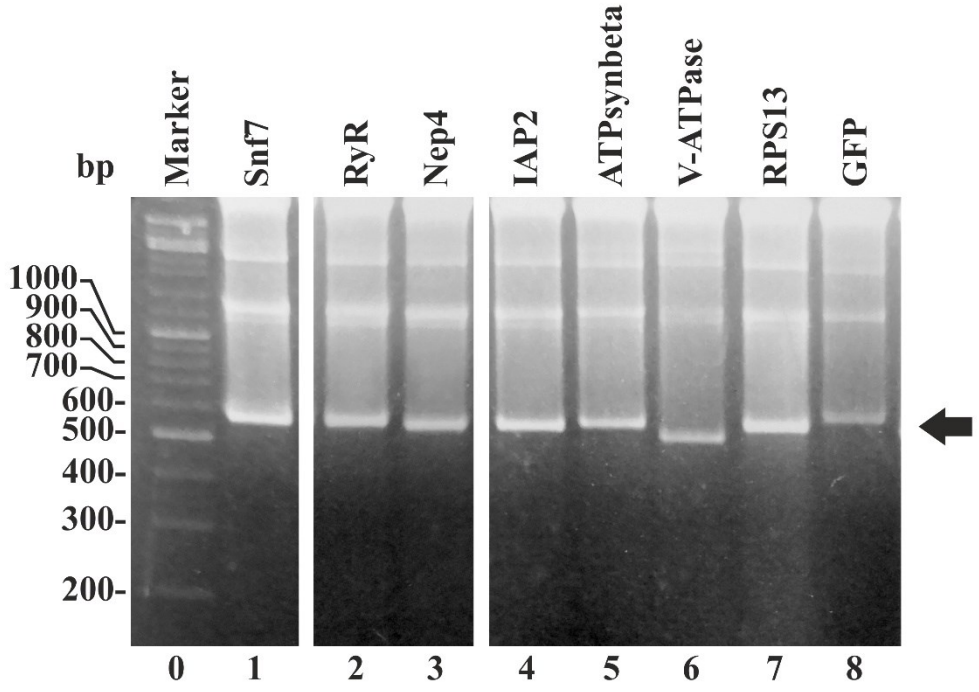
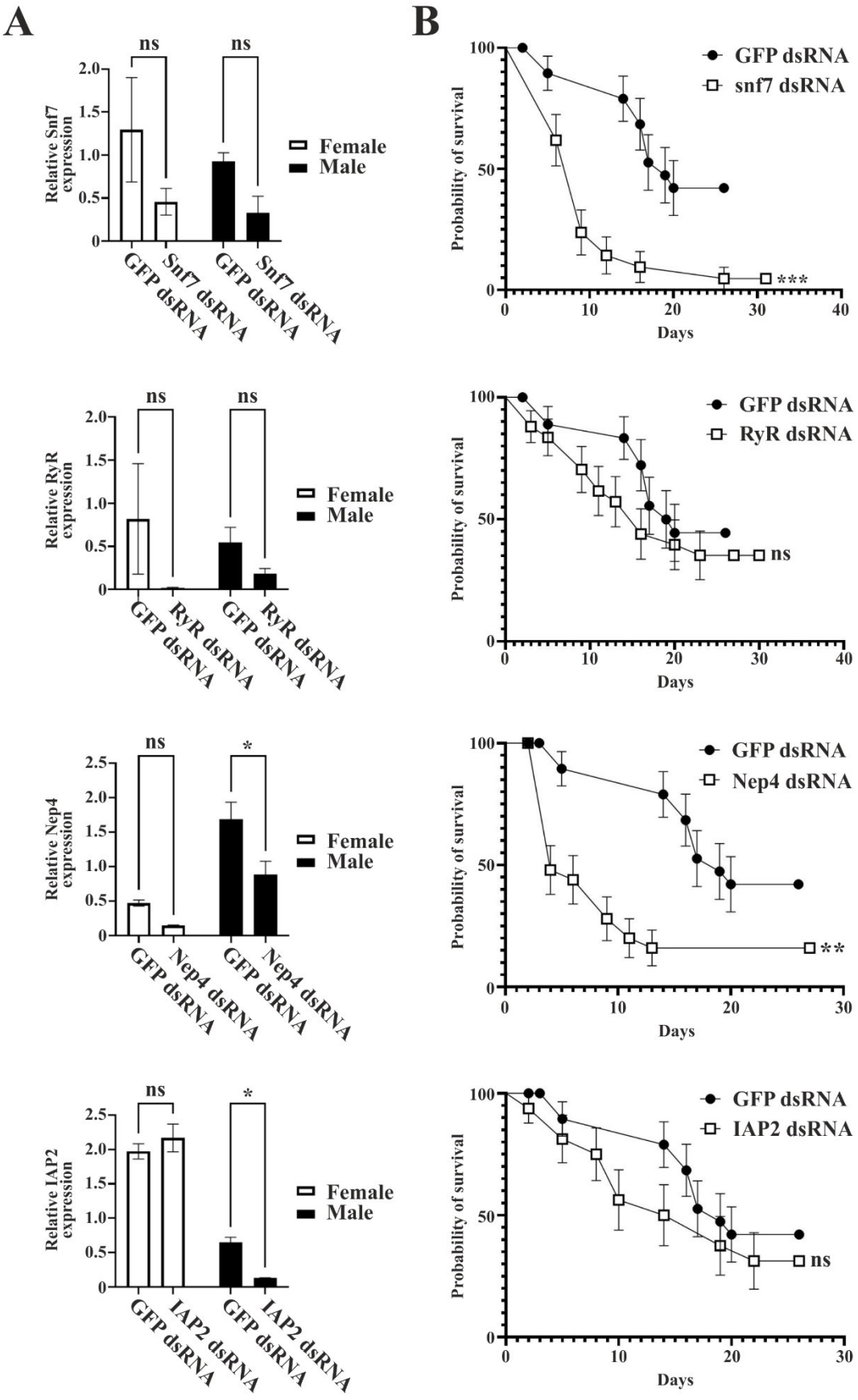


Figure 2. *Ceratitis capitata*-specific hairpins produced by *in vitro* transcription, along with an eGFP control. Plasmids containing cDNA fragments in both orientations of seven medfly mRNAs and eGFP were digested with BsaI and subjected to *in vitro* transcription with T7 RNA polymerase. Aliquots of transcription products were separated by non-denaturing PAGEs and the gels stained with ethidium bromide. Lane 0, DNA marker with sizes (in bp) on the left; lanes 1 to 8, *C. capitata* Snf7, RyR, Nep4, IAP2, ATPsynbeta, V-ATPase, and IRPS13-derived dsRNA. The black arrow points to the positions of the hairpin RNA molecules.

downregulated their target mRNAs in male flies (*Nep4*, *IAP2*, *ATPsynbeta*, *V-ATPase,* and *RPS13*) compared to flies treated with the control dsRNA (GFP). In three of these genes (*ATPsynbeta*, *V-ATPase,* and *RPS13*), the expression was reduced to less than 10%. Shockingly, only one dsRNA (*RPS13*) showed statistically significant downregulation of its target mRNA in females, reducing it to 40%.

We also recorded the fly mortality over time in the first 30 days after injection (**Figure 3B**). A Gehan-Breslow-Wilcoxon test indicated that mortality was


A
B
ns
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Relative Snf7 expression
Female
Male
GFP dsRNA
Snf7 dsRNA
GFP dsRNA
Snf7 dsRNA
Probability of survival
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Relative RyR expression
RyR dsRNA
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Nep4 dsRNA
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IAP2 dsRNA

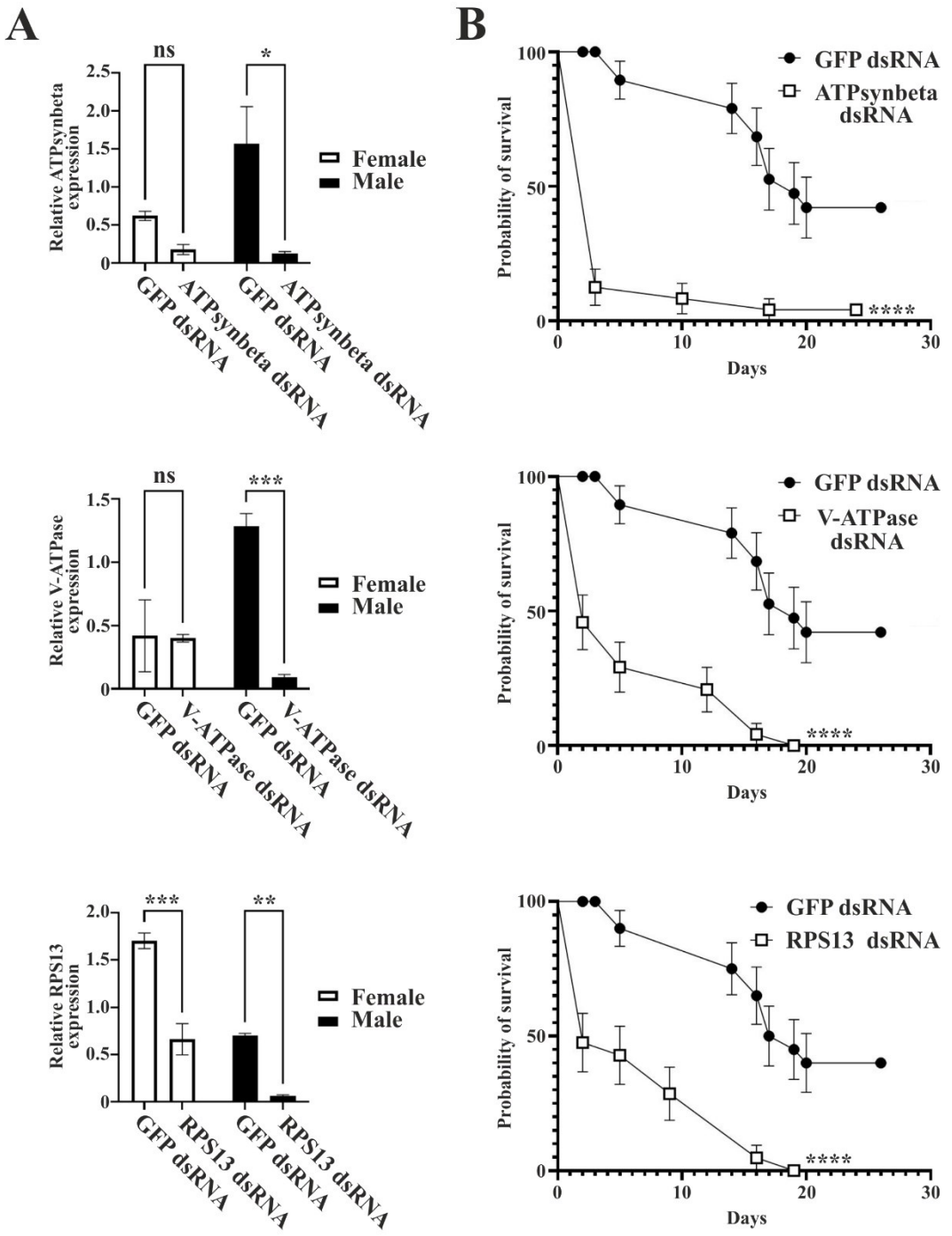

Figure 3. dsRNA injection-based target gene screening for RNAi-mediated control of *C. capitata*. (A) Expression levels of seven targeted genes at 72 h post-injection were determined by RT-qPCR relative to alpha tubulin. All data are reported as means ± SEM of three independent technical replicates of three biological replicates (for each sex and treatment) and normalized against the mean of the controls of both sexes. Female flies are in white bars; males black are in black bars. (B) Kaplan-Meier survival curves for *C. capitata* adults of both sexes (n=20) following the microinjection of 0.5 µg of seven different dsRNA homologous to genes of the medfly (Snf7, RyR, Nep4, IAP2, ATPsynbeta, V-ATPase, and RPS13) compared with flies treated with control dsRNA. Error bars show SEM. ns: not significant, *P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001 in (A) Gehan-Breslow-Wilcoxon tests (B) and unpaired one-tailed t-test.

significantly increased ($p < 0.05$) in five (*Snf7*, *Nep4*, *ATPsynbeta*, *V-ATPase,* and *RPS13*) of the seven tested genes in comparison with GFP control. Tested dsRNAs achieved mortalities circa 100% except in the case of *Nep4*, with a survival probability of 15%. In three genes, namely *ATPsynbeta*, *V-ATPase,* and *RPS13*, these differences were strongly significant ($p < 0.005$), with mortality rates higher than 50% after 48 h post-injection (90, 60, and 55%, respectively). Only two genes (*RyR* and *IAP2*) did not affect insect mortality in our assay conditions. In strikingly difference to gene expression, there were no significant differences in mortality between both sexes. Taken together, these results show that exogenous dsRNAs homologous to some medfly coding sequences can effectively knock down the expression of essential genes and also induce the mortality of adult flies when directly injected into the insect bodies.

Large amounts of recombinant circular dsRNAs can be produced in *E. coli* using a viroid scaffold and two type-I self-splicing introns

Next, we selected the three dsRNAs with the best death-inducing properties (*ATPsynbeta*, *V-ATPase,* and *RPS13*), along with the GFP control, to assess the effectiveness of orally-delivered RNAi. Since dsRNA stability and concentration are essential to reach the insect cells and ensure a substantial RNAi induction, we decided to produce circular versions of the selected dsRNAs using *E. coli* as a biofactory. For this purpose, we employed our recent method that uses two autocatalytic introns, the second in a permuted fashion, to produce large amounts of circular dsRNA in *E. coli* (Ortolá et al., 2021). The cDNA of the first self-splicing intron stabilizes the inverted repeats that will produce both strands of the dsRNA. The second permuted intron flanking the inverted repeats promotes RNA self-circularization removing any remains of the viroid scaffold (**Figure 1B**).

First, we built a series of plasmids (pLPIE-derivatives; **Supplemental Dataset S1**) to constitutively express the circular dsRNAs in *E. coli* under the control of the

constitutive lipoprotein (lpp) promoter. Production was first performed at a low scale in 5 ml culture media for 24 h and later scaled-up in larger flasks with 250 ml culture media. Total *E. coli* RNA was purified, and aliquots were analyzed via non-denaturing PAGE. Prominent bands likely corresponding to the circular dsRNAs were detected for all species in both large and low-scale cultures (**Figure 4**). Analysis of aliquots of the same preparation by denaturing 2-dimension (2D) PAGE confirmed the circularity of the RNAs of interest (**Supplemental Figure S1**). Comparison with previous shorter circular dsRNAs (Ortolá et al., 2021) and non-denaturing PAGE analysis of RNA preparations from *E. coli* expressing deletional versions of pLPIE-*ATPsynbeta* plasmids with shorter dsRNA stretches showed little variation in dsRNA accumulation depending on their lengths, and even a better accumulation of the full-length dsRNAs with respect to the smaller version (100 nt). We also assayed the effect

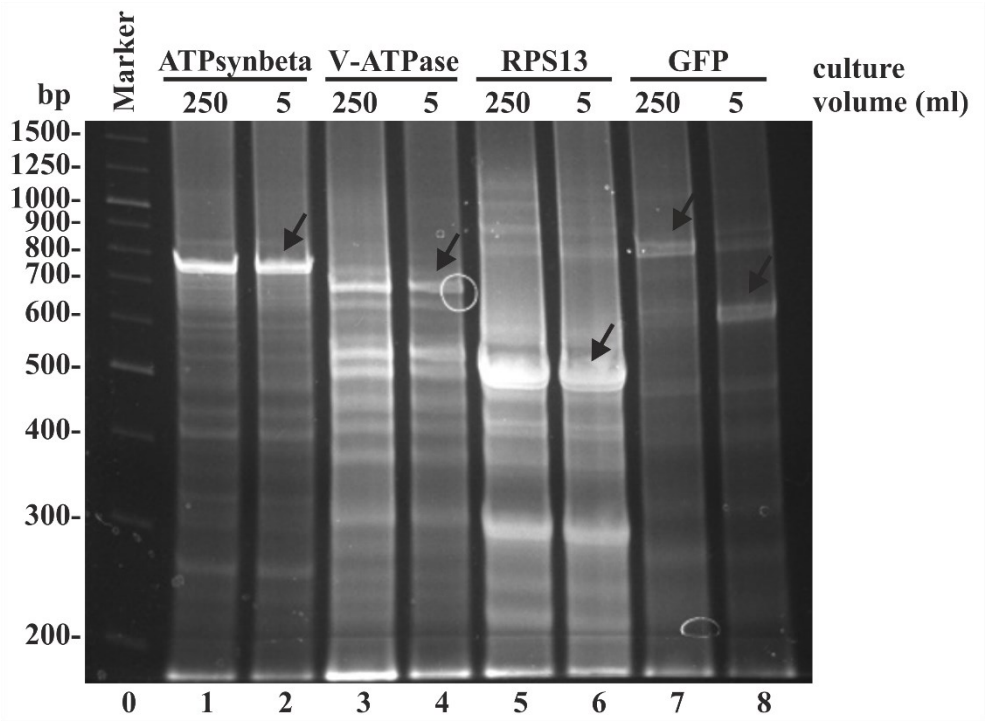

Figure 4. Recombinant circular dsRNAs produced in *E. coli*. Equivalent aliquots from RNA preparations from large (250 ml) and small (5 ml) cultures were separated by non-denaturing PAGE. The gel was stained with ethidium bromide. Lane 0, DNA marker with sizes (in bp) on the left. Lanes 1, 3, 5, and 7 (large cultures) and 2, 4, 6, and 8 (small cultures), RNAs from *E. coli* clones to express ATPsynbeta, V-ATPase, and RPS13, respectively.

of the promoter that drives the expression of the dsRNA precursor. We built a new plasmid to produce *RPS13* circular dsRNA in which the constitutive lpp promoter was replaced with an inducible T7 bacteriophage RNA polymerase promoter. The same *E. coli* strain was transformed with both plasmids. Selected recombinant clones were grown to OD_{600} 0.6 in liquid cultures and, that corresponding to the inducible T7 promoter, made 0.4 mM IPTG to induce T7-driven expression. A time-course analysis of total RNA showed no substantial difference in the level of circular dsRNA accumulation with the two promoters (**Supplemental Figure S2**).

Thus, we produced the molecules to be orally tested under the best experimental conditions. We grew cultures of bacteria transformed with the pLPIE-derivatives under lpp promoter in 250 ml of TB media for 24 h, 37ºC, and 250 rpm. Total bacterial RNA was extracted with phenol:chloroform and re-extracted with chloroform. The non-denaturing PAGE analysis of aliquots of the total RNA showed that the bacteria produced the recombinant circular dsRNA in high quantities. Gel quantification of the produced RNAs via dilution analysis and comparison to standards of known concentration showed a production of up to 50 mg of dsRNA in 250 ml culture in 24 h (**Supplemental Figure S3**). This RNA was precipitated and resuspended in a small volume of 30% sucrose to maximize RNA species' concentration with possible insecticidal activity.

Feeding *C. capitata* adults with bacteria-produced circular dsRNA strongly down-regulate target genes

C. capitata adult flies were fed for three days with the 30% sucrose solutions that contained the circular dsRNAs at 1 µg/µl. Diet solutions were replaced daily. Transcript levels downregulation 72 h post-treatment were analyzed in six randomly picked flies of both sexes as previously detailed. The oral delivery of circular dsRNA

induced a significant reduction of transcript levels for *V-ATPase* and *RPS13* (with a reduction in the expression to 48 and 6%, respectively) after three days of feeding, while *ATPsynbeta* mRNA was not significantly affected (**Figure 5**). Non-statistical differences in the basal expression but significant differences in their reductions were observed between both sexes in the case of *RPS13*. Neither of these differences was observed in the *V-ATPase* treatment. It should also be noted that no significant

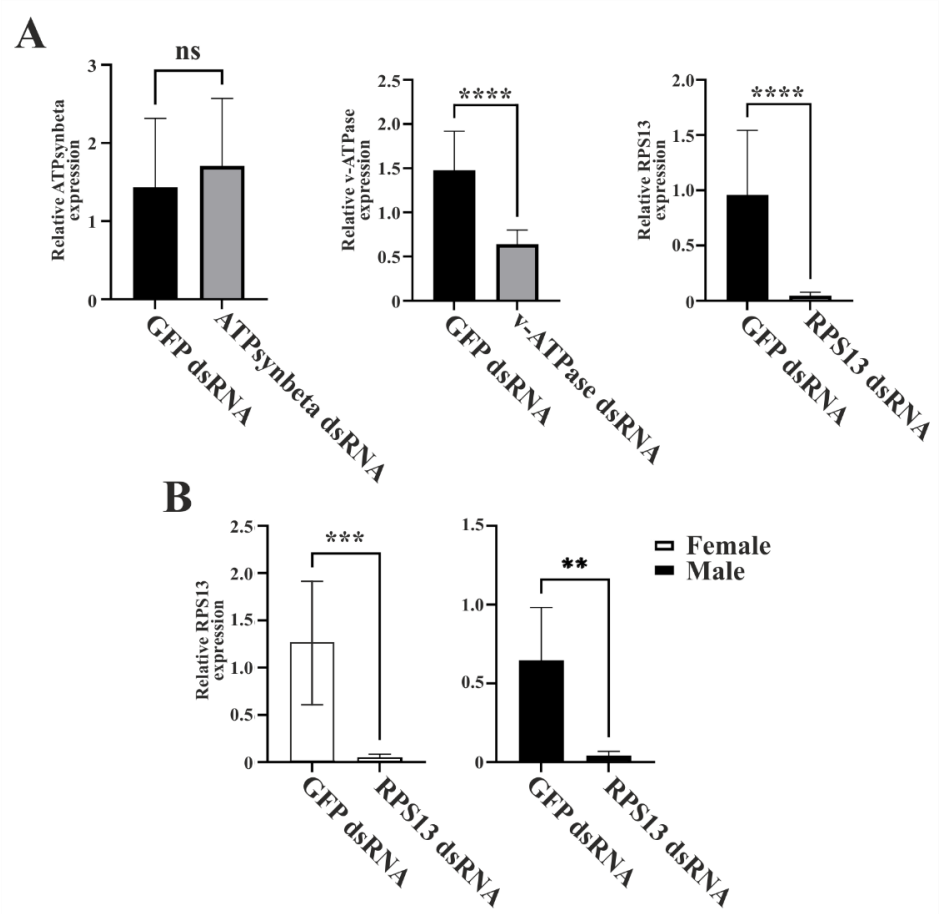

Figure 5. Effect of orally-supplied, gene-specific dsRNAs on the accumulation levels of *C. capitata ATPsynbeta*, *V-ATPase,* and *RPS13 genes*. Equal amounts of circular dsRNAs with homology to three endogenous genes or eGFP, as a control, produced in *E. coli* were dissolved in 30% sucrose and used as a sole nutritional source for adult medflies. 72 h after the end of the treatment, six individuals of each sex were collected for RNAi-mediated knockdown analysis. The relative expression levels of the three genes were determined by RT-qPCR. All data are reported as means ± SEM of twelve biological replicates (6 for each sex and treatment) and normalized against the mean of the controls of both sexes. Asterisks denote significant difference in unpaired one-tailed t-test (ns: not significant, **P<0.01, ***P<0.001, ****P< 0.0001). For each gene, dark bars represent the expression of flies treated with the control (GFP), while the light bars show those related to specific treatments.

differences were found between the expression levels of flies fed with 30% sucrose and flies treated with the control dsRNA (*GFP*), ruling out experimental artifacts. However, feeding assays did not significantly increase fly mortality frequencies for any tested genes.

These results highlight the feasibility of using dsRNA molecules as an orally-supplied insecticide to control *C. capitata*. The downregulation of crucial medfly mRNAs was achieved in this work using circular dsRNAs easily obtained in *E. coli* using a viroid-scaffold-two-self-splicing-intron system.

Discussion

Since the discovery in the model organism *Caenorhabditis elegans* (Fire et al., 1998), RNAi has become a basic strategy in the toolset for the study of gene function and the starting point of potential biotechnological applications. Pest control using RNAi is a topic of current interest (Mezzetti et al., 2020; Willow et al., 2022), with hundreds of studies in various stages of development in many different species, including one treatment approved for its use in the field (Fletcher et al., 2020; Hernández-Soto and Chacón-Cerdas, 2021; Darlington et al., 2022). Here we show this strategy's feasibility for controlling the worldwide distributed pest *C. capitata*, as orally-supplied dsRNA can significantly reduce the expression of insect-targeted genes.

This work used two different methodologies to produce dsRNA based on our recently described viroid-intron-based system (Ortolá et al., 2021). On the one hand, the straightforward assembly of transcription plasmid derivatives with a single self-splicing intron allows the construction of libraries for producing hairpins containing a dsRNA stretch under standard reaction conditions for high-throughput RNAi screening assays. The resulting dsRNAs are constituted of a single molecule and not hybridized

ssRNAs, thus reducing unnecessary labor and variability resulting from hybridization. From our view, this is an exciting alternative to classical *in vitro* synthesis using convergent T7 promoters. On the other hand, the *E. coli* production system, including a second permuted intron, results in circular dsRNA and seems very promising for producing the large quantities required for pest control. Using multicopy plasmids (with pUC replication origin) containing a strong constitutive *E. coli* promoter (lpp) seems to ensure high and constant transcription levels. The circularity and compaction of these molecules must contribute to circumvent degradation and gradually accumulate, reaching a maximum concentration at the stationary phase. Our effort to further optimize production showed that accumulation of the circular dsRNA is not improved using a strong inducible promoter such as that from bacteriophage T7. In addition to transcription, our *in vivo* system also relies on the activity of two self-splicing type-I introns. Although this type of introns only requires Mg^{2+} and guanosine to self-cleave, certain protein factors have been shown to accelerate the functional folding of the structure, thus resulting in saturable reactions (Tijerina et al., 2006; Bhaskaran and Russell, 2007; Jarmoskaite et al., 2014, 2021). This could be especially relevant given the presence of two intron copies in the RNA precursors, one of which is permuted, hindering even more its correct folding. This maximum rate can also explain the size-dependent expression differences in the benefit of longer RNAs, as the shortest dsRNA precursors are transcribed at a faster rate, surpassing those of the self-processing activity and resulting in unprocessed molecules likely prone to degradation. Therefore, the dsRNAs size could be further increased, if necessary, without expected reductions in production. In addition, the system maintains a large yield of the RNAs of interest when the culture is upscaled 50 times. Thus, it is expected that the production should increase proportionally in larger culture volumes. This property, in combination with transcription from constitutive promoter and accumulation at the stationary phase, supports indisputable industrial applicability.

Gene target screening assay showed increased silencing and mortality when affecting a broad spectrum of functions, such as energy metabolism, membrane transporters, detoxification, and translation factors. Intriguing differences in the basal levels of expression and its silencing between males and females were observed without implying variation in mortality. Whether experimental parameters or different treatment responses cause this variation, it remains undetermined. Regardless, the robust mortality effect achieved with three of these RNAs (*ATPsynbeta*, *V-ATPase,* and *RPS13*) stimulated us to take a step forward and further analyze if some of those dsRNAs were suitable for oral RNAi-derived medfly control. All tested dsRNAs lengthened 400-500 bp; this is a key parameter as RNA length is correlated positively with traversing through plasma membranes (Saleh et al., 2006; Bolognesi et al., 2012) and greater diversity of siRNAs generated, but also negatively with intra- and inter-species off-target and resistance development (Bolognesi et al., 2012). The RNAi feeding assay yielded two genes whose silencing was significantly reduced with only 3 days of treatment compared with the *GFP* control. The first gene encodes a subunit of a vacuolar ATPase (*V-ATPase*), ubiquitous membrane protein complexes present in both plasmatic and vesicular membranes and functioning as ATP-consuming proton pumps with multiple cellular functions (Pamarthy et al., 2018). Multiple genes usually encode vacuolar ATPase subunits with splicing variants, some with specific spatio-temporal expression patterns (Allan et al., 2005). We here targeted subunit A, which belongs to the complex's hydrophilic ATP-hydrolyzing (V1) region. *C. capitata* has three different A subunits; our target here (homologous to *D. melanogaster Vha68-2*) exhibits the highest expression among them (Allan et al., 2005). This subunit has also three splicing variants that encode the same polypeptide. The second gene, *RPS13*, encodes the ribosomal protein S13, one of the many proteins associated with rRNAs, to form ribosomal subunits. Specifically, RPS13 is an essential structural element of the 30S ribosomal subunit since it seems responsible for establishing a communication

network not only for the correct assembly of the ribosome but also for its proper translocation (Cukras et al., 2003; Cukras and Green, 2005). Several dozens of genes encode this type of small protein, but unlike vacuolar ATPases, most are single-copy genes, such is the case of our *RPS13* target.

Under our experimental conditions, we achieved both silencing and mortality by microinjection but only targeted knockdown in feeding bioassays. However, the levels of gene silencing achieved with these assays suggest a strong systemic response to introduced dsRNAs in this species. While minor differences in the downregulation of the vacuolar ATPase subunit-A (**Figures 3A** and **9**, ~90% by injection versus ~50% orally) could partly explain the difference in mortality, the *RPS13* results are certainly intriguing, given the silencing similarity between both methods (circa 90%). Experimental factors such as side-effects of microinjection, and the functional supplementation with other isoforms or related proteins due to the slower development of the silencing in the oral experiment, may cause a reduction in mortality. Nevertheless, it should be noted that sublethal exposure to both dsRNA (as more likely would occur in field conditions) might still reduce crop damage in sustainable crop protection approaches.

Vacuolar ATPase complexes and ribosomal proteins such as RPS13 have been implicated in developing and maintaining female reproductive organs and embryonic and juvenile development. The dsRNA-mediated silencing of various vacuolar ATPase subunits in immature and adult females resulted in deformed ovaries and oocytes (Yao et al., 2013; Ghazy and Suzuki, 2022), leading to increased embryonic necrosis, reduced egg laying, with a higher rate of deformity and less hatching (Basnet and Kamble, 2018). Similar phenotypes are generated by silencing ribosomal proteins, such as a reduction in egg production and hatching thus highly affecting fecundity (Kurscheid et al., 2009; Wu and Hoy, 2014; Estep et al., 2016). Affectations to the larval development

of the treated females could be expected since, in *D. melanogaster*, defects in protein biosynthesis as a result of reduction of the expression of ribosomal proteins such as RPS13 leads to the appearance of characteristic phenotypes as larval development delay, poor viability, altered body size, and reduced fertility (Sæbøe-Larssen and Lambertsson, 1996; Marygold et al., 2007). Vacuolar ATPase silencing in nymphs and larvae reduced their food intake, decreased larval growth, adult development, and emergence, and even increased mortality (Liu et al., 2022), probably by affecting the integrity and function of the larval midgut and Malpighian tubules (Mao et al., 2015; Zeng et al., 2021, 2022).

To sum up, this work shows that orally delivered circular dsRNAs induce strong silencing of crucial genes in the devastating pest *C. capitata*. This kind of RNAi-inducing molecules can be reliably produced in *E. coli* bio-factories using a viroid-scaffold-two-intron system for sustainable crop protection programs. Nonetheless, many factors remain to be still analyzed, such as the viability of deriving direct mortality from silencing other genes or the potential benefits of dsRNA encapsulation.

Interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supporting information

Supplemental Dataset S1. Nucleotide sequences and elements of pMT7 plasmid and its derivatives pMT7-CcSnf7, -CcRyR, -CcNep4, -CcIAP2, -CcATPsynbeta, -CcV-ATPase, -CcRPS13, -eGFP, and pLPIE-LacZ plasmid and its derivatives pLPIE-CcATPsynbeta, -CcV-ATPase, -CcRPS13, -eGFP and pLPIE-T7-CcRPS13.

>pMT7 (1981 nt)

TAATACGACTCACTATAGgggaaagagGTCTTCGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCA
TTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG
AAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGGTACCCCAATTCGCCC
TATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTT
AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTG
CGCAGCCTGAATGGCGAATGGGACGCGGAAGACcttttctccccctGAGACCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCC
CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC
CCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAA
GCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG
AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGC
CACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA
ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT
CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGAT
CTCAAGAAGATCCTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTG
CCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAGC
CACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTAT
CCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTG
CCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAG
TTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAG
TGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG
AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCG
CGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT
TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAG
CAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTC
AATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT

In red, minimal T7 RNAP promoter sequence with the first transcribed nucleotide underlined. Highlighted in green, recognition sites for type IIS restriction enzyme BpiI, with the cleavage sites underlined. In blue, 399 nt cDNA coding for LacZ gene. Highlighted in yellow, recognition site for type IIS restriction enzyme BsaI, with the cleavage site underlined. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray.

The expression cassettes of the **pMT7-derivative plasmids** are detailed below. All cassettes contain an inverted repeat (>400 nt) of the corresponding *C. capitata* gene (highlighted in green) surrounding the cDNA of the group-I *Tetrahymena termophila* 26S rRNA intron (in green) with the 10 nts of both flanking exons underlined. The cassettes are located between the T7 RNAP promoter (in red) and the BsaI recognition site (highlighted in yellow).

>pMT7-CcSnf7 (2865 nt) [437 nt fragment of the Snf7 gene]

TAATACGACTCACTATAGgggCTGGCGAGGCTATACAGAAACTTCGTGAAACGGAAAATATGCTTATTAAAAAACAAGAGT
TTCTGGAGTCTAAAATCGAAGAAGAATTAAATATTGCTAGGAAAAATGCATCGAAAAACAAAAGAGTGGCGTTGCAAGCGC
TTAAAAAAAAGAAGCGTTTGGAGAAGCAGTTACAGCAAATAGATGGAACGTTGTCTACAATTGAGATGCAGCGTGAGGCTC
TGGAAAGCGCCAATACAAATACCGCAGTTTTGACCACGATGAAAAATGCGGCTGATGCACTGAAGGCCGCACATAAAAATA
TGGATGTGGATAATGTGCATGATATGATGGATGACATTGCCGAGCAACAAGATGTGGCGCGTGAAATTTCTGACGCTATAT
CAAACCCCGTCGCGTTTGGTGCTGACCTAGATGATGAGGACCTAGAGCGTGAAATGACTCTCTAAATAGCAATATTTACCT
TTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCG
TCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGG
TAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCAC
AGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAA
GTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCC
TTCACGCTCTAGGTCCTCATCATCTAGGTCAGCACCAAACGCGACGGGGTTTGATATAGCGTCAGAAATTTCACGCGCCAC
ATCTTGTTGCTCGGCAATGTCATCCATCATATCATGCACATTATCCACATCCATATTTTTATGTGCGGCCTTCAGTGCATC
AGCCGCATTTTTCATCGTGGTCAAAACTGCGGTATTTGTATTGGCGCTTTCCAGAGCCTCACGCTGCATCTCAATTGTAGA
CAACGTTCCATCTATTTGCTGTAACTGCTTCTCCAAACGCTTCTTTTTTTTAAGCGCTTGCAACGCCACTCTTTTGTTTTT
CGATGCATTTTTCCTAGCAATATTTAATTCTTCTTCGATTTTAGACTCCAGAAACTCTTGTTTTTTAATAAGCATATTTTC
CGTTTCACGAAGTTTCTGTATAGCCTCGCCAGcccctGAGACC...

>pMT7-CcRyR (2879 nt) [444 nt fragment of the CcRyR gene]

TAATACGACTCACTATAGgggCACGTGGACGAGGACTTCTTTTACATGGCTCATGTGATACGTATTGCGGCATTTTTGCAC
AGCTTAGTCTCCCTTGCCATGCTAATTGCATACTATCATTTAAAAGTGCCTTTAGCTATTTTTAAGAGGGAGAAAGAAATT
GCACGTCGTTTGGAATTTGATGGCCTTTTCATTGCTGATCAACCGGAAGATGATGATTTTAAGTCACATTGGGATAAGTTA
GTGATTTCCGCTAAATCATTCCCCGTTAATTATTGGGACAAATTTGTAAAGAAGAAAGTGAGACAAAAGTATAGTGAAACT
TATGACTTCGACTCTATTTCAAATCTTCTGGGTATGGAGAAAAGCGCTTTCATGGCTCAAGAGAGCGAAGAAGGTGGTTTG
GTTAAATACATAATGAATATAGATTGGCGGTACCAGGTGTGGAAAGCAGGCGTTACGTTCATGACTCTCTAAATAGCAATA
TTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGC
AAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAG
GGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGC
AGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGC
GGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAA
GGTAGCCGAACGTAACGCCTGCTTTCCACACCTGGTACCGCCAATCTATATTCATTATGTATTTAACCAAACCACCTTCTT
CGCTCTCTTGAGCCATGAAAGCGCTTTTCTCCATACCCAGAAGATTTGAAATAGAGTCGAAGTCATAAGTTTCACTATACT
TTTGTCTCACTTTCTTCTTTACAAATTTGTCCCAATAATTAACGGGGAATGATTTAGCGGAAATCACTAACTTATCCCAAT
GTGACTTAAAATCATCATCTTCCGGTTGATCAGCAATGAAAAGGCCATCAAATTCCAAACGACGTGCAATTTCTTTCTCCC
TCTTAAAAATAGCTAAAGGCACTTTTAAATGATAGTATGCAATTAGCATGGCAAGGGAGACTAAGCTGTGCAAAAATGCCG
CAATACGTATCACATGAGCCATGTAAAAGAAGTCCTCGTCCACGTGcccctGAGACC...

>pMT7-CcNep4 (2849 nt) [429 nt fragment of the Nep4 gene]

TAATACGACTCACTATAGgggATTCAAACGAAGCGCCAATAAAAGCAAAATACCTCTATCAATCCTGTATAAATACAAAGC
TTCTAGGTAAGCGTGGGATTAAACCTCTCTTAAAATTGATTGATAGTCTTGGAGGTTGGCCTGTACTAAATCCTAGTTGGA
ATCCCCAACACTTTGATTGGTTAAATTTAACTGCACAACTTCGACGATATAACAACGATATACTCATCGTAGAATGGGTAG
GCCCAGATATTAAAAACTCAGACGAAAATATCATACAGTTTGACCAGACTAGTCTAGGACTGCCGACACGTGAATATTATC
TTCAAGATATGAACGCACGTTATTTGATGGCTTACCAGTTATTTATGTCTGAAATAATGCAAAAATTAGGGGCATCTCGTG
ATCGAGCTATCAAAACGGCCACAGATGTTGTGGGTTTCGAAACACATGACTCTCTAAATAGCAATATTTACCTTTGGAGGG
AAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTG
CGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGC
TGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAA
TGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCA
ACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCGTGTTTCG
AAACCCACAACATCTGTGGCCGTTTTGATAGCTCGATCACGAGATGCCCCTAATTTTTGCATTATTTCAGACATAAATAAC

TGGTAAGCCATCAAATAACGTGCGTTCATATCTTGAAGATAATATTCACGTGTCGGCAGTCCTAGACTAGTCTGGTCAAAC
TGTATGATATTTTCGTCTGAGTTTTTAATATCTGGGCCTACCCATTCTACGATGAGTATATCGTTGTTATATCGTCGAAGT
TGTGCAGTTAAATTTAACCAATCAAAGTGTTGGGGATTCCAACTAGGATTTAGTACAGGCCAACCTCCAAGACTATCAATC
AATTTTAAGAGAGGTTTAATCCCACGCTTACCTAGAAGCTTTGTATTTATACAGGATTGATAGAGGTATTTTGCTTTTATT
GGCGCTTCGTTTGAATccccctGAGACC...

>pMT7-CcIAP2 (2887 nt) [448 nt fragment of the IAP2 gene]

TAATACGACTCACTATAGgggCCCTACGATACTGTTGAGGATTTAATAAAGGCTGTTTTTGGTGATACAGAAGACGCCGAA
CCAATCGCAAGTGGTCAATGCACAGAAGCTGTTAGTGCTTCTTTGGCTGGTGATGTTTCACGGCTGATAAATATGCTCGAC
GAAGCTGGTGTCAGTGGTGGGAATTATGTCGGAACCCCAAGCAACACATACAACAATACGATAGCAATGAGTCAGAATAAT
GTGCAAAATCAAATGCATGTGAATGTGTTGAATGGACCAAGCTGTTCAAACGCTTTAACGAACGCGTTGCAAGCGAAAAGT
GCAGGCGATAATTTAAAAACGCCCGCGGAAGAAGTTAAATGCGAAGAAGTGAACACCAAACAGCCAAATGAGCAAACATTG
AATAGAGCTTTGTCGTTGGAGGAGGAGAATCGTAAACTCAAAGATGCTCGCCTCTGTAAGGTTTATGACTCTCTAAATAGC
AATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAA
AGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGC
AAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGG
ATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGC
TAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTC
GTAAGGTAGCCAAACCTTACAGAGGCGAGCATCTTTGAGTTTACGATTCTCCTCCTCCAACGACAAAGCTCTATTCAATGT
TTGCTCATTTGGCTGTTTGGTGTTCACTTCTTCGCATTTAACTTCTTCCGCGGGCGTTTTTAAATTATCGCCTGCACTTTT
CGCTTGCAACGCGTTCGTTAAAGCGTTTGAACAGCTTGGTCCATTCAACACATTCACATGCATTTGATTTTGCACATTATT
CTGACTCATTGCTATCGTATTGTTGTATGTGTTGCTTGGGGTTCCGACATAATTCCCACCACTGACACCAGCTTCGTCGAG
CATATTTATCAGCCGTGAAACATCACCAGCCAAAGAAGCACTAACAGCTTCTGTGCATTGACCACTTGCGATTGGTTCGGC
GTCTTCTGTATCACCAAAAACAGCCTTTATTAAATCCTCAACAGTATCGTAGGGccccctGAGACC...

>pMT7-CcATPsynbeta (2919 nt) [464 nt fragment of the ATPsynbeta gene]

TAATACGACTCACTATAGgggTTGGTATACGGTCAGATGAACGAACCCCCAGGCGCCCGAGCTCGTGTTGCATTGACTGGA
TTGACTGTTGCCGAATATTTCCGTGATCAGGAAGGACAAGACGTGTTACTTTTTATTGATAATATCTTCCGATTTACACAA
GCTGGCTCAGAAGTATCAGCTTTGTTGGGCCGTATTCCATCTGCAGTAGGTTATCAACCAACTTTAGCTACTGACATGGGC
TCCATGCAAGAGCGTATTACCACTACCAAAAAAGGCTCAATCACATCAGTGCAAGCTATTTATGTTCCAGCTGACGATTTG
ACTGATCCTGCCCCAGCTACAACATTTGCTCACTTGGATGCTACAACTGTATTGTCGCGCGCTATTGCGGAATTGGGCATC
TACCCAGCTGTAGATCCCTTAGATTCTACATCACGAATTATGGATCCTAATATTATTGGCCTAGAGCACTACAACGTAGCA
TGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAG
ATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAAC
TTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGA
TCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACC
TCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGAT
TAGTTTTGGAGTACTCGTAAGGTAGCCGCTACGTTGTAGTGCTCTAGGCCAATAATATTAGGATCCATAATTCGTGATGTA
GAATCTAAGGGATCTACAGCTGGGTAGATGCCCAATTCCGCAATAGCGCGCGACAATACAGTTGTAGCATCCAAGTGAGCA
AATGTTGTAGCTGGGGCAGGATCAGTCAAATCGTCAGCTGGAACATAAATAGCTTGCACTGATGTGATTGAGCCTTTTTTG
GTAGTGGTAATACGCTCTTGCATGGAGCCCATGTCAGTAGCTAAAGTTGGTTGATAACCTACTGCAGATGGAATACGGCCC
AACAAAGCTGATACTTCTGAGCCAGCTTGTGTAAATCGGAAGATATTATCAATAAAAAGTAACACGTCTTGTCCTTCCTGA
TCACGGAAATATTCGGCAACAGTCAATCCAGTCAATGCAACACGAGCTCGGGCGCCTGGGGGTTCGTTCATCTGACCGTAT
ACCAAccccctGAGACC...

>pMT7-CcVATPase (2841 nt) [425 nt fragment of the V-ATPase gene]

TAATACGACTCACTATAGgggTTGTGAACCCACGCACCAAGGGTACCGTACGTTATATCGCCCCAACCGGTAACTACAAAG
TCGACGATGTCGTCTTGGAAACCGAATTCGATGGTGAGATCACCAAACACACTATGTTGCAAGTGTGGCCTGTGCGTCAAC
CTCGTCCAGTCACCGAGAAATTGCCCGCCAACCATCCTCTATTGACCGGTCAACGTGTATTGGATTCGCTTTTCCCCTGTG

TACAAGGTGGTACCACCGCCATTCCCGGAGCTTTCGGTTGTGGCAAAACTGTCATCTCACAGGCTTTGTCCAAGTACTCCA
ACTCTGATGTCATTATTTACGTCGGTTGCGGTGAACGTGGTAACGAGATGTCTGAAGTATTGCGTGATTTCCCCGAATTGT
CTGTTGAAATTGACGGTGTCACCGAATCTATCATGAAACGTATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAA
GTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGG
AAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGAC
GGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTC
GGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACAC
TGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCACGTTTCATGAT
AGATTCGGTGACACCGTCAATTTCAACAGACAATTCGGGGAAATCACGCAATACTTCAGACATCTCGTTACCACGTTCACC
GCAACCGACGTAAATAATGACATCAGAGTTGGAGTACTTGGACAAAGCCTGTGAGATGACAGTTTTGCCACAACCGAAAGC
TCCGGGAATGGCGGTGGTACCACCTTGTACACAGGGGAAAAGCGAATCCAATACACGTTGACCGGTCAATAGAGGATGGTT
GGCGGGCAATTTCTCGGTGACTGGACGAGGTTGACGCACAGGCCACACTTGCAACATAGTGTGTTTGGTGATCTCACCATC
GAATTCGGTTTCCAAGACGACATCGTCGACTTTGTAGTTACCGGTTGGGGCGATATAACGTACGGTACCCTTGGTGCGTGG
GTTCACAAcccctGAGACC...

>pMT7-CcRPS13 (2909 nt) [459 nt fragment of the RPS13 gene]

TAATACGACTCACTATAGgggATGGGTCGTATGCACGCTCCTGGCAAGGGTATTTCCCAATCAGCGCTCCCGTACAGGCGC
ACTGTGCCTTCATGGCTGAAATTGAATGCCGAAGATGTAAAAGAACACATAAAGAAATTGGGCAAAAAGGGCATGACCCCT
TCCAAGATTGGTATCATTCTCCGTGATTCCCATGGTGTTGCTCAGGTACGCTTTGTTAATGGTAACAAAATTCTGCGCATC
ATGAAATCAGTTGGTTTGAAACCTGACATCCCCGAAGATTTATACCACATGATTAAGAAAGCCGTTGCTATCCGCAAGCAT
TTGGAGCGTAATCGCAAGGATAAGGATGGCAAATTCCGATTGATTCTTGTTGAGTCTAGAATCCATCGTTTGGCCCGCTAC
TACAAAACCAAGAGTGTTCTGCCACCTAACTGGAAATACGAATCCAGCACTGCATCTGCTTTGGTTGCTTAGAAAATGACT
CTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGC
ATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGA
GATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTC
TGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCC
TTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTT
TTGGAGTACTCGTAAGGTAGCCTTTCTAAGCAACCAAAGCAGATGCAGTGCTGGATTCGTATTTCCAGTTAGGTGGCAGAA
CACTCTTGGTTTTGTAGTAGCGGGCCAAACGATGGATTCTAGACTCAACAAGAATCAATCGGAATTTGCCATCCTTATCCT
TGCGATTACGCTCCAAATGCTTGCGGATAGCAACGGCTTTCTTAATCATGTGGTATAAATCTTCGGGGATGTCAGGTTTCA
AACCAACTGATTTCATGATGCGCAGAATTTTGTTACCATTAACAAAGCGTACCTGAGCAACACCATGGGAATCACGGAGAA
TGATACCAATCTTGGAAGGGGTCATGCCCTTTTTGCCCAATTTCTTTATGTGTTCTTTTACATCTTCGGCATTCAATTTCA
GCCATGAAGGCACAGTGCGCCTGTACGGGAGCGCTGATTGGGAAATACCCTTGCCAGGAGCGTGCATACGACCCATccccT
GAGACC...

>pMT7-eGFP (2967 nt) [488 nt fragment of the eGFP gene]

TAATACGACTCACTATAGgggGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC
CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCC
GCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAG
TTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG
CTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATC
CGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCGACAACCACTACCTGAGATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCT
GGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGT
TCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCAC
GCAGCCAAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATT
CTTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTA
ATTTGTATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCCTCAGGTAGTGGTTGTCGGGCAGCAGCACG
GGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTG
AAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGC
CCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACC
TCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTG

AAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAGGGTGGGC
CAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCccctGAGACC...

>pLPIE-LacZ (3969 pb)

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTGCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGA
TATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGA
AAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCacgtcGAGACGGAAAGCGGGCAGTGAGCGCAACGCAATTAAT
GTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGA
TAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTG
GGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCT
AGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACA
ACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAG
CGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCGTCTCgtgacATGACT
CTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGC
ATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGA
GATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATCTTC
TGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGTCGGACCTCTCC
TTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTT
TTGGAGTACTCGTAAGGTAGCCacgtcgGTCTTCGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTC
ATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG
GAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCCCCC
TCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCG
CGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA
AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC
GATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGGAAGACgctgacATGACTCTCTAAATAGCAATA
TTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGC
AAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAG
GGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGATGAAATCATCCTTAGCGAAA
GCTAAGGATTTTTTTTATCTGAAATGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCG
ACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTC
TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACG
CTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTG
CGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAG
GATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGT
ATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGC
TGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATG
CTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAAC
TACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATC
AGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG
TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC
ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAA
AAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGA
ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGT
GCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCAC
TCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGG
TTATTGTCTCAT

In red, *E. coli* murein lipoprotein promoter. In blue, two copies of the cDNA coding for LacZ gene, each one surrounded by recognition sites for two diferent type-IIS restriction enzymes: the first one for Esp3I, highlighted in yellow; the second one for BpiI, highlighted in red. In both cases, the cleavage

sites are underlined. In green, a group-I *Tetrahymena termophila* 26S rRNA autocatalytic intron separating the two LacZ copies, with both 10 nt flanking exons underlined. In orange, an additional copy of the same intron in an intron-exon permuted fashion between T235 and C236 surrounding the two LacZ copies, with both 10 nt flanking exons underlined. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray.

The expression cassettes of the **pLPIE-derivative plasmids** are detailed below. All cassettes contain an inverted repeat of the corresponding *C. capitata* gene (highlighted in green) surrounding the cDNA of the group-I *Tetrahymena termophila* 26S rRNA intron (in green) with the 10 nts of both flanking exons underlined. The cassettes are located between the *E. coli* murein lipoprotein promoter (in red) and ribosomal rrnC terminator (in fuchsia).

>pLPIE-CcATPsynbeta (3458 pb) [464 nt fragment of the ATPsynbeta gene]

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTGCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGA
TATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGTATGCGA
AAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCTTGGTATACGGTCAGATGAACGAACCCCCAGGCGCCCGAGCT
CGTGTTGCATTGACTGGATTGACTGTTGCCGAATATTTCCGTGATCAGGAAGGACAAGACGTGTTACTTTTTATTGATAAT
ATCTTCCGATTTACACAAGCTGGCTCAGAAGTATCAGCTTTGTTGGGCCGTATTCCATCTGCAGTAGGTTATCAACCAACT
TTAGCTACTGACATGGGCTCCATGCAAGAGCGTATTACCACTACCAAAAAAGGCTCAATCACATCAGTGCAAGCTATTTAT
GTTCCAGCTGACGATTTGACTGATCCTGCCCCAGCTACAACATTTGCTCACTTGGATGCTACAACTGTATTGTCGCGCGCT
ATTGCGGAATTGGGCATCTACCCAGCTGTAGATCCCTTAGATTCTACATCACGAATTATGGATCCTAATATTATTGGCCTA
GAGCACTACAACGTAGCATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGC
TAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGTTCAGTA
CCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCC
AAGTCCTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTC
ATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGCAACACTGGAGCCGCTGGGAACTAATTTGT
ATGCGAAAGTATATTGATTAGTTTTGGAGTACTCGTAAGGTAGCCGCTACGTTGTAGTGCTCTAGGCCAATAATATTAGGA
TCCATAATTCGTGATGTAGAATCTAAGGGATCTACAGCTGGGTAGATGCCCAATTCCGCAATAGCGCGCGACAATACAGTT
GTAGCATCCAAGTGAGCAAATGTTGTAGCTGGGGCAGGATCAGTCAAATCGTCAGCTGGAACATAAATAGCTTGCACTGAT
GTGATTGAGCCTTTTTTGGTAGTGGTAATACGCTCTTGCATGGAGCCCATGTCAGTAGCTAAAGTTGGTTGATAACCTACT
GCAGATGGAATACGGCCCAACAAAGCTGATACTTCTGAGCCAGCTTGTGTAAATCGGAAGATATTATCAATAAAAAGTAAC
ACGTCTTGTCCTTCCTGATCACGGAAATATTCGGCAACAGTCAATCCAGTCAATGCAACACGAGCTCGGGCGCCTGGGGGT
TCGTTCATCTGACCGTATACCAAATGACTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCT
GGTAGCTAGTCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAAAGGGGTCAACAGCCGT
TCAGTACCAAGTCTCAGGGGAAACTTTGAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCAC
GCAGCCAAGTCCTAAGTCAACAGATGAAATCATCCTTAGCGAAAGCTAAGGATTTTTTTTATCTGAAAT...

>pLPIE-CcvATPase (3380 pb) [425 nt fragment of the V-ATPase gene]

CGATGCTTCTTTGAGCGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAAT
ACTTGTAACGCTGCTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGA
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>pLPIE-CcRPS13 (3448 pb) [459 nt fragment of the RPS13 gene]

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>pLPIE-eGFP (Control) (2967 nt) [488 nt fragment of the eGFP gene]

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>pLPIE-T7-CcRPS13 (3372 pb)

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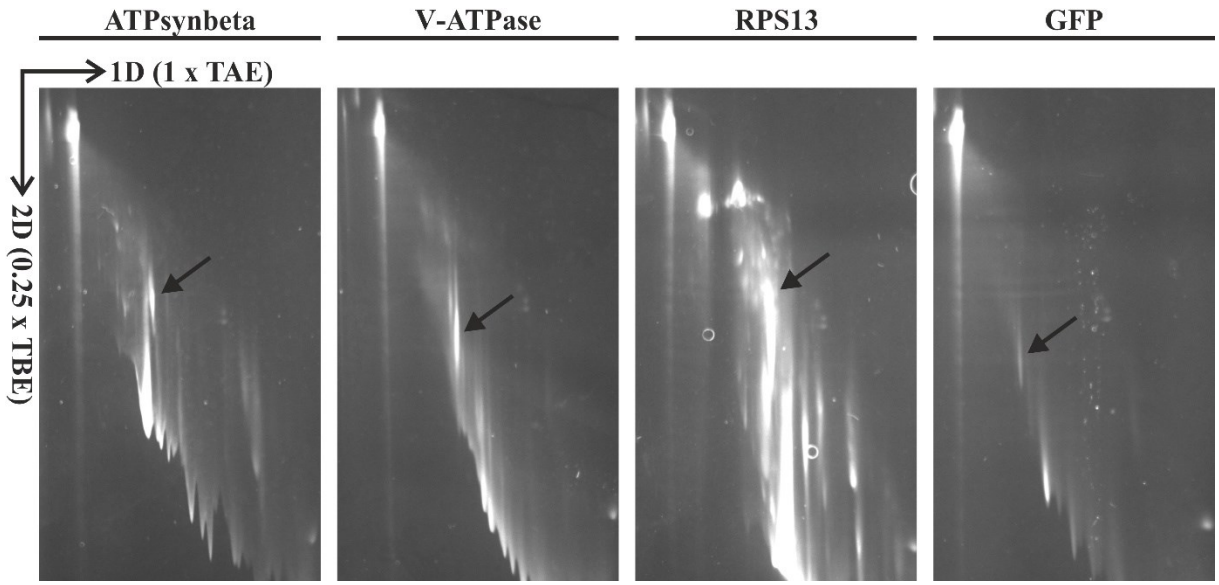
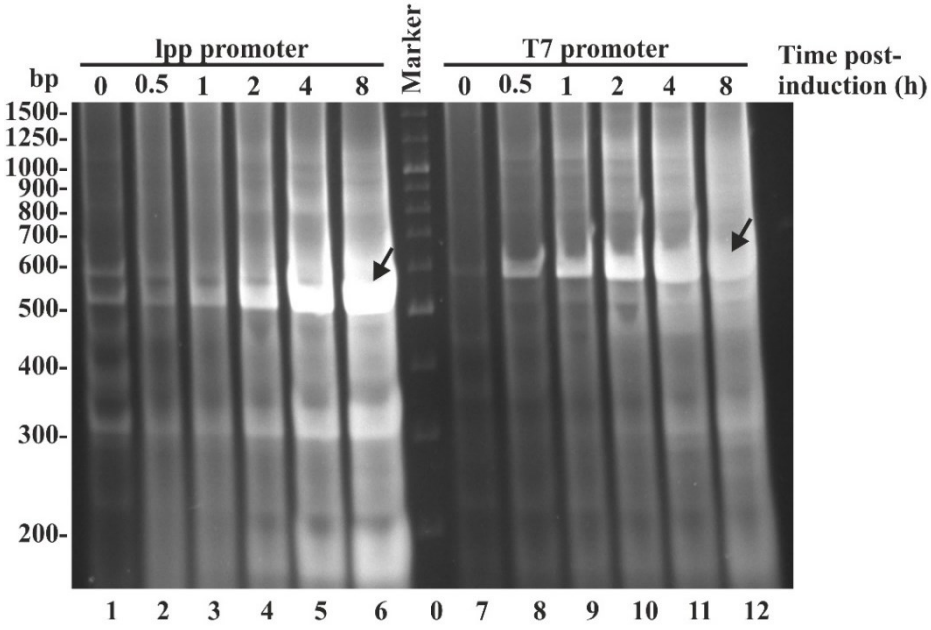
CTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT

In red, minimal T7 RNAP promoter sequence with the first transcribed nucleotide underlined. In orange, a group-I *Tetrahymena termophila* 26S rRNA autocatalytic intron in an intron-exon permuted fashion between T235 and C236, with both 10 nt flanking exons underlined. Highlighted in green, inverted repeat of an 459 nt fragment of the RPS13 gene. In green, between the two copies of the gene, the cDNA of the group-I *Tetrahymena termophila* 26S rRNA intron, with the 10 nts of both flanking exons underlined. In fuchsia, *E. coli* ribosomal rrnC terminator. In gray, pUC replication origin. Highlighted in light grey, ampicillin resistance gene (in inverse orientation), with the promoter highlighted in dark gray.

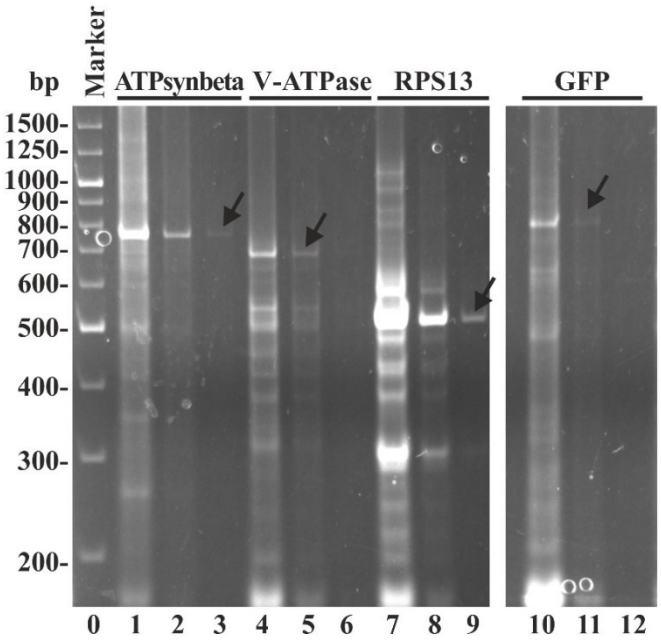
Supplemental Figures S1-S3



Supplemental Figure S1. Analysis of the circularity of the recombinant RNA molecules produced in *E. coli*. Equivalent aliquots from *E. coli* RNA preparations (as indicated) were separated by two-dimension PAGE, and the gels were stained with ethidium bromide. The first and second dimensions were sequentially run under high and low ionic conditions. The directions of migration of the two electrophoretic separations are indicated. The positions of the circular dsRNAs are marked with an arrow.



Supplemental Figure S2. Comparative analysis of recombinant circular dsRNA accumulation in *E. coli* depending on the promoter that drives transcription. Recombinant bacteria transformed with two pLPIE-derivatives expressing dsRNA either under the control of the constitutive *E. coli* murein lipoprotein (lpp) promoter or an IPTG-inducible T7 RNA polymerase promoter were grown until reaching an OD_{600} 0.6. T7-based expression was induced in the second culture by adding IPTG to 0.4 mM. Aliquots of the cultures were taken at the indicated time intervals. *E. coli* was extracted and analyzed by non-denaturing PAGE followed by ethidium bromide staining of the gels. Lane 0, DNA marker with sizes (in bp) on the left. Lanes 1 to 6 and lanes 7 to 12, RNAs from aliquots taken at 0, 1, 2, 4, and 8 h post-induction of the T7 promoter, respectively. A black arrow marks the positions of the circular dsRNA.



Supplemental Figure S3. Quantification of the dsRNA preparations obtained in large-scale *E. coli* cultures. Non-denaturing PAGEs separated serial dilutions of RNA preparations, and the gels were stained with ethidium bromide. Concentration was calculated from band intensity compared with a calibration curve obtained with an *E. coli* 5S rRNA standard. Lane 0, DNA marker with sizes (in bp) on the left. Lanes 1 to 3, 4 to 6, 7 to 9, and 10 to 12, 1:5 serial dilutions of ATPsynbeta, V-ATPase, IRPS13, and eGFP dsRNA preparations, respectively. Arrows indicate the positions of the dsRNAs.

Supplemental Tables S1-S2

Supplemental Table S1. Primers used to construct plasmids for the *in vitro* and *in vivo* production of dsRNA against *C. capitata.*

Name	Sequence (5' to 3')	Target*
D3133	ATGACTCTCTAAATAGCAATATTTACC	*T. thermophila* intron [F]
D3134	GGCTACCTTACGAGTACTCCA	*T. thermophila* intron [R]
D3870	aatacgactcactatagggCTGGCGAGGCTATACAGAAAC	Snf7-T7 [S,F]
D3890	attgctatttagagagtcatTTCACGCTCTAGGTCCTC	Snf7-T7 [S,R]
D3893	ggagtactcgtaaggtagccTTCACGCTCTAGGTCCTC	Snf7-T7 [As,F]
D3894	acgccagcaacgcggtctcaggggCTGGCGAGGCTATACAGAAAC	Snf7-T7 [As,R]
D3872	aatacgactcactatagggCACGTGGACGAGGACTTC	RyR-T7 [S,F]
D3993	attgctatttagagagtcatGAACGTAACGCCTGCTTTC	RyR-T7 [S,R]
D3994	ggagtactcgtaaggtagccGAACGTAACGCCTGCTTTC	RyR-T7 [As,F]
D3995	acgccagcaacgcggtctcaggggCACGTGGACGAGGACTTC	RyR-T7 [As,R]
D3876	aatacgactcactatagggATTCAAACGAAGCGCCAATAAAAG	Nep4-T7 [S,F]
D3996	attgctatttagagagtcatGTGTTTCGAAACCCACAAC	Nep4-T7 [S,R]
D3997	ggagtactcgtaaggtagccGTGTTTCGAAACCCACAAC	Nep4-T7 [As,F]
D3998	acgccagcaacgcggtctcaggggATTCAAACGAAGCGCCAATAAAAG	Nep4-T7 [As,R]
D3882	aatacgactcactatagggCCCTACGATACTGTTGAGG	IAP2-T7 [S,F]
D3999	attgctatttagagagtcatAAACCTTACAGAGGCGAG	IAP2-T7 [S,R]
D4000	ggagtactcgtaaggtagccAAACCTTACAGAGGCGAG	IAP2-T7 [As,F]
D4001	acgccagcaacgcggtctcaggggCCCTACGATACTGTTGAGG	IAP2-T7 [As,R]
D3884	aatacgactcactatagggTTGGTATACGGTCAGATGAAC	ATPsynbeta-T7 [S,F]
D4002	attgctatttagagagtcatGCTACGTTGTAGTGCTCTAG	ATPsynbeta-T7 [S, R]
D4003	ggagtactcgtaaggtagccGCTACGTTGTAGTGCTCTAG	ATPsynbeta-T7 [As,F]
D4004	acgccagcaacgcggtctcaggggTTGGTATACGGTCAGATGAAC	ATPsynbeta-T7 [As,R]
D4454	ggagtactcgtaaggtagccTTGGTATACGGTCAGATG	ATPsynbeta-PIE [S,F]
D4455	attgctatttagagagtcatTTGGTATACGGTCAGATG	ATPsynbeta-PIE [As,R]
D3886	aatacgactcactatagggTTGTGAACCCACGCACCAAG	V-ATPase-T7 [S,F]
D4005	attgctatttagagagtcatACGTTTCATGATAGATTCGGTGAC	V-ATPase-T7 [S,R]
D4006	ggagtactcgtaaggtagccACGTTTCATGATAGATTCGGTGAC	V-ATPase-T7 [As,F]
D4007	acgccagcaacgcggtctcaggggTTGTGAACCCACGCACCAAG	V-ATPase-T7 [As,R]
D4456	ggagtactcgtaaggtagccTTGTGAACCCACGCACCAAG	V-ATPase-PIE [S,F]
D4457	attgctatttagagagtcatTTGTGAACCCACGCACCAAG	VATPase-PIE [As,R]
D3888	aatacgactcactatagggATGGGTCGTATGCACGCTC	RPS13-T7 [S,F]
D4008	attgctatttagagagtcatTTTCTAAGCAACCAAAGCAGATG	RPS13-T7 [S,R]
D4009	ggagtactcgtaaggtagccTTTCTAAGCAACCAAAGCAGATG	RPS13-T7 [As,F]
D4010	acgccagcaacgcggtctcaggggATGGGTCGTATGCACGCTC	RPS13-T7 [As,R]
D4458	ggagtactcgtaaggtagccATGGGTCGTATGCACGCTC	RPS13-PIE [S,F]
D4459	attgctatttagagagtcatATGGGTCGTATGCACGCTC	RPS13-PIE [As,R]
D4073	aatacgactcactatagggGGCAAGCTGACCCTGAAG	eGFP-T7 [S,F]
D4074	attgctatttagagagtcatCTCAGGTAGTGGTTGTCG	eGFP-T7 [S,R]
D4075	ggagtactcgtaaggtagccCTCAGGTAGTGGTTGTCG	eGFP-T7 [As,F]
D4076	acgccagcaacgcggtctcaggggGGCAAGCTGACCCTGAAG	eGFP-T7 [As,R]
D4462	ggagtactcgtaaggtagccGGCAAGCTGACCCTGAAG	eGFP-PIE [S,F]
D4463	attgctatttagagagtcatGGCAAGCTGACCCTGAAG	eGFP-PIE [As,R]
D3715	actatagggagaCTTCTGTTGATATGGATGC	pLPIE-CcRPS13 [F]
D3189	gagtcgtattaATGAGACAATAACCCTGATAAATG	pLPIE-CcRPS13 [R]

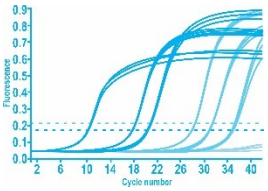
*S and As, primers for gene amplification in sense and antisense orientations, respectively. F and R,

forward and reverse primers, respectively. -T7 and -PIE suffixes indicates that the 5' end of the primer (in lowercase) is complementary to sequences of the pMT7 and pLPIE plasmids, respectively. Primers D3189 and D3715 were used to replace the lpp promoter to an inducible T7 promoter in pLPIE-CcRPS13.

Supplemental Table S2. Primers used to analyze the expression levels of *C. capitata* targeted genes by RT-qPCR.

Name	Sequence (5' to 3')	Target*
D3007	GGTGCCCTACCCACGTATTC	Alpha tubulin [F]
D3008	ACCATCTGGTTGGCTGGTTC	Alpha tubulin [R]
D4077	ACCTTGACTGCAGAAGAGGAAAA	Snf7 [F]
D4078	CTGTCGCCTACAGTGCTCAT	Snf7 [R]
D4079	CGAGTCTCCGAGTTTTGCTGA	RyR [F]
D4080	TCCATTTCCTCCGGTGTGTA	RyR [R]
D4081	GGGGAGAGTACCCAAGGTGA	Nep4 [F]
D4082	ATTCCGGGGAGACGTTCATC	Nep4 [R]
D4083	GAGCATGCCAAGTGGTTTCC	IAP2 [F]
D4084	AGCACTGGTGCAGTCATCAT	IAP2 [R]
D4085	TTCTATGTAGGAGCAACCGTGG	ATPsynbeta [F]
D4086	GGTGGCAAGTCAACATCGAAC	ATPsynbeta [R]
D4087	TATCCGGTCCTGTCGTCACA	V-ATPase [F]
D4088	GTTTGCCAGTACGCAAGACG	V-ATPase [R]
D4089	AGTCTCCACTTCCGTTCCGT	RPS13 [F]
D4090	GGAAATACCCTTGCCAGGAGC	RPS13 [R]

*F and R, forward and reverse primers, respectively.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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fluorescence
2
6
10
14
18
22
26
30
34
40
Cycle number

Discussion

The traditional notion of RNA as no more than an intermediary between genetic information and protein synthesis has been largely overcome; the development of high-throughput techniques, especially massive sequencing and computational tools, have demonstrated the participation of RNA in almost any biological process. Despite the simplicity of their composition (four structurally simple precursor molecules), these polymers form highly variable and dynamic structures that interact with cellular components (especially other nucleic acids and proteins) with which they establish their function. RNA plays central roles in genome regulation, maintenance, and defense in all organisms, being also able to catalyze chemical reactions (Cech and Steitz, 2014). Thus, it is not surprising that the focus of the composition and targets of new treatments for human diseases is shifting towards RNA (Coelho et al., 2013; Solano et al., 2014; Polack et al., 2020; Baden et al., 2021). Something similar occurs with circular RNAs: initially its function was limited to pathogenic genomes, as circular RNA is more stable than its linear equivalent. Lacking degradation-sensitive termini, circular RNAs are ideal for storing genetic information in primitive life forms, in which circularization was mediated by intrinsic RNA activities (Petkovic and Müller, 2015). The low expression level of circular RNAs in higher organisms made initially think of them as products of aberrant splicing, only recently beginning to receive the deserved attention (Haque and Harries, 2017; Haddad and Lorenzen, 2019).

Likely remnants of these primitive life forms are the viroids, whose interactions with cellular components are necessary for their infectivity. Previous works

demonstrated the participation of host ligases in the circularization of viroids from both families and the likely involvement of viroid sequences or structures in its ligation, thus not being only consequence of having suitable ends for the ligases (Martínez et al., 2009; Nohales et al., 2012a and b). In order to analyze the interactions between ELVd and tRNA ligase, the use of *E. coli* bacteria as a heterologous experimental system was proposed, by co-expressing the enzyme and a direct HHR repeat flanking a single copy of the viroid. The processing of viroid precursors and their ligation in the bacteria is so efficient that the system was adapted and optimized to produce RNAs of interest inserted into the circular viroid RNA molecule (Daròs et al., 2015, 2018; Cordero et al., 2018; Ortolá and Daròs, 2021). In this Thesis, the same expression system is used to address RNA circularity from two different points of view: to analyze the enzymatic ligation of ELVd and to develop bacterial biofactories to produce dsRNA with pesticide activity.

Here we propose the double function of the ELVd HHRs to mediate the processing of the linear concatemers produced during the rolling circle replication and to acquire additional structures to interact with the host enzyme for its circularization. Possible structures mimicked by HHR may include, but not be restricted to, the canonical substrate for these enzymes, the anticodon loop of tRNA. This being the simplest explanation for the enzymatic ligation of the viroid, in fact certain structural and sequence characteristics seem to be conserved between viroid HHRs and the tRNA anticodon loop (**Figure 12A**). Thus, a proposed model for the ELVd circularization mechanism is depicted in **Figure 12B**. The mimicry of these structures, highly evolutionarily conserved in all living organisms, is a very robust strategy for parasitizing key host proteins such as the ligase, since it leaves the host with no options to evade infection. The conservation of motifs across all viroid HHRs suggests that this interaction also occurs in viroids of - polarity, as well as in other economically relevant members of the family *Avsunviroidae*. In addition, the eggplant tRNA ligase

circularizes *in vitro* monomers of both polarities from three other members of the family (Nohales et al., 2012b). Similarly, nuclear viroids appear to require unknown structures or sequences for their enzymatic circularization. As occurs in ELVd, the opening of PSTVd (+) in sites other than the wild type prevents its circularization *in vitro* (Nohales et al., 2012a and b). We propose that a similar approach to that of these

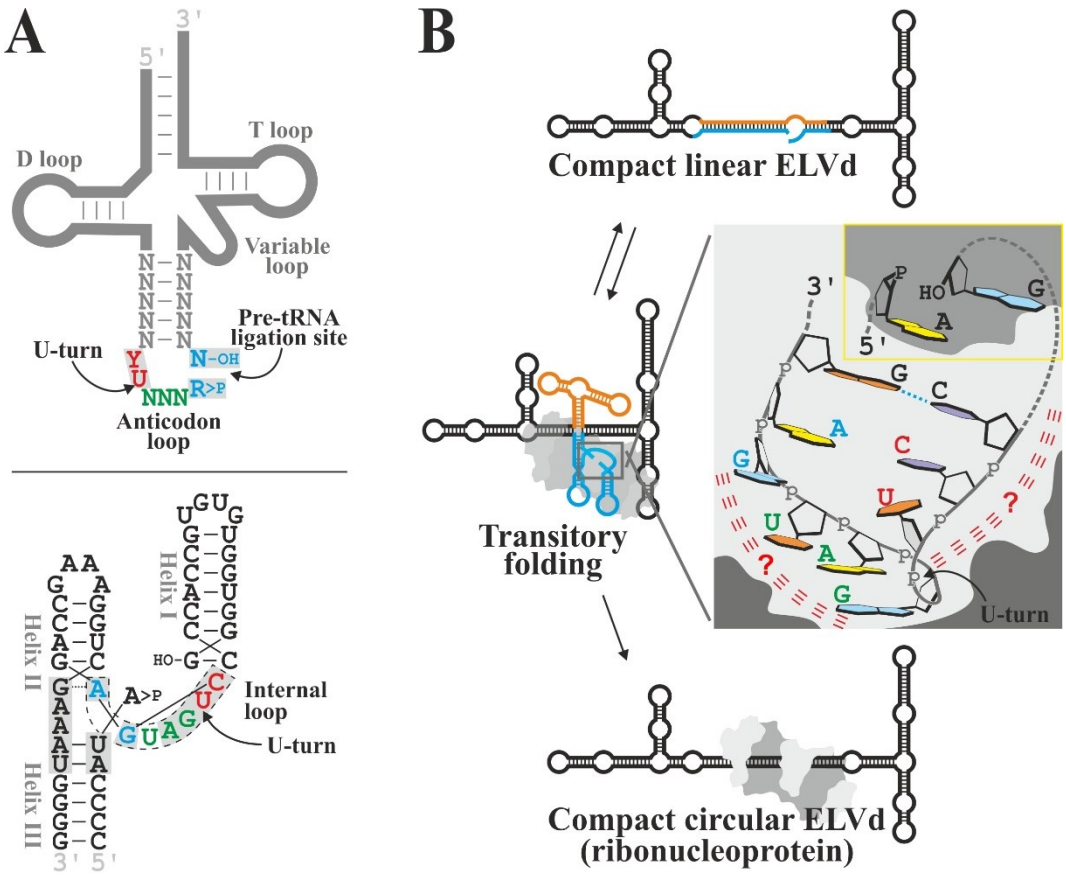


Figure 12. Proposed mechanism for the circularization of ELVd and other chloroplastic viroids. (A) Generic cloverleaf structure of tRNAs, focusing the anticodon loop (top) and secondary structures of ELVd HHR functional structure after cleavage (bottom). Conserved nucleotides of each RNA are shown in light grey boxes; the analogous nucleotides between both RNAs share colors. (B) The natural viroid ligation site resides in a quasi-double-stranded structure formed by the hybridization of + and - HHR domains in the central region of the molecule. It is likely that this region could form an alternative, less compact structure in which a characteristic folding of both ribozyme domains allows the access of the eggplant tRNA ligase to the terminal nucleotides for the ligation. The model proposes a role for the ribozyme in the enzymatic ligation process. The HHR internal loop might adopt a fold like that of the tRNA anticodon loop that is recognized by the tRNA ligase via unknown interactions (red lines). In the model, the terminal nucleotides, located in the proximity of the catalytic pocket, are correctly positioned in the ligase catalytic center (yellow insert) for its ligation.

works (that is, opening the viroid in different parts of the molecule), as well as additional mutations affecting the HHR structure, in combination with our heterologous *in vivo* system will serve to further analyze the interactions between the ELVd and tRNA ligase regarding the involvement of the HHR in this process and the alternative structures that it acquires. Engineered versions of the viroid, with HHR-containing ligation sites at different points of viroid RNA, could reveal whether sequences in the vicinity of the HHR can stablish interactions with this domain, thus avoiding the formation of the structures recognized by the enzyme. In addition, by varying the viroid and the corresponding ligase, this system has the potential to being useful to analyze the particularities of their circularization and infer the evolution of other chloroplastic and nuclear viroids to sequester their respective enzymes.

It has been described that a chaperone assists in the folding of the ASBVd ribozyme during cleavage (Daròs and Flores, 2002). Although ELVd is correctly processed in the bacteria, we cannot rule out the participation of additional plant factors that stabilize additional viroid RNA structures, stimulating the circularization, or on the contrary, unknown host factors that increase the specificity of the enzyme with respect to its substrates, reducing the circularization of the studied mutants. The model plant *N. benthamiana*, whose ligase circularizes ELVd *in vivo* (Nohales et al., 2012b), may be interesting for a rapid analysis of these processes, while the eggplant, its natural host, may also inform about the effect of the modifications in the ligation site on other viroid processes. For example, the left half of the viroid is involved in the movement between intracellular compartments, while the transcription start site is located next to the ribozyme sequence of - polarity (Gómez and Pallás, 2010, 2012; López-Carrasco et al., 2016); these regions have been likely modified in our analyses.

Plant and fungal tRNA ligases leave a 2' monophosphate group instead of the hydroxyl on the 5' ligated nucleotide (Konarska et al., 1981; Greer et al., 1983). The

monophosphate is removed from tRNAs, and possibly viroids, by 2'-phosphotransferases, that are also expressed in chloroplasts (McCraith and Phizicky, 1990; Englert et al., 2007; Banerjee et al., 2019). Thus, it is likely that HHR structures may be involved in an additional step of viroid processing, probably through interactions similar to those that are stablished with the tRNA ligase, as both processes are intimately related. However, the biological relevance of this step in viroid replication is unknown. It has been speculated that the additional phosphate group in a small fraction of the viroid population induces polymerase jumping during reverse transcription, thus resulting in non-infective clones (Ambrós et al., 1998; de La Peña et al., 1999); if a similar process occurs *in vivo*, the removal of the 2' residue would be essential during viroid replication. Additional research may help to uncover the importance of this process and to detail the viroid-enzyme interactions, a possible additional point for the control of these pathogens.

In this respect, lacking self-coded proteins, which would allow stop the infection by interrupting their functions, viroids only can be controlled by affecting their RNA and their interactions with host factors. Finding suitable targets to control pathogenic infections can be difficult, especially in chloroplast viroids given their high mutation rate (Gago et al., 2009), which might facilitate the evasion of control strategies. However, this rate is lower in certain regions such as the central viroid region that includes the HHRs of both polarities (López-Carrasco et al., 2017). The selective pressure of this region must be greater than that of other viroid regions since conserved sequences participate in the HHR catalysis mechanism, while non-conserved surrounding nucleotides are needed to establish the three HHR stems. The involvement of additional structures adopted by this region in the ligation (and probably the 2' monophosphate group removal) would further restrict the variability of this region, making it ideal a target for viroid control. Thus, the selective blockade of the foldings in this region could likely prevent viroid replication. In this sense, compounds capable

of blocking HHR activity by competing with metal ions for binding these ribozymes have been described (Clouet-d'Orval et al., 1995; Stage et al., 1995; Murray and Arnold, 1996; Feig et al., 1998; Tor et al., 1998; Horton and DeRose, 2000), as well as nucleoside analogs that can be incorporated into the ribozyme during its transcription (Yen et al., 2004, 2006). Direct interaction of polyamines with CChMVd ribozymes appears to induce structural changes that prevent HHR function (Kaddour et al., 2014). Since polyamines are ubiquitous in plant cells, the authors propose to combat viroids by modulating polyamine biosynthesis or by its exogenous supply. It has been also demonstrated the efficient blocking of self-cleavage using oligonucleotides complementary to the sequence where cleavage takes place (Yen et al., 2004). Therefore, it is necessary to analyze the viability of this type of strategies *in vivo*, as well as to identify new molecules with adequate inhibition and cytotoxic properties, able to overcome the low permeability of chloroplast membranes.

The proposed double function of the HHR arises new questions regarding the evolution of the enzyme and its different substrates. Did the adaptation of HHR to be recognized by the tRNA ligase occur when viroids became plant parasites, or is this relationship evolutionarily older? Proto-tRNAs have been proposed to act as a bridge between the RNA and ribonucleoprotein worlds, guiding the evolution of the genetic code and translation (de Farias et al., 2016; de Farias and José, 2020). At the interface between both worlds, it is consistent to think that proto-tRNAs presented one or several ribozyme activities to carry out their function. In fact, among the various models on the appearance and evolution of tRNAs, the possible nature of a *Bacillus subtilis* proto-tRNA as a viroid-like replicable ribozyme with a self-cleaving HHR structure is included (Ohnishi et al., 2005). Also, introns located in the anticodon loop are evolutionarily ancient and may have existed at the beginning of life (Kanai, 2013), while group I and II autocatalytic introns are also present in a wide variety of organisms, such as cyanobacteria and plants (Vogel and Hess, 2001; Mohanta et al.,

2019). Thus, selfish genetic elements containing HHR could have been common in this loop. The presence of these elements during tRNA evolution could have modulated the appearance of tRNA ligases and the general morphology of tRNAs. Despite the additional energy requirement, introns seem to entail a series of benefits, allowing the maintenance of the enzymes necessary for their elimination and consequently facilitating viroid parasitism. Probably these characteristics are shared with other cell RNAs, thus being processes by the tRNA ligase (Mori et al., 2010; Cervera et al., 2016; Nagashima et al., 2016; Cervera and de La Peña, 2020). In addition, recent reassessment of sequence databases has revealed more than a million new viruses present in diverse biological samples, some of which have small circular RNA genomes containing ribozymes (including those with hammerhead features) (Edgar et al., 2022). In this sense, unveil the interactions between HHRs and circularizing enzymes may have implications for understanding health and disease phenomena in plants, humans and other metazoans.

It is expected that HHR self-ligation in primitive viroid forms would be greater to form circular genomes. The efficiency of the cleavage and especially the ligation increases when comparing minimal ribozymes to those that are physiologically active (Nelson et al., 2005; Canny et al., 2007). This is likely caused by the stabilization of the longest helices and interactions that occur between them. It is possible that the ligase encoded by the plants parasitized by viroids allowed them to acquire smaller, less active versions of the HHR, making viroids more compact parasites for example by allowing sequence variation to hybridize the HHR of both polarities in the monomeric viroid. Consequently, our ELVd production system in *E. coli* requires the co-expression of host proteins. It would be of interest to analyze modifications of the ELVd endogenous HHR able to increase its self-ligation capacity without destabilizing the resulting molecule, simplifying the system and allowing the reconstruction of a possible evolutionary past of chloroplast viroids.

In the mature viroid RNA, the complete sequence of the ribozyme is present, so a balance in the ribozyme self-cleavage and enzymatic re-ligation reactions would be expected. However, this is not apparent in our system, as the equilibrium is shifted towards maintaining the circular structure, especially with the expression using Terrific Broth medium, which possibly allows greater accumulation of the ligase. Hybridization of ribozymes of both polarities in the wild-type viroid may be more stable than the catalytic folding of the ribozyme. In addition, the suboptimal structure of HHRs can also affect cleavage, requiring additional cofactors not accessible to the compact structure. This may also be caused by the formation of the proposed ribonucleoprotein complex with the ligase (Daròs et al., 2018) or by the low stability of the linear monomeric ELVd molecules, which could rapidly degrade in the bacteria.

The stability of dsRNA, and specially the vulnerability of RNA to degradation by their ends, both during its production in the bacteria and its application, is the main limitations for the use of RNAi in various approaches, together with the need of high yields of dsRNA. In recent years, great strides have been made to develop a variety of efficient expression systems. Most of them express hairpins or complementary ssRNAs that hybridize to form dsRNAs; that is, molecules with termini susceptible to exonuclease degradation. These strategies usually depend on nanomaterials with which they are formulated to protect the dsRNA. Although this strategy has been very useful, it is also interesting that the produced molecules intrinsically possess certain resistance to degradation. In this regard, improving the stability of dsRNAs can be achieved through chemical modifications on the phosphate backbone or the ribose moieties, although certain modifications can decrease RNAi activity and/or increase cytotoxicity (Abe et al., 2007, 2011); alternatively, RNA nanostructures can be rationally designed. These latter authors were pioneers in generating covalently closed dsRNA molecules that increase stability against 3' exonucleases. They expressed ssRNAs with a complementary central region and additional sequences to generate loops at both ends,

that are ligated by T4 RNA ligase; these molecules are correctly processed by the RNAi machinery. Since then, multiple strategies have been developed for the synthesis of covalently closed circles and other structures that provide protection to RNA (Grabow et al., 2011; Lee et al., 2012; Kim et al., 2016). Circular dsRNAs not only allow greater bioavailability (and consequently a more powerful effect) but also greater cell uptake and longer silencing times, probably by the slower production of siRNAs (Winkle et al., 2021). In this work, we used two different strategies to obtain covalently closed dsRNAs that can be used in pest control. In both of them, we use an autocatalytic intron to separate the inverted repeats of the target gene, while circularization is achieved through the intrinsic properties of viroids or an additional intron copy in permuted conformation. Gnanamony et al. (2021) described, in parallel to our work, the production of circular dsRNAs with the use of permuted autocatalytic introns. However, they expressed microRNAs of ~100 nts, whose imperfectly paired hairpin structure makes dispensable the use of additional spacers.

Although the double intron strategy would be chosen for general RNAi applications, in certain cases it may be useful to express RNAs inserted in the ELVd molecule. Its great potential is that viroids of the family *Avsunviroidae* are the only known pathogens capable of efficiently entering the chloroplast, an organelle of biotechnological interest. Chloroplasts lack RNAi machinery, thus dsRNAs accumulating in there are not processed by the plant and their uptake by insects not hindered. Our production system could be adapted to express chimeras in the plant nucleus, which accumulate intact and in great amounts in the chloroplast. In addition, general research on viroids has led to important discoveries on RNA and plant biology (Ma et al., 2022b), and the utility for dissecting gene functions in eggplant has recently been shown for ELVd clones carrying plant sequences (Márquez-Molins et al., 2022a). In the same way, it would be interesting to exploit both versions of the system for other aspects of plant protection and improvement, such as inducing resistance to

viruses, abiotic stresses or increasing productivity (Tenllado et al., 2003; Vargas et al., 2008; Necira et al., 2021; Sharma et al., 2022; Ullah et al., 2022). Although the use of perfectly matched hairpins or dsRNA, both processed by the siRNA pathway, is the most common methodology for insect control, there are some examples of other RNAi pathways exploited for the same purpose (Muerdter et al., 2012; Ye et al., 2017; Bally et al., 2020; Mondal et al., 2020; Zhang et al., 2021a). Given the RNA nature of the precursors of these pathways, and especially the highly structured pri- and pre-miRNAs, their expression is compatible with our system, so it would be interesting to analyze their use for pest control. Therefore, the toolbox involving the ELVd-based system can be widely expanded.

It is expected the main limitation of our *E. coli* production system being the accumulation of the ligase and its effect on the bacteria. Therefore, analyzing the circularization process with additional RNA ligases, including those of smaller size, may allow for increased bacterial growth and accumulation of the viroid-RNA chimeras of interest, as well as provide additional data on enzyme-viroid interactions. In this sense, plant tRNA ligase can functionally substitute the activity of the smaller yeast counterpart in tRNA splicing (but not in other non-canonical processes), and vice versa (Wang et al., 2006a; Mori et al., 2010). In other domains of life, a different clade of ligases is involved in the processing of tRNAs (Gerber et al., 2022). Despite the absence of tRNAs interrupted by introns, *E. coli* contains a small protein (RtcB) with homology to those that in vertebrates catalyze the ligation of tRNA exons, expressing it only in response to certain stresses (Tanaka and Shuman, 2011). These enzymes have a different catalytic mechanism and they do not generate the 2' monophosphate characteristic of plant and fungal tRNA ligases but recognize and ligate the same termini. RtcB in *E. coli* can carry out the ligation of broken tRNA stem-loops, so along with the low self-ligation activity of HHRs may be responsible for the residual ELVd circularization observed in negative controls without ligase. In addition, recent work

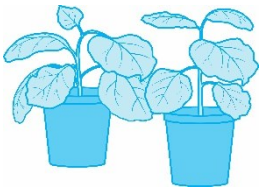
demonstrates that this enzyme mediates the circularization of RNAs flanked by twister-type ribozymes in human cells encoding a RtcB homologue (Litke and Jaffrey, 2019), pointing to lower sequence and/or structure requirements for the substrates of these enzymes.

As a central part of our dsRNA expression system, the autocatalytic intron can also be optimized to further improve the production. Since our work points to a saturable autocatalytic reaction, probably due to the participation of chaperones that mediate correct intron folding (Bhaskaran and Russell, 2007), it would be interesting to analyze the use of introns with lower requirements, and therefore higher reaction speed and efficiency. Also, the use of shorter but similarly efficient introns could help to optimize the bacteria's resources. For example, smaller group I natural introns can be found in *Phialophora americana* fungi and is composed solely of 67 nt (Harris and Rogers, 2008), a sequence <20% of that used in our works. Furthermore, detailed function analysis of the hairpins of various introns have shown the presence of a minimal catalytic unit while other domains play a mainly structural role, so a rational design of smaller functional structures is possible (Doudna and Szostak, 1989; Ikawa et al., 2000). On the other hand, the main limitation of using these introns in biotechnological applications is the need of incorporating small exonic segments of the original gene to establish interactions with an intron internal guide and define the 5' and 3' splicing sites (Rausch et al., 2021). In our molecules, the resulting exonic loops are unstructured ssRNAs, likely more sensitive to degradation. Through deletional analysis we were able to shorten both exons to 2+2 nt without reducing splicing efficiency; however, the interactions described for this intron are as higher as 6 base pairs between exon 1 and the guide sequence, and 7 between the guide and the second exon (Michel and Westhof, 1990). It is possible that the intrinsic characteristics of the sequences of our analysis are responsible for the efficient processing, allowing these interactions to be established. We cannot rule out that our flanking sequences, an

extensive region of perfectly complementary dsRNA, position and stabilize the ends to be correctly processed and/or provide a very stable base that facilitates the adequate co-transcriptional folding of the intron, increasing circularization efficiency despite imperfect interactions with the recombinant exons. In any case, strategies for optimizing the processing of any sequence should be explored. The rational selection of the intron-flanking sequences of the inverted repeats, along with modifications in the guide sequence, should allow the generation of exonic loops of smaller sizes with high efficiency (Rausch et al., 2021). In the same way, the use of other small autocatalytic introns, both of group I and II, able to self-splice with less exonic requirements could be useful (Mikheeva et al., 1997; Rausch et al., 2021).

The ability of both types of molecules orally delivered to silence endogenous genes has been demonstrated in *D. virgifera virgifera* and *C. capitata*. While in the first species the RNAi strategy is well established and studied, with the first GM crop variety producing dsRNAs against the pest approved for commercialization, the process in the second is largely unknown. The variability of the cell machinery between insect species has an enormous impact on the success of RNAi strategies. Thus, the use of suitable nanomaterials may be needed to compensate these particularities. Further studies are required to analyze aspects such as the ability of the nucleases of the digestive system and hemolymph of these insects to degrade our RNAs or the specific mechanisms by which they are imported into the cell cytoplasm and distributed systemically. In this regard, endocytosis mediated by clathrin and scavenger receptors is the main mechanism of dsRNA uptake in Diptera, but molecules with an architecture similar to ours (dsRNA fragments closed by single-stranded loops on both sides) are also internalized independently of this route (Abbasi et al., 2020), so our produced dsRNAs could be of interest in some insects recalcitrant to RNAi.

To recapitulate, the heterologous expression system of circular RNAs in *E. coli* has allowed us to analyze the circularization process of ELVd and propose the involvement of its HHR in the process, probably by mimicking the tRNA anticodon loop to be recognized by the eggplant tRNA ligase, as well as to efficiently produce compact and stable dsRNA circular molecules, with or without sequences of such viroid, capable of inducing gene silencing in pests of economic relevance when administered orally. This experimental system has the potential to allow the development and be part of effective strategies for the control of various pathogens and pests in complex ecological environments.



Conclusions

1. Viroid hammerhead ribozyme (HHR) domains are required for the processing of linear concatemers to monomeric units during replication and, at least in the case of eggplant latent viroid (ELVd) expressed in a heterologous expression system, also for their circularization as they have the capacity to recruit the co-expressed chloroplastic eggplant tRNA ligase.

2. Different folds of this domain are likely necessary to mediate HHR activity in each process. The conservation of motifs and structures between HHR and tRNAs lead us to propose that HHR might mimic the anticodon loop of pre-tRNAs, the natural substrates of these enzymes, for its recognition.

3. Given the conservation of both the viroid HHR characteristics and the enzymes involved in the process, this HHR-guided, enzyme-mediated process must also occur in other viroids of the family *Avsunviroidae*.

4. Group I autocatalytic introns allows the production of double-stranded RNA (dsRNA) hairpins in prokaryotic systems lacking splicing machinery (such as *Escherichia coli*), solving the instability of DNA sequences with inverted repeats by separating them with sequences that are removed from the primary transcript.

5. This strategy, when combined with the intrinsic characteristics of ELVd or with the circularization capacity of these introns in permuted configuration,

allows the obtention of circular and compact dsRNA molecules, more likely resistant to degradation.

6. Both types of molecules (with and without viroid scaffolding) have proven *in vivo* insecticidal capacity in *Diabrotica virgifera virgifera* and *Ceratitis capitata*, respectively. Thus, our production system is useful for screening insect susceptible genes for pesticidal approaches and has potential for field application.

7. Both strategies presented here are powerful tools for the biofactories of the future. They provide high yield of dsRNA, thus being a fast and cheap alternative for RNA synthesis. Its scalability, robustness and flexibility make this system industrially applicable to produce substantial amounts of dsRNA for the control of these insects and other pests and pathogens, as well as other RNAi applications.

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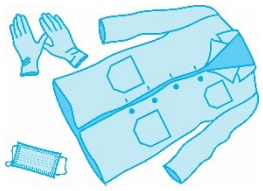

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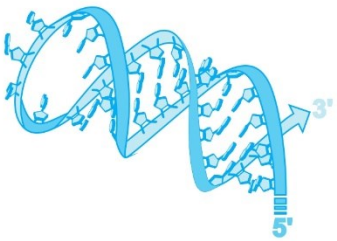

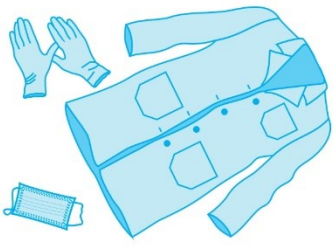

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