biology

MDPI

Review

RNA Interference in Insects: From a Natural Mechanism of Gene Expression Regulation to a Biotechnological Crop Protection Promise

Beltrán Ortolá and José-Antonio Daròs *

Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universitat Politècnica de València, 46022 Valencia, Spain
* Correspondence: jadaros@ibmcp.upv.es

Simple Summary: Large amounts of conventional insecticides are used to control insect pests in agricultural production worldwide. New sustainable strategies, more specific and environmentally friendly, are urgently needed to combat insect pests. RNA interference (RNAi) is a natural mechanism of gene expression regulation that has been repurposed for biotechnological applications. Double-stranded RNAs homologous to endogenous genes, which could be produced in the crop plant or exogenously synthesized and applied onto crops, efficiently trigger gene silencing in insects, reducing pest damage. Consequently, these molecules are envisioned as the new generation of insecticidal compounds.

Abstract: Insect pests rank among the major limiting factors in agricultural production worldwide. In addition to direct effect on crops, some phytophagous insects are efficient vectors for plant disease transmission. Large amounts of conventional insecticides are required to secure food production worldwide, with a high impact on the economy and environment, particularly when beneficial insects are also affected by chemicals that frequently lack the desired specificity. RNA interference (RNAi) is a natural mechanism gene expression regulation and protection against exogenous and endogenous genetic elements present in most eukaryotes, including insects. Molecules of double-stranded RNA (dsRNA) or highly structured RNA are the substrates of cellular enzymes to produce several types of small RNAs (sRNAs), which play a crucial role in targeting sequences for transcriptional or post-transcriptional gene silencing. The relatively simple rules that underlie RNAi regulation, mainly based in Watson–Crick complementarity, have facilitated biotechnological applications based on these cellular mechanisms. This includes the promise of using engineered dsRNA molecules, either endogenously produced in crop plants or exogenously synthesized and applied onto crops, as a new generation of highly specific, sustainable, and environmentally friendly insecticides. Fueled on this expectation, this article reviews current knowledge about the RNAi pathways in insects, and some other applied questions such as production and delivery of recombinant RNA, which are critical to establish RNAi as a reliable technology for insect control in crop plants.

Keywords: RNAi interference; insect pest; small RNA; double-stranded RNA; recombinant RNA; RNA delivery

Citation: Ortolá, B.; Daròs, J.-A. RNA Interference in Insects: From a Natural Mechanism of Gene Expression Regulation to a Biotechnological Crop Protection Promise. *Biology* **2024**, *13*, 137. https://doi.org/10.3390/biology13030137

Academic Editor: Ming Wang

Received: 14 December 2023
Revised: 14 February 2024
Accepted: 19 February 2024
Published: 21 February 2024

1. Introduction

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 [1,2], 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 [3–5]. 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 [6]. 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 or releasing of sterile insects and pest predators, parasitoids and pathogens [7]. The development of genetically edited plants capable of providing protection against insects, by modifying mixtures of volatiles or expressing *Bacillus thuringiensis* entomotoxins against specific pests, is also promising [8]. 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.

RNAi describes a series of mechanisms highly conserved in eukaryotes, regulating gene expression and protecting against exogenous and endogenous genetic elements, such as viruses, viroids, 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 and post-transcriptional level; the first involves epigenetic modifications in DNA and histones that repress the transcription process, and the second, mRNA degradation or translational repression.

2. RNAi Discovery

The discovery of the RNAi mechanistic bases is attributed to the work of Andrew Z. Fire and Craig C. Mello with the nematode and model organism *Caenorhabditis elegans* [9], which earned them 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 [10]. Their discovery served to explain previous phenomena of unexpected silencing in various organisms. For example, in the nematode, similar levels of silencing were achieved by using antisense and sense RNAs (the latter frequently used as control in antisense strategies) [11,12]. Other examples were described in plants and fungi, in which the use of transgenes to overexpress endogenous or exogenous proteins (with high sequence homology) sometimes resulted in the silencing of these genes [13–17]. All these experimental procedures 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* [18–21], 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 inspired many biotechnological applications.

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. They include small interfering RNAs (siRNAs), microRNAs (miRNAs), and P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs). sRNAs can be exogenous (foreign genetic elements, such as viruses with dsRNA genomes, dsRNA replication intermediates or highly structured RNAs, 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 has evolved to positively regulate the expression of target sequences at the transcriptional level in many eukaryotes [22,23].

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 [24,25], although multiple subtypes of siRNAs with endogenous origin (esiRNA) exist. esiRNAs can derive from transposable elements, genomic regions with inverted repeats and from mRNAs with overlapping sequences (natural antisense siRNA, natsiRNA) [26–30]. esiRNAs are involved in maintaining genome stability [26–28] and regulation of gene expression in certain cellular processes, such as energy homeostasis or the response to several stresses [31–33].

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 [34–37]. In *Drosophila melanogaster* and presumably all insects, there are two different Dicer genes, Dicer-2 being responsible for specifically producing siRNAs [38]. *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 [39,40]. This enzyme can process dsRNAs according to their characteristics following two different mechanisms [40–43]. In the first mechanism, 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 dsRNA. In the second mechanism, the PAZ/Platform domain interacts directly with dsRNAs containing 2 nt 3′ overhangs (that are characteristic of cleavage by RNase III) independently of ATP. After each cleavage, the dsRNA dissociates, and its protruding ends can be recognized again. Both mechanisms are typical, but not exclusive, for the described molecules [44]. The recognition and cleavage of dsRNAs may also depend on the involvement of dsRNA binding proteins (dsRBPs). For example, it has been described that R2D2 (protein with two dsRBDs associated with Dicer-2) prevents Dicer-2 from processing miRNA precursors *in vitro*, increasing its affinity for long dsRNAs [42], while Loquacious-PD (Loqs-PD) is necessary for processing certain esiRNAs but not for viral siRNAs [45–48]. It has been proposed that Loqs-PD promotes the use of suboptimal dsRNAs by altering the Dicer-2 dependence of terminal structures [43,44,49,50].

The length of the produced RNAs is characteristic of the Dicer protein, since it depends on the distance between the PAZ domain and the active center of the RNase domains, acting as a sort of molecular rule [51–53]. Thus, typical insect siRNAs are 20–22 nt long [54]. The presence of two RNase III domains and their characteristic positioning generates 2 nt overhanging 3′ ends, with 5′ phosphate and 3′ hydroxyl termini [42–50,55].

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 [56]; the other strand (passenger strand) is usually degraded [57–59]. Strand selection follows the thermodynamic asymmetry rule, whereby the strand with its 5′ termini less stably paired with the complementary strand is preferentially selected [60,61]. The selection occurs in the so-called RISC loading complex, consisting of Dicer-2, R2D2 and possibly additional factors [62,63]. R2D2 interacts with the more thermodynamically stable end of the duplex and tightly binds the 5′ end of the passenger strand, restricting Dicer-2 to the opposite end of the siRNA duplex [64]. This function can be replaced by Loqs-PD in certain esiRNAs [64,65].

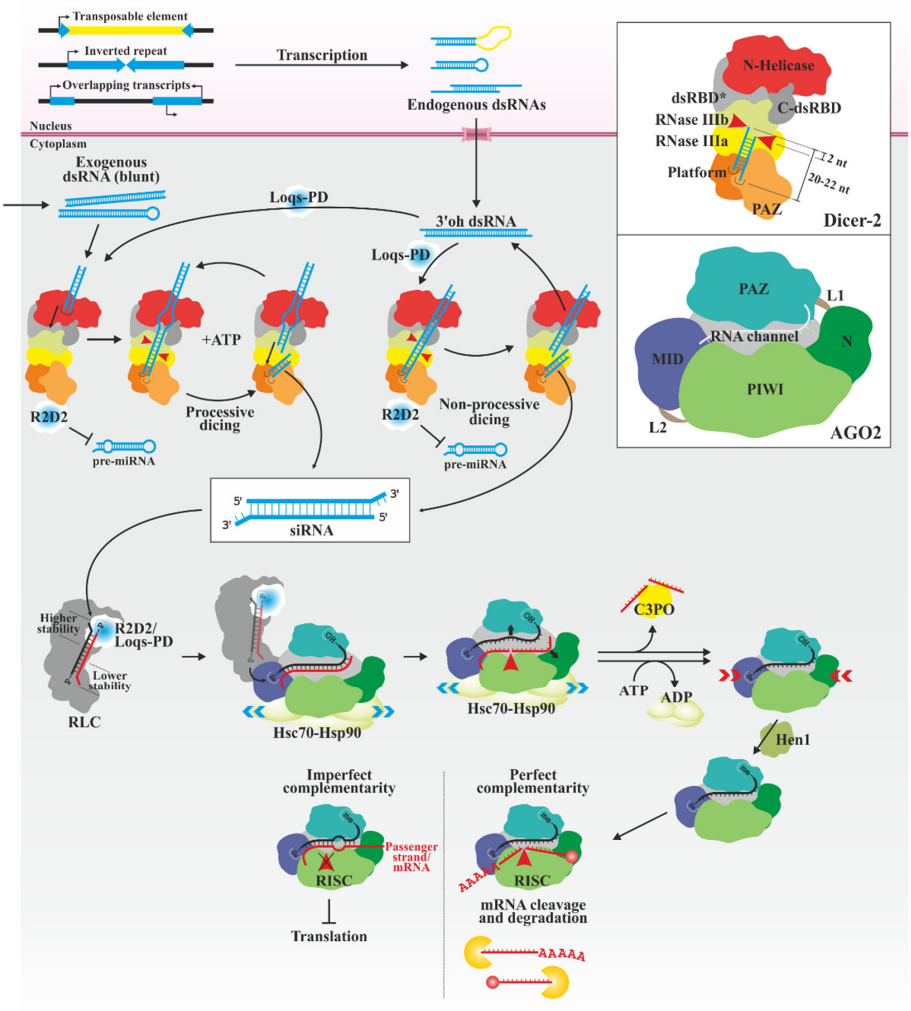

Figure 1. siRNA pathway in insects. Perfectly complementary long dsRNAs of exogenous or endogenous origin (depicted in blue) are differentially processed in the cytoplasm by Dicer-2 depending on the nature of their termini. Additional factors mediate the selection of suitable substrates in both processes. siRNAs of 20–22 bp are generated. Dicer-2 and R2D2 (or 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 (also in red, depicted with 5′ cap and 3′ poly(A) tail), marking them for further degradation. The presence of mismatches with the target 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. -me, -OH and -P, 3′ 2′-O-methyl, 3′-hydroxyl and 5′-phosphate termini, respectively; 3′oh, 2 nt 3′ overhang termini; AGO2, Argonaute 2; C-dsRBD, carboxyl-terminal dsRNA-binding domain; C3PO, component 3 promoter of RISC; dsRBD*, atypical dsRNA-binding domain; Hsc70-Hsp90, complex of 70 kDa heat shock analog protein and 90 kDa heat shock protein, respectively; L1 y L2, linker 1 and 2, respectively; Loqs-PD, Loquacious isoform PD; MID, middle domain; N, amino-terminal variable domain; N-helicase, amino-terminal helicase domain; PAZ, Piwi/Argonaute/Zwille domain; pre-miRNA, micro RNA precursor; R2D2, protein with two dsRNA-binding domains associated with Dicer-2; RISC, RNA-induced silencing complex; RLC, RISC loading complex; and siRNA, small interfering RNA.

The cellular effector of the silencing is a protein of the Argonaute (AGO) family [35,56,66,67], which forms RISC together with accessory proteins and the RNA. In insects, two proteins of the AGO subfamily have been described [66,68,69]. They are multidomain proteins orga-

nized in two lobes: the N-terminal contains the variable N- and PAZ domains, connected by a linker (L1); a second linker (L2) connects this lobe to the C-terminal lobe, which contains the middle (MID) and PIWI domains [67,70–72]. 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 [24,28,30,73,74]. 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 [75–81].

The loading of the duplex requires Dicer-2/R2D2 (or Loqs-PD) and AGO2 [58,62,63,82,83]. Their interaction allows the MID domain to recognize and bind the 5′ phosphate end of the guide strand, transferring the duplex [83,84]. Weaker interactions are established between PAZ and the 3′ overhang end [67,85]. The duplex is then unwound, presumably by the N and PAZ domains [63,73,86–88], and the PIWI domain cleaves the passenger strand [57–59], which is rapidly removed by the C3PO (component 3 promoter of RISC) endonuclease [89]. The 3′ terminal nucleotide is methylated at the 2′-O position by RNA methyltransferase Hen1 [90], 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 [83,91,92], 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 guide RNA. PIWI is similar in structure to RNase H, which typically catalyzes the hydrolytic cleavage of RNA in RNA/DNA duplexes [70,93]. The cleavage generates 5′-phosphate and 3′-hydroxyl ends [94], 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 [95]. The AGO2-mediated cleavage, both in the passenger strand and in target mRNAs, does not occur if there are mismatches between the guide and its complementary strand, partially explaining the differential loading of RNAs in both AGO isoforms [58]. In this case, the elimination of the passenger strand is slower, and silencing is established by blocking protein synthesis.

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 [96–99]. They are usually expressed as polycistronic RNAs from intergenic regions of the genome carrying their own promoters or from intragenic regions co-expressed with the gene they regulate [100]. They are generally transcribed by RNA polymerase II as primary transcripts (pri-miRNA) with typical mRNA modifications (5′ cap and 3′ poly(A) tail) [101–103] and a general hairpin structure with a long complementary double-stranded region flanked by a terminal loop and two unstructured single-stranded segments (ssRNA).

The pri-miRNAs are processed in the nucleus [104] by the type-III ribonuclease Drosha, with the help of Pasha, a dsRBPs with which it forms the Microprocessor complex [105–107]. The processing of pri-miRNAs is especially studied with the human counterpart of the Microprocessor, probably occurring similarly in insects [108–113]. Two Pasha proteins recognize and bind the terminal loop of the pri-miRNA, and their dsRBDs interact with part of the dsRNA region. Drosha dsRBD binds the other half of the dsRNA, and additional interactions are established with the terminal ssRNA-dsRNA region. 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 bp. Additional RNA motifs may affect processing efficiency [114]. The two RNase III domains of Drosha eliminate the basal single-stranded segments and part of the dsRNA (~11 nt), generating miRNA precursors (pre-miRNAs), hairpins of about 70 nt with one of the characteristic 2 nt protruding 3′ ends of miRNAs. As an alternative to this canonical

biogenesis pathway, 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 the Microprocessor, directly entering the pathway as pre-miRNAs with a hairpin structure [115,116].

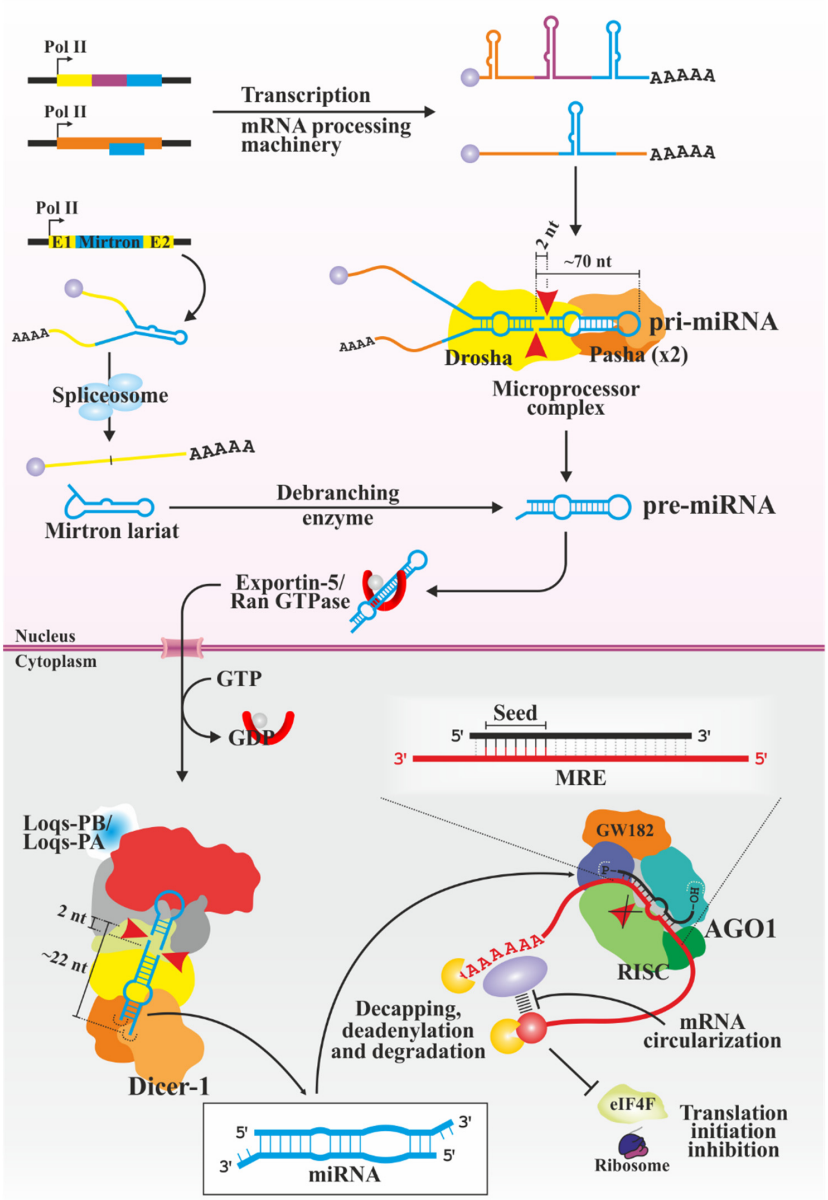

Figure 2. miRNA pathway in insects. miRNA-containing genomic loci are transcribed by RNA polymerase II generating long, partially dsRNA primary precursors (depicted in blue) that are trimmed into shorter hairpins by the Microprocessor complex Drosha/Pasha in the nucleus. This step is not necessary in 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. The miRNA guide strand (black) loaded into AGO1 induces cleavage-independent mRNA (in red, depicted with 5′ cap and 3′ poly(A) tail) degradation and translation suppression of the partially complementary mRNAs. -OH and -P, 3′-hydroxyl and 5-phosphate termini, respectively; AGO1, Argonaute 1; E1/E2, exon 1 and 2, respectively; eIF4F, eukaryotic initiation factor 4F; Loqs-PA/-PB, Loquacious isoforms PA and PB, respectively; miRNA, micro RNA; MRE, microRNA recognition elements; Pol II, RNA polymerase II; pre- and pri-miRNA, precursor and primary micro RNA, respectively; and RISC, RNA-induced silencing complex.

The next step in miRNA processing occurs in the cytoplasm [104]. Exportin-5 (Exp-5) mediates pre-miRNAs translocation to the cytoplasm through the nuclear pores in combination with Ran GTPase [117,118]. The efficient transport of precursors by Exp-5 may depend largely on the presence of 2 nt 3′ overhangs and, to a lesser extent, on the characteristics of the apical loop, as occurs in mammals [119–121]. Release of pre-miRNA into the cytosol requires hydrolysis of GTP by Ran GTPase. There, they are processed by Dicer-1 [38,75]. The PAZ domain recognizes and binds to the 3′ overhang generated by Drosha while the helicase domain binds to the loop region [122], the latter being not functional in this isoform [38]. The helicase senses the loop size while PAZ act as a molecular ruler measuring the distance from the loop to the 3′ overhang. Thus, tandem RNase III domains only cleave substrates with adequate distance between the 3′ overhang and the terminal loop [122]. 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 is involved in the processing of most but not all miRNAs, possibly stabilizing RNAs with unstable structures at the cleavage site [123–127].

Next, the miRNA duplex is loaded onto RISC containing AGO1 [73], and the strand selection occurs following the same rules as for siRNAs. Unlike siRNas, in which R2D2 or Loqs-PD are required for AGO2 loading, Loqs-PB seems to be dispensable for the duplex transfer to AGO1 [127], 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 [88,128]. It is worth mentioning that miRNAs and their non-mature forms can be edited, adding or modifying nucleotides that are critical for their maturation, regulation and functionality [129,130].

In animals, the miRNAs recognition elements (MREs) are usually found in the 3′ untranslated region (3′ UTR) of mRNAs [131]. Their interaction is usually imperfect but with a characteristic pattern: nucleotides 2–8 of the miRNA 5′ end (called seed region) have perfect complementarity with the mRNA and is sufficient for its function, although additional pairing may participate in the process [132–134]. This short sequence allows a miRNA to regulate several mRNAs and different miRNAs can act on a single mRNA [135,136]. In *D. melanogaster*, both AGOs have cleavage activity; however, the catalytic rate of AGO1 is limited by the inefficient dissociation of the reaction product [75]. Thus, AGO1 induces silencing in several ways in which GW182 proteins generally participate by recruiting cell factors and serving as scaffolding. It can inhibit translation by preventing the 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 [137–139]. It can recruit deadenylases and decapping enzymes [137,140–143], making the mRNA sensitive to the action of the exoribonuclease 5′-3′ XRN1 [144].

3.3. piRNAs

The piRNA pathway (Figure 3) is mediated by proteins of the AGO 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 [145,146]. piRNAs also participate, in germ and somatic cells, in processes such as fertility, maintenance and differentiation of stem cells or defense against some viruses [147–151].

Unlike siRNAs and miRNAs, piRNAs are processed from single-stranded precursors that are transcribed by RNA pol II and processed and exported as typical mRNAs [152,153]. In germline, the expression of precursors from bidirectional clusters is common [146,154,155], involving the same polymerase but requiring 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 the mRNA nuclear export factor complexNxf3-Nxt1, that mediates export of precursors to the cytoplasm [155–161].

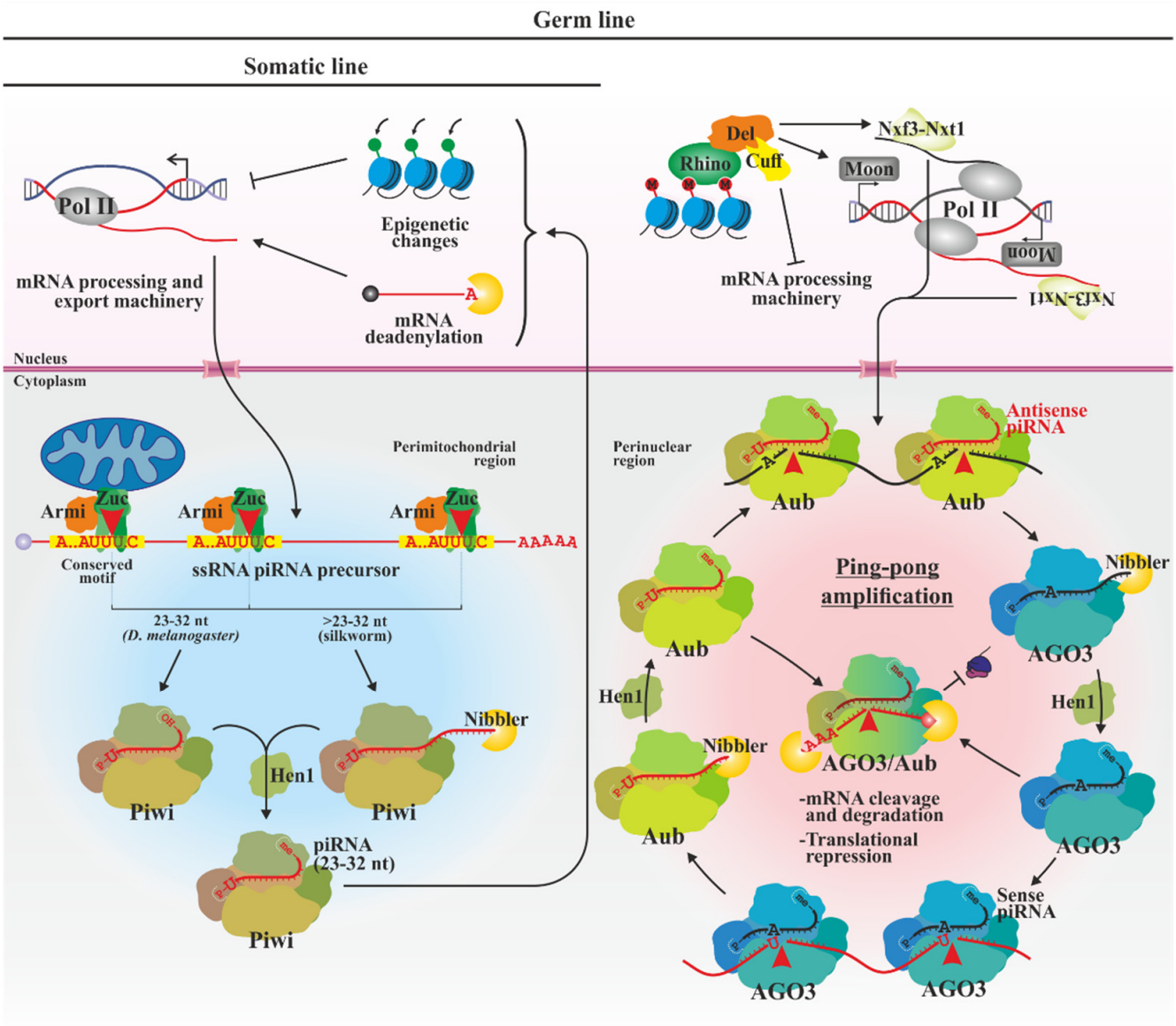

Figure 3. piRNA pathway in both, insects' somatic and germline cells. In somatic cells, piRNA precursors are transcribed from unidirectional piRNA clusters. Linear precursors are exported to cytoplasmic perimitochondrial regions where Zucchini and in some cases additional exonucleases generate single-stranded 23–32 nt piRNAs that are loaded on Piwi proteins and methylated at their 3′ end. In germline, the expression of specialized transcription machinery also allows the generation of piRNAs of both senses from bidirectional piRNA clusters. Protein-loaded sense piRNAs participate in the generation of antisense piRNAs and vice versa (ping-pong cycle) in perinuclear regions. Aub/AGO3 differentially load RNAs of both polarities that are also terminally trimmed and 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 (also in red, depicted with 5′ cap and 3′ poly(A) tail) and repressing translation. -me, -OH and -P, 3′ 2′-O-methyl, 3′-hydroxyl and 5′-phosphate termini, respectively; AGO3, Argonaute 3; Armi, Armitage; Aub, Aubergine; Cuff, Cutoff; Del, Deadlock; M, H3K9me3 epigenetic marker; Moon, Moonshiner transcription factor; Nxf3-Nxt1, mRNA nuclear export factor complex; piRNA, PIWI-interacting RNAs; Pol II, RNA polymerase II; and Zuc, Zucchini.

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 [162–164]. In germline cells, the 5′ phosphate end of piRNAs is generated by Aub proteins containing antisense guide RNAs that recognize and cleave sense precursors, 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 [165–169]. Only the second pathway occurs in the somatic line, since the machinery required for generating complementary piRNAs is not expressed. The 5′-phosphate intermediates generated in both pathways are loaded onto Piwi proteins (somatic cells) and also in AGO3 (in germline) [146]. Mature piRNAs are slightly longer than miRNAs and siRNAs (23–32 nt). The 3′ end of piRNAs is generated by additional downstream action of Zuc or Aub proteins. In some species such as silkworm, the 3′ ends are trimmed by a 3′-5′ exonuclease to generate optimal RNAs to be fully accommodated by PIWI subfamily proteins [170,171]. In *D. melanogaster*, it has been suggested that the Nibbler exonuclease is dispensable if the 3′ cleavage is generated by Zuc, which would directly generate the appropriate end of the piRNA [172]. The maturation of the piRNAs concludes with the 2′-O-methylation of its 3′ end by the Hen1 methyltransferase [90,173], probably being required for its correct interaction with the PAZ domain, as in mammals [174].

In germline, sense precursors loaded to AGO3 serve as a guide for the cleavage of homologous antisense transcripts [146,175]. The new molecule is recognized, loaded and processed in Aub. piRNA-Aub, in turn, recognizes and cuts transcripts from the piRNA cluster itself, generating new sense piRNAs, thus amplifying the silencing signal with the generation of secondary piRNAs in the so-called "ping-pong mechanism". Due to the cleavage pattern, the molecules loaded in AGO3 have 10 nt homology to the antisense piRNA, and most of them have an adenine in the tenth position. Also, the additional activity of Zuc/Aub/AGO3 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 [165,167–169].

To carry out their silencing activity, Aub and AGO3 remain in the cytoplasm where they mediate post-transcriptional gene silencing by cleaving target RNAs [146,175], repressing translation and promoting mRNA degradation [176–178]. Conversely, Piwi develops its function mainly in the nucleus, inducing epigenetic changes [179–181] and deadenylating mRNAs in sites of active transcription of transposable elements [182].

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) [183] (Figure 4). Both processes require additional cellular machinery that seems 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 knowledge about non-cell autonomous RNAi comes from *C. elegans*, in which it 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; 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 [184–187]. Two endosomal vesicle-associated proteins, SID-5 [188] and SID-3 [189,190], are also required in both dsRNA uptake and systemic distribution by mediating the import and export of vesicles containing such dsRNAs.

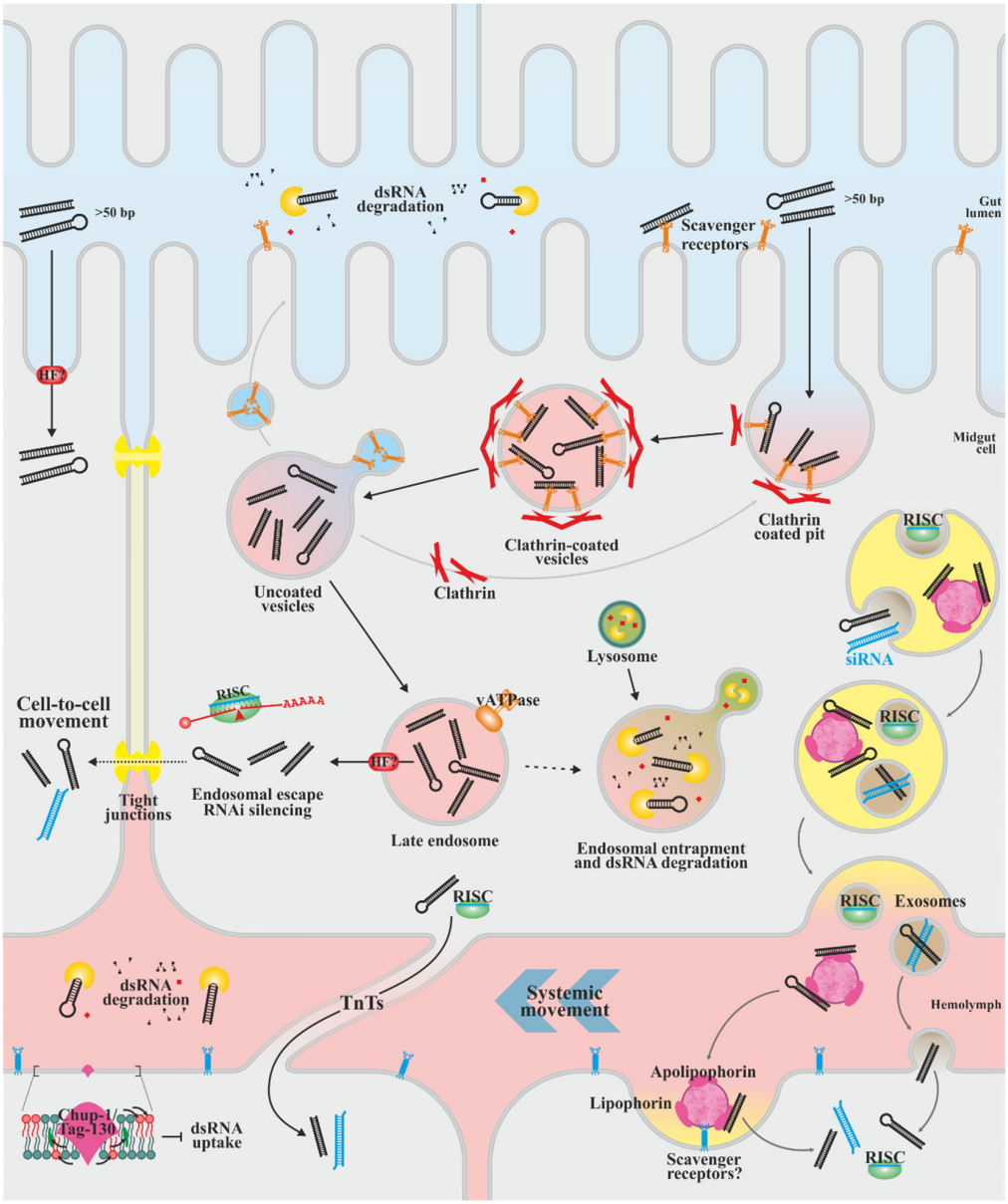

Figure 4. Proposed mechanisms of exogenous dsRNA cell-uptake, cell-to-cell and systemic movement in insects. Scavenger receptor-mediated clathrin-dependent endocytosis is the main pathway for cellular uptake of dsRNAs in midgut cells, although additional unknown factors may be involved in this process. 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 it 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. Chup-1/Tag-130, cholesterol uptake protein 1; HF?, unknown host factor; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; TnTs, tunneling nanotubes; and vATPase, Vacuolar-type ATPase.

Other than *C. elegans*, SID-2 homologs have only been found within the genus *Caenorhabditis*, in species resistant to environmental RNAi [185]. SID-1 homologous genes have

been identified in most insects, except for those of the superorder Antliophora (Diptera, Mecoptera and Siphonaptera) [191]. However, these genes may have more homology with tag-130/CHUP-1, a SID-1 paralogue that does not participate in RNAi in *C. elegans* but is involved in cholesterol internalization [192,193]. 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 from subsequent exposures to environmental dsRNA [194]. This is consistent with the elusive role of this protein in insects, since there is no straightforward association between absence, presence and number of Sid-1-like genes with the efficient uptake and systemic distribution of dsRNAs [192,195,196]. 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 [197,198]. In addition, chemical blockade of pattern recognition receptors disrupts uptake [197], and two scavenger receptors, SR-CI and Eater, have been implicated as the main mediators of the process [198]. A similar role for these receptors and clathrin-mediated endocytosis has been demonstrated in several insect species [193,195,199–203], while Eater/SR-CI have also been involved in the clathrin-independent phagocytosis of dsRNAs encapsulated in bacteria [204]. However, several of these works show that silencing or blocking these receptors does not completely interrupt the 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 [203].

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 [197,205,206], which can be a disadvantage in many dsRNA delivery methodologies. Another factor that limits the development of this type of strategy is the variable capacity of RNases in the digestive tract of different insect species to degrade exogenous dsRNAs [207–210]. Some insects also have extremely alkaline midguts, thus inducing alkaline hydrolysis of dsRNAs. Similarly, efficient endosomal escape of dsRNAs is required for developing the RNAi response. For example, some species have low sensitivity to RNAi, at least in part, due to dsRNA entrapment in endosomes and degradation after fusing with lysosomes [211,212]. Blocking the interaction of late endosomes with lysosomes enhances si- and miRNA-mediated silencing in *D. melanogaster*, while blocking late endosome formation limits silencing [213], thus restricting dsRNA escape between late endosome formation and lysosomal fusion. The activity of the vacuolar H^+-ATPase has been related to dsRNA cell entry in several species [197,201,214]; 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 [215]. In addition, as occurs in mammals [216] movement of the RNAi signal through tight junctions could be possible, although not yet demonstrated. Over long distances, dsRNA movement appears to occur through hemolymph. In certain insects, hemolymph nucleases efficiently degrade dsRNA [217–219], 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* [220] and *Schistocerca gregaria* (and probably in species of the orders Orthoptera, Blattodea and Diptera) [221] 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 [199]. RNAs are also carried by extracellular vesicles. miRNA-containing vesicles have been identified in *D. melanogaster* cell cultures [222], and their occurrence in vivo has been proposed [223]. Furthermore, viral infection in this species generate viral siRNAs that are packed in vesicles that circulate through hemolymph, systemically diffusing the RNAi signal [224]. 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 [225,226]. In the latter, some of the factors related to 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.

Additionally, in some insects, the silencing effects are not restricted to the treated insect but also appear in its progeny, even some time after the administration has stopped [227–230]. This is the so-called parental RNAi, of which most of the mechanistic details of the transfer are unknown.

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 [231,232] (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). This is SMARTSTAX PRO corn (Bayer), that produces dsRNA against the Snf7 gene of *Diabrotica virgifera virgifera* [233]. Its commercialization is scheduled to start soon. However, multiple limitations hold back the development of this kind of technology. On the one hand, generation of genetically modified organisms (GMOs) is laborious, expensive due to the rigid commercialization regulations [234], and currently have scant public acceptance [235]. On the other hand, plant RNAi machinery recognizes the produced dsRNAs, generating siRNAs [236], which can negatively affect their uptake by the insect. A possibility to circumvent this problem consists of expressing dsRNAs in chloroplasts or other compartments lacking RNAi machinery [237–239], although their accumulation is very size-sensitive [240,241], and their usefulness with sap feeding Hemiptera is limited [242]. The use of RNAs with structures resistant to the plant RNAi machinery, such as artificial pre-miRNAs, has also been proposed [243].

Other approaches not requiring plant modification have been developed. Plants infected with modified viruses have been widely used for screening potential RNAi target genes [244–247], and similar viruses may also confer protection to plants against fungi and nematodes [248,249]. The virus acts as a dsRNA factory during its replication in the plant. 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 in functional genetics, and their use for pest control has been proposed [250–255]. 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 [253,255]. An interaction between both types of viruses is found in the Flock House virus, which replicates in insects and plants [256–258]. Although viruses represent an interesting insecticidal strategy, the cross-kingdom status of the silencing suppressors encoded in both virus types must be considered [259,260]. 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. VLPs are usually produced in plants

to produce recombinant proteins, but also have been used to induce resistance against viruses and insects [261,262]. 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 [263–267].

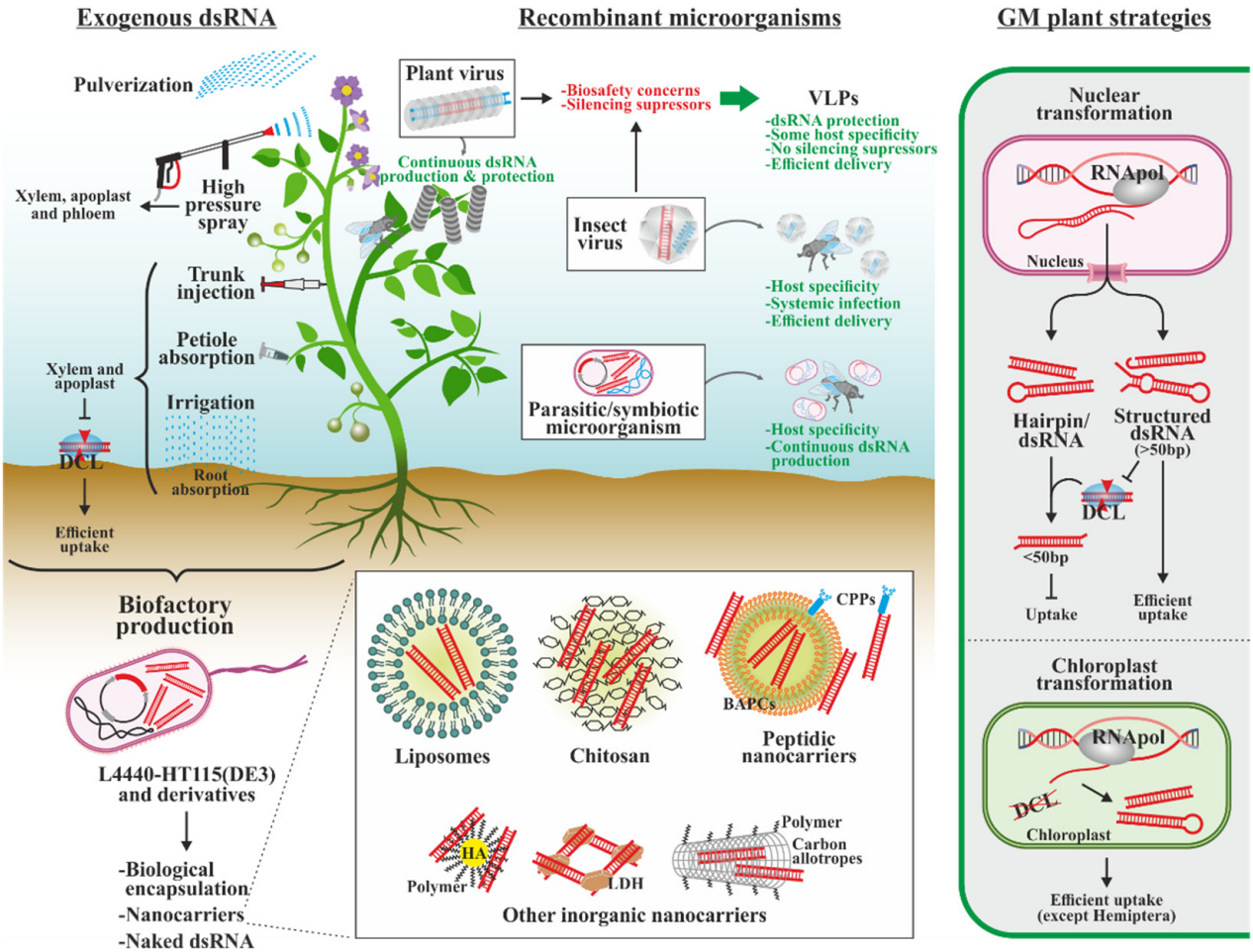

Figure 5. Proposed strategies for supplying dsRNAs to insects for RNAi-mediated pest control in the field. dsRNAs can be produced in biofactories and used, alone or in combination with nanomaterials (insert), with several application strategies. The use of plant and insect viruses, VLPs derived from them, as well as other insect pathogens or symbionts, taking advantage of their intrinsic characteristics, has been also studied. The development of nuclear and chloroplast transformants allows the continuous production of dsRNAs in genetically modified plants. BAPCs, branched amphipathic peptide capsules; CPPs, cell-penetrating peptides; DCL, Dicer-like proteins; GM, genetically modified; HA, hydroxyapatite; LDH, layered double hydroxide; RNApol, RNA polymerase; and VLPs, virus-like particles.

The exogenous application of dsRNAs as a non-transformative alternative for plant protection was pioneered against viral diseases [268], while the use of dsRNAs pulverized as a conventional pesticide was later proposed [269]. The RNA can also be internalized in the plant by roots and petiole or trunk injection [270–273]. 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 and insects [274–277], in a process in which dsRNAs have also been detected in the phloem. These strategies

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 [278,279], 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 [280,281] 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. [282–288]. To put these advances in perspective, researchers initially reported the production of 4 µg of dsRNA per ml of culture with the L4440-HT115(DE3) system [268], while other researchers recently achieved larger than 1 mg/mL using a modified strain of *Corynebacterium glutamicum* expressing a high copy number L4440-derivative [286]. As a result of these improvements and the advent of novel systems, the price per gram of dsRNA produced has been reported to fall from over $12,000 in 2008 to about $1 today, and up to half of that in cell-free systems [279]. Our research group has recently developed a system to overproduce RNAs in *E. coli* based on the intrinsic properties of viroids [289–291], obligate plant parasites with minimal genomes of non-coding, single-stranded but highly structured circular RNA. Expressing RNAs of interest within the eggplant latent viroid (ELVd) (+) RNA increases their half-life and accumulation in the bacteria due to the circular viroid scaffold, compact and possibly associated with the ligase [289–291]. To produce dsRNAs, the incorporation of a self-processing group-I intron cDNA between the inverted repeat is needed to stabilize the expression plasmids, while the intron RNA is efficiently excised from the final chimera, contributing to its compaction. An additional sequence of the intron in a permuted configuration flanking the inverted repeat allows the production of circular dsRNAs without the viroid scaffold [292,293]. This system is also interesting as some viroids such as ELVd are the only known pathogens able to infect the chloroplast. Lacking RNAi machinery, nucleus-expressed but chloroplast-accumulated chimeras could be a potential new strategy for effective pest control. It is also worth highlighting that although the use of perfectly complementary hairpins or dsRNA, both processed by the siRNA pathway, is the most common methodology for insect control and genetic studies, there are some examples of the miRNA and piRNa pathways being exploited for the same purposes [243,294–297].

Naked dsRNA molecules are prone 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 [298]. One common strategy is to encapsulate dsRNA in liposomes, lipid bilayer spherical structures [299–303]. 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 [303–306]. Extensive modifications to these natural polymers have been made to improve their stability and cell delivery in pest control and other applications [307–309]. Several inorganic nanoparticles can also be used, such as hydroxyapatite, silica, phosphate calcium, carbon allotropes or quantum dots [303,305,310–312]. 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. [313,314]. 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, fungi and also for pest control applications [315–318]. 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 as BAPCs, have been described as potentially more biocompatible and biodegradable than synthetic polymers, and more stable than those composed of lipids and polysaccharides [319]. They have been used to enhance the RNAi response in insects such as *T. castaneum*, *Acyrthosiphon pisum* [320], and *Spodoptera frugiperda* [321]. In the latter case, clathrin-mediated endocytosis and macropinocytosis uptake has been described, along with high endosomal escape and an increase in dsRNA transcytosis to hemolymph, improving the systemic response. On the other hand, peptides can be used as uptake mediators. Cell-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 recalcitrances to any specific entry pathway, as reviewed extensively for human therapy [322]. This strategy has been successfully used in insects, using a CPP fused with a dsRBD to improve the silencing effect in *Anthonomus grandis* [323]. A similar improvement is obtained with the fusion of a dsRBD with agglutinin in *Spodoptera exigua* [324].

Additional strategies propose increasing RNA bioavailability in cells by reducing dsRNA degradation by combining it with nuclease inhibitors such as EDTA or divalent ions [301,325] or chemically modifying the RNA [326], but also improving the endocytosis process by altering the membrane composition with hydrogen peroxide or arachidonic acid [194,327].

6. RNAi in Pest Control: Challenges and Future Directions

The current challenges for exploiting RNAi as an insecticidal strategy can be grouped into (i) the variable efficiency of RNAi among pests, and (ii) the cost-effective production of dsRNA.

It is well known that not all insects are equally susceptible to dsRNA, with enormous differences between insects of different orders but also within closely related species. Additionally, variable silencing efficiency in the same insect is commonly obtained depending on development stages, target tissues and/or delivery systems. Although progress has been made in recent years to unravel the molecular determinants of the RNAi efficiency, being a set of highly interrelated factors, they are still not fully understood. Despite several exceptions reported, generally orthopteran, blattodean and coleopteran insects are considered to be very susceptible to exogenous dsRNAs, while in hemipteran species, the RNAi efficiency is highly variable, and lepidopterans and dipterans usually have much lower efficiencies.

For efficient insect control, the appropriate target gene has to be selected. Silencing and mortality have been described by affecting a wide spectrum of gene functions such as energy metabolism, membrane transporters, detoxification, structural proteins, etc. Ideally, gene silencing should lead to the death of the insect in the shortest time and with the minimum dose of dsRNA. Thus, it must encode ubiquitously expressed proteins with a short half-life originating from abundantly transcribed mRNAs with high turnover. An

important detail is that under ideal experimental conditions, insect food intake is controlled and restricted to sources with dsRNA, while in the field, the insect will have food sources beyond those that supply the dsRNA, thus hampering the silencing process. In this scenario, the timeframe in which compensatory upregulation of the target gene or paralogs that could functionally supplement said gene would increase, allowing the compensation of the silencing phenotype. The sublethal effects of RNAi on pests [328,329], as well as parental RNAi, may become essential to reduce crop damage in the field; the mechanisms involved in the latter process must be explored. However, it is also possible that sublethal treatments could facilitate the appearance of refractoriness in some insects, protecting them from subsequent exposures. Worryingly, the first case of resistance development after RNAi treatment has been reported [330]. The resistance was located at a single autosomal locus and inherited recessively; however, we are still clueless of how this resistance occurs and how to minimize it. It is also important to mention that additional factors, such as the environmental fate of RNA and its effects, including the repercussions on non-target species, have to be taken into account. Reassuringly, a myriad of studies showed that pest-specific dsRNAs do not seem to have a negative impact on unrelated species. It is expected that the establishment of high-throughput strategies, along with -omics technologies, will provide the tools to select the best target genes, and rational designs of the silencing strategies must ameliorate some of the mentioned problems. For example, it is believed that increasing homologous sequence of the dsRNA (and therefore, the diversity of derived siRNAs) can hinder the development of resistance, while off-target effects can be reduced by selecting less evolutionarily conserved sequences. In addition, the synergistic effects of targeting multiple essential genes may be interesting to increase the likelihood of insect mortality, avoiding functional supplementation and resistance development. We must also not forget the benefits that the combination of RNAi with other control strategies can provide. In any case, an adequate risk assessment is needed.

A determinant of RNAi efficiency is dsRNA degradation, partially explaining the differences between delivery methods, as in some species, effective silencing by injection is correlated with oral insensitivity. This is especially relevant in lepidopterans, hemipterans and dipterans. Lepidopterans have the highest degradation capacity in insects and express specific nucleases not seen in any other order, reducing the efficiency of RNAi in these insects [209], while Hemipterans are the only reported order to degrade dsRNA in saliva [210]. In addition, dsRNase expression can vary between life stages (resulting in the stage-dependent silencing results). Several "RNAi-of-RNAi" studies showed that silencing mRNAs of specific nucleases managed to increase the induced silencing [331–334]. Thus, co-delivering strategies have been proposed to extend the scope of this experimental strategy to pest control. It would also be interesting to explore new rationally designed RNA nanostructures to increase the half-life of the dsRNA (both in the field and within the insect) [335–338], along with the previously mentioned use of nanoparticles or formulations.

Deficient dsRNA uptake limits RNAi responses in dipterans and in certain tissues of orthopterans and hymenopterans, while impaired endosomal escape of dsRNA appears to be limiting in lepidopterans. The mechanisms governing these processes, however, are mostly unclear, possibly involving different mechanisms in different species, stages and/or tissues. Thus, further work is needed to uncover the cell factors involved in how to enhance the uptake. RNAi-of-RNAi strategies could be employed to co-silence factors (such as tag-130/CHUP-1) that hinder RNA uptake if multiple rounds of feeding are going to be required for effective silencing; also, as certain factors are known that mediate endosomal-lysosomal fusion [339], their co-silencing could minimize the fusion, thus increasing the time frame of RNA entry into the cytosol. In this regard, small molecules such as chloroquine have been reported to promote the cytosolic translocation of endocytosed nucleotides for human therapeutics *in vitro* [340]. Again, novel dsRNA structures could help in the uptake processes as previously reported [203], along with the use of nanoparticles or formulations.

Systemic distribution of the silencing signal is generally required to achieve phenotypic effects. But unlike nematodes, fungi or plants, the RNAi signal is not amplified as many insects lack endogenous RNA-dependent RNA polymerases (RdRp) [341]. Thus, all silencing derives from the initial introduced RNA, making adequate concentrations of dsRNA essential, which can be difficult for its application as they are usually high. While it has been speculated that RNA is possibly transported packaged in protective structures such as exosomes, again, the mediators of this process are largely unknown. Extensive research is needed to fully understand the cellular pathways involved and how it can be hijacked to our favor with the use of nanocarriers, small molecules or novel strategies to enhance dsRNA propagation. As an alternative for the most recalcitrant insects, it is expected that potent and localized silencing in the dsRNA-capturing cells could reduce the damage caused by insects. For example, disrupting the proper functioning of midgut cells via RNAi could limit insect feeding and even induce death [342]. On a more positive note, virus-infection-derived secondary sRNAs has been found in *D. melanogaster* [224], being generated in hemocytes through viral DNA synthesis by endogenous reverse transcriptase, followed by transcription and dicing, secreted in exosome-like vesicles and conferring systemic protection. Therefore, it would be interesting to study its occurrence in other insects and if this mechanism could be exploited to enhance the silencing signal.

The RNAi pathways described in Section 3 come mostly from studies in model species such as the insect *D. melanogaster*, or the mammal counterparts, but the core enzymes have been identified in an increasing number of species. Interestingly, its genes have suffered duplications and deletions [191], and its basal expression varies between tissue and stage, thus partially explaining RNAi variability [343–346]. Furthermore, Dicer and AGO of certain insects may be not equally functional due to different evolution of their structures. For example, a recent study identified variability in certain conserved domains and loop regions of the RNAi machinery, especially in Lepidoptera [347]. Even less known are the accessory protein factors involved in siRNA; they may not be as conserved as the core enzymes, and we may still not know factors relevant to the process. All these aspects could explain, for example, the differential base bias in dsRNA processing between species, affecting the RNAi efficiency [348]. Curiously, it has been proposed that in Lepidoptera (and to a lesser extent in Diptera) the prevalence of viral infections has led to evolutionarily replace the siRNA pathway as the prime antiviral strategy in favor of alternative defense mechanisms that cannot be overcome by viral silencing suppressors (thus diminishing expression/function of core RNAi factors and dsRNA uptake components, and increasing nucleases) [349]. In sum, the mechanistic details of one species cannot be directly extrapolated to other insects. Species-specific studies must be conducted to assess the basal expression (and possible upregulation after treatment) of core siRNA enzymes to target (if possible) life stages and tissues with the highest core expression as well as to identify variations in its mode of function to tailor the characteristics of the trigger dsRNA molecules.

Finally, it must be mentioned that we are currently lacking a clear regulatory framework that guides and facilitates the development of this new kind of pesticide. Approaches such as plant transformation or modified insect viruses or symbionts can be quite restricted by the rigid actual regulations. Thus, the main strategy available is the exogenous application of RNA molecules. However, it is likely that we are still far from having the capacity to profitably produce the enormous quantities of RNA needed to support global agricultural production. The development of methods for overproduction of RNA has not historically accompanied that of DNA and proteins, possibly due to the difficulty related with the short half-life of RNA and the relatively minor role formerly attributed to these molecules. It was not until recently that a true revolution in exogenous dsRNA production systems began, achieving progressively higher yields at increasingly more affordable prices for large-scale use as an insecticide. Therefore, it is expected that the extensive application of RNAi strategies will come hand-in-hand with future and improved strategies based on biofactories.

7. Conclusions

Biotechnological applications based on RNAi may contribute to counteracting current challenges imposed by insect pests in global food production. Endogenously produced in crop plants or exogenously applied, properly engineered dsRNA molecules may substitute classic insecticides to fight insect pests in a more specific, sustainable, and environmentally friendly manner. However, for this to be true, we need to keep improving our knowledge about the insect endogenous RNAi pathways, including RNA intake and systemic movement, and to refine technologies for recombinant RNA production and delivery.

Author Contributions: Conceptualization, B.O. and J.-A.D.; writing—original draft preparation, B.O. and J.-A.D.; writing—review and editing, B.O. and J.-A.D.; funding acquisition, B.O. and J.-A.D. Both authors have read and agreed to the published version of the manuscript. Parts of this manuscript correspond to B.O. Ph.D. dissertation. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grant PID2020-114691RB-I00 from the Spanish Ministerio de Ciencia, Innovación y Universidades through the Agencia Estatal de Investigación (MCIU/AEI/10.13039/501100011033). B.O. was the recipient of a predoctoral contract from Universitat Politècnica de València (PAID-01-17).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Culliney, T.W. Crop Losses to Arthropods. In *Integrated Pest Management: Pesticide Problems*; Springer: Amsterdam, The Netherlands, 2014; Volume 3, pp. 201–225. ISBN 9789400777965.
2. Sharma, S.; Kooner, R.; Arora, R. Insect Pests and Crop Losses. In *Breeding Insect Resistant Crops for Sustainable Agriculture*; Springer: Singapore, 2017; pp. 45–66. ISBN 9789811060564.
3. Escobar-Bravo, R.; Alba, J.M.; Pons, C.; Granell, A.; Kant, M.R.; Moriones, E.; Fernández-Muñoz, R. A Jasmonate-Inducible Defense Trait Transferred from Wild into Cultivated Tomato Establishes Increased Whitefly Resistance and Reduced Viral Disease Incidence. *Front. Plant Sci.* **2016**, *7*, 1732. [CrossRef] [PubMed]
4. Monci, F.; García-Andrés, S.; Sánchez-Campos, S.; Fernández-Muñoz, R.; Díaz-Pendón, J.A.; Moriones, E. Use of Systemic Acquired Resistance and Whitefly Optical Barriers to Reduce Tomato Yellow Leaf Curl Disease Damage to Tomato Crops. *Plant Dis.* **2019**, *103*, 1181–1188. [CrossRef] [PubMed]
5. Fortes, I.M.; Fernández-Muñoz, R.; Moriones, E. Host Plant Resistance to *Bemisia tabaci* to Control Damage Caused in Tomato Plants by the Emerging Crinivirus Tomato Chlorosis Virus. *Front. Plant Sci.* **2020**, *11*, 585510. [CrossRef] [PubMed]
6. Chala, B.; Hamde, F. Emerging and Re-Emerging Vector-Borne Infectious Diseases and the Challenges for Control: A Review. *Front. Public. Health* **2021**, *9*, 1466. [CrossRef] [PubMed]
7. Deguine, J.P.; Atiama-Nurbel, T.; Aubertot, J.N.; Augusseau, X.; Atiama, M.; Jacquot, M.; Reynaud, B. Agroecological Management of Cucurbit-Infesting Fruit Fly: A Review. *Agron. Sustain. Dev.* **2015**, *35*, 937–965. [CrossRef]
8. Tyagi, S.; Kesiraju, K.; Saakre, M.; Rathinam, M.; Raman, V.; Pattanayak, D.; Sreevathsa, R. Genome Editing for Resistance to Insect Pests: An Emerging Tool for Crop Improvement. *ACS Omega* **2020**, *5*, 20674–20683. [CrossRef] [PubMed]
9. Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans. *Nature* **1998**, *391*, 806–811. [CrossRef] [PubMed]
10. Izant, J.G.; Weintraub, H. Inhibition of Thymidine Kinase Gene Expression by Anti-Sense RNA: A Molecular Approach to Genetic Analysis. *Cell* **1984**, *36*, 1007–1015. [CrossRef] [PubMed]
11. Fire, A.; Albertson, D.; Harrison, S.W.; Moerman, D.G. Production of Antisense RNA Leads to Effective and Specific Inhibition of Gene Expression in C. Elegans Muscle. *Development* **1991**, *113*, 503–514. [CrossRef]
12. Guo, S.; Kemphues, K.J. Par-1, a Gene Required for Establishing Polarity in C. Elegans Embryos, Encodes a Putative Ser/Thr Kinase That Is Asymmetrically Distributed. *Cell* **1995**, *81*, 611–620. [CrossRef]
13. Matzke, M.A.; Primig, M.; Trnovsky, J.; Matzke, A.J.M. Reversible Methylation and Inactivation of Marker Genes in Sequentially Transformed Tobacco Plants. *EMBO J.* **1989**, *8*, 643. [CrossRef]
14. Napoli, C.; Lemieux, C.; Jorgensen, R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in Trans. *Plant Cell* **1990**, *2*, 279–289. [CrossRef]

15. van der Krol, A.R.; Mur, L.A.; Beld, M.; Mol, J.N.M.; Stuitje, A.R. Flavonoid Genes in Petunia: Addition of a Limited Number of Gene Copies May Lead to a Suppression of Gene Expression. *Plant Cell* **1990**, *2*, 291–299. [CrossRef]
16. Romano, N.; Macino, G. Quelling: Transient Inactivation of Gene Expression in Neurospora Crassa by Transformation with Homologous Sequences. *Mol. Microbiol.* **1992**, *6*, 3343–3353. [CrossRef] [PubMed]
17. Cogoni, C.; Irelan, J.T.; Schumacher, M.; Schmidhauser, T.J.; Selker, E.U.; Macino, G. Transgene Silencing of the Al-1 Gene in Vegetative Cells of Neurospora Is Mediated by a Cytoplasmic Effector and Does Not Depend on DNA-DNA Interactions or DNA Methylation. *EMBO J.* **1996**, *15*, 3153–3163. [CrossRef] [PubMed]
18. Kennerdell, J.R.; Carthew, R.W. Use of DsRNA-Mediated Genetic Interference to Demonstrate That Frizzled and Frizzled 2 Act in the Wingless Pathway. *Cell* **1998**, *95*, 1017–1026. [CrossRef]
19. Hamilton, A.J.; Baulcombe, D.C. A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants. *Science* **1999**, *286*, 950–952. [CrossRef]
20. Wianny, F.; Zernicka-Goetz, M. Specific Interference with Gene Function by Double-Stranded RNA in Early Mouse Development. *Nat. Cell Biol.* **1999**, *2*, 70–75. [CrossRef] [PubMed]
21. Drinnenberg, I.A.; Weinberg, D.E.; Xie, K.T.; Mower, J.P.; Wolfe, K.H.; Fink, G.R.; Bartel, D.P. RNAi in Budding Yeast. *Science* **2009**, *326*, 544–550. [CrossRef]
22. Li, L.C.; Okino, S.T.; Zhao, H.; Pookot, D.; Place, R.F.; Urakami, S.; Enokida, H.; Dahiya, R. Small DsRNAs Induce Transcriptional Activation in Human Cells. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17337. [CrossRef]
23. de Hayr, L.; Asad, S.; Hussain, M.; Asgari, S. RNA Activation in Insects: The Targeted Activation of Endogenous and Exogenous Genes. *Insect Biochem. Mol. Biol.* **2020**, *119*, 103325. [CrossRef] [PubMed]
24. Wang, X.H.; Aliyari, R.; Li, W.X.; Li, H.W.; Kim, K.; Carthew, R.; Atkinson, P.; Ding, S.W. RNA Interference Directs Innate Immunity against Viruses in Adult Drosophila. *Science* **2006**, *312*, 452–454. [CrossRef] [PubMed]
25. Galiana-Arnoux, D.; Dostert, C.; Schneemann, A.; Hoffmann, J.A.; Imler, J.L. Essential Function in Vivo for Dicer-2 in Host Defense against RNA Viruses in Drosophila. *Nat. Immunol.* **2006**, *7*, 590–597. [CrossRef] [PubMed]
26. Chung, W.J.; Okamura, K.; Martin, R.; Lai, E.C. Endogenous RNA Interference Provides a Somatic Defense against Drosophila Transposons. *Curr. Biol.* **2008**, *18*, 795–802. [CrossRef]
27. Czech, B.; Malone, C.D.; Zhou, R.; Stark, A.; Schlingeheyde, C.; Dus, M.; Perrimon, N.; Kellis, M.; Wohlschlegel, J.A.; Sachidanandam, R.; et al. An Endogenous Small Interfering RNA Pathway in Drosophila. *Nature* **2008**, *453*, 798–802. [CrossRef] [PubMed]
28. Ghildiyal, M.; Seitz, H.; Horwich, M.D.; Li, C.; Du, T.; Lee, S.; Xu, J.; Kittler, E.L.W.; Zapp, M.L.; Weng, Z.; et al. Endogenous SiRNAs Derived from Transposons and MRNAs in Drosophila Somatic Cells. *Science* **2008**, *320*, 1077–1081. [CrossRef]
29. Okamura, K.; Balla, S.; Martin, R.; Liu, N.; Lai, E.C. Two Distinct Mechanisms Generate Endogenous SiRNAs from Bidirectional Transcription in Drosophila Melanogaster. *Nat. Struct. Mol. Biol.* **2008**, *15*, 581–590. [CrossRef]
30. Okamura, K.; Chung, W.J.; Ruby, J.G.; Guo, H.; Bartel, D.P.; Lai, E.C. The Drosophila Hairpin RNA Pathway Generates Endogenous Short Interfering RNAs. *Nature* **2008**, *453*, 803–806. [CrossRef]
31. Lucchetta, E.M.; Carthew, R.W.; Ismagilov, R.F. The Endo-SiRNA Pathway Is Essential for Robust Development of the Drosophila Embryo. *PLoS ONE* **2009**, *4*, e7576. [CrossRef]
32. Lim, D.H.; Oh, C.T.; Lee, L.; Hong, J.S.; Noh, S.H.; Hwang, S.; Kim, S.; Han, S.J.; Lee, Y.S. The Endogenous SiRNA Pathway in Drosophila Impacts Stress Resistance and Lifespan by Regulating Metabolic Homeostasis. *FEBS Lett.* **2011**, *585*, 3079–3085. [CrossRef]
33. Lim, D.H.; Lee, L.; Oh, C.T.; Kim, N.H.; Hwang, S.; Han, S.J.; Lee, Y.S. Microarray Analysis of Drosophila Dicer-2 Mutants Reveals Potential Regulation of Mitochondrial Metabolism by Endogenous SiRNAs. *J. Cell Biochem.* **2013**, *114*, 418–427. [CrossRef]
34. Zamore, P.D.; Tuschl, T.; Sharp, P.A.; Bartel, D.P. RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of MRNA at 21 to 23 Nucleotide Intervals. *Cell* **2000**, *101*, 25–33. [CrossRef]
35. Hammond, S.M.; Bernstein, E.; Beach, D.; Hannon, G.J. An RNA-Directed Nuclease Mediates Post-Transcriptional Gene Silencing in Drosophila Cells. *Nature* **2000**, *404*, 293–296. [CrossRef]
36. Elbashir, S.M.; Lendeckel, W.; Tuschl, T. RNA Interference Is Mediated by 21- and 22-Nucleotide RNAs. *Genes. Dev.* **2001**, *15*, 188. [CrossRef]
37. Bernstein, E.; Caudy, A.A.; Hammond, S.M.; Hannon, G.J. Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference. *Nature* **2001**, *409*, 363–366. [CrossRef] [PubMed]
38. Lee, Y.S.; Nakahara, K.; Pham, J.W.; Kim, K.; He, Z.; Sontheimer, E.J.; Carthew, R.W. Distinct Roles for Drosophila Dicer-1 and Dicer-2 in the SiRNA/MiRNA Silencing Pathways. *Cell* **2004**, *117*, 69–81. [CrossRef]
39. Lau, P.W.; Guiley, K.Z.; De, N.; Potter, C.S.; Carragher, B.; MacRae, I.J. The Molecular Architecture of Human Dicer. *Nat. Struct. Mol. Biol.* **2012**, *19*, 436–440. [CrossRef] [PubMed]
40. Sinha, N.K.; Iwasa, J.; Shen, P.S.; Bass, B.L. Dicer Uses Distinct Modules for Recognizing DsRNA Termini. *Science* **2018**, *359*, 329–334. [CrossRef]
41. Welker, N.C.; Maity, T.S.; Ye, X.; Aruscavage, P.J.; Krauchuk, A.A.; Liu, Q.; Bass, B.L. Dicer's Helicase Domain Discriminates DsRNA Termini to Promote an Altered Reaction Mode. *Mol. Cell* **2011**, *41*, 589–599. [CrossRef] [PubMed]
42. Cenik, E.S.; Fukunaga, R.; Lu, G.; Dutcher, R.; Wang, Y.; Tanaka Hall, T.M.; Zamore, P.D. Phosphate and R2D2 Restrict the Substrate Specificity of Dicer-2, an ATP-Driven Ribonuclease. *Mol. Cell* **2011**, *42*, 172–184. [CrossRef]

43. Sinha, N.K.; Trettin, K.D.; Aruscavage, P.J.; Bass, B.L. Drosophila Dicer-2 Cleavage Is Mediated by Helicase- and DsRNA Termini-Dependent States That Are Modulated by Loquacious-PD. *Mol. Cell* **2015**, *58*, 406–417. [CrossRef]
44. Naganuma, M.; Tadakuma, H.; Tomari, Y. Single-Molecule Analysis of Processive Double-Stranded RNA Cleavage by Drosophila Dicer-2. *Nat. Commun.* **2021**, *12*, 4268. [CrossRef] [PubMed]
45. Zhou, R.; Czech, B.; Brennecke, J.; Sachidanandam, R.; Wohlschlegel, J.A.; Perrimon, N.; Hannon, G.J. Processing of Drosophila Endo-SiRNAs Depends on a Specific Loquacious Isoform. *RNA* **2009**, *15*, 1886–1895. [CrossRef] [PubMed]
46. Hartig, J.V.; Esslinger, S.; Böttcher, R.; Saito, K.; Förstemann, K. Endo-SiRNAs Depend on a New Isoform of Loquacious and Target Artificially Introduced, High-Copy Sequences. *EMBO J.* **2009**, *28*, 2932–2944. [CrossRef] [PubMed]
47. Miyoshi, K.; Miyoshi, T.; Hartig, J.V.; Siomi, H.; Siomi, M.C. Molecular Mechanisms That Funnel RNA Precursors into Endogenous Small-Interfering RNA and MicroRNA Biogenesis Pathways in Drosophila. *RNA* **2010**, *16*, 506–515. [CrossRef] [PubMed]
48. Marques, J.T.; Wang, J.P.; Wang, X.; de Oliveira, K.P.V.; Gao, C.; Aguiar, E.R.G.R.; Jafari, N.; Carthew, R.W. Functional Specialization of the Small Interfering RNA Pathway in Response to Virus Infection. *PLoS Pathog.* **2013**, *9*, e1003579. [CrossRef]
49. Trettin, K.D.; Sinha, N.K.; Eckert, D.M.; Apple, S.E.; Bass, B.L. Loquacious-PD Facilitates Drosophila Dicer-2 Cleavage through Interactions with the Helicase Domain and DsRNA. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E7939–E7948. [CrossRef] [PubMed]
50. Jonely, M.; Singh, R.K.; Donelick, H.M.; Bass, B.L.; Noriega, R. Loquacious-PD Regulates the Terminus-Dependent Molecular Recognition of Dicer-2 toward Double-Stranded RNA. *Chem. Commun.* **2021**, *57*, 10879–10882. [CrossRef]
51. MacRae, I.J.; Zhou, K.; Li, F.; Repic, A.; Brooks, A.N.; Cande, W.Z.; Adams, P.D.; Doudna, J.A. Structural Basis for Double-Stranded RNA Processing by Dicer. *Science* **2006**, *311*, 195–198. [CrossRef]
52. MacRae, I.J.; Zhou, K.; Doudna, J.A. Structural Determinants of RNA Recognition and Cleavage by Dicer. *Nat. Struct. Mol. Biol.* **2007**, *14*, 934–940. [CrossRef]
53. Kandasamy, S.K.; Fukunaga, R. Phosphate-Binding Pocket in Dicer-2 PAZ Domain for High-Fidelity SiRNA Production. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 14031–14036. [CrossRef] [PubMed]
54. Santos, D.; Mingels, L.; Vogel, E.; Wang, L.; Christiaens, O.; Cappelle, K.; Wynant, N.; Gansemans, Y.; van Nieuwerburgh, F.; Smagghe, G.; et al. Generation of Virus- and DsRNA-Derived SiRNAs with Species-Dependent Length in Insects. *Viruses* **2019**, *11*, 738. [CrossRef]
55. Zhang, H.; Kolb, F.A.; Jaskiewicz, L.; Westhof, E.; Filipowicz, W. Single Processing Center Models for Human Dicer and Bacterial RNase III. *Cell* **2004**, *118*, 57–68. [CrossRef] [PubMed]
56. Martinez, J.; Patkaniowska, A.; Urlaub, H.; Lührmann, R.; Tuschl, T. Single-Stranded Antisense SiRNAs Guide Target RNA Cleavage in RNAi. *Cell* **2002**, *110*, 563–574. [CrossRef] [PubMed]
57. Rand, T.A.; Petersen, S.; Du, F.; Wang, X. Argonaute2 Cleaves the Anti-Guide Strand of SiRNA during RISC Activation. *Cell* **2005**, *123*, 621–629. [CrossRef] [PubMed]
58. Matranga, C.; Tomari, Y.; Shin, C.; Bartel, D.P.; Zamore, P.D. Passenger-Strand Cleavage Facilitates Assembly of SiRNA into Ago2-Containing RNAi Enzyme Complexes. *Cell* **2005**, *123*, 607–620. [CrossRef] [PubMed]
59. Miyoshi, K.; Tsukumo, H.; Nagami, T.; Siomi, H.; Siomi, M.C. Slicer Function of Drosophila Argonautes and Its Involvement in RISC Formation. *Genes. Dev.* **2005**, *19*, 2837–2848. [CrossRef]
60. Khvorova, A.; Reynolds, A.; Jayasena, S.D. Functional SiRNAs and MiRNAs Exhibit Strand Bias. *Cell* **2003**, *115*, 209–216. [CrossRef]
61. Schwarz, D.S.; Hutvágner, G.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P.D. Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell* **2003**, *115*, 199–208. [CrossRef]
62. Liu, X.; Jiang, F.; Kalidas, S.; Smith, D.; Liu, Q. Dicer-2 and R2D2 Coordinately Bind SiRNA to Promote Assembly of the SiRISC Complexes. *RNA* **2006**, *12*, 1514–1520. [CrossRef]
63. Tomari, Y.; Matranga, C.; Haley, B.; Martinez, N.; Zamore, P.D. A Protein Sensor for SiRNA Asymmetry. *Science* **2004**, *306*, 1377–1380. [CrossRef]
64. Mirkovic-Hösle, M.; Förstemann, K. Transposon Defense by Endo-SiRNAs, PiRNAs and Somatic PilRNAs in Drosophila: Contributions of Loqs-PD and R2D2. *PLoS ONE* **2014**, *9*, e84994. [CrossRef]
65. Tants, J.N.; Fesser, S.; Kern, T.; Stehle, R.; Geerlof, A.; Wunderlich, C.; Juen, M.; Hartlmüller, C.; Böttcher, R.; Kunzelmann, S.; et al. Molecular Basis for Asymmetry Sensing of SiRNAs by the Drosophila Loqs-PD/Dcr-2 Complex in RNA Interference. *Nucleic Acids Res.* **2017**, *45*, 12536–12550. [CrossRef]
66. Hammond, S.M.; Boettcher, S.; Caudy, A.A.; Kobayashi, R.; Hannon, G.J. Argonaute2, a Link between Genetic and Biochemical Analyses of RNAi. *Science* **2001**, *293*, 1146–1150. [CrossRef]
67. Song, J.J.; Liu, J.; Tolia, N.H.; Schneiderman, J.; Smith, S.K.; Martienssen, R.A.; Hannon, G.J.; Joshua-Tor, L. The Crystal Structure of the Argonaute2 PAZ Domain Reveals an RNA Binding Motif in RNAi Effector Complexes. *Nat. Struct. Mol. Biol.* **2003**, *10*, 1026–1032. [CrossRef]
68. Kataoka, Y.; Takeichi, M.; Uemura, T. Developmental Roles and Molecular Characterization of a Drosophila Homologue of Arabidopsis Argonaute1, the Founder of a Novel Gene Superfamily. *Genes. Cells* **2001**, *6*, 313–325. [CrossRef]
69. Rubio, M.; Maestro, J.L.; Piulachs, M.D.; Belles, X. Conserved Association of Argonaute 1 and 2 Proteins with MiRNA and SiRNA Pathways throughout Insect Evolution, from Cockroaches to Flies. *Biochim. Biophys. Acta (BBA)—Gene Regul. Mech.* **2018**, *1861*, 554–560. [CrossRef]

70. Song, J.J.; Smith, S.K.; Hannon, G.J.; Joshua-Tor, L. Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity. *Science* **2004**, *305*, 1434–1437. [CrossRef]
71. Schirle, N.T.; MacRae, I.J. The Crystal Structure of Human Argonaute2. *Science* **2012**, *336*, 1037–1040. [CrossRef]
72. Yamaguchi, S.; Oe, A.; Nishida, K.M.; Yamashita, K.; Kajiya, A.; Hirano, S.; Matsumoto, N.; Dohmae, N.; Ishitani, R.; Saito, K.; et al. Crystal Structure of Drosophila Piwi. *Nat. Commun.* **2020**, *11*, 858. [CrossRef]
73. Okamura, K.; Ishizuka, A.; Siomi, H.; Siomi, M.C. Distinct Roles for Argonaute Proteins in Small RNA-Directed RNA Cleavage Pathways. *Genes. Dev.* **2004**, *18*, 1655–1666. [CrossRef]
74. van Rij, R.P.; Saleh, M.C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA Silencing Endonuclease Argonaute 2 Mediates Specific Antiviral Immunity in Drosophila Melanogaster. *Genes. Dev.* **2006**, *20*, 2985–2995. [CrossRef]
75. Förstemann, K.; Horwich, M.D.; Wee, L.M.; Tomari, Y.; Zamore, P.D. Drosophila MicroRNAs Are Sorted into Functionally Distinct Argonaute Complexes after Production by Dicer-1. *Cell* **2007**, *130*, 287–297. [CrossRef]
76. Tomari, Y.; Du, T.; Zamore, P.D. Sorting of Drosophila Small Silencing RNAs. *Cell* **2007**, *130*, 299–308. [CrossRef]
77. Czech, B.; Zhou, R.; Erlich, Y.; Brennecke, J.; Binari, R.; Villalta, C.; Gordon, A.; Perrimon, N.; Hannon, G.J. Hierarchical Rules for Argonaute Loading in Drosophila. *Mol. Cell* **2009**, *36*, 445–456. [CrossRef]
78. Okamura, K.; Liu, N.; Lai, E.C. Distinct Mechanisms for MicroRNA Strand Selection by Drosophila Argonautes. *Mol. Cell* **2009**, *36*, 431–444. [CrossRef]
79. Ghildiyal, M.; Xu, J.; Seitz, H.; Weng, Z.; Zamore, P.D. Sorting of Drosophila Small Silencing RNAs Partitions MicroRNA* Strands into the RNA Interference Pathway. *RNA* **2010**, *16*, 43–56. [CrossRef]
80. Ameres, S.L.; Hung, J.H.; Xu, J.; Weng, Z.; Zamore, P.D. Target RNA-Directed Tailing and Trimming Purifies the Sorting of Endo-SiRNAs between the Two Drosophila Argonaute Proteins. *RNA* **2011**, *17*, 54–63. [CrossRef]
81. Nishida, K.M.; Miyoshi, K.; Ogino, A.; Miyoshi, T.; Siomi, H.; Siomi, M.C. Roles of R2D2, a Cytoplasmic D2 Body Component, in the Endogenous SiRNA Pathway in Drosophila. *Mol. Cell* **2013**, *49*, 680–691. [CrossRef]
82. Liu, Q.; Rand, T.A.; Kalidas, S.; Du, F.; Kim, H.E.; Smith, D.P.; Wang, X. R2D2, a Bridge between the Initiation and Effector Steps of the Drosophila RNAi Pathway. *Science* **2003**, *301*, 1921–1925. [CrossRef]
83. Iwasaki, S.; Sasaki, H.M.; Sakaguchi, Y.; Suzuki, T.; Tadakuma, H.; Tomari, Y. Defining Fundamental Steps in the Assembly of the Drosophila RNAi Enzyme Complex. *Nature* **2015**, *521*, 533–536. [CrossRef]
84. Wang, Y.; Sheng, G.; Juranek, S.; Tuschl, T.; Patel, D.J. Structure of the Guide-Strand-Containing Argonaute Silencing Complex. *Nature* **2008**, *456*, 209–213. [CrossRef]
85. Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. Structure and Nucleic-Acid Binding of the Drosophila Argonaute 2 PAZ Domain. *Nature* **2003**, *426*, 465–469. [CrossRef]
86. Kwak, P.B.; Tomari, Y. The N Domain of Argonaute Drives Duplex Unwinding during RISC Assembly. *Nat. Struct. Mol. Biol.* **2012**, *19*, 145–151. [CrossRef]
87. Gu, S.; Jin, L.; Huang, Y.; Zhang, F.; Kay, M.A. Slicing-Independent RISC Activation Requires the Argonaute PAZ Domain. *Curr. Biol.* **2012**, *22*, 1536–1542. [CrossRef]
88. Park, J.H.; Shin, C. Slicer-Independent Mechanism Drives Small-RNA Strand Separation during Human RISC Assembly. *Nucleic Acids Res.* **2015**, *43*, 9418–9433. [CrossRef] [PubMed]
89. Liu, Y.; Ye, X.; Jiang, F.; Liang, C.; Chen, D.; Peng, J.; Kinch, L.N.; Grishin, N.V.; Liu, Q. C3PO, an Endoribonuclease That Promotes RNAi by Facilitating RISC Activation. *Science* **2009**, *325*, 750–753. [CrossRef] [PubMed]
90. Horwich, M.D.; Li, C.; Matranga, C.; Vagin, V.; Farley, G.; Wang, P.; Zamore, P.D. The Drosophila RNA Methyltransferase, DmHen1, Modifies Germline PiRNAs and Single-Stranded SiRNAs in RISC. *Curr. Biol.* **2007**, *17*, 1265–1272. [CrossRef]
91. Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 Chaperone Machinery Mediates ATP-Dependent RISC Loading of Small RNA Duplexes. *Mol. Cell* **2010**, *39*, 292–299. [CrossRef] [PubMed]
92. Miyoshi, T.; Takeuchi, A.; Siomi, H.; Siomi, M.C. A Direct Role for Hsp90 in Pre-RISC Formation in Drosophila. *Nat. Struct. Mol. Biol.* **2010**, *17*, 1024–1026. [CrossRef] [PubMed]
93. Liu, J.; Carmell, M.A.; Rivas, F.V.; Marsden, C.G.; Thomson, J.M.; Song, J.J.; Hammond, S.M.; Joshua-Tor, L.; Hannon, G.J. Argonaute2 Is the Catalytic Engine of Mammalian RNAi. *Science* **2004**, *305*, 1437–1441. [CrossRef] [PubMed]
94. Martinez, J.; Tuschl, T. RISC Is a 5′ Phosphomonoester-Producing RNA Endonuclease. *Genes. Dev.* **2004**, *18*, 975–980. [CrossRef] [PubMed]
95. Lima, W.F.; de Hoyos, C.L.; Liang, X.H.; Crooke, S.T. RNA Cleavage Products Generated by Antisense Oligonucleotides and SiRNAs Are Processed by the RNA Surveillance Machinery. *Nucleic Acids Res.* **2016**, *44*, 3351–3363. [CrossRef] [PubMed]
96. Brennecke, J.; Hipfner, D.R.; Stark, A.; Russell, R.B.; Cohen, S.M. Bantam Encodes a Developmentally Regulated MicroRNA That Controls Cell Proliferation and Regulates the Proapoptotic Gene Hid in Drosophila. *Cell* **2003**, *113*, 25–36. [CrossRef] [PubMed]
97. Song, J.; Li, W.; Zhao, H.; Gao, L.; Fan, Y.; Zhou, S. The MicroRNAs Let 7 and Mir 278 Regulate Insect Metamorphosis and Oogenesis by Targeting the Juvenile Hormone Early Response Gene Krüppel Homolog. *Development* **2018**, *145*, dev.170670. [CrossRef]
98. Kang, L.; Wang, M.; Cao, X.; Tang, S.; Xia, D.; Shen, X.; Zhao, Q. Inhibition of Expression of BmNPV Cg30 by Bmo-MiRNA-390 Is a Host Response to Baculovirus Invasion. *Arch. Virol.* **2018**, *163*, 2719–2725. [CrossRef]
99. Ma, K.; Li, F.; Tang, Q.; Liang, P.; Liu, Y.; Zhang, B.; Gao, X. CYP4CJ1-Mediated Gossypol and Tannic Acid Tolerance in Aphis Gossypii Glover. *Chemosphere* **2019**, *219*, 961–970. [CrossRef]

100. Aravin, A.A.; Lagos-Quintana, M.; Yalcin, A.; Zavolan, M.; Marks, D.; Snyder, B.; Gaasterland, T.; Meyer, J.; Tuschl, T. The Small RNA Profile during Drosophila Melanogaster Development. *Dev. Cell* **2003**, *5*, 337–350. [CrossRef]
101. Cai, X.; Hagedorn, C.H.; Cullen, B.R. Human MicroRNAs Are Processed from Capped, Polyadenylated Transcripts That Can Also Function as MRNAs. *RNA* **2004**, *10*, 1957–1966. [CrossRef]
102. Church, V.A.; Pressman, S.; Isaji, M.; Truscott, M.; Cizmecioglu, N.T.; Buratowski, S.; Frolov, M.V.; Carthew, R.W. Microprocessor Recruitment to Elongating RNA Polymerase II Is Required for Differential Expression of MicroRNAs. *Cell Rep.* **2017**, *20*, 3123–3134. [CrossRef]
103. Xiong, H.; Qian, J.; He, T.; Li, F. Independent Transcription of MiR-281 in the Intron of ODA in Drosophila Melanogaster. *Biochem. Biophys. Res. Commun.* **2009**, *378*, 883–889. [CrossRef]
104. Lee, Y.; Jeon, K.; Lee, J.T.; Kim, S.; Kim, V.N. MicroRNA Maturation: Stepwise Processing and Subcellular Localization. *EMBO J.* **2002**, *21*, 4663–4670. [CrossRef]
105. Landthaler, M.; Yalcin, A.; Tuschl, T. The Human DiGeorge Syndrome Critical Region Gene 8 and Its D. Melanogaster Homolog Are Required for MiRNA Biogenesis. *Curr. Biol.* **2004**, *14*, 2162–2167. [CrossRef] [PubMed]
106. Denli, A.M.; Tops, B.B.J.; Plasterk, R.H.A.; Ketting, R.F.; Hannon, G.J. Processing of Primary MicroRNAs by the Microprocessor Complex. *Nature* **2004**, *432*, 231–235. [CrossRef]
107. Lee, Y.; Ahn, C.; Han, J.; Choi, H.; Kim, J.; Yim, J.; Lee, J.; Provost, P.; Rådmark, O.; Kim, S.; et al. The Nuclear RNase III Drosha Initiates MicroRNA Processing. *Nature* **2003**, *425*, 415–419. [CrossRef]
108. Ma, H.; Wu, Y.; Choi, J.G.; Wu, H. Lower and Upper Stem-Single-Stranded RNA Junctions Together Determine the Drosha Cleavage Site. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 20687–20692. [CrossRef]
109. Nguyen, T.A.; Jo, M.H.; Choi, Y.G.; Park, J.; Kwon, S.C.; Hohng, S.; Kim, V.N.; Woo, J.S. Functional Anatomy of the Human Microprocessor. *Cell* **2015**, *161*, 1374–1387. [CrossRef]
110. Kwon, S.C.; Nguyen, T.A.; Choi, Y.G.; Jo, M.H.; Hohng, S.; Kim, V.N.; Woo, J.S. Structure of Human DROSHA. *Cell* **2016**, *164*, 81–90. [CrossRef]
111. Partin, A.C.; Zhang, K.; Jeong, B.C.; Herrell, E.; Li, S.; Chiu, W.; Nam, Y. Cryo-EM Structures of Human Drosha and DGCR8 in Complex with Primary MicroRNA. *Mol. Cell* **2020**, *78*, 411–422.e4. [CrossRef]
112. Jin, W.; Wang, J.; Liu, C.P.; Wang, H.W.; Xu, R.M. Structural Basis for Pri-MiRNA Recognition by Drosha. *Mol. Cell* **2020**, *78*, 423–433.e5. [CrossRef]
113. Herbert, K.M.; Sarkar, S.K.; Mills, M.; De La Herran, H.C.D.; Neuman, K.C.; Steitz, J.A. A Heterotrimer Model of the Complete Microprocessor Complex Revealed by Single-Molecule Subunit Counting. *RNA* **2016**, *22*, 175–183. [CrossRef]
114. Fang, W.; Bartel, D.P. The Menu of Features That Define Primary MicroRNAs and Enable de Novo Design of MicroRNA Genes. *Mol. Cell* **2015**, *60*, 131–145. [CrossRef] [PubMed]
115. Ruby, J.G.; Jan, C.H.; Bartel, D.P. Intronic MicroRNA Precursors That Bypass Drosha Processing. *Nature* **2007**, *448*, 83–86. [CrossRef]
116. Okamura, K.; Hagen, J.W.; Duan, H.; Tyler, D.M.; Lai, E.C. The Mirtron Pathway Generates MicroRNA-Class Regulatory RNAs in Drosophila. *Cell* **2007**, *130*, 89–100. [CrossRef] [PubMed]
117. Shibata, S.; Sasaki, M.; Miki, T.; Shimamoto, A.; Furuichi, Y.; Katahira, J.; Yoneda, Y. Exportin-5 Orthologues Are Functionally Divergent among Species. *Nucleic Acids Res.* **2006**, *34*, 4711–4721. [CrossRef] [PubMed]
118. Singh, C.P.; Singh, J.; Nagaraju, J. A Baculovirus-Encoded MicroRNA (MiRNA) Suppresses Its Host MiRNA Biogenesis by Regulating the Exportin-5 Cofactor Ran. *J. Virol.* **2012**, *86*, 7867–7879. [CrossRef] [PubMed]
119. Zeng, Y.; Cullen, B.R. Structural Requirements for Pre-MicroRNA Binding and Nuclear Export by Exportin 5. *Nucleic Acids Res.* **2004**, *32*, 4776–4785. [CrossRef] [PubMed]
120. Okada, C.; Yamashita, E.; Lee, S.J.; Shibata, S.; Katahira, J.; Nakagawa, A.; Yoneda, Y.; Tsukihara, T. A High-Resolution Structure of the Pre-Microrna Nuclear Export Machinery. *Science* **2009**, *326*, 1275–1279. [CrossRef]
121. Zhang, X.; Liu, F.; Yang, F.; Meng, Z.; Zeng, Y. Selectivity of Exportin 5 Binding to Human Precursor MicroRNAs. *RNA Biol.* **2021**, *18*, 730–737. [CrossRef]
122. Tsutsumi, A.; Kawamata, T.; Izumi, N.; Seitz, H.; Tomari, Y. Recognition of the Pre-MiRNA Structure by Drosophila Dicer-1. *Nat. Struct. Mol. Biol.* **2011**, *18*, 1153–1158. [CrossRef]
123. Lim, M.Y.T.; Ng, A.W.T.; Chou, Y.; Lim, T.P.; Simcox, A.; Tucker-Kellogg, G.; Okamura, K. The Drosophila Dicer-1 Partner Loquacious Enhances MiRNA Processing from Hairpins with Unstable Structures at the Dicing Site. *Cell Rep.* **2016**, *15*, 1795–1808. [CrossRef] [PubMed]
124. Jiang, F.; Ye, X.; Liu, X.; Fincher, L.; McKearin, D.; Liu, Q. Dicer-1 and R3D1-L Catalyze MicroRNA Maturation in Drosophila. *Genes. Dev.* **2005**, *19*, 1674–1679. [CrossRef]
125. Förstemann, K.; Tomari, Y.; Du, T.; Vagin, V.V.; Denli, A.M.; Bratu, D.P.; Klattenhoff, C.; Theurkauf, W.E.; Zamore, P.D. Normal MicroRNA Maturation and Germ-Line Stem Cell Maintenance Requires Loquacious, a Double-Stranded RNA-Binding Domain Protein. *PLoS Biol.* **2005**, *3*, e236. [CrossRef]
126. Saito, K.; Ishizuka, A.; Siomi, H.; Siomi, M.C. Processing of Pre-MicroRNAs by the Dicer-1–Loquacious Complex in Drosophila Cells. *PLoS Biol.* **2005**, *3*, e235. [CrossRef]
127. Liu, X.; Park, J.K.; Jiang, F.; Liu, Y.; Mckearin, D.; Liu, Q. Dicer-1, but Not Loquacious, Is Critical for Assembly of MiRNA-Induced Silencing Complexes. *RNA* **2007**, *13*, 2324–2329. [CrossRef]

128. Kawamata, T.; Seitz, H.; Tomari, Y. Structural Determinants of MiRNAs for RISC Loading and Slicer-Independent Unwinding. *Nat. Struct. Mol. Biol.* **2009**, *16*, 953–960. [CrossRef]
129. Chawla, G.; Sokol, N.S. ADAR Mediates Differential Expression of Polycistronic MicroRNAs. *Nucleic Acids Res.* **2014**, *42*, 5245–5255. [CrossRef]
130. Reimão-Pinto, M.M.; Ignatova, V.; Burkard, T.R.; Hung, J.H.; Manzenreither, R.A.; Sowemimo, I.; Herzog, V.A.; Reichholf, B.; Fariña-Lopez, S.; Ameres, S.L. Uridylation of RNA Hairpins by Tailor Confines the Emergence of MicroRNAs in Drosophila. *Mol. Cell* **2015**, *59*, 203–216. [CrossRef]
131. Agarwal, V.; Subtelny, A.O.; Thiru, P.; Ulitsky, I.; Bartel, D.P. Predicting MicroRNA Targeting Efficacy in Drosophila. *Genome Biol.* **2018**, *19*, 152. [CrossRef]
132. Lai, E.C. Micro RNAs Are Complementary to 3′ UTR Sequence Motifs That Mediate Negative Post-Transcriptional Regulation. *Nat. Genet.* **2002**, *30*, 363–364. [CrossRef] [PubMed]
133. Stark, A.; Brennecke, J.; Russell, R.B.; Cohen, S.M. Identification of Drosophila MicroRNA Targets. *PLoS Biol.* **2003**, *1*, e60. [CrossRef] [PubMed]
134. Bartel, D.P. MicroRNAs: Target Recognition and Regulatory Functions. *Cell* **2009**, *136*, 215–233. [CrossRef]
135. Kuzin, A.; Kundu, M.; Brody, T.; Odenwald, W.F. The Drosophila Nerfin-1 MRNA Requires Multiple MicroRNAs to Regulate Its Spatial and Temporal Translation Dynamics in the Developing Nervous System. *Dev. Biol.* **2007**, *310*, 35–43. [CrossRef] [PubMed]
136. Lee, G.J.; Hyun, S. Multiple Targets of the MicroRNA MiR-8 Contribute to Immune Homeostasis in Drosophila. *Dev. Comp. Immunol.* **2014**, *45*, 245–251. [CrossRef] [PubMed]
137. Zekri, L.; Huntzinger, E.; Heimstädt, S.; Izaurralde, E. The Silencing Domain of GW182 Interacts with PABPC1 to Promote Translational Repression and Degradation of MicroRNA Targets and Is Required for Target Release. *Mol. Cell Biol.* **2009**, *29*, 6220–6231. [CrossRef]
138. Zdanowicz, A.; Thermann, R.; Kowalska, J.; Jemielity, J.; Duncan, K.; Preiss, T.; Darzynkiewicz, E.; Hentze, M.W. Drosophila MiR2 Primarily Targets the M7GpppN Cap Structure for Translational Repression. *Mol. Cell* **2009**, *35*, 881–888. [CrossRef]
139. Fukaya, T.; Iwakawa, H.O.; Tomari, Y. MicroRNAs Block Assembly of EIF4F Translation Initiation Complex in Drosophila. *Mol. Cell* **2014**, *56*, 67–78. [CrossRef]
140. Behm-Ansmant, I.; Rehwinkel, J.; Doerks, T.; Stark, A.; Bork, P.; Izaurralde, E. MRNA Degradation by MiRNAs and GW182 Requires Both CCR4:NOT Deadenylase and DCP1:DCP2 Decapping Complexes. *Genes. Dev.* **2006**, *20*, 1885–1898. [CrossRef]
141. Eulalio, A.; Rehwinkel, J.; Stricker, M.; Huntzinger, E.; Yang, S.F.; Doerks, T.; Dorner, S.; Bork, P.; Boutros, M.; Izaurralde, E. Target-Specific Requirements for Enhancers of Decapping in MiRNA-Mediated Gene Silencing. *Genes. Dev.* **2007**, *21*, 2558–2570. [CrossRef]
142. Braun, J.E.; Huntzinger, E.; Fauser, M.; Izaurralde, E. GW182 Proteins Directly Recruit Cytoplasmic Deadenylase Complexes to MiRNA Targets. *Mol. Cell* **2011**, *44*, 120–133. [CrossRef]
143. Nishihara, T.; Zekri, L.; Braun, J.E.; Izaurralde, E. MiRISC Recruits Decapping Factors to MiRNA Targets to Enhance Their Degradation. *Nucleic Acids Res.* **2013**, *41*, 8692–8705. [CrossRef]
144. Rehwinkel, J.; Behm-Ansmant, I.; Gatfield, D.; Izaurralde, E. A Crucial Role for GW182 and the DCP1:DCP2 Decapping Complex in MiRNA-Mediated Gene Silencing. *RNA* **2005**, *11*, 1640–1647. [CrossRef]
145. Vagin, V.V.; Sigova, A.; Li, C.; Seitz, H.; Gvozdev, V.; Zamore, P.D. A Distinct Small RNA Pathway Silences Selfish Genetic Elements in the Germline. *Science* **2006**, *313*, 320–324. [CrossRef]
146. Brennecke, J.; Aravin, A.A.; Stark, A.; Dus, M.; Kellis, M.; Sachidanandam, R.; Hannon, G.J. Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. *Cell* **2007**, *128*, 1089–1103. [CrossRef]
147. Gonzalez, J.; Qi, H.; Liu, N.; Lin, H. Piwi Is a Key Regulator of Both Somatic and Germline Stem Cells in the Drosophila Testis. *Cell Rep.* **2015**, *12*, 150–161. [CrossRef]
148. Klein, J.D.; Qu, C.; Yang, X.; Fan, Y.; Tang, C.; Peng, J.C. C-Fos Repression by Piwi Regulates Drosophila Ovarian Germline Formation and Tissue Morphogenesis. *PLoS Genet.* **2016**, *12*, e1006281. [CrossRef]
149. Dietrich, I.; Shi, X.; McFarlane, M.; Watson, M.; Blomström, A.L.; Skelton, J.K.; Kohl, A.; Elliott, R.M.; Schnettler, E. The Antiviral RNAi Response in Vector and Non-Vector Cells against Orthobunyaviruses. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0005272. [CrossRef]
150. Katsuma, S.; Kawamoto, M.; Shoji, K.; Aizawa, T.; Kiuchi, T.; Izumi, N.; Ogawa, M.; Mashiko, T.; Kawasaki, H.; Sugano, S.; et al. Transcriptome Profiling Reveals Infection Strategy of an Insect Maculavirus. *DNA Res.* **2018**, *25*, 277–286. [CrossRef]
151. Kotov, A.A.; Adashev, V.E.; Godneeva, B.K.; Ninova, M.; Shatskikh, A.S.; Bazylev, S.S.; Aravin, A.A.; Olenina, L.v. PiRNA Silencing Contributes to Interspecies Hybrid Sterility and Reproductive Isolation in Drosophila Melanogaster. *Nucleic Acids Res.* **2019**, *47*, 4255–4271. [CrossRef]
152. Goriaux, C.; Desset, S.; Renaud, Y.; Vaury, C.; Brasset, E. Transcriptional Properties and Splicing of the Flamenco PiRNA Cluster. *EMBO Rep.* **2014**, *15*, 411–418. [CrossRef]
153. Dennis, C.; Brasset, E.; Sarkar, A.; Vaury, C. Export of PiRNA Precursors by EJC Triggers Assembly of Cytoplasmic Yb-Body in Drosophila. *Nat. Commun.* **2016**, *7*, 13739. [CrossRef]
154. Malone, C.D.; Brennecke, J.; Dus, M.; Stark, A.; McCombie, W.R.; Sachidanandam, R.; Hannon, G.J. Specialized PiRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary. *Cell* **2009**, *137*, 522–535. [CrossRef]
155. Mohn, F.; Sienski, G.; Handler, D.; Brennecke, J. The Rhino-Deadlock-Cutoff Complex Licenses Noncanonical Transcription of Dual-Strand PiRNA Clusters in Drosophila. *Cell* **2014**, *157*, 1364–1379. [CrossRef]

156. Klattenhoff, C.; Xi, H.; Li, C.; Lee, S.; Xu, J.; Khurana, J.S.; Zhang, F.; Schultz, N.; Koppetsch, B.S.; Nowosielska, A.; et al. The Drosophila HP1 Homolog Rhino Is Required for Transposon Silencing and PiRNA Production by Dual-Strand Clusters. *Cell* **2009**, *138*, 1137–1149. [CrossRef]
157. Andersen, P.R.; Tirian, L.; Vunjak, M.; Brennecke, J. A Heterochromatin-Dependent Transcription Machinery Drives PiRNA Expression. *Nature* **2017**, *549*, 54–59. [CrossRef]
158. Chen, Y.C.A.; Stuwe, E.; Luo, Y.; Ninova, M.; le Thomas, A.; Rozhavskaya, E.; Li, S.; Vempati, S.; Laver, J.D.; Patel, D.J.; et al. Cutoff Suppresses RNA Polymerase II Termination to Ensure Expression of PiRNA Precursors. *Mol. Cell* **2016**, *63*, 97–109. [CrossRef] [PubMed]
159. Zhang, Z.; Wang, J.; Schultz, N.; Zhang, F.; Parhad, S.S.; Tu, S.; Vreven, T.; Zamore, P.D.; Weng, Z.; Theurkauf, W.E. The HP1 Homolog Rhino Anchors a Nuclear Complex That Suppresses PiRNA Precursor Splicing. *Cell* **2014**, *157*, 1353–1363. [CrossRef]
160. ElMaghraby, M.F.; Andersen, P.R.; Pühringer, F.; Hohmann, U.; Meixner, K.; Lendl, T.; Tirian, L.; Brennecke, J. A Heterochromatin-Specific RNA Export Pathway Facilitates PiRNA Production. *Cell* **2019**, *178*, 964–979.e20. [CrossRef]
161. Kneuss, E.; Munafò, M.; Eastwood, E.L.; Deumer, U.S.; Preall, J.B.; Hannon, G.J.; Czech, B. Specialization of the Drosophila Nuclear Export Family Protein Nxf3 for PiRNA Precursor Export. *Genes. Dev.* **2019**, *33*, 1208–1220. [CrossRef]
162. Ai, K.L.; Kai, T. Unique Germ-Line Organelle, Nuage, Functions to Repress Selfish Genetic Elements in Drosophila Melanogaster. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 6714–6719. [CrossRef]
163. Qi, H.; Watanabe, T.; Ku, H.Y.; Liu, N.; Zhong, M.; Lin, H. The Yb Body, a Major Site for Piwi-Associated RNA Biogenesis and a Gateway for Piwi Expression and Transport to the Nucleus in Somatic Cells. *J. Biol. Chem.* **2011**, *286*, 3789–3797. [CrossRef] [PubMed]
164. Szakmary, A.; Reedy, M.; Qi, H.; Lin, H. The Yb Protein Defines a Novel Organelle and Regulates Male Germline Stem Cell Self-Renewal in Drosophila Melanogaster. *J. Cell Biol.* **2009**, *185*, 613–627. [CrossRef]
165. Gainetdinov, I.; Colpan, C.; Arif, A.; Cecchini, K.; Zamore, P.D. A Single Mechanism of Biogenesis, Initiated and Directed by PIWI Proteins, Explains PiRNA Production in Most Animals. *Mol. Cell* **2018**, *71*, 775–790.e5. [CrossRef]
166. Nishida, K.M.; Sakakibara, K.; Iwasaki, Y.W.; Yamada, H.; Murakami, R.; Murota, Y.; Kawamura, T.; Kodama, T.; Siomi, H.; Siomi, M.C. Hierarchical Roles of Mitochondrial Papi and Zucchini in Bombyx Germline PiRNA Biogenesis. *Nature* **2018**, *555*, 260–264. [CrossRef]
167. Izumi, N.; Shoji, K.; Suzuki, Y.; Katsuma, S.; Tomari, Y. Zucchini Consensus Motifs Determine the Mechanism of Pre-PiRNA Production. *Nature* **2020**, *578*, 311–316. [CrossRef]
168. Han, B.W.; Wang, W.; Li, C.; Weng, Z.; Zamore, P.D. Noncoding RNA. PiRNA-Guided Transposon Cleavage Initiates Zucchini-Dependent, Phased PiRNA Production. *Science* **2015**, *348*, 817–821. [CrossRef]
169. Mohn, F.; Handler, D.; Brennecke, J. Noncoding RNA. PiRNA-Guided Slicing Specifies Transcripts for Zucchini-Dependent, Phased PiRNA Biogenesis. *Science* **2015**, *348*, 812–817. [CrossRef] [PubMed]
170. Kawaoka, S.; Izumi, N.; Katsuma, S.; Tomari, Y. 3′ End Formation of PIWI-Interacting RNAs in Vitro. *Mol. Cell* **2011**, *43*, 1015–1022. [CrossRef] [PubMed]
171. Izumi, N.; Shoji, K.; Sakaguchi, Y.; Honda, S.; Kirino, Y.; Suzuki, T.; Katsuma, S.; Tomari, Y. Identification and Functional Analysis of the Pre-PiRNA 3′ Trimmer in Silkworms. *Cell* **2016**, *164*, 962–973. [CrossRef]
172. Hayashi, R.; Schnabl, J.; Handler, D.; Mohn, F.; Ameres, S.L.; Brennecke, J. Genetic and Mechanistic Diversity of PiRNA 3′-End Formation. *Nature* **2016**, *539*, 588–592. [CrossRef]
173. Saito, K.; Sakaguchi, Y.; Suzuki, T.; Suzuki, T.; Siomi, H.; Siomi, M.C. Pimet, the Drosophila Homolog of HEN1, Mediates 2′-O-Methylation of Piwi- Interacting RNAs at Their 3′ Ends. *Genes. Dev.* **2007**, *21*, 1603–1608. [CrossRef] [PubMed]
174. Tian, Y.; Simanshu, D.K.; Ma, J.B.; Patel, D.J. Structural Basis for PiRNA 2′-O-Methylated 3′-End Recognition by Piwi PAZ (Piwi/Argonaute/Zwille) Domains. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 903–910. [CrossRef] [PubMed]
175. Gunawardane, L.S.; Saito, K.; Nishida, K.M.; Miyoshi, K.; Kawamura, Y.; Nagami, T.; Siomi, H.; Siomi, M.C. A Slicer-Mediated Mechanism for Repeat-Associated SiRNA 5′ End Formation in Drosophila. *Science* **2007**, *315*, 1587–1590. [CrossRef] [PubMed]
176. Rouget, C.; Papin, C.; Boureux, A.; Meunier, A.C.; Franco, B.; Robine, N.; Lai, E.C.; Pelisson, A.; Simonelig, M. Maternal MRNA Deadenylation and Decay by the PiRNA Pathway in the Early Drosophila Embryo. *Nature* **2010**, *467*, 1128–1132. [CrossRef] [PubMed]
177. Rojas-Ríos, P.; Chartier, A.; Pierson, S.; Simonelig, M. Aubergine and PiRNAs Promote Germline Stem Cell Self-Renewal by Repressing the Proto-Oncogene Cbl. *EMBO J.* **2017**, *36*, 3194–3211. [CrossRef] [PubMed]
178. Ai, K.L.; Tao, L.; Kai, T. PiRNAs Mediate Posttranscriptional Retroelement Silencing and Localization to Pi-Bodies in the Drosophila Germline. *J. Cell Biol.* **2009**, *186*, 333–342. [CrossRef]
179. Klenov, M.S.; Sokolova, O.A.; Yakushev, E.Y.; Stolyarenko, A.D.; Mikhaleva, E.A.; Lavrov, S.A.; Gvozdev, V.A. Separation of Stem Cell Maintenance and Transposon Silencing Functions of Piwi Protein. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 18760–18765. [CrossRef] [PubMed]
180. Sienski, G.; Dönertas, D.; Brennecke, J. Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. *Cell* **2012**, *151*, 964–980. [CrossRef]
181. Shpiz, S.; Ryazansky, S.; Olovnikov, I.; Abramov, Y.; Kalmykova, A. Euchromatic Transposon Insertions Trigger Production of Novel Pi- and Endo-SiRNAs at the Target Sites in the Drosophila Germline. *PLoS Genet.* **2014**, *10*, e1004138. [CrossRef]

182. Kordyukova, M.; Sokolova, O.; Morgunova, V.; Ryazansky, S.; Akulenko, N.; Glukhov, S.; Kalmykova, A. Nuclear Ccr4-Not Mediates the Degradation of Telomeric and Transposon Transcripts at Chromatin in the Drosophila Germline. *Nucleic Acids Res.* **2020**, *48*, 141–156. [CrossRef]
183. Whangbo, J.S.; Hunter, C.P. Environmental RNA Interference. *Trends Genet.* **2008**, *24*, 297–305. [CrossRef]
184. Hunter, C.P.; Winston, W.M.; Molodowitch, C.; Feinberg, E.H.; Shih, J.; Sutherlin, M.; Wright, A.J.; Fitzgerald, M.C. Systemic RNAi in Caenorhabditis Elegans. *Cold Spring Harb. Symp. Quant. Biol.* **2006**, *71*, 95–100. [CrossRef]
185. Winston, W.M.; Sutherlin, M.; Wright, A.J.; Feinberg, E.H.; Hunter, C.P. Caenorhabditis Elegans SID-2 Is Required for Environmental RNA Interference. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 10565–10570. [CrossRef] [PubMed]
186. Winston, W.M.; Molodowitch, C.; Hunter, C.P. Systemic RNAi in C. Elegans Requires the Putative Transmembrane Protein SID-1. *Science* **2002**, *295*, 2456–2459. [CrossRef] [PubMed]
187. Feinberg, E.H.; Hunter, C.P. Transport of DsRNA into Cells by the Transmembrane Protein SID-1. *Science* **2003**, *301*, 1545–1547. [CrossRef]
188. Hinas, A.; Wright, A.J.; Hunter, C.P. SID-5 Is an Endosome-Associated Protein Required for Efficient Systemic RNAi in C. Elegans. *Curr. Biol.* **2012**, *22*, 1938–1943. [CrossRef]
189. Jose, A.M.; Kim, Y.A.; Leal-Ekman, S.; Hunter, C.P. Conserved Tyrosine Kinase Promotes the Import of Silencing RNA into Caenorhabditis Elegans Cells. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 14520–14525. [CrossRef]
190. Gao, J.; Zhao, L.; Luo, Q.; Liu, S.; Lin, Z.; Wang, P.; Fu, X.; Chen, J.; Zhang, H.; Lin, L.; et al. An EHBP-1-SID-3-DYN-1 Axis Promotes Membranous Tubule Fission during Endocytic Recycling. *PLoS Genet.* **2020**, *16*, e1008763. [CrossRef]
191. Dowling, D.; Pauli, T.; Donath, A.; Meusemann, K.; Podsiadlowski, L.; Petersen, M.; Peters, R.S.; Mayer, C.; Liu, S.; Zhou, X.; et al. Phylogenetic Origin and Diversification of RNAi Pathway Genes in Insects. *Genome Biol. Evol.* **2016**, *8*, 3784–3793. [CrossRef]
192. Tomoyasu, Y.; Miller, S.C.; Tomita, S.; Schoppmeier, M.; Grossmann, D.; Bucher, G. Exploring Systemic RNA Interference in Insects: A Genome-Wide Survey for RNAi Genes in Tribolium. *Genome Biol.* **2008**, *9*, R10. [CrossRef]
193. Pinheiro, D.H.; Vélez, A.M.; Fishilevich, E.; Wang, H.; Carneiro, N.P.; Valencia-Jiménez, A.; Valicente, F.H.; Narva, K.E.; Siegfried, B.D. Clathrin-Dependent Endocytosis Is Associated with RNAi Response in the Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS ONE* **2018**, *13*, e0201849. [CrossRef]
194. Dong, X.; Li, X.; Li, Q.; Jia, H.; Zhang, H. The Inducible Blockage of RNAi Reveals a Role for Polyunsaturated Fatty Acids in the Regulation of DsRNA-Endocytic Capacity in *Bactrocera dorsalis*. *Sci. Rep.* **2017**, *7*, 5584. [CrossRef]
195. Cappelle, K.; de Oliveira, C.F.R.; van Eynde, B.; Christiaens, O.; Smagghe, G. The Involvement of Clathrin-Mediated Endocytosis and Two Sid-1-like Transmembrane Proteins in Double-Stranded RNA Uptake in the Colorado Potato Beetle Midgut. *Insect Mol. Biol.* **2016**, *25*, 315–323. [CrossRef]
196. Yoon, J.S.; Shukla, J.N.; Gong, Z.J.; Mogilicherla, K.; Palli, S.R. RNA Interference in the Colorado Potato Beetle, *Leptinotarsa decemlineata*: Identification of Key Contributors. *Insect Biochem. Mol. Biol.* **2016**, *78*, 78–88. [CrossRef] [PubMed]
197. Saleh, M.C.; van Rij, R.P.; Hekele, A.; Gillis, A.; Foley, E.; O'Farrell, P.H.; Andino, R. The Endocytic Pathway Mediates Cell Entry of DsRNA to Induce RNAi Silencing. *Nat. Cell Biol.* **2006**, *8*, 793–802. [CrossRef] [PubMed]
198. Ulvila, J.; Parikka, M.; Kleino, A.; Sormunen, R.; Ezekowitz, R.A.; Kocks, C.; Rämet, M. Double-Stranded RNA Is Internalized by Scavenger Receptor-Mediated Endocytosis in Drosophila S2 Cells. *J. Biol. Chem.* **2006**, *281*, 14370–14375. [CrossRef] [PubMed]
199. Aung, K.M.; Boldbaatar, D.; Umemiya-Shirafuji, R.; Liao, M.; Xuenan, X.; Suzuki, H.; Galay, R.; Tanaka, T.; Fujisaki, K. Scavenger Receptor Mediates Systemic RNA Interference in Ticks. *PLoS ONE* **2011**, *6*, e28407. [CrossRef] [PubMed]
200. Wynant, N.; Santos, D.; van Wielendaele, P.; vanden Broeck, J. Scavenger Receptor-Mediated Endocytosis Facilitates RNA Interference in the Desert Locust, *Schistocerca gregaria*. *Insect Mol. Biol.* **2014**, *23*, 320–329. [CrossRef] [PubMed]
201. Xiao, D.; Gao, X.; Xu, J.; Liang, X.; Li, Q.; Yao, J.; Zhu, K.Y. Clathrin-Dependent Endocytosis Plays a Predominant Role in Cellular Uptake of Double-Stranded RNA in the Red Flour Beetle. *Insect Biochem. Mol. Biol.* **2015**, *60*, 68–77. [CrossRef]
202. Meng, F.; Yang, M.; Li, Y.; Li, T.; Liu, X.; Wang, G.; Wang, Z.; Jin, X.; Li, W. Functional Analysis of RNA Interference-Related Soybean Pod Borer (Lepidoptera) Genes Based on Transcriptome Sequences. *Front. Physiol.* **2018**, *9*, 383. [CrossRef]
203. Abbasi, R.; Heschuk, D.; Kim, B.; Whyard, S. A Novel Paperclip Double-Stranded RNA Structure Demonstrates Clathrin-Independent Uptake in the Mosquito Aedes Aegypti. *Insect Biochem. Mol. Biol.* **2020**, *127*, 103492. [CrossRef] [PubMed]
204. Rocha, J.J.E.; Korolchuk, V.I.; Robinson, I.M.; O'Kane, C.J. A Phagocytic Route for Uptake of Double-Stranded RNA in RNAi. *PLoS ONE* **2011**, *6*, e19087. [CrossRef] [PubMed]
205. Bolognesi, R.; Ramaseshadri, P.; Anderson, J.; Bachman, P.; Clinton, W.; Flannagan, R.; Ilagan, O.; Lawrence, C.; Levine, S.; Moar, W.; et al. Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *PLoS ONE* **2012**, *7*, e47534. [CrossRef] [PubMed]
206. Miller, S.C.; Miyata, K.; Brown, S.J.; Tomoyasu, Y. Dissecting Systemic RNA Interference in the Red Flour Beetle *Tribolium castaneum*: Parameters Affecting the Efficiency of RNAi. *PLoS ONE* **2012**, *7*, e47431. [CrossRef] [PubMed]
207. Singh, I.K.; Singh, S.; Mogilicherla, K.; Shukla, J.N.; Palli, S.R. Comparative Analysis of Double-Stranded RNA Degradation and Processing in Insects. *Sci. Rep.* **2017**, *7*, 17059. [CrossRef]
208. Wang, K.; Peng, Y.; Pu, J.; Fu, W.; Wang, J.; Han, Z. Variation in RNAi Efficacy among Insect Species Is Attributable to DsRNA Degradation in Vivo. *Insect Biochem. Mol. Biol.* **2016**, *77*, 1–9. [CrossRef]
209. Guan, R.B.; Li, H.C.; Fan, Y.J.; Hu, S.R.; Christiaens, O.; Smagghe, G.; Miao, X.X. A Nuclease Specific to Lepidopteran Insects Suppresses RNAi. *J. Biol. Chem.* **2018**, *293*, 6011–6021. [CrossRef]

210. Lomate, P.R.; Bonning, B.C. Distinct Properties of Proteases and Nucleases in the Gut, Salivary Gland and Saliva of Southern Green Stink Bug, *Nezara viridula*. *Sci. Rep.* **2016**, *6*, 27587. [CrossRef]
211. Shukla, J.N.; Kalsi, M.; Sethi, A.; Narva, K.E.; Fishilevich, E.; Singh, S.; Mogilicherla, K.; Palli, S.R. Reduced Stability and Intracellular Transport of DsRNA Contribute to Poor RNAi Response in Lepidopteran Insects. *RNA Biol.* **2016**, *13*, 656–669. [CrossRef] [PubMed]
212. Yoon, J.S.; Gurusamy, D.; Palli, S.R. Accumulation of DsRNA in Endosomes Contributes to Inefficient RNA Interference in the Fall Armyworm, *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* **2017**, *90*, 53–60. [CrossRef] [PubMed]
213. Lee, Y.S.; Pressman, S.; Andress, A.P.; Kim, K.; White, J.L.; Cassidy, J.J.; Li, X.; Lubell, K.; Lim, D.H.; Cho, I.S.; et al. Silencing by Small RNAs Is Linked to Endosomal Trafficking. *Nat. Cell Biol.* **2009**, *11*, 1150–1156. [CrossRef]
214. Shi, X.; Liu, X.; Cooper, A.M.W.; Silver, K.; Merzendorfer, H.; Zhu, K.Y.; Zhang, J. Vacuolar (H+)-ATPase Subunit c Is Essential for the Survival and Systemic RNA Interference Response in *Locusta migratoria*. *Pest. Manag. Sci.* **2022**, *78*, 1555–1566. [CrossRef]
215. Karlikow, M.; Goic, B.; Mongelli, V.; Salles, A.; Schmitt, C.; Bonne, I.; Zurzolo, C.; Saleh, M.C. Drosophila Cells Use Nanotube-like Structures to Transfer DsRNA and RNAi Machinery between Cells. *Sci. Rep.* **2016**, *6*, 27085. [CrossRef] [PubMed]
216. Valiunas, V.; Polosina, Y.Y.; Miller, H.; Potapova, I.A.; Valiuniene, L.; Doronin, S.; Mathias, R.T.; Robinson, R.B.; Rosen, M.R.; Cohen, I.S.; et al. Connexin-Specific Cell-to-Cell Transfer of Short Interfering RNA by Gap Junctions. *J. Physiol.* **2005**, *568*, 459–468. [CrossRef] [PubMed]
217. Garbutt, J.S.; Bellés, X.; Richards, E.H.; Reynolds, S.E. Persistence of Double-Stranded RNA in Insect Hemolymph as a Potential Determiner of RNA Interference Success: Evidence from *Manduca sexta* and *Blattella germanica*. *J. Insect Physiol.* **2013**, *59*, 171–178. [CrossRef] [PubMed]
218. Ma, Z.; Zheng, Y.; Chao, Z.; Chen, H.; Zhang, Y.; Yin, M.; Shen, J.; Yan, S. Visualization of the Process of a Nanocarrier-Mediated Gene Delivery: Stabilization, Endocytosis and Endosomal Escape of Genes for Intracellular Spreading. *J. Nanobiotechnol.* **2022**, *20*, 124. [CrossRef] [PubMed]
219. Li, J.; Du, J.; Li, S.; Wang, X. Identification and Characterization of a Double-Stranded RNA Degrading Nuclease Influencing RNAi Efficiency in the Rice Leaf Folder *Cnaphalocrocis medinalis*. *Int. J. Mol. Sci.* **2022**, *23*, 3961. [CrossRef] [PubMed]
220. Sakashita, K.; Tatsuke, T.; Masaki, Y.; Lee, M.; Kawaguch, Y.; Kusakabe, T. DsRNA Binding Activity of Silkworm Larval Hemolymph Is Mediated by Lipophorin Complex. *J. Fac. Agric. Kyushu Univ.* **2009**, *54*, 401–406. [CrossRef]
221. Wynant, N.; Duressa, T.F.; Santos, D.; van Duppen, J.; Proost, P.; Huybrechts, R.; vanden Broeck, J. Lipophorins Can Adhere to DsRNA, Bacteria and Fungi Present in the Hemolymph of the Desert Locust: A Role as General Scavenger for Pathogens in the Open Body Cavity. *J. Insect Physiol.* **2014**, *64*, 7–13. [CrossRef] [PubMed]
222. van den Brande, S.; Gijbels, M.; Wynant, N.; Santos, D.; Mingels, L.; Gansemans, Y.; van Nieuwerburgh, F.; vanden Broeck, J. The Presence of Extracellular MicroRNAs in the Media of Cultured Drosophila Cells. *Sci. Rep.* **2018**, *8*, 17312. [CrossRef]
223. Dhahbi, J.M.; Atamna, H.; Li, R.; Yamakawa, A.; Guerrero, N.; Lam, H.T.; Mote, P.; Spindler, S.R. MicroRNAs Circulate in the Hemolymph of Drosophila and Accumulate Relative to Tissue Micrornas in an Age-Dependent Manner. *Genom. Insights* **2016**, *9*, 29–39. [CrossRef]
224. Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. *Cell* **2017**, *169*, 314–325.e13. [CrossRef]
225. Mingels, L.; Wynant, N.; Santos, D.; Peeters, P.; Gansemans, Y.; Billen, J.; van Nieuwerburgh, F.; vanden Broeck, J. Extracellular Vesicles Spread the RNA Interference Signal of *Tribolium castaneum* TcA Cells. *Insect Biochem. Mol. Biol.* **2020**, *122*, 103377. [CrossRef] [PubMed]
226. Yoon, J.S.; Kim, K.; Palli, S.R. Double-Stranded RNA in Exosomes: Potential Systemic RNA Interference Pathway in the Colorado Potato Beetle, *Leptinotarsa decemlineata*. *J. Asia Pac. Entomol.* **2020**, *23*, 1160–1164. [CrossRef]
227. Bucher, G.; Scholten, J.; Klingler, M. Parental RNAi in Tribolium (Coleoptera). *Curr. Biol.* **2002**, *12*, R85–R86. [CrossRef] [PubMed]
228. Horn, T.; Narov, K.D.; Panfilio, K.A. Persistent Parental RNAi in the Beetle *Tribolium castaneum* Involves Maternal Transmission of Long Double-Stranded RNA. *Adv. Genet.* **2022**, *3*, 2100064. [CrossRef] [PubMed]
229. Khajuria, C.; Vélez, A.M.; Rangasamy, M.; Wang, H.; Fishilevich, E.; Frey, M.L.F.; Carneiro, N.P.; Gandra, P.; Narva, K.E.; Siegfried, B.D. Parental RNA Interference of Genes Involved in Embryonic Development of the Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte. *Insect Biochem. Mol. Biol.* **2015**, *63*, 54–62. [CrossRef] [PubMed]
230. Vicari, P.E.; Chang, E.S.; Perondini, A.L.P.; Selivon, D. Parental RNAi Silencing of the Transformer-2 Gene in a Species of the Anastrepha Fraterculus Complex of Cryptic Species (Diptera, Tephritidae). *J. Agric. Sci.* **2021**, *13*, p70. [CrossRef]
231. Baum, J.A.; Bogaert, T.; Clinton, W.; Heck, G.R.; Feldmann, P.; Ilagan, O.; Johnson, S.; Plaetinck, G.; Munyikwa, T.; Pleau, M.; et al. Control of Coleopteran Insect Pests through RNA Interference. *Nat. Biotechnol.* **2007**, *25*, 1322–1326. [CrossRef] [PubMed]
232. Mao, Y.B.; Cai, W.J.; Wang, J.W.; Hong, G.J.; Tao, X.Y.; Wang, L.J.; Huang, Y.P.; Chen, X.Y. Silencing a Cotton Bollworm P450 Monooxygenase Gene by Plant-Mediated RNAi Impairs Larval Tolerance of Gossypol. *Nat. Biotechnol.* **2007**, *25*, 1307–1313. [CrossRef]
233. Head, G.P.; Carroll, M.W.; Evans, S.P.; Rule, D.M.; Willse, A.R.; Clark, T.L.; Storer, N.P.; Flannagan, R.D.; Samuel, L.W.; Meinke, L.J. Evaluation of SmartStax and SmartStax PRO Maize against Western Corn Rootworm and Northern Corn Rootworm: Efficacy and Resistance Management. *Pest. Manag. Sci.* **2017**, *73*, 1883–1899. [CrossRef]
234. AgbioInvestor. *Time and Cost to Develop a New GM Trait*; AgbioInvestor: Pathhead, UK, 2022.

235. Sikora, D.; Rzymski, P. Public Acceptance of GM Foods: A Global Perspective (1999–2019). In *Policy Issues in Genetically Modified Crops*; Academic Press: Cambridge, MA, USA, 2021; pp. 293–315.
236. Gordon, K.H.J.; Waterhouse, P.M. RNAi for Insect-Proof Plants. *Nat. Biotechnol.* **2007**, *25*, 1231–1232. [CrossRef]
237. Jin, S.; Singh, N.D.; Li, L.; Zhang, X.; Daniell, H. Engineered Chloroplast DsRNA Silences *Cytochrome P450 Monooxygenase*, *V-ATPase* and *Chitin synthase* Genes in the Insect Gut and Disrupts *Helicoverpa armigera* Larval Development and Pupation. *Plant Biotechnol. J.* **2015**, *13*, 435–446. [CrossRef]
238. Zhang, J.; Khan, S.A.; Hasse, C.; Ruf, S.; Heckel, D.G.; Bock, R. Pest Control. Full Crop Protection from an Insect Pest by Expression of Long Double-Stranded RNAs in Plastids. *Science* **2015**, *347*, 991–994. [CrossRef]
239. Bally, J.; McIntyre, G.J.; Doran, R.L.; Lee, K.; Perez, A.; Jung, H.; Naim, F.; Larrinua, I.M.; Narva, K.E.; Waterhouse, P.M. In-Plant Protection against *Helicoverpa armigera* by Production of Long HpRNA in Chloroplasts. *Front. Plant Sci.* **2016**, *7*, 1453. [CrossRef]
240. Burke, W.G.; Kaplanoglu, E.; Kolotilin, I.; Menassa, R.; Donly, C. RNA Interference in the Tobacco Hornworm, Manduca Sexta, Using Plastid-Encoded Long Double-Stranded RNA. *Front. Plant Sci.* **2019**, *10*, 313. [CrossRef]
241. He, W.; Xu, W.; Xu, L.; Fu, K.; Guo, W.; Bock, R.; Zhang, J. Length-Dependent Accumulation of Double-Stranded RNAs in Plastids Affects RNA Interference Efficiency in the Colorado Potato Beetle. *J. Exp. Bot.* **2020**, *71*, 2670–2677. [CrossRef] [PubMed]
242. Kaplanoglu, E.; Kolotilin, I.; Menassa, R.; Donly, C. Plastid Transformation of Micro-Tom Tomato with a Hemipteran Double-Stranded RNA Results in RNA Interference in Multiple Insect Species. *Int. J. Mol. Sci.* **2022**, *23*, 3918. [CrossRef] [PubMed]
243. Bally, J.; Fishilevich, E.; Doran, R.L.; Lee, K.; de Campos, S.B.; German, M.A.; Narva, K.E.; Waterhouse, P.M. Plin-AmiR, a Pre-MicroRNA-Based Technology for Controlling Herbivorous Insect Pests. *Plant Biotechnol. J.* **2020**, *18*, 1925–1932. [CrossRef] [PubMed]
244. Kumar, P.; Pandit, S.S.; Baldwin, I.T. Tobacco Rattle Virus Vector: A Rapid and Transient Means of Silencing *Manduca sexta* Genes by Plant Mediated RNA Interference. *PLoS ONE* **2012**, *7*, e31347. [CrossRef] [PubMed]
245. Wuriyanghan, H.; Falk, B.W. RNA Interference towards the Potato Psyllid, *Bactericera cockerelli*, Is Induced in Plants Infected with Recombinant *Tobacco mosaic* Virus (TMV). *PLoS ONE* **2013**, *8*, e66050. [CrossRef]
246. Ramos, J.E.; Jain, R.G.; Powell, C.A.; Dawson, W.O.; Gowda, S.; Borovsky, D.; Shatters, R.G., Jr. Crowdsourced Identification of Potential Target Genes for CTV Induced Gene Silencing for Controlling the Citrus Greening Vector *Diaphorina citri*. *Front Physiol.* **2021**, *12*, 571826. [CrossRef]
247. Khan, A.M.; Ashfaq, M.; Khan, A.A.; Naseem, M.T.; Mansoor, S. Evaluation of Potential RNA-Interference-Target Genes to Control Cotton Mealybug, *Phenacoccus solenopsis* (Hemiptera: Pseudococcuidae). *Insect Sci.* **2018**, *25*, 778–786. [CrossRef]
248. Valentine, T.A.; Randall, E.; Wypijewski, K.; Chapman, S.; Jones, J.; Oparka, K.J. Delivery of Macromolecules to Plant Parasitic Nematodes Using a Tobacco Rattle Virus Vector. *Plant Biotechnol. J.* **2007**, *5*, 827–834. [CrossRef] [PubMed]
249. Dubreuil, G.; Magliano, M.; Dubrana, M.P.; Lozano, J.; Lecomte, P.; Favery, B.; Abad, P.; Rosso, M.N. Tobacco Rattle Virus Mediates Gene Silencing in a Plant Parasitic Root-Knot Nematode. *J. Exp. Bot.* **2009**, *60*, 4041–4050. [CrossRef]
250. Hajós, J.P.; Vermunt, A.M.W.; Zuidema, D.; Kulcsár, P.; Varjas, L.; de Kort, C.A.D.; Závodszky, P.; Vlak, J.M. Dissecting Insect Development: Baculovirus-Mediated Gene Silencing in Insects. *Insect Mol. Biol.* **1999**, *8*, 539–544. [CrossRef] [PubMed]
251. Uhlirova, M.; Foy, B.D.; Beaty, B.J.; Olson, K.E.; Riddiford, L.M.; Jindra, M. Use of Sindbis Virus-Mediated RNA Interference to Demonstrate a Conserved Role of Broad-Complex in Insect Metamorphosis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15607–15612. [CrossRef]
252. Huang, Y.; Deng, F.; Hu, Z.; Vlak, J.M.; Wang, H. Baculovirus-Mediated Gene Silencing in Insect Cells Using Intracellularly Produced Long Double-Stranded RNA. *J. Biotechnol.* **2007**, *128*, 226–236. [CrossRef] [PubMed]
253. Gu, J.; Liu, M.; Deng, Y.; Peng, H.; Chen, X. Development of an Efficient Recombinant Mosquito Densovirus-Mediated RNA Interference System and Its Preliminary Application in Mosquito Control. *PLoS ONE* **2011**, *6*, e21329. [CrossRef]
254. Kontogiannatos, D.; Swevers, L.; Maenaka, K.; Park, E.Y.; Iatrou, K.; Kourti, A. Functional Characterization of a Juvenile Hormone Esterase Related Gene in the Moth *Sesamia nonagrioides* through RNA Interference. *PLoS ONE* **2013**, *8*, e73834. [CrossRef]
255. Taning, C.N.T.; Christiaens, O.; Li, X.X.; Swevers, L.; Casteels, H.; Maes, M.; Smagghe, G. Engineered Flock House Virus for Targeted Gene Suppression through RNAi in Fruit Flies (*Drosophila melanogaster*) in Vitro and in Vivo. *Front. Physiol.* **2018**, *9*, 805. [CrossRef] [PubMed]
256. Selling, B.H.; Allison, R.F.; Kaesberg, P. Genomic RNA of an Insect Virus Directs Synthesis of Infectious Virions in Plants. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 434–438. [CrossRef] [PubMed]
257. Dasgupta, R.; Garcia, B.H.; Goodman, R.M. Systemic Spread of an RNA Insect Virus in Plants Expressing Plant Viral Movement Protein Genes. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4910–4915. [CrossRef] [PubMed]
258. Zhou, Y.; Maharaj, P.D.; Mallajosyula, J.K.; McCormick, A.A.; Kearney, C.M. In Planta Production of Flock House Virus Transencapsidated RNA and Its Potential Use as a Vaccine. *Mol. Biotechnol.* **2015**, *57*, 325–336. [CrossRef] [PubMed]
259. Li, H.; Li, W.X.; Ding, S.W. Induction and Suppression of RNA Silencing by an Animal Virus. *Science* **2002**, *296*, 1319–1321. [CrossRef] [PubMed]
260. Hajeri, S.; Killiny, N.; El-Mohtar, C.; Dawson, W.O.; Gowda, S. *Citrus tristeza* Virus-Based RNAi in Citrus Plants Induces Gene Silencing in *Diaphorina citri*, a Phloem-Sap Sucking Insect Vector of Citrus Greening Disease (Huanglongbing). *J. Biotechnol.* **2014**, *176*, 42–49. [CrossRef]
261. Niehl, A.; Soininen, M.; Poranen, M.M.; Heinlein, M. Synthetic Biology Approach for Plant Protection Using DsRNA. *Plant Biotechnol. J.* **2018**, *16*, 1679–1687. [CrossRef]

262. Xue, Q.; Samakovli, D.; Swevers, L.; Taning, C.N.T. Drosophila X Virus-like Particles as Efficient DsRNA Carriers for Improved RNAi against the Invasive Species, *Drosophila suzukii*. *J. Pest Sci.* **2023**, *97*, 429–443. [CrossRef]
263. Taracena, M.L.; Oliveira, P.L.; Almendares, O.; Umaña, C.; Lowenberger, C.; Dotson, E.M.; Paiva-Silva, G.O.; Pennington, P.M. Genetically Modifying the Insect Gut Microbiota to Control Chagas Disease Vectors through Systemic RNAi. *PLoS Negl. Trop. Dis.* **2015**, *9*, e0003358. [CrossRef]
264. Whitten, M.M.A.; Facey, P.D.; del Sol, R.; Fernández-Martínez, L.T.; Evans, M.C.; Mitchell, J.J.; Bodger, O.G.; Dyson, P.J. Symbiont-Mediated RNA Interference in Insects. *Proc. R. Soc. B Biol. Sci.* **2016**, *283*, 20160042. [CrossRef] [PubMed]
265. Chen, X.; Li, L.; Hu, Q.; Zhang, B.; Wu, W.; Jin, F.; Jiang, J. Expression of DsRNA in Recombinant *isaria fumosorosea* Strain Targets the *TLR7* Gene in *Bemisia tabaci*. *BMC Biotechnol.* **2015**, *15*, 64. [CrossRef] [PubMed]
266. Hu, Q.; Wu, W. Recombinant Fungal Entomopathogen RNAi Target Insect Gene. *Bioengineered* **2016**, *7*, 504–507. [CrossRef] [PubMed]
267. Murphy, K.A.; Tabuloc, C.A.; Cervantes, K.R.; Chiu, J.C. Ingestion of Genetically Modified Yeast Symbiont Reduces Fitness of an Insect Pest via RNA Interference. *Sci. Rep.* **2016**, *6*, 22587. [CrossRef] [PubMed]
268. Tenllado, F.; Martínez-García, B.; Vargas, M.; Díaz-Ruíz, J.R. Crude Extracts of Bacterially Expressed DsRNA Can Be Used to Protect Plants against Virus Infections. *BMC Biotechnol.* **2003**, *3*, 3. [CrossRef] [PubMed]
269. San Miguel, K.; Scott, J.G. The next Generation of Insecticides: DsRNA Is Stable as a Foliar-Applied Insecticide. *Pest. Manag. Sci.* **2016**, *72*, 801–809. [CrossRef] [PubMed]
270. Hunter, W.B.; Glick, E.; Paldi, N.; Bextine, B.R. Advances in RNA Interference: DsRNA Treatment in Trees and Grapevines for Insect Pest Suppression. *Southwest. Entomol.* **2012**, *37*, 85–87. [CrossRef]
271. Li, H.; Guan, R.; Guo, H.; Miao, X. New Insights into an RNAi Approach for Plant Defence against Piercing-Sucking and Stem-Borer Insect Pests. *Plant Cell Environ.* **2015**, *38*, 2277–2285. [CrossRef] [PubMed]
272. Dalakouras, A.; Jarausch, W.; Buchholz, G.; Bassler, A.; Braun, M.; Manthey, T.; Krczal, G.; Wassenegger, M. Delivery of Hairpin Rnas and Small Rnas into Woody and Herbaceous Plants by Trunk Injection and Petiole Absorption. *Front. Plant Sci.* **2018**, *9*, 1253. [CrossRef]
273. Pampolini, F.; Rodrigues, T.B.; Leelesh, R.S.; Kawashima, T.; Rieske, L.K. Confocal Microscopy Provides Visual Evidence and Confirms the Feasibility of DsRNA Delivery to Emerald Ash Borer through Plant Tissues. *J. Pest Sci.* **2020**, *93*, 1143–1153. [CrossRef]
274. Dalakouras, A.; Wassenegger, M.; McMillan, J.N.; Cardoza, V.; Maegele, I.; Dadami, E.; Runne, M.; Krczal, G.; Wassenegger, M. Induction of Silencing in Plants by High-Pressure Spraying of in Vitro-Synthesized Small RNAs. *Front. Plant Sci.* **2016**, *7*, 1327. [CrossRef]
275. Koch, A.; Biedenkopf, D.; Furch, A.; Weber, L.; Rossbach, O.; Abdellatef, E.; Linicus, L.; Johannsmeier, J.; Jelonek, L.; Goesmann, A.; et al. An RNAi-Based Control of *Fusarium graminearum* Infections through Spraying of Long DsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathog.* **2016**, *12*, e1005901. [CrossRef] [PubMed]
276. Biedenkopf, D.; Will, T.; Knauer, T.; Jelonek, L.; Furch, A.C.U.; Busche, T.; Koch, A. Systemic Spreading of Exogenous Applied Rna Biopesticides in the Crop Plant Hordeum Vulgare. *ExRNA* **2020**, *2*, 12. [CrossRef]
277. Dalakouras, A.; Ganopoulos, I. Induction of Promoter DNA Methylation upon High-Pressure Spraying of Double-Stranded RNA in Plants. *Agronomy* **2021**, *11*, 789. [CrossRef]
278. Das, P.R.; Sherif, S.M. Application of Exogenous DsRNAs-Induced RNAi in Agriculture: Challenges and Triumphs. *Front. Plant Sci.* **2020**, *11*, 946. [CrossRef] [PubMed]
279. Taning, C.N.T.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Mezzetti, B.; Sabbadini, S.; Sorteberg, H.G.; Sweet, J.; Ventura, V.; et al. RNA-Based Biocontrol Compounds: Current Status and Perspectives to Reach the Market. *Pest. Manag. Sci.* **2020**, *76*, 841–845. [CrossRef]
280. Timmons, L.; Court, D.L.; Fire, A. Ingestion of Bacterially Expressed DsRNAs Can Produce Specific and Potent Genetic Interference in Caenorhabditis Elegans. *Gene* **2001**, *263*, 103–112. [CrossRef] [PubMed]
281. Timmons, L.; Fire, A. Specific Interference by Ingested DsRNA. *Nature* **1998**, *395*, 854. [CrossRef] [PubMed]
282. Papić, L.; Rivas, J.; Toledo, S.; Romero, J. Double-Stranded RNA Production and the Kinetics of Recombinant *Escherichia coli* HT115 in Fed-Batch Culture. *Biotechnol. Rep.* **2018**, *20*, e00292. [CrossRef]
283. Ahn, S.-J.; Donahue, K.; Koh, Y.; Martin, R.R.; Choi, M.-Y. Microbial-Based Double-Stranded RNA Production to Develop Cost-Effective RNA Interference Application for Insect Pest Management. *Int. J. Insect Sci.* **2019**, *11*, 1179543319840323. [CrossRef]
284. Ma, Z.Z.; Zhou, H.; Wei, Y.L.; Yan, S.; Shen, J. A Novel Plasmid-*Escherichia coli* System Produces Large Batch DsRNAs for Insect Gene Silencing. *Pest. Manag. Sci.* **2020**, *76*, 2505–2512. [CrossRef]
285. Park, M.G.; Kim, W.J.; Choi, J.Y.; Kim, J.H.; Park, D.H.; Kim, J.Y.; Wang, M.; Je, Y.H. Development of a Bacillus Thuringiensis Based DsRNA Production Platform to Control Sacbrood Virus in *Apis Cerana*. *Pest. Manag. Sci.* **2020**, *76*, 1699–1704. [CrossRef] [PubMed]
286. Hashiro, S.; Chikami, Y.; Kawaguchi, H.; Krylov, A.A.; Niimi, T.; Yasueda, H. Efficient Production of Long Double-Stranded RNAs Applicable to Agricultural Pest Control by Corynebacterium Glutamicum Equipped with Coliphage T7-Expression System. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 4987–5000. [CrossRef] [PubMed]
287. Islam, M.T.; Davis, Z.; Chen, L.; Englaender, J.; Zomorodi, S.; Frank, J.; Bartlett, K.; Somers, E.; Carballo, S.M.; Kester, M.; et al. Minicell-Based Fungal RNAi Delivery for Sustainable Crop Protection. *Microb. Biotechnol.* **2021**, *14*, 1847–1856. [CrossRef]

288. Figueiredo Prates, L.H.; Merlau, M.; Rühl-Teichner, J.; Schetelig, M.F.; Häcker, I. An Optimized/Scale Up-Ready Protocol for Extraction of Bacterially Produced DsRNA at Good Yield and Low Costs. *Int. J. Mol. Sci.* **2023**, *24*, 9266. [CrossRef]
289. Cordero, T.; Aragonés, V.; Daròs, J.A. Large-Scale Production of Recombinant RNAs on a Circular Scaffold Using a Viroid-Derived System in *Escherichia coli*. *J. Vis. Exp.* **2018**, *141*, e58472. [CrossRef]
290. Daròs, J.A.; Aragonés, V.; Cordero, T. A Viroid-Derived System to Produce Large Amounts of Recombinant RNA in *Escherichia coli*. *Sci. Rep.* **2018**, *8*, 1904. [CrossRef]
291. Ortolá, B.; Daròs, J.-A. Production of Recombinant RNA in *Escherichia coli* Using Eggplant Latent Viroid as a Scaffold. In *Viroids: Methods and Protocols*; Rao Ayala, L.N., Lavagi-Craddock, I., Georgios, V., Eds.; Springer: New York, NY, USA, 2021; ISBN 978-1-0716-1463-1.
292. Ortolá, B.; Cordero, T.; Hu, X.; Daròs, J.A. Intron-Assisted, Viroid-Based Production of Insecticidal Circular Double-Stranded RNA in *Escherichia coli*. *RNA Biol.* **2021**, *18*, 1846–1857. [CrossRef]
293. Ortolá, B.; Urbaneja, A.; Eiras, M.; Pérez-Hedo, M.; Daròs, J.A. RNAi-Mediated Silencing of Mediterranean Fruit Fly (*Ceratitis capitata*) Endogenous Genes Using Orally-Supplied Double-Stranded RNAs Produced in *Escherichia coli*. *Pest. Manag. Sci.* **2024**, *80*, 1087–1098. [CrossRef]
294. Muerdter, F.; Olovnikov, I.; Molaro, A.; Rozhkov, N.V.; Czech, B.; Gordon, A.; Hannon, G.J.; Aravin, A.A. Production of Artificial PiRNAs in Flies and Mice. *RNA* **2012**, *18*, 42–52. [CrossRef] [PubMed]
295. Ye, W.; Liu, X.; Guo, J.; Sun, X.; Sun, Y.; Shen, B.; Zhou, D.; Zhu, C. PiRNA-3878 Targets P450 (CpCYP307B1) to Regulate Pyrethroid Resistance in Culex Pipiens Pallens. *Parasitol. Res.* **2017**, *116*, 2489–2497. [CrossRef] [PubMed]
296. Mondal, M.; Brown, J.K.; Flynt, A. Exploiting Somatic PiRNAs in *Bemisia tabaci* Enables Novel Gene Silencing through RNA Feeding. *Life Sci. Alliance* **2020**, *3*, e202000731. [CrossRef] [PubMed]
297. Zhang, Q.; Dou, W.; Taning, C.N.T.; Smagghe, G.; Wang, J.J. Regulatory Roles of MicroRNAs in Insect Pests: Prospective Targets for Insect Pest Control. *Curr. Opin. Biotechnol.* **2021**, *70*, 158–166. [CrossRef] [PubMed]
298. Han, X.; Mitchell, M.J.; Nie, G. Nanomaterials for Therapeutic RNA Delivery. *Matter* **2020**, *3*, 1948–1975. [CrossRef]
299. Whyard, S.; Singh, A.D.; Wong, S. Ingested Double-Stranded RNAs Can Act as Species-Specific Insecticides. *Insect Biochem. Mol. Biol.* **2009**, *39*, 824–832. [CrossRef] [PubMed]
300. Taning, C.N.T.; Christiaens, O.; Berkvens, N.; Casteels, H.; Maes, M.; Smagghe, G. Oral RNAi to Control *Drosophila suzukii*: Laboratory Testing against Larval and Adult Stages. *J. Pest Sci.* **2016**, *89*, 803–814. [CrossRef]
301. Castellanos, N.L.; Smagghe, G.; Sharma, R.; Oliveira, E.E.; Christiaens, O. Liposome Encapsulation and EDTA Formulation of DsRNA Targeting Essential Genes Increase Oral RNAi-Caused Mortality in the Neotropical Stink Bug Euschistus Heros. *Pest. Manag. Sci.* **2019**, *75*, 537–548. [CrossRef]
302. Gurusamy, D.; Mogilicherla, K.; Shukla, J.N.; Palli, S.R. Lipids Help Double-Stranded RNA in Endosomal Escape and Improve RNA Interference in the Fall Armyworm, *Spodoptera frugiperda*. *Arch. Insect Biochem. Physiol.* **2020**, *104*, e21678. [CrossRef]
303. Wang, K.; Peng, Y.; Chen, J.; Peng, Y.; Wang, X.; Shen, Z.; Han, Z. Comparison of Efficacy of RNAi Mediated by Various Nanoparticles in the Rice Striped Stem Borer (*Chilo Suppressalis*). *Pestic. Biochem. Physiol.* **2020**, *165*, 104467. [CrossRef]
304. Zhang, X.; Zhang, J.; Zhu, K.Y. Chitosan/Double-Stranded RNA Nanoparticle-Mediated RNA Interference to Silence Chitin Synthase Genes through Larval Feeding in the African Malaria Mosquito (*Anopheles Gambiae*). *Insect Mol. Biol.* **2010**, *19*, 683–693. [CrossRef]
305. Das, S.; Debnath, N.; Cui, Y.; Unrine, J.; Palli, S.R. Chitosan, Carbon Quantum Dot, and Silica Nanoparticle Mediated DsRNA Delivery for Gene Silencing in Aedes Aegypti: A Comparative Analysis. *ACS Appl. Mater. Interfaces* **2015**, *7*, 19530–19535. [CrossRef]
306. Gurusamy, D.; Mogilicherla, K.; Palli, S.R. Chitosan Nanoparticles Help Double-Stranded RNA Escape from Endosomes and Improve RNA Interference in the Fall Armyworm, *Spodoptera frugiperda*. *Arch. Insect Biochem. Physiol.* **2020**, *104*, e21677. [CrossRef]
307. Dhandapani, R.K.; Gurusamy, D.; Howell, J.L.; Palli, S.R. Development of CS-TPP-DsRNA Nanoparticles to Enhance RNAi Efficiency in the Yellow Fever Mosquito, Aedes Aegypti. *Sci. Rep.* **2019**, *9*, 8775. [CrossRef]
308. Dou, T.; Wang, J.; Han, C.; Shao, X.; Zhang, J.; Lu, W. Cellular Uptake and Transport Characteristics of Chitosan Modified Nanoparticles in Caco-2 Cell Monolayers. *Int. J. Biol. Macromol.* **2019**, *138*, 791–799. [CrossRef] [PubMed]
309. Huang, G.; Chen, Q.; Wu, W.; Wang, J.; Chu, P.K.; Bai, H.; Tang, G. Reconstructed Chitosan with Alkylamine for Enhanced Gene Delivery by Promoting Endosomal Escape. *Carbohydr. Polym.* **2020**, *227*, 115339. [CrossRef] [PubMed]
310. Elhaj Baddar, Z.; Gurusamy, D.; Laisney, J.; Tripathi, P.; Palli, S.R.; Unrine, J.M. Polymer-Coated Hydroxyapatite Nanocarrier for Double-Stranded RNA Delivery. *J. Agric. Food Chem.* **2020**, *68*, 6811–6818. [CrossRef]
311. Edwards, C.H.; Christie, C.R.; Masotti, A.; Celluzzi, A.; Caporali, A.; Campbell, E.M. Dendrimer-Coated Carbon Nanotubes Deliver DsRNA and Increase the Efficacy of Gene Knockdown in the Red Flour Beetle *Tribolium castaneum*. *Sci. Rep.* **2020**, *10*, 12422. [CrossRef] [PubMed]
312. Henry, D.; Lacarriere, V.C. Alkali-Resistant Calcium Phosphate/Nucleic Acids Hybrid Carrier for Pest Control and Method to Produce the Particles. U.S. Patent Application US20210169083A1, 10 June 2021.
313. He, B.; Chu, Y.; Yin, M.; Müllen, K.; An, C.; Shen, J. Fluorescent Nanoparticle Delivered DsRNA toward Genetic Control of Insect Pests. *Adv. Mater.* **2013**, *25*, 4580–4584. [CrossRef]
314. Christiaens, O.; Tardajos, M.G.; Reyna, Z.L.M.; Dash, M.; Dubruel, P.; Smagghe, G. Increased RNAi Efficacy in Spodoptera Exigua via the Formulation of DsRNA with Guanylated Polymers. *Front. Physiol.* **2018**, *9*, 316. [CrossRef]

315. Mitter, N.; Worrall, E.A.; Robinson, K.E.; Li, P.; Jain, R.G.; Taochy, C.; Fletcher, S.J.; Carroll, B.J.; Lu, G.Q.; Xu, Z.P. Clay Nanosheets for Topical Delivery of RNAi for Sustained Protection against Plant Viruses. *Nat. Plants* **2017**, *3*, 16207. [CrossRef]
316. Worrall, E.A.; Bravo-Cazar, A.; Nilon, A.T.; Fletcher, S.J.; Robinson, K.E.; Carr, J.P.; Mitter, N. Exogenous Application of RNAi-Inducing Double-Stranded RNA Inhibits Aphid-Mediated Transmission of a Plant Virus. *Front. Plant Sci.* **2019**, *10*, 265. [CrossRef]
317. Sundaresha, S.; Sharma, S.; Bairwa, A.; Tomar, M.; Kumar, R.; Bhardwaj, V.; Jeevalatha, A.; Bakade, R.; Salaria, N.; Thakur, K.; et al. Spraying of DsRNA Molecules Derived from *Phytophthora infestans*, along with Nanoclay Carriers as a Proof of Concept for Developing Novel Protection Strategy for Potato Late Blight. *Pest. Manag. Sci.* **2022**, *78*, 3183–3192. [CrossRef]
318. Jain, R.G.; Fletcher, S.J.; Manzie, N.; Robinson, K.E.; Li, P.; Lu, E.; Brosnan, C.A.; Xu, Z.P.; Mitter, N. Foliar Application of Clay-Delivered RNA Interference for Whitefly Control. *Nat. Plants* **2022**, *8*, 535–548. [CrossRef]
319. Gudlur, S.; Sukthankar, P.; Gao, J.; Avila, L.A.; Hiromasa, Y.; Chen, J.; Iwamoto, T.; Tomich, J.M. Peptide Nanovesicles Formed by the Self-Assembly of Branched Amphiphilic Peptides. *PLoS ONE* **2012**, *7*, e45374. [CrossRef]
320. Avila, L.A.; Chandrasekar, R.; Wilkinson, K.E.; Balthazor, J.; Heerman, M.; Bechard, J.; Brown, S.; Park, Y.; Dhar, S.; Reeck, G.R.; et al. Delivery of Lethal DsRNAs in Insect Diets by Branched Amphiphilic Peptide Capsules. *J. Control. Release* **2018**, *273*, 139–146. [CrossRef]
321. McGraw, E.; Roberts, J.D.; Kunte, N.; Westerfield, M.; Streety, X.; Held, D.; Avila, L.A. Insight into Cellular Uptake and Transcytosis of Peptide Nanoparticles in *Spodoptera frugiperda* Cells and Isolated Midgut. *ACS Omega* **2022**, *7*, 10933–10943. [CrossRef] [PubMed]
322. Falato, L.; Gestin, M.; Langel, Ü. Cell-Penetrating Peptides Delivering SiRNAs: An Overview. In *Design and Delivery of SiRNA Therapeutics*; Humana Press Inc.: Totowa, NJ, USA, 2021; Volume 2282, pp. 329–352.
323. Gillet, F.X.; Garcia, R.A.; Macedo, L.L.P.; Albuquerque, E.V.S.; Silva, M.C.M.; Grossi-de-Sa, M.F. Investigating Engineered Ribonucleoprotein Particles to Improve Oral RNAi Delivery in Crop Insect Pests. *Front. Physiol.* **2017**, *8*, 256. [CrossRef]
324. Martinez, Z.; de Schutter, K.; van Damme, E.J.M.; Vogel, E.; Wynant, N.; vanden Broeck, J.; Christiaens, O.; Smagghe, G. Accelerated Delivery of DsRNA in Lepidopteran Midgut Cells by a Galanthus Nivalis Lectin (GNA)-DsRNA-Binding Domain Fusion Protein. *Pestic. Biochem. Physiol.* **2021**, *175*, 104853. [CrossRef] [PubMed]
325. Cooper, A.M.W.; Song, H.; Yu, Z.; Biondi, M.; Bai, J.; Shi, X.; Ren, Z.; Weerasekara, S.M.; Hua, D.H.; Silver, K.; et al. Comparison of Strategies for Enhancing RNA Interference Efficiency in *Ostrinia nubilalis*. *Pest. Manag. Sci.* **2021**, *77*, 635–645. [CrossRef] [PubMed]
326. Howard, J.D.; Beghyn, M.; Dewulf, N.; De Vos, Y.; Philips, A.; Portwood, D.; Kilby, P.M.; Oliver, D.; Maddelein, W.; Brown, S.; et al. Chemically Modified DsRNA Induces RNAi Effects in Insects in Vitro and in Vivo: A Potential New Tool for Improving RNA-Based Plant Protection. *J. Biol. Chem.* **2022**, *298*, 102311. [CrossRef] [PubMed]
327. Li, X.; Dong, X.; Zou, C.; Zhang, H. Endocytic Pathway Mediates Refractoriness of Insect *Bactrocera dorsalis* to RNA Interference. *Sci. Rep.* **2015**, *5*, 8700. [CrossRef] [PubMed]
328. Cedden, D.; Güney, G.; Scholten, S.; Rostás, M. Lethal and Sublethal Effects of Orally Delivered Double-Stranded RNA on the Cabbage Stem Flea Beetle, *Psylliodes chrysocephala*. *Pest. Manag. Sci.* 2023; *early view*. [CrossRef]
329. Ullah, F.; Gul, H.; Tariq, K.; Hafeez, M.; Desneux, N.; Gao, X.; Song, D. RNA Interference-Mediated Silencing of Ecdysone Receptor (EcR) Gene Causes Lethal and Sublethal Effects on Melon Aphid, Aphis Gossypii. *Entomol. Gen.* **2022**, *42*, 791–797. [CrossRef]
330. Khajuria, C.; Ivashuta, S.; Wiggins, E.; Flagel, L.; Moar, W.; Pleau, M.; Miller, K.; Zhang, Y.; Ramaseshadri, P.; Jiang, C.; et al. Development and Characterization of the First DsRNA-Resistant Insect Population from Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS ONE* **2018**, *13*, e0197059. [CrossRef]
331. Zhang, X.; Fan, Z.; Wang, Q.; Kong, X.; Liu, F.; Fang, J.; Zhang, S.; Zhang, Z. RNAi Efficiency through DsRNA Injection Is Enhanced by Knockdown of DsRNA Nucleases in the Fall Webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae). *Int. J. Mol. Sci.* **2022**, *23*, 6182. [CrossRef]
332. Prentice, K.; Smagghe, G.; Gheysen, G.; Christiaens, O. Nuclease Activity Decreases the RNAi Response in the Sweetpotato Weevil Cylas Puncticollis. *Insect Biochem. Mol. Biol.* **2019**, *110*, 80–89. [CrossRef] [PubMed]
333. Tayler, A.; Heschuk, D.; Giesbrecht, D.; Park, J.Y.; Whyard, S. Efficiency of RNA Interference Is Improved by Knockdown of DsRNA Nucleases in Tephritid Fruit Flies. *Open Biol.* **2019**, *9*, 190198. [CrossRef] [PubMed]
334. Fan, Y.H.; Song, H.F.; Abbas, M.; Wang, Y.L.; Li, T.; Ma, E.B.; Cooper, A.M.W.; Silver, K.; Zhu, K.Y.; Zhang, J.Z. A DsRNA-Degrading Nuclease (DsRNase2) Limits RNAi Efficiency in the Asian Corn Borer (*Ostrinia furnacalis*). *Insect Sci.* **2021**, *28*, 1677–1689. [CrossRef]
335. Kim, J.S.; Park, J.; Choi, J.H.; Kang, S.; Park, N. RNA–DNA Hybrid Nano-Materials for Highly Efficient and Long Lasting RNA Interference Effect. *RSC Adv.* **2023**, *13*, 3139–3146. [CrossRef] [PubMed]
336. Kim, H.; Lee, J.S.; Lee, J.B. Generation of SiRNA Nanosheets for Efficient RNA Interference. *Sci. Rep.* **2016**, *6*, 25146. [CrossRef]
337. Abe, N.; Abe, H.; Nagai, C.; Harada, M.; Hatakeyama, H.; Harashima, H.; Ohshiro, T.; Nishihara, M.; Furukawa, K.; Maeda, M.; et al. Synthesis, Structure, and Biological Activity of Dumbbell-Shaped Nanocircular RNAs for RNA Interference. *Bioconjug Chem.* **2011**, *22*, 2082–2092. [CrossRef]
338. Grabow, W.W.; Zakrevsky, P.; Afonin, K.A.; Chworos, A.; Shapiro, B.A.; Jaeger, L. Self-Assembling RNA Nanorings Based on RNAI/II Inverse Kissing Complexes. *Nano Lett.* **2011**, *11*, 878–887. [CrossRef]

339. Lund, V.K.; Madsen, K.L.; Kjaerulff, O. Drosophila Rab2 Controls Endosome-Lysosome Fusion and LAMP Delivery to Late Endosomes. *Autophagy* **2018**, *14*, 1520–1542. [CrossRef]
340. Johannes, L.; Lucchino, M. Current Challenges in Delivery and Cytosolic Translocation of Therapeutic RNAs. *Nucleic Acid. Ther.* **2018**, *28*, 178–193. [CrossRef]
341. Pinzón, N.; Bertrand, S.; Subirana, L.; Busseau, I.; Escrivá, H.; Seitz, H. Functional Lability of RNA-Dependent RNA Polymerases in Animals. *PLoS Genet.* **2019**, *15*, e1007915. [CrossRef]
342. Hu, X.; Richtman, N.M.; Zhao, J.Z.; Duncan, K.E.; Niu, X.; Procyk, L.A.; Oneal, M.A.; Kernodle, B.M.; Steimel, J.P.; Crane, V.C.; et al. Discovery of Midgut Genes for the RNA Interference Control of Corn Rootworm. *Sci. Rep.* **2016**, *6*, 30542. [CrossRef]
343. Wynant, N.; Verlinden, H.; Breugelmans, B.; Simonet, G.; Vanden Broeck, J. Tissue-Dependence and Sensitivity of the Systemic RNA Interference Response in the Desert Locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* **2012**, *42*, 911–917. [CrossRef]
344. Cooper, A.M.W.; Song, H.; Shi, X.; Yu, Z.; Lorenzen, M.; Silver, K.; Zhang, J.; Zhu, K.Y. Characterization, Expression Patterns, and Transcriptional Responses of Three Core RNA Interference Pathway Genes from *Ostrinia nubilalis*. *J. Insect Physiol.* **2021**, *129*, 104181. [CrossRef]
345. Guo, W.C.; Fu, K.Y.; Yang, S.; Li, X.X.; Li, G.Q. Instar-Dependent Systemic RNA Interference Response in *Leptinotarsa decemlineata* Larvae. *Pestic. Biochem. Physiol.* **2015**, *123*, 64–73. [CrossRef]
346. Bansal, R.; Michel, A.P. Core RNAi Machinery and Sid1, a Component for Systemic RNAi, in the Hemipteran Insect, *Aphis glycines*. *Int. J. Mol. Sci.* **2013**, *14*, 3786–3801. [CrossRef] [PubMed]
347. Arraes, F.B.M.; Martins-de-Sa, D.; Noriega Vasquez, D.D.; Melo, B.P.; Faheem, M.; de Macedo, L.L.P.; Morgante, C.V.; Barbosa, J.A.R.G.; Togawa, R.C.; Moreira, V.J.V.; et al. Dissecting Protein Domain Variability in the Core RNA Interference Machinery of Five Insect Orders. *RNA Biol.* **2021**, *18*, 1653–1681. [CrossRef] [PubMed]
348. Guan, R.; Hu, S.; Li, H.; Shi, Z.; Miao, X. The in Vivo DsRNA Cleavage Has Sequence Preference in Insects. *Front. Physiol.* **2018**, *9*, 1768. [CrossRef] [PubMed]
349. Swevers, L.; Broeck, J.V.; Smagghe, G. The Possible Impact of Persistent Virus Infection on the Function of the RNAi Machinery in Insects: A Hypothesis. *Front. Physiol.* **2013**, *4*, 66388. [CrossRef] [PubMed]