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Nontransformative Strategies for RNAi in Crop Protection

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Abstract

RNAi in crop protection can be achieved not only by plant-incorporated protectants through plant transformation (transgenic) but also by nontransformative strategies such as formulations of sprayable dsRNAs used as direct control agents, resistance factor repressors, or developmental disruptors. Therefore, the RNAi-based biopesticides are expected to reach the market also in the form of nontransgenic strategies such as sprayable products, stem injection, root drenching, seed treatment, or powder/granule. While the delivery of dsRNA by transgenic expression is well established, it requires generations of crop plants and is costly, which may take years and delays for practical application, depending on the regulatory rules, plant transformability, genetic stability, and public acceptance of genetically modified crop species. DsRNA delivery as a nontransgenic approach was already published as a proof-of-concept work, so it is time to point out some directions on how the real potential for agriculture and crop protection is.

Keywords: RNAi-based, biopesticides, nontransgenic approaches, dsRNA, nontransformative plant protection

1. Crop protection and RNAi

The beginning of human civilization can be traced back to the ability of cultivating crops. This has allowed that a higher number of people could be supported in the same environment; however, it also brought several crop protection challenges that mankind has been facing continuously. To ensure sufficient food production, since the earliest days of agriculture, farmers

have had a history of using agrochemicals to protect their crops against yield loss from a vast range of organisms including pest insects, mites, fungi, weeds, and others.

The modern era of synthetic pesticides began in the 1930s, and with insects, fungal pathogens, and weeds, destroying each one more than 13% before harvest and about 10% in postharvest [1], the pesticide use has become fundamental to modern crop protection technology. The increasing resistance [2] of weeds, pest insects, and fungi to established agrochemical compound classes, stringent regulatory environment rules, and market growth has stimulated demand for more selective, safer, and cost-effective pest control methods. Crop protection scientists have allocated a great deal of intellectual energy into seeking of more refined strategies to reduce crop losses such as transgenic crops expressing *Bacillus thuringiensis* (Bt) [3] toxins and more recently gene silencing through RNAi (RNA interference) [4, 5].

RNAi is a natural process present in eukaryotic cells for gene regulation and antiviral defense. Although, from a crop protection perspective, RNAi refers to double-stranded RNA (dsRNA)-mediated gene silencing that involves the blocking of the expression of specific target genes by destroying the corresponding mRNA molecules affecting only the translation process. Due to its sequence-dependent mode of action, the RNAi technology, as referred nowadays by industry, has a vast range of potential crop protection application, including genetic studies and pest control research in insects [6–12], mites [13–15] and ticks [16], plant pathogens [17–23], termites, nematodes, and weeds [2, 24, 25] in a range of crops. These RNAi practical applications have been pursuing over the last decade for the development of novel crop protection methods.

The application of this technology did not go unnoticed in agriculture; hence, since the discovery of RNAi and its regulatory potentials, it has become evident that RNAi has immense potential in opening a new vista for crop protection. Nevertheless, one of the biggest challenges for the RNAi technology is to make possible that target organisms (i.e., pest insects, plant pathogens, nematodes, viruses) uptake intact and active molecules that will trigger an RNAi pathway. Delivery of dsRNA to a target organism is the easiest through transformative RNAi approach (i.e., transgenic plants) [26, 27], but it is not practical to every target and crop. Therefore, the development of nontransformative approach (i.e., sprayable dsRNA) [9, 11, 28] for RNAi delivery will boost up its use in the field.

2. RNAi mechanism in brief

The cellular mechanics of gene silencing by RNAi was largely misunderstood or even unknown until the work of Andrew Fire and Craig Mello with the nematode *Caenorhabditis elegans* [5]. RNAi regulates gene expression through small noncoding RNAs (sRNAs). The sRNAs of ~21–25 bp long dsRNA molecules have ~2 nt 3′ overhangs that allow them to be recognized by enzymes from the RNAi machinery, which subsequently leads to homology-dependent degradation of target mRNA. There are two primary classes for sRNAs in the RNAi pathway, the micro-RNAs (miRNAs) and the short-interfering RNAs (siRNAs). The miRNAs are derived from endogenously expressed products and from stem-loop precursors with incomplete double-stranded character, whereas siRNAs are primarily exogenous

in origin from viruses or transposons and from long, fully complementary double-stranded RNAs (dsRNAs) [29]. Briefly, both siRNA and miRNA molecules are initially generated from longer dsRNAs processed by the ribonuclease III enzyme dicer into 20–30 nucleotide duplexes. Subsequently, an argonaute family protein (AGO), which is the catalytic component of the RNA-induced silencing complex (RISC), is incorporated. The RISC mediates either the degradation of mRNA or the repression of translation. In most RNAi-competent eukaryotes, with notable exceptions of insects and vertebrates, the primary dsRNA trigger induces the synthesis of secondary siRNAs through the action of RNA-dependent RNA polymerase (RdRP) enzymes. The three classes of molecules, namely dicer, argonaute, and 20–30 nucleotide duplexes of RNA, are heralded as the signature components of RNA silencing of genes, comprehensively reviewed in several articles [8, 9, 12, 29–35].

3. Transformative versus nontransformative RNAi

As aforementioned for field applications, the transformative RNAi includes the plant-incorporated protectants (PIPs; i.e., transgenic plants/RNAi-based plant traits), whereas non-PIP dsRNA-containing end-use products (dsRNA-EPs) might be formulated as dsRNA active ingredient such as a raw material for insecticide, antifungal, or antiviral with variable delivery modes (see Section 4).

The RNAi mechanism works at mRNA level exploring a sequence-dependent mode of action, which makes it unique in potency and selectivity compared to any regular agrochemicals. Therefore, one advantage of RNAi either by transformative or by nontransformative approach is that it would allow farmers to target pests more specifically. The technology can be designed by using sequences of RNA that match very specific gene sequences in a target pest; hence, RNAi should leave other species unharmed. The careful selection of unique regions of pest genes results in effects highly targeted, avoiding unintended effects.

The transformative RNAi strategy through transgenic plants known as host-induced gene silencing (HIGS) has proved to be successful in the protection of crops against their specific pest insects [26, 36–38], plant pathogens [18, 19], viruses [22, 39–41], and nematodes [42, 43], recently reviewed [44].

A proof-of-concept milestone paper of Baum et al. [36] demonstrated that a dsRNA construct in a genetically engineered plant could provoke larval mortality in western corn rootworm (WCR), *Diabrotica virgifera*. This research was fundamental to spread the idea on the potential of dsRNA as a new pest control agent through transgenic plants. In September 2016, the Canadian Food Inspection Agency (CFIA) had announced the approval of the RNAi-based corn event Monsanto MON87411, the "SmartStax PRO" (expressing Cry3Bb1, Cry34Ab1/ Cry35Ab1, and DvSnf7) [38], containing a *D. virgifera* dsSnf7 construct in combination with two *Bt* constructs, for commercialization and release. Also, the US EPA had confirmed in June 2017 the approval of this event for commercial planting.

The development of a transgenic plant expressing dsRNA as a strategy for plant protection is straightforward, but it is not practical to every pest organism and crop [9, 10, 12]. Although

the delivery of dsRNA by transgenic expression is well established, it requires generations of crop plants, which may take years and delays for practical application, depending on the regulatory rules, plant transformability, genetic stability, and public acceptance of genetically modified (GM) crop species [45].

While RNAi-based crops are expensive to produce and have a high risk of resistance break-down, topical application is underway as a nontransformative approach that might enable RNAi-based pest management. Therefore, triggering an RNAi pathway in a pest organism may also be possible through a spray-induced gene silencing (SIGS) approach, without changing the plant DNA. The SIGS as a nontransgenic approach for pest control was already published [46] as a proof-of-concept work and recently reviewed [9, 11]. Because of using this approach to silence genes without introducing heritable changes into the genome, it may not be regulated as a GM product. A dsRNA spray can be used almost immediately as a regular pesticide without having to go through several years involved in the development of a GM or conventionally bread crop. Besides, in several countries due to the slow regulatory procedure to approve transgenic crops, nontransformative RNAi strategies with similar results such as some of those demonstrated above could be applied. Still, the main drawback of nontrans-formative RNAi strategy is that as a plant grows, new leaves have to be sprayed to guarantee protection, so this implies in possible higher costs to farmers, whereas transgenic plants will produce dsRNA continuously. However, the vascular system of plants naturally translocates RNAs [47]. Therefore, sprays on leaves, injection in trunks, or soil application of dsRNA can travel long distances through plant vessels; hence, this can be exploited for the development of pest control strategies [11, 28].

The idea to use sprayable dsRNA was followed by an underlying supposition that this type of molecule would have a short half-life for an effective crop protection agent [48], and the short half-life of dsRNA in soil and by UV light has been confirmed [10]. This apparent challenge posed by SIGS approach is that the effects on plants last only a few days because unprotected RNAs break down soon. Farmers may not want to apply extensive sprays to keep plants pro-tected; however, there are some positive issues because the sprays of dsRNA can be quickly tai-lored toward a pest organism, much faster than a GM crop, and last only a few days or weeks different from most regular pesticides. Crop protectors should bear in mind that there is no need for a pest control agent persist active for months to become an efficient pest control agent.

Regardless of the target species, for a successful nontransformative RNAi strategy, it is of paramount importance to identify unique regions in very essential target genes, so that brief changes in the level of expression can provoke severe consequences as well as delivery of suf-ficient amount of intact dsRNA. Alternatively to transgenic plants, the delivery of dsRNA can be through other routes including dsRNA sprays, dsRNA expression in bacteria, trunk injec-tion, and engineered viruses, among others. For example, to control plant viruses, farmers are obligated to either grow varieties with resistance to viruses or try to kill the organism that spread, such as aphids or hemipterans. Sprays with dsRNA might be rapidly tailored against existing or new type of virus, and the gene-silencing effects of RNAi will last only a few days, enough to suppress virus replication. Overall, the SIGS approach opens up a range of possi-bilities for several pest insects that are difficult to control such as root-feeding and sap-feeding

insects, plant viruses, and plant pathogens, especially in perennial crops (e.g., fruits such as grapes, apples, and citrus), where plant transformation takes years to develop and is costly.

4. Successes of RNAi through nontransformative approaches

The delivery of dsRNA through nontransformative approaches is likely to hit the market in four categories: (i) direct control agents; (ii) resistance factor repressors; (iii) developmental disruptors; and (iv) growth enhancers [9–11, 49–52]. As a direct control agent, nontransgenic approaches were successfully managed to achieve long-lasting gene silencing [9, 10, 18, 19, 41].

In some experiments, full-sized citrus and grapevine trees were treated with dsRNA using foliar sprays, root drenching, or trunk injections. Two hemipteran insects, a xylem- and a phloem-feeding, and a coleopteran chewing insect took up the dsRNA after feeding on plants previously treated with dsRNA [11, 28]. Similarly, rice plants were able to take up dsRNAs when their roots were soaked in dsRNA solution showing resistance against piercing-sucking and stem-borer pest insects [53] and also mites [15]. Altogether, these experiments are clear demonstrations that drench/soak roots, trunk injections, and sprays on leaves are success strategies for delivery of dsRNA molecules without any modification on plants DNA.

Plant diseases caused by virus have a tremendous impact in food production and quality, being responsible for loses in several crops, fruits, and vegetables worldwide. Coherent with an ancient role to protect genome from invasive viruses, the RNAi mechanism can be reprogrammed to work by destroying any virus RNA. Without viral RNA, no viral proteins are made, thus preventing virus replication and plant diseases. Some studies have already been conducted on topical application of dsRNA to control plant viruses [41, 54]. However, a major limitation in the practical application of dsRNA to control viruses is that RNAs face a hostile environment where it is rapidly degraded with not only low uptake into plants, but also the short virus protection window of few days postspray. There are some rumors that the initiated pipeline branded "BioDirect" by Monsanto controls pest insects and plant viruses with sprays of dsRNA, but details on this probably are not publically available. To address some of these limitations, a layered double hydroxide (LDH) clay nanosheet, called "BioClay," was developed [55] and combined with dsRNA molecules. The clay nanoparticles are positively charged and so bind and protect the negatively charged RNAs; delivery occurs when atmospheric carbon dioxide and moisture react with clay nanoparticles breaking down LDH, gradually releasing RNAs. Using this dsRNA-LDH complex was possible to achieve long-lasting gene silencing, protecting tobacco plants from a virus for 20 days with a single spray [55, 56], thus extending the period of 5–7 days using naked dsRNA. The complex dsRNA-LDH protected plants in both local lesions and systematically. Also using RNAi to control plant viruses, the mechanism of dsRNA uptake into the leaf was investigated. It was reported a rapid systemic spread of dsRNA when leaves of *Nicotiana tabacum* cv. Xanthi were mechanically inoculated with naked dsRNA homologous to tobacco mosaic virus (TMV) [57]. From these experiments, we can conclude that topical application of dsRNA targeting virus genes can induce a systemic RNAi toward virus resistance.

Direct spray of dsRNA was also used experimentally to control the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, under greenhouse conditions [10]. The naked dsRNA molecules in foliar application were sufficiently stable for at least 28 days, enough to control the CPB. The authors also investigated the RNA degradation under UV light, where they concluded that an exposition of 1–2 h is needed for dsRNAs to become inactive in feeding assays. The long biological activity (28 days) during greenhouse feeding experiments suggests that naked dsRNA is more stable in leaf surface than in a glass surface used for the UV stability studies.

The fungi kingdom consists of a large and diverse group of eukaryotes, and plant diseases caused by fungi exert particular and agronomic impact on global grain and food production. Generally, the proteins dicer, argonaute, and RdRP, which are some of the major components of RNAi pathways, are present in most fungi species [58]. Therefore, the RNAi pathways can be harnessed to control plant diseases [22]. Sprays of CYP3-dsRNAs, targeting simultaneously three fungal ergosterol biosynthesis genes (P450 lanosterol C-14α-demethylases—*CYP51A*, *CYP51B*, *CYP51C*), on barley leaves were used to control *Fusarium graminearum* infections in the local areas, where dsRNA was sprayed, but strikingly also in unsprayed distal leaf parts, showing that dsRNA was systematically translocated within the plant [18]. The example above was a proof-of-concept article showing that after spray an even long dsRNA molecule (791 nt) could be taken up by the plant and transferred as unprocessed dsRNA via plant vascular system to infection sites, where it was processed by the fungal RNAi machinery to carry out its antifungal activity. The same authors also demonstrated that green fluorescent protein (GFP) from jellyfish was silenced in a *Fusarium graminearum* strain expressing GFP, suggesting that sprays of dsRNA are not sequence selective and thus with the potential for targeting any gene across several plant pathogens. Similar study [19] showed that dsRNA and sRNAs targeting dicer-like protein genes DCL1 and DCL2 of *Botrytis cinerea* were externally applied on fruits, vegetables, flower petals, and *Arabidopsis* leaves, followed with *B. cinerea* infection. The authors showed that *B. cinerea* was able to take up dsRNA and sRNAs from the environment, inhibiting gray mold disease.

The study with full-sized citrus trees (2.5 m tall) was performed with 2 g of dsRNA in 15 l of water applied by root drench and injections [46]. The dsRNA was detected in psyllids and leafhoppers 5–8 days postingestion from plants and for at least for 57 days in the citrus trees; this allows the development of an area-wide pest suppression approach. Similarly, Koch et al. [18] showed that the CYP3-dsRNA labeled with the green fluorescent dye (ATTO 488) was detected in the vascular tissue 24 h after spraying leaves. Also, the leaf sections demonstrated that the fluorescence was detected in the xylem, in the apoplast and symplast of phloem parenchyma cells, companion cells, mesophyll cells, as well as in trichomes and stomata. The labeled dsRNA was detected also inside fungal conidia and germ tubes as well as in the fungal mycelium. These experiments conducted by Koch et al. [18] using sprays on barley leaf surface are the first examples of active dsRNA uptake by plant cells.

The uptake of RNAs from the environment, a phenomenon known as environmental RNAi [8], has not yet been observed in mammals. This phenomenon was observed in *C. elegans*, others nematodes and insects [10, 59]. However, until recently, it was not clear whether plants

and fungi could take up RNAs from the environment. From what we know so far, it is worth noting that plants and fungi are indeed capable to take up dsRNAs and sRNAs applied externally [18, 19]. As described above, the locally applied dsRNA can inhibit pathogen growth also at distal unsprayed leaves, so these RNAs should be able to spread systematically across plant cells and tissues [18, 55, 60]. The nematode *C. elegans* is able to take up environmental dsRNAs that are longer than 50 bp, where the shorter dsRNAs cannot be taken up [59]. Generally, plant-feeding insects are able to take up dsRNAs that are longer than 50–60 pb, but not shorter dsRNAs or sRNAs [61, 62], while fungi and plants can take up both external sRNAs and long dsRNAs [18, 19]. The differences in the uptake of RNA species between plants/fungi and insects suggest that entry/uptake channels or pathways may differ among organisms. In the light of this, the uptake mechanisms that the externally applied RNAs may be translocated into plant pathogens and/or herbivorous insects could have at least two possible routes for entry. First, for insects, RNAs could be directly taken up during herbivory or through the cuticle to get into insect cells; similarly for fungi, RNAs could be taken up directly into fungal cells after spray. Second, the RNAs could be taken up by plant cells first and then move into insect/fungal cells indirectly (**Figure 1**).
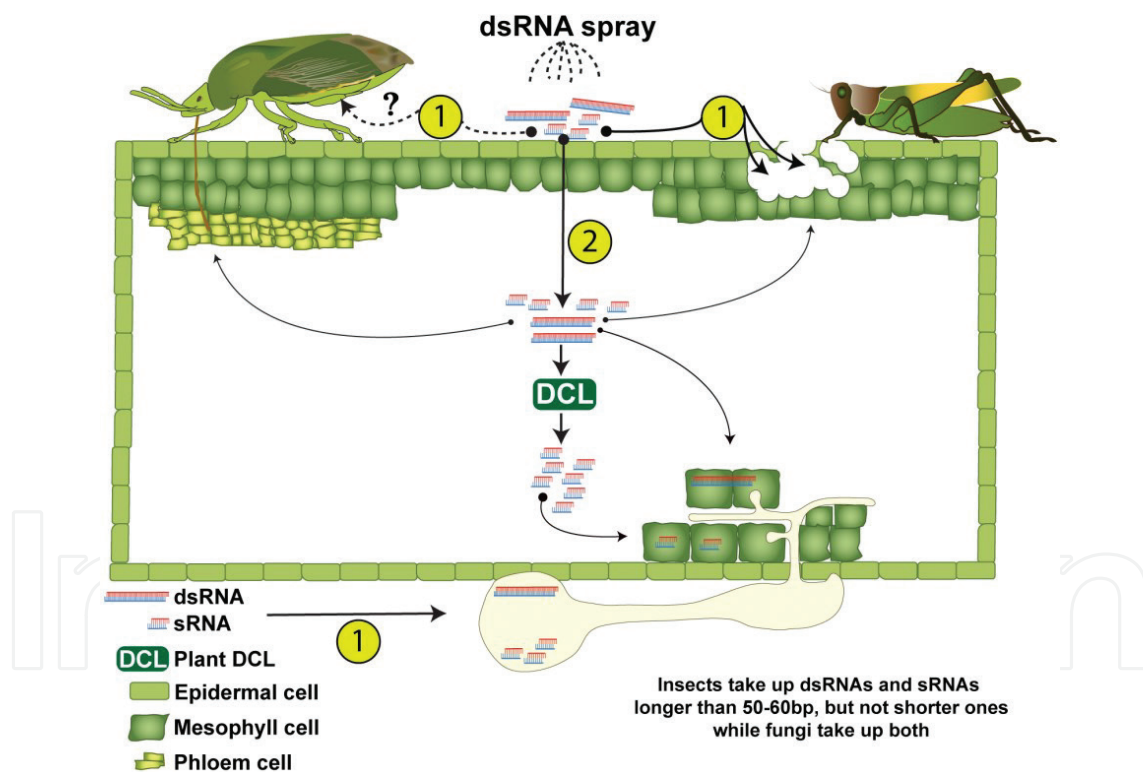

Figure 1. Two possible pathways of silencing insect and fungal genes induced by sprays of dsRNAs and sRNAs. There are at least two possible routes for RNAs to get into insect/fungal cells. Pathway 1: Insects and fungi directly take up sprayed RNAs. The up taken dsRNAs may be sliced into sRNAs by fungal or insect DCL proteins. Pathway 2: Externally applied dsRNAs and sRNAs are taken up by plant cells and then transferred into insect or fungal cells. The long dsRNAs may be sliced into sRNAs by plant dicer like (DCL). In both possible pathways, fungi take up longer and shorter dsRNAs, while insects take up dsRNAs longer than 50–60 bp in length. For phloem-feeding insects such as stinkbugs and aphids, sprayed RNAs may prove difficult to get into insect cell directly (pathway 1), while for chewing insects such as grasshoppers and caterpillar, RNAs are taken up easily during herbivory.

One obstacle, if not the biggest, is the cost for the mass production of dsRNA. While the issues of environmental stability and delivery are being addressed with creative innovations such as BioClay, making mass amounts of RNA is still expansive. Indeed, cost-effective methods will allow real-world applications of exogenous dsRNA for RNAi-mediated crop protection. To our knowledge, currently, there are no commercial RNAi-based products that utilize dsRNA as a spray for crop protection. Since the discovery of dsRNA and its potential for crop protection, some companies and academic scientists are seeking to develop more cost-efficient methods for large production of dsRNA. Similarly, RNAi to control devastating pests such as the Colorado potato beetle has obviously attracted attention in private research and development. As mentioned before, Monsanto (currently Bayer) and Syngenta (current ChemChina) have allocated major investments toward SIGS technology. Already in mid-2015, Monsanto launched its technology BioDirect, and although the principle was the same as we had seen in academia, these products work differently because they are not expressed in the leaves, but applied exogenously to the plants. Syngenta scientists also are developing lines of biocontrol products based on RNAi (https://www.youtube.com/embed/BiVZbAy4NHw?ecver=1). For example, these dsRNA-based products when sprayed onto the potato plants (field trials) or soy plants targeting genes of Colorado potato beetle and stink bug, *Nezara viridula*, respectively, suppress efficiently plant defoliation. Additionally, these products indicated that beneficial species even closely relate species that are not harmed [63].

The *in vitro* transcription and the *in vivo* syntheses are basically the two nonchemical sources of pure dsRNA with potential for mass production. Both strategies are based on annealing of two single-stranded RNAs (ssRNAs) enzymatically synthetized. Therefore, the annealing of ssRNAs may be performed *in vitro* [18, 19, 41, 57] or *in vivo* using bacterial cells deficient of enzyme RNase III that degrades dsRNAs [35, 64, 65]; both approaches have advantages and disadvantages (**Table 1**). For example, there are possible hybridizations of two complementary ssRNA molecules that often result in a low final production of the correct and fully dsRNA duplexes. Moreover, the *in vivo* production may contain bacterial homologous DNA molecules that will affect RNA quality and its applicability.

In the last few years, we have experienced an ever-growing interest in the market for dsRNA that has pushed long-established companies and startups toward better production, cost-efficient, and stable delivery systems. In instance, the cost to produce 1 g of dsRNA (100 up to 800 pb) has dropped from $12,500 USD in 2008 to $100 USD in 2016, and to less than $60 USD today (July 2018) (http://www.agrorna.com/sub_05.html). The agroRNA [67] produces bulk amounts of dsRNA that could be used in agriculture; however, it is worth noting that naked dsRNA as sold by agroRNA needs to be formulated if the objective is a long-lasting

Approach	Cost	Purification	Labor demand	Mass production
In vivo	High	Hard	High	Possible
In vitro	Moderate	Easy	Medium	Maybe

*References: http://www.agrorna.com/ [9, 66].

Table 1. Common strategies* for mass production of dsRNA with pros and cons.

crop protection; otherwise, the dsRNAs will last only a few days. For crop protection, dsRNA does not need to be as pure as for medical application; however, at least for gene silencing in insects, the efficacy of dsRNA increased using purified RNA.

Considering the rapid half-life of dsRNA mainly regulated due to action of RNases and sunlight in the hostile environment, a biotechnology company RNAagri (former APSE) developed a technology "Apse RNA Containers" (ARCs) that allows the mass production of encapsulated ready-to-spray dsRNA with costs near $1 USD per 1 g [68]. In brief, this technology is based on plasmids engineered to produce naturally occurring proteins such as capsids that are cotransformed with another plasmid coding for the target dsRNA with a sequence called the "packing site." The proteins produced by bacteria self-assemble around RNAs, resulting in RNA protected and resistant to environmental hostile conditions. For long-lasting crop protection with exogenous applications, the dsRNAs should be protected with coating of nanoparticles, liposomes, or polymers, which will increase the efficacy by reducing dsRNA degradation [9].

Alternatively to pure dsRNA, the *Escherichia coli* [HT115(DE3)] strain can be used to produce large quantities of dsRNA. The crude extracts of bacterially expressed dsRNA can be sprayed on crops to protect against pest insects and plant pathogens [9, 10, 35]. Also, symbionts have shown to be a promising dsRNA delivery method [69]. These naturally occurring organisms such as virus/bacteria can be engineered to generate a symbiont-mediated RNAi system to continue produce dsRNA in the host. In perennial crops, there is a risk that the viral/bacterial genome could lose the dsRNA construct and revert to the wild type, while for annual crops, the area could be treated once and then deliver dsRNA during the entire production season.

The virus-induced gene silencing (VIGS) has also a great potential [70–72] to transiently silence target genes of insects or pathogens on host plants. Therefore, if an insect or pathogen-specific RNAi inducer sequence is introduced into an engineered plant virus, siRNAs specific for insect/pathogen targets will be produced upon plant infection [18, 73].

5. Postharvest protection using nontransformative RNAi

Yearly, vegetables, grains, flowers, and fresh fruits are damaged by microbial pathogens and insects. Sprays of dsRNA may also be efficient on postharvest products [19] to protect them during processing, transportation, and storage. Indeed, spraying *B. cinerea* dicer-like1/2 dsR-NAs or sRNAs on the surface of fruits, vegetables, and flowers significantly inhibits gray mold diseases. Sprays of regular fungicides/insecticides commonly control insects and microbial pathogens attacking postharvest products, but sometimes these left residues on food. Also, mycotoxins, which are considered carcinogenic, are produced by fungal pathogens such as *Aspergillus* and *Fusarium* while proliferating on postharvest products. Sprays of dsRNA may also be used to control postharvest pest insects and pathogens as a new generation of sustainable and environmentally friendly products. It is worth to remind that postharvest products are not exposed to severe environment conditions such as sunlight, which contributes to reduced dsRNA degradation and long-lasting protection of postharvest products.

6. Other applications

RNAi naturally protects the cell from invasive viruses. Therefore, beyond the application of dsRNA sprays for pest and pathogen control, there is also a potential for the protection of beneficial insects such as bees from viral diseases. For example, the Israeli acute paralysis virus (IAPV) [74] is a single-stranded RNA virus in the family Dicistroviridae that increases bee mortality. The ingestion of dsRNAs from two regions of the IAPV genome protected bees from subsequent IAPV infection. The success of this experiment has encouraged field trials [75]. Large-scale field trials tested the efficiency of a dsRNA product, Rembee™ (Beeologics, LLC, Miami, FL, USA), in protecting honeybees from IAPV infection. The result was twice as many bees in the dsRNA-treated hives when compared to untreated hives. Additionally, dsRNA-treated hives produced threefold more honey than the untreated hives infected with IAPV. Similar results are also observed. A similar result was observed in bumblebees (*Bombus terrestris*) upon feeding of IAPV virus-specific dsRNAs that rescued the workers from mortality [76]. Also RNAi was efficient against the internal microsporidian parasites *Nosema apis* and *Nosema ceranae* [77] as well as Varroa mite *Varroa destructor* [78] and other mites [15], thus improving the honeybee health.

7. Conclusions

Crop protection against pathogens and pest insects relies mostly on the widespread use of chemical pesticides that are applied to the environment in large amounts yearly; some of these chemicals are in use for almost half a century. Therefore, there is a need for novel tools more sustainable and less detrimental to the environment. Therefore, scientists have harnessed RNAi to turn off genes that they are studying. RNAi through nontransformative strategies will demand mass production of dsRNA, efficient delivery methods, and methods to validate its environmental stability.

A large number of studies have demonstrated the feasibility and efficacy of RNAi-based approaches, and some transgenic plants have been approved for commercialization and release [38, 39, 79, 80]. However, unlike these strategies, which depend on plant transformation, the spray of dsRNA externally realizes crop protection without changing the plant DNA. The dsRNA-containing end-use products, nevertheless, will be differently regulated when compared to transgenic plants such as *Bacillus thuringiensis* crops. Moreover, chemical compounds act through a structure-dependent mechanism, and dsRNA acts though a tailored species-specific sequence. Clearly, the dsRNA has more changes to act only against the target species. Also, multiple target genes could be silenced simultaneously by fusing dsRNA sequences to generate a pyramidic plant protection approach, without any modification of the plant genome.

It is worth to remind that a specific dsRNA exerts its mode of action throughout entire sequence length by generating a large pool of target-specific siRNAs [29, 30, 32]. This large pool of siRNAs for a single target increases target specificity and largely reduces evolution of mutations and resistance in the targeted organism. Indeed, the dsRNA is designed to match a long nucleotide sequences in the target organism (i.e., insects, pathogens, or viruses). The

effectiveness of a long dsRNA will remain even when parts of this sequence mutate. So that it is believed that it is unlike to face resistance evolution that commonly makes a chemical pesticide ineffective. Resistance development toward RNAi has not been documented in insects and fungi, but as a famous artist says, "*life finds a way*," these organisms have a great phenotypic and genetic plasticity and relatively short life cycle contributing for that some individuals/strains could be more or less sensitive to RNAi. For example, issues such as malfunction of dsRNA uptake or nuclease upregulation and/or processing dsRNA and systemic spread of RNAi signaling could stop the initiation and spread of RNAi response [45]. At least for arthropod species as recently reviewed [45], the potential degradation of dsRNA prior to ingestion, breakdown by nucleases in saliva and/or in the gastrointestinal tract, degradation of dsRNA in the hemolymph, and/or transport mechanisms of dsRNA within the organism are some of several barriers to physiological exposure that may lead to resistance.

The sprayed dsRNAs, different from regular pesticides, are biocompatible compounds as they occur naturally in the nature as well as inside/outside body of organisms and in food. The dsRNA ultimately is a regular RNA molecule that enters naturally within plants and other organisms. These molecules are subject of pathways from RNAi silencing mechanism, converted into siRNA and finally degraded by natural cell processes. In water and soil, dsR-NAs are rapidly degraded as regular RNA molecules do [81], so unlike to left considerably novel residues in food products.

New genomic tools will allow the development of technologies such as dsRNA sprays that increase crop resistance against insects, pathogens, and viruses; these could even replace chemical pesticides in some applications.

Acknowledgements

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