Improving RNAi efficiency for pest control in crop species

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ABSTRACT

The application of RNAi promotes the development of novel approaches toward plant protection in a sustainable way. Genetically modified crops expressing dsRNA have been developed as commercial products with great potential in insect pest management. Alternatively, some nontransformative approaches, including foliar spray, irrigation and trunk injection, are favorable in actual utilization. In this review, we summarize the recent progress and successful cases of RNAi-based pest management strategy, explore essential implications and possibilities to improve RNAi efficiency by delivery of dsRNA through transformative and nontransformative approaches, and highlight the remaining challenges and important issues related to the application of this technology.

To meet the increasing global demands for food and energy, mankind is facing the biggest challenge: how to increase crop yields in a profitable, efficient and sustainable way. There are several issues constraining agricultural productivity, such as damage by insect pests, diseases and weeds [1]. For instance, insect pests can cause direct and indirect crop damages mainly through loss in yield or quality owing to their adaptive ecological and physiological characteristics, which will be an important constraint to the supply of food in the next 40−50 years [2]. The estimates on potential yield loss of major crops by insect pests reach approximately 18% [3]. Furthermore, climate change can lead to some potential impacts on insect pests. The global yield losses of main crops such as rice, maize and wheat are projected to increase by 10−25% per degree of global mean surface warming [4,5].

Currently, chemical pesticides remain the major approach for suppressing insect pests owing to their well-controlled effect. Unfortunately, the excessive application of chemical pesticides has caused some serious problems threatening the environment and human health [6−8]. Therefore, a great demand for novel and effective alternative approaches has developed in recent years owing to growing consumer awareness and pressure for safer and healthier food. A pest management strategy should be economically, environmentally and farmer friendly. RNAi, first described in *Caenorhabditis elegans*, is known as post-transcriptional gene silencing because exogenous RNAs can induce sequence-specific mRNA degradation [9]. Among the mature biotechnological tools, RNAi has not only provided a novel and powerful reverse genetics tool for identifying gene functions, but also showed a great potential in pest management [10−13].

One promising area is the development of transgenic crops expressing dsRNA against key genes of insect pests, which are now realized by some commercial products [14−16]. Alternatively, there is also a great demand for nontransformative approaches. For instance, nontransformative products for foliar application, trunk injection, root dipping and seed treatment have their advantages [17−19]. However, there remain some issues hindering the practice and development of transformative and nontransformative RNAi products. When these products were designed for pest management, RNAi efficiency was the biggest technical bottleneck that needed to be overcome before products for pest control could be implemented. In this review, we summarize the successful applications of RNAi-based pest management strategy in crop species, discuss the possibility to further increase RNAi efficiency and propose the remaining challenges and possible solutions.

RNAi PATHWAYS & MECHANISMS FOR PEST MANAGEMENT

There are three RNAi pathways in insects: the dsRNA/siRNA-mediated siRNA pathway, miRNA-mediated miRNA pathway and piwi-interacting RNA (piRNA)-mediated piRNA pathway [11,20−22]. These pathways play different roles, such as in defense against viruses and transposable elements (transposons) via the siRNA pathway [23,24], regulation of gene expression via the miRNA pathway [25,26] and suppression of germ-line transposon expression via the piRNA pathway [21,27]. The core machinery genes of RNAi pathways may vary among different insect species. Insect pests tend to take up dsRNA rather than siRNA in some coleopteran and dipteran species through clathrin-dependent endocytosis [28−31]; however, siRNA works in some other insect species [32−34]. Therefore, gene silencing is usually triggered by supplying exogenous dsRNA in insects, although some insects are insensitive to RNAi through feeding. However, it is a bit different for fungi and plants, in which siRNAs are usually provided to trigger

RNAi [35–37]. When long dsRNA enters the target cells, it is cleaved by Dcr2 in association with R2D2 in the exo-siRNA pathway or Loqs in the endo-siRNA pathway into siRNA, which is loaded into Ago2 to assemble the RNA-induced silencing complex to degrade the complementary mRNA [38,39].

As shown in Figure 1, when insecticidal dsRNAs are applied in practical production, they need to enter target cells to work. Genetically modified (GM) crops and topical application, known as direct uptake, possess good prospects for wider application, and there have been some successful cases [15,40–42]. However, dsRNA delivery efficiency is often low in topical application, and during conventional transgenesis it is difficult to produce sufficient amounts of stable dsRNA owing to the plant RNAi machinery. However, dsRNAs can also enter the plant vascular system and then undergo uptake by insect pests [43], known as indirect uptake. However, dsRNAs may be restricted to the xylem vessels, and dsRNA delivery inside the plant vascular system is limited [44]. Therefore, some limitations exist when applying this indirect uptake method. Introduction of transplastomic technology and nanotechnology may overcome these current difficulties, and could improve RNAi efficiency, promoting the development and practice of RNAi-based pest management strategies (Figure 1).

APPLICATION OF TRANSGENIC CROPS FOR PEST MANAGEMENT

Conventional transgenic crops

GM crops engineered to express Bt toxins have become the most successfully commercialized transgenic crops for pest management [45,46]. Meanwhile, plant-mediated RNAi has developed rapidly in recent years attributed to the great advantage that almost any lethal gene can be targeted in insect pests. The most inspiring breakthrough was the development of GM maize expressing *vATPaseA* dsRNA to efficiently control the western corn rootworm (WCR, *Diabrotica virgifera virgifera*). This pest is susceptible to dsRNA supplied in an artificial diet, which can lead to larval stunting and mortality [15]. Another example targeting a *cytochrome P450* gene encoding a detoxifying enzyme is the Cotton bollworm (*Helicoverpa armigera*), which can resist

gossypol and related sesquiterpene aldehydes that are toxic to many organisms. Mao *et al.* [47] constructed GM plants expressing hairpin RNA (hpRNA) to successfully suppress the *CYP6AE14* expression in cotton bollworm, which reduced the larval tolerance to gossypol. Other successful cases exist in the application of GM crops expressing dsRNAs, such as the GM wheat against aphids [48,49], the GM potato against *Leptinotarsa decemlineata* [50], the GM cotton against *Tetranychus cinnabarinus* [51] and *H. armigera* [52], and the GM tobacco against *Myzus persicae* [53] and *H. armigera* [54].

Factors determining RNAi efficiency of transgenic crops

Several factors influence RNAi efficiency in insect pests. The success of plant-mediated RNAi for pest management first relies on the stable expression of dsRNA, as GM crops should provide enough dsRNAs to trigger a strong RNAi response. Conventional GM crops use nuclear transformation, and the expressed hpRNAs enter cytoplasm and are usually processed into siRNAs by plant RNAi machinery. RNAi efficiency is clearly dependent on the dsRNA dose, and the desired pest control effect needs to be determined experimentally against various target genes. The length of expressed dsRNA is an important factor affecting RNAi efficiency in some insect species. dsRNAs are taken up by an active process involving the receptor-mediated endocytosis, and insects are more responsive to longer dsRNA. In *Drosophila* S2 cells, dsRNAs of 1000 and 200 bp can induce a significant gene silencing; however, 21 bp siRNAs cannot result in any significant silencing [29]. In WCR, dsRNAs longer than or equal to 60 bp are required for an efficient RNAi, whereas 21 bp siRNAs cannot trigger RNAi [55]. RNAi efficiency is also dependent on insect species that possess different abilities of dsRNA degradation [56]. The activity of dsRNases that can efficiently cleave dsRNA has been identified in several insect species [57–59]. Suppression of specific *dsRNase* genes can lead to the reduction of dsRNA degrading activity and improve RNAi efficiency in *Cylas puncticollis* [60], *Locusta migratoria* [61], *Schistocerca gregaria* [59], *Ostrinia furnacalis* [62] and *L. decemlineata* [63].

Transplastomic crops with higher RNAi efficiency

Transformation of chloroplast DNA, also referred to as transplastomic crops, overcomes many current difficulties and has a good potential application [64,65]. The high transgene expression levels from chloroplast genome make transplastomic technology an attractive choice in herbicide and insect resistance engineering [66–70]. The greatest advantage in applying chloroplast-expressing dsRNAs is that it permits the accumulation of much higher amounts of stable dsRNA in the chloroplast, and therefore is not cleaved by the plant RNAi machinery [71,72]. In addition, the transplastomic technology provides an environmentally benign method, because plastids are maternally inherited in most crops and, therefore, constrain the pollen-mediated gene flow to decrease the potential environmental risk [64,73,74].

The RNAi efficiencies of nuclear- and chloroplast-transformed potatoes targeting the β-*actin* gene of *L. decemlineata* have been compared [71]. In transplastomic potato, the dsRNAs accumulated to as much as 0.4% of the total cellular RNA, whereas the nuclear-transformed potato produced much less dsRNAs. Meanwhile, the siRNAs specific to the target gene were detected in the beetles feeding on transplastomic potato, but no detectable siRNAs were found in the beetles feeding on nuclear-transformed potato. Reasonably, the transplastomic potato exhibited higher gene silencing and better pest control effects. Similarly, the hpRNAs, targeting the *acetylcholinesterase2* gene of *H. armigera*, were integrated into either the nuclear or chloroplast genome of *Nicotiana benthamiana*. The hpRNAs accumulated in transplastomic *N. benthamiana* to confer a strong resistance to *H. armigera*, whereas the hpRNAs produced by nuclear-transformed crops were cleaved into siRNAs, exhibiting a more modest antifeeding activity [75]. *Chitin synthase*, *cytochrome P450 monooxygenase* and *vATPase* dsRNA expressed via the chloroplast genome decreased the target gene expression and showed a strong resistance to *H. armigera* [76]. These results demonstrate that there is less or no RNAi machinery in chloroplast, and that the dsRNAs produced within chloroplast do not enter the cytoplasm, but can be taken up by the insect midgut cells
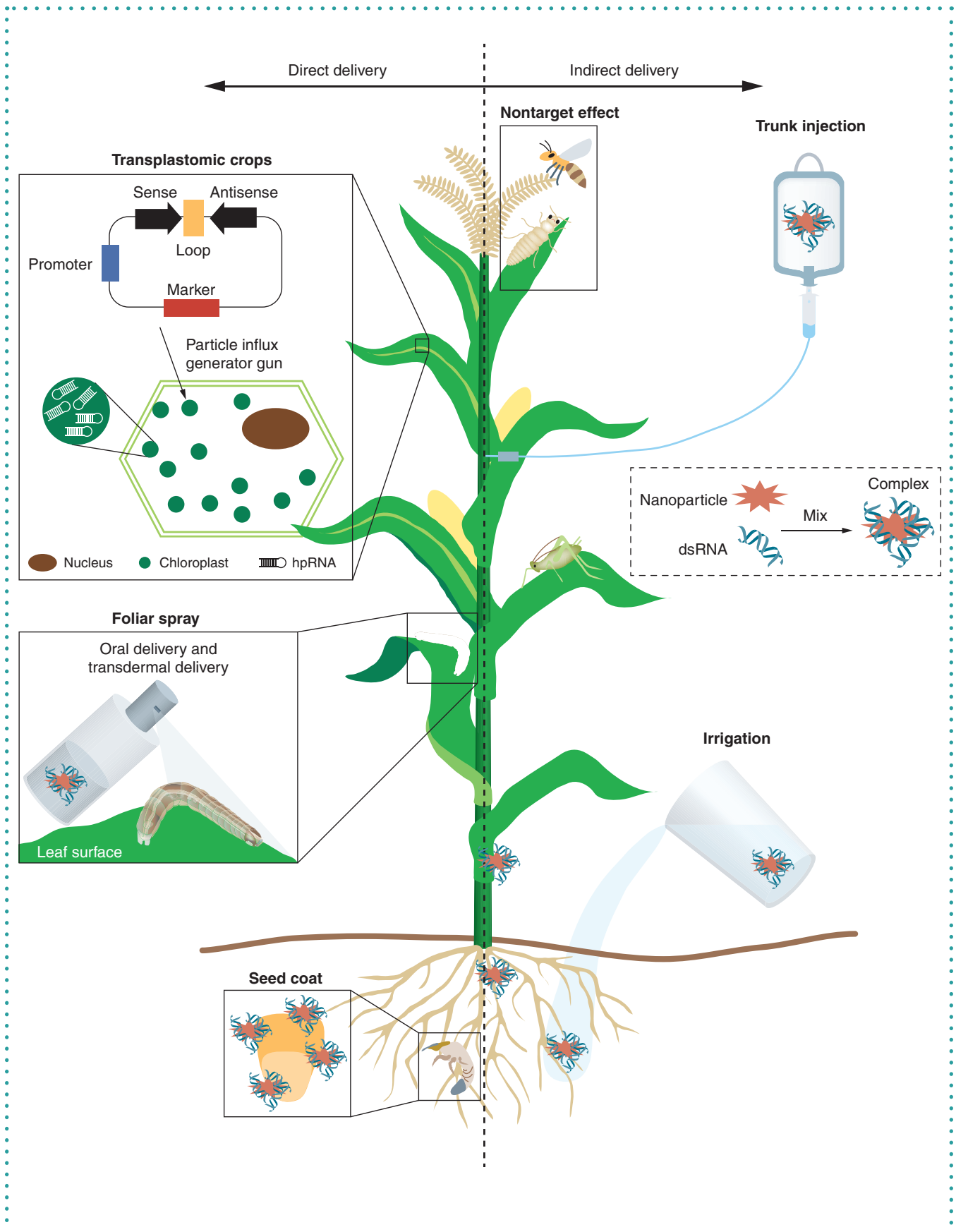
Figure 1. Application of transplastomic technology and nanotechnology to improve RNAi efficiency for insect pest management.

to trigger RNAi, making chloroplast a good tool for dsRNA expression.

APPLICATION OF NONTRANSFORMATIVE DELIVERY FOR PEST MANAGEMENT

Foliar spray

Sprayable RNAi-based products can be suitable for suppressing pests on stems, foliage or fruits. The dsRNA formulation can be directly sprayed on insect pests, which may penetrate the cuticle to induce lethal effects, and also be sprayed on the crops to feed pests. One famous study exploring the application of sprayable dsRNA formulation was conducted to control *L. decemlineata* [77]. Second instar *L. decemlineata* could not survive to fourth instar on potato plants treated with *actin* dsRNA. The dsRNA was sufficiently stable for at least 4 weeks under greenhouse conditions. However, dsRNAs may be degraded by nuclease in the field, and the stability of dsRNA should be evaluated owing to the complex factors in the environment. Another study was conducted using six siRNAs targeting *acetylcholine esterase* genes of *Plutella xylostella* [33]. The best insecticidal activity with 89% mortality rate was observed when second instar *P. xylostella* were fed with *Brassica* spp. leaves sprayed with siRNAs. Furthermore, the dsRNAs, targeting three functional domains of the *Ostrinia furnacalis methionine-rich storage protein* gene, were sprayed directly on *O. furnacalis* and *H. armigera*, and the dsRNA targeting the C-terminal domain caused high mortality rates in both insect pests [78]. There are also some studies reporting that dsRNA can penetrate insect cuticle to mediate gene silencing. A droplet of 0.5 μl dsRNA targeting *TAP1* gene was applied to the dorsal thorax of *Aedes aegypti*, and the mortality rate caused by the combination of three dsRNAs reached 42% at 24 h post-topical application [40]. Two papers from the same study group revealed that the topically applied dsRNA could penetrate the cuticle of *Diaphorina citri*, revealing the dsRNA products can be sprayed directly on some insect species [41,79]. Topical RNAi-mediated gene silencing seems also to work in aphids [80]. However, the penetration ability of dsRNA into the cuticle is different among insects, and the topical application is not fit for all insect pests.

Irrigation

The delivery of dsRNA via crop roots can trigger RNAi in insect pests, and the irrigation of RNAi-based products seems to be an alternative for suppressing pests feeding/growing in stems and fruits. The longevity of the dsRNAs applied through the root drench seems suitable to develop an area-wide pest suppression approach. The persistence of dsRNA in citrus trees was detectable at least 57 d post-treatment, whereas in psyllids and leafhoppers the detection was 5–8 d post-treatment [81]. One study exploring the applications of dsRNA via crop roots was conducted against the brown planthopper and the Asian corn borer [82]. When *Nilaparvata lugens* fed on rice that had been irrigated with *carboxylesterase* dsRNAs, the mortality rate reached nearly 50% at 5 d post-treatment. Meanwhile, the 5-d mortality rate was more than 45% when *O. furnacalis* was fed on dsRNA-treated maize. A method for RNAi bioassay was developed by feeding dsRNA via plants. The plant stem was detached and inserted into a centrifuge tube containing dsRNA, and the insects were released and reared on the stems and observed for the RNAi effects. This method has already been applied in the evaluation of RNAi effects using the citrus stem for *Toxoptera citricida*, tomato leaf for *Bemisia tabaci*, bean leaf for *Acyrthosiphon pisum* and *Brassica* leaf for *M. persicae* [83–87]. The irrigation is a simple yet practical method to deliver dsRNA; however, dsRNA may be degraded within approximately 2 d after the application to soil, regardless of texture, pH, clay content and other soil differences [88]. Thus, the success of this delivery strategy relies on the advances of formulations to protect dsRNA from degradation.

Trunk injection

The efficiencies of foliar spray and irrigation are relatively low, and sometimes this method is impractical for trees. Trunk injection is a promising method to deliver agrochemicals in many tree species while reducing the environmental impacts, risk for users and consumer exposure [89,90]. Phloem is considered a preferential channel for the transport of dsRNA/siRNA where it can remain stable for long periods, owing to the RNase-free environment in phloem sap [91,92]. Trunk injection can deliver dsRNAs into the vascular plant systems of xylem and phloem, and the Arborjet® is available and may be applied to deliver dsRNA [93]. Citrus trees (2.5-m height) and grapevines were treated with dsRNA via root drench and trunk injection, and the dsRNA was taken up into the whole plant system over 3 months to suppress insect pests [81]. The control of some insect pests has been difficult, especially for underground root-feeding pests, and the trunk injection may solve this problem. This strategy may be more effective for sap-sucking pests than for chewing pests feeding largely on leaves [93].

Improved delivery efficiency of dsRNA by nanoparticles

Nanoparticles are defined as any particle between 1 and 100 nm [94]. In addition to shielding and protecting the dsRNA from environmental nuclease degradation, nanoparticles promote the translocation of dsRNA across the peritrophic membrane, cell membrane and insect cuticle [95–98]. In most cases, the nanoparticle combines with dsRNA into the nanoparticle/dsRNA complex through the electrostatic interactions between the cationic groups in the nanoparticle and the phosphate groups in the dsRNA [99,100]. The complex usually retains a net positive charge that facilitates the interaction with negatively charged cell membrane surface [96]. When the complex is bound to the cell membrane, it can penetrate the cell membrane into the cytoplasm through endocytosis [29–31]. Complexation of the nanoparticle and dsRNA can avoid the degradation within the endocytic vesicles (early endosomes, late endosomes and lysosomes), and the nanoparticle can escape early and late endosomes through a process known as the sponge effect [101–103].

Nanoparticle-mediated dsRNA delivery has the potential to become a more sustainable and eco-friendly pest management method. The first attempt to study nanoparticle-mediated dsRNA delivery was conducted to silence *chitin synthase* genes in *Anopheles gambiae* using chitosan, and the improved RNAi efficiency was observed [104]. Similarly, the nanoparticle-mediated RNAi was then tested in *Spodoptera frugiperda* via a synthetic cationic polymer [105], *A. aegypti* via chitosan, carbon quantum dot and silica [106], *S. exigua* via a

guanylated polymer [107] and *Euschistus heros* and *Blattella germanica* via the liposome [108,109]. He *et al.* [100] designed a cationic core-shell fluorescent nanoparticle to deliver *CHT10* dsRNA through orally feeding and efficiently inhibited the normal development of *O. furnacalis*. A similar study was also performed in *Agrotis ypsilon* [110]. Zheng *et al.* [98] applied a fluorescent nanoparticle to deliver dsRNA to penetrate the aphid cuticle within 1 h. RNAi efficiency reaches 95.4%, and aphid population control effect reaches 80.5%. The transdermal dsRNA delivery system is a benefit for the development of sprayable RNA pesticides, which can be simply applied as chemical pesticides to achieve a high lethal effect. A star polycation was constructed recently as a highly efficient gene and botanical pesticide vector to increase pesticidal activities [97,111,112]. This nanoparticle/dsRNA formulation was sprayed directly on soybean seedlings with *Aphis glycines*, which resulted in a high mortality up to 78.5% [97]. The application of this nanoparticle to deliver pesticide and dsRNA at the same time may be a good option for foliar spray. In addition, nanoparticles can also facilitate the delivery of dsRNA in the *Arabidopsis* plant through the root tip [113], which is beneficial for the development of irrigation and trunk injection.

CURRENT CHALLENGES IN APPLICATION OF RNAi-BASED PRODUCTS

High production cost of dsRNA
The large-scale production of dsRNA, with low cost and high efficiency, must be developed for field application, and the dsRNA expression in bacteria seems to be a good alternative. Expression of dsRNA in bacteria strains deficient for RNaseIII is the major method, and the L1440-HT115(DE3) system is the most widely used dsRNA expression system that has been successfully applied in the RNAi of *Mythimna separate* [114], *L. decemlineata* [115] and *Bactrocera dorsalis* [116]. We applied the Scarless Cas9-assisted recombineering system to knock out the *rnc* gene in *Escherichia coli* BL21(DE3) and matched with the RNAi expression vector containing a single T7 promoter to construct a novel dsRNA expression system. The dsRNA expression efficiency of our system was about three-

times that of the L1440-HT115(DE3) system (unpublished data); however, the production efficiency of this system still needs improvement to meet the actual demands.

Nontarget & off-target effects of siRNA
Nonspecific binding of siRNA may occur within the target and nontarget genomes. The off-target effects may be not a problem in pest management; however, the binding that occurs in nontarget organisms, such as predators and honeybees, may lead to some sublethal effects, which is difficult to predict [17]. The specificity of siRNA for corresponding mRNA is related to sequence homology, and the substantial sequence diversity between two molecules does not preclude gene silencing, which has been confirmed by Baum *et al.* [15]. The *vATPaseA* and *vATPaseE* sequences from *L. decemlineata* and WCR shared 83 and 79% nucleotide-sequence identities, respectively, and the dsRNAs targeting WCR *vATPaseA* and *vATPaseE* also reduced the fitness of *L. decemlineata*. As expected, the *L. decemlineata* dsRNAs appeared more activity than the orthologous WCR dsRNAs. So far, some computational design tools have been developed for the accurate and systemic evaluation of RNAi nontarget and off-target effects, but they should be used with proper bioassays to protect the exposed nontarget organisms.

Potential RNAi resistance
Insect pests can develop resistance to RNAi-based products through various mechanisms as they do for conventional pesticides, including the mutations of target genes or core RNAi machinery genes, enhanced dsRNA degradation and lower dsRNA uptake [22,96]. Furthermore, the sequence polymorphism of target genes can cause the mismatch between dsRNA and mRNA, which can be potentially selected and lead to the evolution of resistance [117]. The WCR fed on transgenic maize expressing *DvSnf7* dsRNA was proved to exhibit resistance to dsRNA owing to the impaired luminal uptake, and this resistance was not *DvSnf7* dsRNA-specific, as indicated by cross-resistance to all other tested dsRNAs [118]. An RNAi-resistant cell strain of *L. decemlineata* expresses a low-level expression of coleopteran-specific StaufenC, which is required for RNAi and is a potential target for RNAi

resistance [119]. The commercialization of transplastomic crops may give pests a stronger selection pressure than nontransformative RNAi-based products, which may lead to resistance faster.

Limitations of transgenic crops
The US Environmental Protection Agency and Canadian Food Inspection Agency have approved the application of transgenic crops based on RNAi technology. However, there are several issues to consider about environmental safety, especially foreign gene escape that may lead to some serious ecological consequences [73,120–122]. Although transplastomic crops are regarded as safe, even several magnitudes safer than nuclear-transformed crops attributed to their extranuclear inheritance, we should pay more attention to the horizontal transfer of antibiotic resistant marker genes that may result in some negative effects on humans and microorganisms in the natural environment [123,124]. Furthermore, the lack of proper transformation and especially selection and regeneration protocols to obtain fertile homoplastic crops are major problems in applying transplastomic technology in several major crops [124]. In addition, GM crops still cost more to produce and take a longer time for development [18].

Potential risk of nanoparticle-mediated dsRNA delivery
The introduction of nanoparticles may bring potential risks for humans and environmental health, including the contamination of water sources and residues on food products [125]. So far, the development of nanopesticides and nanofertilizers has received less or at least delayed attention, and these nanoagrochemicals may be regarded as an intentional diffuse source of engineered nanoparticles in the environment [126]. A tiered approach focusing on key drivers of impact is typically used during their risk assessments, and each titer involves the estimation of a predicted environmental concentration, including the estimated concentration of the active substance in surface water, groundwater and soil [127]. Low cytotoxicity is a vital parameter for ideal dsRNA carrier. A series of fluorescent and nonfluorescent nanoparticles constructed by the group of Shen and Yin showed biocompatibility *in* ▶

vitro and *in vivo*, with a much lower cytotoxicity than PEI [97,110,128–130], revealing their safety to some extent. However, studies on the environmental risk of nanoparticle/dsRNA formulation are very limited.

FUTURE PERSPECTIVE

During the past two decades, RNAi has become an effective tool in functional genomics studies. Fast forward to today, the application of RNAi has helped scientists to find a possible solution to the global problems of agricultural losses attributed to insects and pathogens in a sustainable way. Recent studies reveal that this technology has raised enough attention and received ample funding support [131]. For GM crops expressing dsRNA, transplastomic crops seem to be a preferable strategy to achieve the improved effects. However, they are still considered a GM product in most countries, which requires the crops to undergo a rigorous evaluation before approval, and the extensive regulatory process is constraining the extension of transplastomic technology. The commercialization of SMARTSTAX PRO maize seems to be a good beginning. Furthermore, scientists should develop new chloroplast transformation protocols for major crops to promote the expansion of chloroplast-transformed crop range. For nontransformative RNAi products, the supply of dsRNAs associated with nanoparticles, through foliar spray, irrigation and trunk injection would be a great strategy to improve insecticidal activity, and other delivery methods, especially seed coats, still need to be evaluated. Bacteria-based expression of dsRNA is regarded as the most cost-effective method to produce large batch dsRNA, and some biotech companies are investing in this production method to produce affordable dsRNA for small and large farms [93]. Scientists should also pay more attention to public concerns regarding the specificity of dsRNA, fate of nanoparticle/dsRNA formulation in the environment, effects of RNAi-based products on nontarget organisms, and so on.

AUTHOR CONTRIBUTIONS

S Yan and J Shen conceived the idea and reviewed the literature. S Yan, J Shen, B Zeng and B Ren revised the manuscript. S Yan wrote the manuscript and contributed to the generation of figures.

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