RNAi technology for plant protection and its application in wheat

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Abstract The RNAi technology takes advantage of the intrinsic RNA interference (RNAi) mechanism that exists in nearly all eukaryotes in which target mRNAs are degraded or functionally suppressed. Significant progress has been made in recent years where RNAi technology is applied to several crops and economic plants for protection against diseases like fungi, pests, and nematode. RNAi technology is also applied in controlling pathogen damages in wheat, one of the most important crops in the world. In this review, we first give a brief introduction of the RNAi technology and the underneath mechanism. We then review the recent progress of its utilization in crops, particular wheat. Finally, we discuss the existing challenges and prospect future development of this technology in crop protection.

Keywords Double-stranded RNA, Pathogens, Pests, Nematodes, RNA interference, Small RNA, Wheat

INTRODUCTION

Wheat (Triticum aestivum L.) contributes more than 20% of the total dietary calories and proteins for humans worldwide (Shiferaw et al. 2013). It plays a pivotal role in securing the global food demand. The increase of wheat yield, however, has slowed down in recent years partly due to newly emerging varieties of various diseases—pathogens, pests and nematodes (Rosegrant and Cline 2003). On the other hand, the overuse of pesticides for disease control has posed substantial risks to food safety, the environment, and all living organisms (Ali 2014). The transgenic crops expressing insecticidal proteins from Bacillus thuringiensis (Bt) effectively reduced the insecticide usage and increased crop yields. However, the limited scope of Bt crops and the appearance of Bt-resistant pests in fields call for new technologies for pest control (Carriere et al. 2015; Jin et al. 2015; Tabashnik et al. 2013). The phenomenon of RNA interference (RNAi) is widely found in eukaryotes (plants, fungi, insects, animals, and nematodes etc.) and has been developed as a promising technology for crop health protection (Zhang et al. 2017). RNAi is a natural process that involves the regulation of gene expression by several manners: effective post-transcriptional gene silencing (PTGS), translational inhibition, RNA destabilization, and/or transcriptional gene silencing (TGS) by directing DNA methylation (Fire et al. 1998; Coleman et al. 2015; Ghildiyal et al. 2008; Huvenne and Smagghe 2010; Jones-Rhoeades et al. 2006; Liu et al. 2020; Mao et al. 2007; Sherman et al. 2015). Here, we review recent progress in the development of RNAi-based plant protection technologies, particularly on its application in
wheat. We discuss its potential for the control of fungal pathogens, pests and nematodes, as well as current challenges facing RNAi strategy. We also prospect the future improvement in delivery methods for effective applications of this technology in crop protection.

THE MECHANISM OF RNAI TECHNOLOGY

RNAi is a self-protection mechanism in eukaryotic cells and is triggered by double-stranded RNA (dsRNA) when present in a cell. dsRNA is processed by the ribonuclease III enzyme Dicer or Dicer-like enzymes to produce small interfering RNAs (siRNAs) of 20–30 nucleotide (nt) long. These small RNA (sRNA) are bound to Argonaute family proteins (AGOs), the catalytic components of the RNAi system. The AGO/siRNA complexes are then recruited to the RNA-induced silencing complex (RISC) (Lee et al. 2010), which mediates mRNA degradation, mRNA translation, or chromatin modification (Borges and Martienssen 2015) (Fig. 1). In most eukaryotes, including pathogens and pests, RNA-dependent RNA polymerases (RdRPs) have been identified for secondary dsRNA synthesis and are essential for the systemic effect of RNAi. Two works have specified functions for the RdRP activity in RNAi in Caenorhabditis elegans (Sijen et al. 2001) and fungi (Dang et al. 2011); however, a similar RdRP-based amplification system is yet to be discovered in insects (Zotti et al. 2018). Given the presence of RNAi pathways in pathogens, pests, and nematodes, it is not surprising to take advantage of its working mechanism in crop protection.

DELIVERY OF INTERFERING RNAs

Interspecific transportation of sRNAs takes place naturally. siRNAs can be shuttled between plants and pathogens by secreted vesicles (Cai et al. 2018; Weiberg et al. 2013). In cotton, the production of microRNAs (miRNAs) miR166 and miR159 was increased upon Verticillium dahliae (a vascular fungal pathogen responsible for devastating wilt diseases in many crops) infection and transported to infection sites to silence virulence genes reducing its damage (Zhang et al. 2016). Despite these in vivo mechanisms, the RNAi technology is impeded by in vitro dsRNA delivering efficiency. Numerous efforts on artificial delivery methods have been attempted. The selection of the suitable delivery approaches (e.g. host-induced gene silencing, foliar sprays, recombinant microbes) is in fact determining
the success of the technology (Fig. 1). A few methods have been tested.

The first approach is the application of synthetic dsRNA or sRNA derived from pathogen or pest genes as pesticides on crop leaves. Foliar application with sprayable RNAi-based products, such as sRNAs, is suitable for controlling pests and pathogens on stems, foliage, or fruits. The products are evaluated similarly to topical pesticides where the RNA solution is sprayed on leaves or fed to the target pests, and impacts on insects are then observed (Andrade and Hunter 2017). One of the first case exploring the applications of sprayable RNA molecules to control pests was the use of siRNA against the diamondback moth (Plutella xylostella). Brassica leaves that were sprayed with chemically synthesized siRNAs targeting the acetylcholine esterase gene AchE2 caused high mortality for P. xylostella. (Gong et al. 2013). In another case, foliar application of dsRNA targeting the cytchrome P450 (CYP3) gene of Fusarium graminearum resulted in successful inhibition of fungal growth in directly sprayed leaves as well as the distal non-sprayed leaves in barley plants (Koch et al. 2016). This strategy or so-called spray-induced gene silencing (SIGS) opens an avenue of development of biopesticide which is environmentally friendly. Moreover, since RNAi is highly dependent on the sequence specificity, it has little effects on the non-target microorganisms or non-target pests.

The second method is to use recombinant microbes such as virus and bacteria engineered to produce dsRNA in host crops (Cagliari et al. 2018; Dubrovina and Kiselev 2019; Goulin et al. 2019). Virus-induced gene silencing (VIGS) is a naturally occurring (Baulcombe 2015; Waterhouse et al. 2001). Unlike stable RNAi and mutants, the transiently expressed dsRNA by VIGS does not modify plant genetic composition. For instance, three midgut-expressed CYP genes of the Lepidoptera insect, Manduca sexta were targeted through viral vectors to produce dsRNA in the host plant. The viral vector was engineered using Tobacco Rattle Virus (TRV) to deliver dsRNA into Nicotiana attenuata (Kumar et al. 2012). dsRNA could also be produced in the bacteria (HT115). When the cotton bollworm (Helicoverpa armigera) larvae fed with the artificial diet coated with dsRNA expressing HT115, high mortality was observed after five days. Data showed that inhibition of target gene expression led to significant reductions in body weight, body length, and pupation rate (Ai et al. 2018).

The third approach is host-induced gene silencing (HIGS) which employs transgenic plants to produce dsRNA derived from pathogen or pest genes. RNAi occurs in pests when they ingest sufficient dsRNA or sRNA. Tests have been made for a few pests where persistent effects were obtained for several common species (Baum et al. 2007; Mao and Zeng 2014; Sun et al. 2019; Zhu et al. 2012). The phloem-feeding hemipterans such as aphids with specialized mouthparts (stylets) that penetrate through plant tissues to ingest cell saps. In this case, dsRNA sequences of shp gene effectively reduced the growth, the reproduction, and the survival rate of tested aphids. Remarkably, other developmental aberrations were also observed such as winged adults and delayed maturation (Abdellatief et al. 2015). This method is a complementary tool to Bt-based insect-resistant plants which is not effective for several hemipterans with specialized stylets. Cotton plants constitutively expressing dsRNA from genes encoding the P450 protein CYP6AE14 and NDPH dehydrogenase protein 2 of cotton bollworm (Helicoverpa armigera) significantly improved resistance to this pest, and the dsNDPH cotton is almost equivalent to Bt cottons in resistance efficiency (Mao et al. 2011; Wu et al. 2016). Similarly, dsRNA homologous to V-type ATPase gene of corn root worm (Diabrotica virgifera) in transgenic corn plants rendered significant improvement of insect resistance (Baum et al. 2007).

For woody plants, such as fruit trees, dsRNA can be delivered via insecticidal baits, nanoparticle trunk injection and root soaking. The information of these methods can be found elsewhere for detail (Liu et al. 2020; Zhu and Palli 2019).

THE APPLICATION OF RNAI FOR WHEAT PROTECTION

Management of bacterial and fungal pathogens

In wheat, a few serious wheat diseases, such as Fusarium head blight (FHB) caused by necrotrophic fungi of the genus Fusarium and leaf rust caused by biotrophic fungi of the genus Puccinia (Table 1), have been targeted using RNAi technology. Transgenic wheat plants were engineered to confer three hairpin RNA fragments derived from the Fusarium graminearum chitin synthase gene (Chs3b), which is responsible for the biosynthesis of chitin. These transgenic plants showed strong resistance to FHB and Fusarium seedling blight (FSB) (Cheng et al. 2015). On the other hand, expressing dsRNA complementary to mRNAs of Puccinia triticina MAP-kinase (PtMAPK1, 520 bp) or a cyclophilin (PtCYCl, 501 bp) showed efficient silencing of the corresponding genes in the fungus and significant reduction of the fungal pathogenicity and growth in transgenic wheat. P. triticina is an aggressive fungal pathogen that causes severe leaf rust disease in wheat. P. triticina
Table 1 RNAi target genes tested in pests/pathogens/nematodes

| Organism | Target genes | Assay/method | Effects | References |
|----------|--------------|--------------|---------|------------|
| **Insects** | | | | |
| *Sitobion avenae* | Salivary sheath protein (*SHP*) | HIGS | Mortality/fecundity/transgenetional gene silencing | Abdellatef et al. (2015) |
| *Rhopalosiphum padi* | Acetylcholinesterase gene *RpAce1* | Injection | Susceptibility/fecundity | Xiao et al. (2015) |
| *Sitobion avenae* | Catalase gene *CAT* | Feeding | Mortality | Deng and Zhao (2014) |
| *Sitobion avenae* | Acetylcholinesterase gene *SaAce1* | Injection | Susceptibility/fecundity | Xiao et al. (2015) |
| *Sitobion avenae* | Cytochrome c oxidase subunit VIIc precursor, zinc finger protein, three unknown proteins | Feeding | Mortality/developmental stunting | Zhang et al. (2013) |
| *Sitobion avenae* | Secreted salivary peptide *DSR32*, salivary protein *DSR33*, serine protease 1 *DSR48* | Feeding | Mortality | Wang et al. (2015) |
| *Sitobion avenae* | Olfactory coreceptor gene *SaveOrco* | Feeding | Impaired response | Fan et al. (2015) |
| *Sitobion avenae* | Lipase maturation factor 2-like gene | HIGS | Mortality/fecundity | Xu et al. (2017) |
| *Sitobion avenae* | Laccase 1 (*Lac1*) | Feeding | Mortality | Zhang et al. (2018) |
| *Sitobion avenae* | Zinc finger protein (*SaZFP*) | HIGS | Mortality/transgenetional gene silencing | Sun et al. (2019) |
| *Sitobion avenae* | Ecdysone receptor (*EcR*) and ultraspiracle protein (*USP*) | Feeding | Mortality/fecundity | Yan et al. (2016) |
| *Sitobion avenae* | Chitin synthase 1 (*CHS1*) | HIGS | Mortality/fecundity | Zhao et al. (2018) |
| **Pathogens** | | | | |
| *Fusarium graminearum* | Cytochrome P450 lanosterol C-14α-demethylase (*CYP51*) | HIGS | Inhibiting fungal mycelium formation | Koch et al. (2013) |
| *Fusarium graminearum* | Cytochrome P450 lanosterol C-14α-demethylase (*CYP51*) | SIGS | Inhibition of fungal growth | Koch et al. (2016) |
| *Fusarium graminearum* | Chs3b | HIGS | Restriction of fungal growth through | Cheng et al. (2015) |
| *Blumeria graminis* | Virulence effector (*Avra10*) | HIGS | Reduced fungal development | Nowara et al. (2010) |
| *Fusarium asiaticum* | Myosin 5 | SIGS | Reduced virulence | Song et al. (2018) |
| *B. graminis f. sp. hordei* | Ribonuclease-like protein Ribonuclease-like protein Virulence effector Glucanase Metalloprotease Virulence effector | HIGS | Reduced virulence | Pliego et al. (2013) |
| *Fusarium culmorum* | Secreted lipase (*FgI1*), Mitogen-activated protein (MAP) kinase (*Fmk1*), Beta 1,3-Glucan synthase (*Gls1*) | VIGS and HIGS | Reduced virulence | Chen et al. (2016) |
| *Puccinia striiformis f. sp. tritici* | Calcineurin homologue (*PsCNA1, PsCNB1*) | VIGS | Slower extension of fungal hyphae | Yin et al. (2010) |
| *Puccinia striiformis f. sp. tritici* | Mitogen-activated protein kinase (MAPK1), Cyclinophilin (*CYC1*), Calcineurin regulatory subunit (*CNB*) | VIGS | Reduced virulence | Panwar et al. (2013) |
proliferation was significantly reduced together with decreasing fungal target gene transcript abundance and reduced biomass accumulation in RNAi-based resistant plants (Panwar et al. 2018).

Powdery mildew caused by *Blumeria graminis f. sp. hordet* in barley and *B. graminis f. sp. tritici* in wheat is a serious disease as well. Transgenic barley expressing dsRNA targeting the avirulence gene *Avra10*, which corresponds to the resistance gene *Mla10*, showed reduced fungal gene transcripts and severely affected fungal development (Nowara et al. 2010). Silencing of 1,3-β-glucanosyltransferase genes (*BgGTF1* and *BgGTF2*) via VIGS that was built on the barley stripe mosaic virus (BSMV) significantly slowed down the growth of the powdery mildew fungus (Qi et al. 2019b). Mildew resistance locus o (Mlo) encodes a transmembrane protein (Panstruga et al. 2005) that acts as a negative regulator to suppress plant immunity in uninfected tissues. It is also involved in protection against cell death as well as in responses to biotic and abiotic stresses (Piffanelli et al. 2002). Down-regulation of the *TaMlo* gene via VIGS resulted in the broad-spectrum powdery mildew resistance (Qi et al. 2019b). Mildew resistance locus o (Mlo) encodes a transmembrane protein (Panstruga et al. 2005) that acts as a negative regulator to suppress plant immunity in uninfected tissues. It is also involved in protection against cell death as well as in responses to biotic and abiotic stresses (Piffanelli et al. 2002). Down-regulation of the *TaMlo* gene via VIGS resulted in the broad-spectrum powdery mildew resistance (Qi et al. 2019b).

Management of wheat pests

Several major pests, such as grain aphid (*Sitobion avenae*), bird cherry-oat aphid (*Rhopalosiphum padi*), and wheat aphid (*Schizaphis graminum*), can cause severe yield loss (Table 1) (Peairs 2008; Smith and Chuang 2014; Yu et al. 2014). Transgenic wheat plants expressing a 198 bp fragment of dsRNA complementary to the zinc finger protein (*SaZFP*) of grain aphid can effectively increase its mortality and reduce its daily fecundity (Sun et al. 2019). In barley, dsRNA targeting the grain aphid gene encoding salivary sheath protein (SHP), a pivotal component of the stylet penetration process, effectively reduces the reproduction and survival rates of the aphid and the effect can be transmitted for seven generations (Abdellatef et al. 2015). Effects of additional target genes were also confirmed by feeding or direct injection into grain aphid, such as those encoding catalase, acetylcholinesterase1,
cytochrome c oxidase subunit VIIc precursor, and zinc finger protein, and abnormally high mortality and developmental stunting were observed (Wang et al. 2015; Zhang et al. 2013).

Management of nematodes in wheat

Wheat parasites cause enormous yield losses and threaten the quality of grains, including *Heterodera avenae*, *H. filipjevi* and *H. latipons* (Table 1) (Toumi and Waeyenberge 2013). Targeting of the *Ha18764* effector protein family genes of *H. avenae* by the VIGS-based RNAi approach significantly attenuated the parasitism and reproduction status of *H. avenae* in wheat (Yang et al. 2019). Down-regulation by RNAi of *pat-10* and *unc-87* genes on Thorne’s meadow nematode (*Pratylenchus thornei*), which infects wheat roots, significantly reduced the reproduction of the worms (Tan et al. 2013). Moreover, RNAi in wheat can be stimulated by poly-component biostimulants derived from metabolites of various soil streptomycetes which up-regulate siRNAs and miRNAs in wheat plants. These small RNAs are complementary to cereal cyst nematode mRNA and hence suppress their reproduction providing resistance to wheat plants (Blyuss et al. 2019).

CHALLENGES FOR USING RNAI TECHNOLOGY

While the outlook of using RNAi for plant protection appears to be promising, several issues need to be resolved before efficient practical applications.

The stability of dsRNA

One of the primary concerns for the use of RNA as a biopesticide is their stability, especially for the sprayable dsRNA and siRNA applications. Microorganisms in the environment can degrade dsRNA prior to their uptake by pathogens or pests. Rapid degradation of dsRNA may occur by nucleases in the saliva, gut lumen, and/or haemolymph of pests as well (Allen and Walker III 2012; Chung et al. 2018; CoGuan et al. 2018; Katoch and Thakur 2012; Kennedy et al. 2004; Luo et al. 2013). The high or low pH found in the gut lumens of some pests can also reduce dsRNA stability either directly or indirectly by affecting the activity of gut nucleases (Cooper et al. 2019).

Other environmental factors may exert different effects on the stability of dsRNA and siRNA. Several works show that dsRNA is degraded to undetectable levels within 48 h after their application on three types of soil (silt loam, loamy sand, and clay loam) and within 7 days after their addition to aquatic systems containing natural water and various types of sediment (Albright et al. 2017; Fischer et al. 2017). Despite this, actin-dsRNA derived Colorado potato beetle (CPB) remained active for at least four weeks after application to potato leaves. It suppressed CPB larval weight gain, delayed its development, and increased its mortality (San Miguel and Scott 2016). Therefore, dissecting the process of dsRNA degradation is helpful in evaluating the potential effect of dsRNA in various environments and target organisms.

Cost-effective methods for dsRNA production

For RNAi application to be practical for field use, the major hurdle is to produce sufficient amount of dsRNA. The traditional dsRNA production method in the laboratory is expensive and produces only a limited amount of dsRNA and thus is not practical for large-scale application needs (Ahn et al. 2019). Producing dsRNA in bacterial cells with RNaseIII deficiency seems to be an alternative. However, only a handful works have demonstrated microbial-based dsRNA production. One approach uses L1440-HT115 (DE3) system that has been successfully applied in the RNAi of *Mythimna separate* (Das et al. 2015; Parsons et al. 2018; Zhang et al. 2010). With more research underway, the production efficiency of this system should be augmented to meet market demands.

Off-target effects

RNAi is a sequence homology-dependent mechanism. Several studies show that siRNA is not always specific and can have off-target effects and thus is problematic in disease management (Mamta and Rajam 2017). Some target genes are highly conserved between species which increases the likelihood of off-targets among them. The sequences of *vATPaseA* and *vATPaseE* from *L. decemlineata*, for instance, shared 83% and 79% nucleotide-sequence identities to their counterparts in Western Corn Rootworm (WCR), respectively. dsRNAs from WCR *vATPaseA* and *vATPaseE* could reduce the fitness of Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) in a bioassay (Baum et al. 2007). Computational design program is needed for specific and systemic evaluation of non-target and off-target effects which should be further verified by additional bioassays. In addition, feeding studies revealed that dsRNAs of at least 60 nucleotide (nt) in length are necessary for an efficient RNAi response in *D. virgifera* (Bolognesi et al. 2012) and *Tribolium castaneum* (Wang et al. 2019). A minimum of 21 nt was required for the size of
siRNA for efficient protection against WCR and active orthologs (Bachman et al. 2013).

**RNAi resistance**

Pests and pathogens can develop resistance to RNAi-based products through various mechanisms as they do for conventional biopesticides. Compared to conventional commercialized transgenic crops expressing Bt toxins for pest management (James, 2010), RNAi-based strategy induces down-regulation of the target gene by in-complete resistance in most of cases. This may reduce the selection pressure on the pathogen that may contribute to durable resistance. But genetic variation in pathogenic organisms may also cause single nucleotide polymorphisms (SNPs) in the target gene. The efficiency of RNAi would be cut down owing to the reduction of complementarity between the target gene and the dsRNA. Synonymous SNPs lead to nearly no fitness cost on the pathogens and pests, but the difference between dsRNA and the original gene sequences reduces their complementarity, causing reduced RNAi effect or RNAi resistance (Scott et al. 2013; Yu et al. 2016). Thereby, the potential of RNAi resistance should be taken into consideration in application.

**CONCLUSIONS AND FUTURE PROSPECTS**

In the past few years, we have seen diverse applications of RNAi in crop protection methodologies against pests, pathogens, and nematodes. RNAi technology has emerged as a promising new strategy for wheat protection either. The wide use of HIGS on a commercial scale appears possible soon. The major obstacles for the HIGS strategy will be resolved, by optimal target and fragment selection methods, highly efficient transformation constructs, and stable transgenic systems. To this end, it is worthy to mention that the V-type ATPase-based RNAi technology has passed the GM safety evaluation in eight countries and regions including the United States, Brazil, and Japan. It has also been licensed for planting by the US Environmental Protection Agency (Zotti et al. 2018), painting an elusive picture for the commercialization of the RNAi technology. Technical barriers are being overcome to allow a wide range of applications from laboratory to the field. The technology of encapsulated dsRNA on leaves with SIGS has significantly promoted dsRNA stability in the environment as well as during its uptake by pests enhancing plant protection. Cost-effective approaches for massive production of dsRNA (e.g. bacterial, plant, and synthetic production) are being optimized and will contribute to lowering costs of the technology. There is no doubt that a new era of disease control based on RNAi technology for crop protection is right at the corner.

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**Compliance with ethical standards**

**Conflict of interest** There are no conflicts of interest.

**Ethical approval** We declare that all materials and methods comply with required ethical standards.

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