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Review

RNAi technology extends its reach: Engineering plant resistance against harmful eukaryotes

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RNA interference (RNAi) is a homology-dependent gene silencing technology that is initiated by double stranded RNA (dsRNA). It has emerged as a genetic tool for engineering plants resistance against prokaryotic pathogens such as virus and bacteria. Recent studies broaden the role of RNAi, and many successful examples have described the application of RNAi for engineering plant resistance against a range of eukaryotic organisms. Expression of dsRNA directed against suitable eukaryotic pathogens target genes in transgenic plants has been shown to give protection against harmful eukaryotic species, including nematodes, herbivorous insects, parasitic weeds and fungi. This review addresses the progress of RNAi-based transgenic plant resistance against these four class eukaryotic pests, as well as future challenges and prospects.

Key words: dsRNA, RNAi, crop resistance, biotechnology, nematode, insect, parasitic weed, fungus.

INTRODUCTION

Plant harmful eukaryotes include plant parasitic nematodes, herbivorous insects, parasitic weeds, fungus, oomycetes and other pathogens. Most of them cause significant yield losses due to attacks which occur in the agricultural and horticultural species. At present, there are three strategies to control the detrimental organisms: (a) Crop rotation; growing susceptive plant and non-host crops in the same place in rotation will decrease the population level of pests by eliminating hosts and interrupting pest life cycles (Scholte, 1992; Whiting and Crookston, 1993; Hwang et al., 2009). A complex crop rotation has some disadvantages: Firstly, it reduces profitability because of limited acreage of the most profitable crop. Secondly, relatively greater investments in more crop-specific equipments are required. Thirdly, farmers require more management knowledge and greater skills as more crop species are raised. All of these increase the cost of production and limit the utilization of crop rotation in agriculture. (b) Chemical control; agrochemicals such as nematicides, insecticides, herbicides and antifungal agents had been adopted to control plant pests for a long period. Although the availability of agrochemicals is effective, chemical control is restricted because of the harmful effects and toxicity on humans and the ecosystem (Getsinger, 1998). (c) Breeding of resistant crops; developing resistant cultivars is widely considered to be the most cost-effective and sustainable management tactic for pest control, although the conventional breeding of resistant crops is time consuming and limited by the deficiency of natural resistance gene (Harms, 1992; Johnson, 2000; Stuthman et al., 2007). Unfortunately, most pathogens have the ability of persistent genetic variation and adaptive evolution, virulent biotypes emerge rapidly under the selection pressure of resistance gene generations to overcome the cultivar resistance (Leach et al., 2001; McDonald and Linde, 2002), even the transgenic plant with Bt toxin had been broken out by insect variance (Candas et al., 2003; Janmaat and Myers, 2003). Therefore, alternative pest control strategies are urgently needed for the development of more durable resistant cultivars.

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Abbreviations: RNAi, RNA interference; **amiRNAs,** artificial miRNAs; **UTR,** 5' or 3' untranslated regions; **M6PR,** mannose 6-phosphate reductase; **siRNA,** small interfering RNA; **RT-PCR,** reverse transcription-polymerase chain reaction; **WCR,** western corn rootworm; **dsRNA,** double stranded RNA.

RNA interference (RNAi) is emerging as an alternative genetic tool in the ongoing task of developing pathogen and pest-resistant crops. Since the technology for generating virus resistance in plants was first demonstrated in 1998 (Fire et al., 1998), the strategy has been widely adopted to engineer host resistance against many different kinds of plant viruses (Kasschau and Carrington, 1998; Waterhouse, 2001; Mikhail et al., 2003) and bacteria (Escobar, 2001). It has remained undecided from the beginning about whether RNAi could be used to protect plant invasion or attack on organisms other than viruses or bacteria. Over the past three years, the technology of host plant delivered RNAi has expanded significantly, this broadened engineering resistance against harmful eukaryotic organisms including plant parasitic nematodes, herbivorous insects, parasitic weeds and fungus. In this review, we focused on the recent progress in the development of plant-derived RNAi for resistance against harmful eukaryotes and the future application of the technology.

BACKGROUND OF RNA INTERFERENCE (RNAi)

RNAi is a conserved mechanism in a wide range of eukaryotes, RNAi-related phenomena was initially elucidated in plants and was named post-transcriptional gene silencing (PTGS) (Jorgensen et al., 1996; Waterhouse et al., 1998), although the mechanism was first experimentally dissected in the model organism *Caenorhabditis elegans* (Fire et al., 1998). RNAi is triggered by double-stranded RNA (dsRNA), which is recognized by Dicer enzyme and processed into 21- to 26-nucleotide (nt) dsRNA fragments called short interfering RNAs (siRNAs). These small RNAs are then incorporated into 'RNA-induced silencing complex' (RISC). Then the RISC directs the degradation of endogenous mRNAs that are homologous to the small RNAs (Hannon, 2002; Novina and Sharp, 2004). RNAi is considered to play important roles in suppression of transposon activity (Tabara et al., 1999; Dawe 2003), resistance to dsRNA virus infection (Vance and Vaucheret, 2001; Voinnet, 2001), post-transcriptional and post-translational regulation of gene expression and epigenetic regulation of chromatin structure (Bühler and Moazed, 2007; Grewal and Elgin, 2007), and because of its specialty, high-efficiency and heritability, RNAi has been rapidly adopted as a powerful tool for the reversal of genetics in functional genomics and are used as biotech- nological approaches to plants genetic improvement (Mansoor et al., 2006).

ENGINEERING PARASITIC NEMATODES RESISTANCE IN PLANTS BY RNAi

Although without the latest comprehensive surveys, plant parasitic nematodes have caused damage to world agriculture estimated recently to be worth US$125 billion annually in 2003 (Chitwood, 2003), and the figure keeps increasing in recent years. The vast majority of damage should be ascribed to the sedentary species such as the root knot nematodes (e.g. *Meloidogyne* spp.) and cyst nematodes (e.g. *Heterodera* spp. and *Globodera* spp.). Both of the nematodes invade host roots and release secretions into plant cells to induce physiological and morphological changes, and finally modify them into very specialized and metabolically active cells as feeding site, from which the nematodes continuingly obtain nutrients that are necessary to support their development and reproduction during the later life stages (Davis et al., 2000; Gheysen and Fenoll, 2002; Vanholme et al., 2004).

As early as the RNAi mechanism's first demonstration in 1998, the possibility that dsRNA mediates gene silencing in nematodes other than *C. eleganse* was mentioned by Fire et al. (1998). This is not similar to the free-living nematode *C. elegans*, RNAi in plant parasitic nematodes by injection or soaking is not feasible, because they are too small to be microinjected with dsRNA and do not normally ingest fluid until they have infected a host plant (Bakhetia et al., 2005). The technological bottleneck was not broken open until 2002. Urwin et al. used octopamine to stimulate pharyngeal pumping by pre-parasitic juveniles (J2s) of the cyst nematodes *Globodera pallid* and *Heterodera glycines* leading to uptake of dsRNA from the soaking solution. Oral ingestion was observed in the pharyngeal lumen by monitoring the fluorescent of fluoroscein isothiocyanate (FITC) which was used as a visual marker, and the RNAi effect was confirmed by analysizing the transcript abundance and the silence phenotypes on the development or sexual fate of target genes (Urwin et al., 2002). After then, another two stimulation reagents of resorcinol and serotonin were also used to induce dsRNA uptake by J2s much more effectively (Rosso et al., 2005). Now, the *in vitro* RNAi assays have been establish for sedentary forms including root knot nematodes (e.g. *Meloidogyne*), cyst nematodes (e.g. *Heterodera* and *Globodera*), and migratory pine nematodes (*Bursaphelenchus xylophilus*). A number of genes, expressed in a range of different tissues and cell types, are now been successfully targeted for silencing in different plant parasitic nematodes (PPNs) and all these experiments were well summarized (Lilley et al., 2007; Rosso et al., 2009). All these cases have shown that RNAi is a powerful technique for investigating the function of nematode genes and identifying potential targets for parasite control.

In vitro RNAi data provide support for the application of host-derived RNAi to develop PPNs resistance crops. The first case of which plant source siRNA was active in inducing gene silencing in worm was *C. elegans* (Boutla et al., 2002). In this study, RNA extracts prepared from transgenic GFP silenced plant were injected into a GFP-expressing strain of *C. elegans*, when they induced RNAi, the worm GFP activity was reduced substantially after injection extracts were derived from a silenced plant. It

suggested that plant delivering dsRNA had specific activity in nematode. In theory, transgenic plants expressing dsRNA hairpin structures which target essential gene of PPNs, this dsRNA, or its siRNAs, would then be delivered from the plant to the nematode through ingestion. RNAi response is elicited to silence the target gene in nematode, so the crop resistance would be achieved. The first published report describing successful effects of host-derived RNAi was by Yadav et al. (2006). Two genes-one coding for splicing factor and the other coding for integrase - of the root-knot nematode *Meloidogyne incognita* were selected as targets, the hair-pin-shaped dsRNA expressing constructs were introduced into tobacco plants and almost complete resistance to RKN infection in transgenic plant preceded this study. Soon, transgenic soybeans transformed with an RNAi expression vector containing both sense and anti-sense strands of the major sperm protein gene from *H. glycines*, significantly reducing the reproductive potential of this nematode with 68% reduction in eggs per gram root tissue (Steeves et al., 2006). The root-knot nematode conservative parasitism gene *16D10* encodes a parasitism peptide secreted from the subventral glands of pre-J2 and functions as a ligand for *SCARECROW*-like plant transcription factor. It is thought to have an important role in the early signaling that occurs during feeding-site formation (Huang et al., 2006a). Ingestion of *16D10* dsRNA *in vitro* silenced the target parasitism gene of RKN and resulted in reduced nematode infectivity. *In vivo* expression of *16D10* dsRNA in *Arabidopsis* resulted in resistance effective against the four major RKN species, it showed a 63 - 90% reduction in the number of galls as well as a 69 - 93% reduction in the number of RKN eggs per gram root compared with the control plants (Huang et al., 2006b). Recently, much more efforts have been put into examining the possibility of the utility of RNAi for the control of PPNs on different host plants (Table 1).

Careful target selection plays a key role in the biotechnological application of RNAi towards PPNs control. Two kinds of genes were considered as potential candidate targets, the one kind is essential and conserved genes, of which *C. elegans* orthologs has lethal or highly damaging RNAi effect (Bakhetia et al., 2005). Unlike PPNs, *C. elegans* is a model nematode and a wealth of information exists on lethal mutations and the effects of silencing of specific genes. So it is feasible to identify PPNs homologyes through comparative genomics approach and some attempt has been made on *H. glycines* (Alkharouf et al., 2007) and *M. incognita* (Abad et al., 2008). The other kind of targets were parasitism genes, which are expressed in the esophageal gland cells of PPNs, encode proteins that are secreted into host root. These proteins include cell-wall-modifying enzymes, multiple regulators of host cell cycle and metabolism, suppressors of host defense and mimics of plant molecules. Although multitude genes encoding secreted proteins have been identified in the past few years, only a few numbers were determined in detail (e.g. *16D10* of *M. incognita* and *CBP* of *H. schachti*) (Huang et al., 2006a; Hewezi et al., 2008). Elucidation of the molecular mechanism of action of much more parasitism genes will help to find optimal anti-parasite targets to achieve perfect efficiency of engineering resistance.

The nematode feeding tube acts as a molecular sieve allowing nematode uptake of nutrition with specific size. Using different forms of proteins expressed in feeding site, it appears that the molecular exclusion limit varies between PPNs. Juveniles of *H. schachtii* are unable to take up 28 kDa *GFP* (Bockenhoff and Grundler, 1994), but *G. rostochiensis* and *M. incognita* could ingest a molecular weight of 32 kDa *GFP* and 54 kDa *Cry6A*, respectively (Urwin et al., 1997; Li et al., 2007). This is because no evidence suggests whether its dsRNA or siRNAs in the transgenic plants that trigger silencing of nematode genes. The RNAi construct design must consider host-derived molecules of ingestible size, so that RNA molecules would not be excluded by the feeding tube.

ENGINEERING HERBIVOROUS INSECTS RESISTANCE IN PLANTS BY RNAi

Losses due to insect herbivores is a significant factor in limiting food production, this was estimated at 10 - 20% for major crops (Ferry et al., 2006). Engineering crop plants for endogenous resistance to insect pests has been one of the real successes of GM technology. Many proteins have been employed for developing transgenic plants and they show differing specificities of insecticidal activity toward pests, such as proteinase inhibitors, lectins, cholesterol oxidase, avidin, *Photorhabdus luminescens* insecticidal proteins *tcdA* and *Bacillus thuringiensis* (*Bt*) insecticidal toxins (Gatehouse, 2008). Among which *Bt* toxins have become widely used in commercial transgenic plants successfully. However, some pest species cannot be targeted by the *Bt* toxins and resistant populations of target pests have evolved in field, so developing novel strategies for durable pest control is necessary.

The possibility of plant mediated RNAi to protect plants against insects has also been recognized for many years, but it was considered unfeasible initially, because in the complete genome sequence for the model insect *Drosophila melanogaster* (Schwarz et al., 2002; Roignant et al., 2003) and other insects, there is no homologue encoding RNA dependent RNA polymerase (*RdRP*), which is necessary for the siRNA amplification that leads to persistent and systemic RNAi effects in *C. elegans*. In addition, the homologues of the *C. elegans sid-1* gene have not been identified in the *Drosophila* genome (Piccin et al., 2001; Roignant et al., 2003), which function as a channel for the uptake and release of dsRNA among cells. Both the components were important for systemic RNAi response when dsRNA was delivered orally. Insect systemic RNAi was first documented in another model insect

Table 1. Samples of host generated RNAi targeted PPNs and the resulting phenotype.

Target gene	Target PPN	Host plant	Transgenic phenotype	Reference
Splicing factor	*M. incognita*	Tobacco	Reduction in number of galls and females	Yadav et al., 2006
Integrase	*M. incognita*	Tobacco	Reduction in number of galls and females	Yadav et al., 2006
Secreted peptide 16D10	*M. incognita, M. artiellia, M. javanica, M. hapla*	Arabidopsis	Reduced galling, Decrease in number of established nematodes	Huang et al., 2006b
MSP	*H. glycines*	Soybean	Reduction in eggs	Steeves et al., 2006
MjTis11	*M. javanica*	Tobacco	Down-regulation of transcript levels	Fairbairn et al., 2007
Parasitism gene 3B05	*H. schachtii*	Arabidopsis	Down-regulation of transcript levels, Reduction in number of females	Sindhu et al., 2009
Parasitism gene 4G06	*H. schachtii*	Arabidopsis	Down-regulation of transcript levels, Reduction in number of females	Sindhu et al., 2009
Parasitism gene 8H07	*H. schachtii*	Arabidopsis	Down-regulation of transcript levels, Reduction in number of females	Sindhu et al., 2009
Parasitism gene 10A06	*H. schachtii*	Arabidopsis	Down-regulation of transcript levels, Reduction in number of females	Sindhu et al., 2009
Hg-rps-3a	*H. glycines*	Soybean	Reduction in number of females	Klink et al., 2009
Hg-rps-4	*H. glycines*	Soybean	Reduction in number of females	Klink et al., 2009
Hg-spk-1	*H. glycines*	Soybean	Reduction in number of females	Klink et al., 2009
Hg-snb-1	*H. glycines*	Soybean	Reduction in number of females	Klink et al., 2009
Mi-tnc	*M. incognita*	Tobacco	Reduction of transcripts in the progeny	Dubreuil et al., 2009
Mi-crt	*M. incognita*	Tobacco	Fewer galls, Reduction of eggs hatch and transcripts in the progeny	Dubreuil et al., 2009
Hs4F01	*H. schachtii*	Arabidopsis	Down-regulation of transcript levels, Reduction in number of females	Patel et al., 2010

Tribolium castaneum (flour beetle) and multiple genes such as *Tc-ASH*, Distalless, maxillopedia and proboscipedia were targeted by injection of specific dsRNA (Bucher et al., 2002; Tomoyasu and Denell, 2004). Confirmation of whether RNAi effects could be induced in insects by orally delivered dsRNA is a prerequisite for utilization of RNAi for crop protection against insect pests. Turner offered experimental validation of this strategy on the larval stage of the light brown apple moth (*Epiphyas postvittana*). Transcript level of a larval gut-expressed gene (*EposCXE1*) and adult antennae-expressed gene (*EposPBP1*) were reduced by feeding specific dsRNA (Turner et al., 2006). Now, very recent work indicate that several herbivorous insect pests from different orders can be effectively targeted by oral delivery of dsRNA (Price and Gatehouse, 2008; Huvenne and Smagghe, 2009).

In 2007, great progress was made, two groups successfully achieved managing insect by transgene-encoded RNAi in plants. Mao et al. identified a *P450* monooxygenase from the cotton bollworm (*Helicoverpa armigera*), it was named *CYPAE14* and was involved in detoxification of the otherwise toxic allelochemical- an indiscriminately toxic compound called gossypol-produced by cotton plant. This gene is induced by gossypol and its suppression reduced the larval tolerance to gossypol. The researchers transferred hairpin RNA constructs directed against *CYP6AE14* into plant and fed cotton bollworm with the plant material. The result demonstrated that transgenic plant provided sufficient levels of dsRNA to suppress gene expression in the insect midgut and stunt its growth (Mao et al., 2007). Baum's research team adopted the same strategy for defense against coleopteran and lepidopteran pests; these included western corn rootworm (WCR), southern corn root-worm and Colorado potato beetle. For screening of candidate targets, the researchers fed larvae with artificial diet supplemented with dsRNAs specific to a large number of essential insect genes, then 14 genes whose knock-down exhibited a dramatic suppression efficacy of both larval stunting and mortality were identified. To test whether corn plants expressing a WCR-derived dsRNA were protected from rootworm feeding damage, the

hpRNA expression cassette of *V-ATPase A* dsRNA was assembled and transferred into corn. Transgenic corn showed suppression of mRNA in the insect and reduction in feeding damage by WCR infestation comparable to that provided by a *Bt* transgene (Baum et al., 2007). These findings strongly suggest that plant-expressed dsRNA can be delivered into insects and trigger systemic silencing, although some significant challenges remain, there is no doubt that the plant-induced RNAi could be an alternative pest-control strategies.

ENGINEERING PARASITIC WEEDS RESISTANCE

Parasitic weeds are wide distribution in many countries. The most economically damaging genera are broomrapes (*Orobanche* spp., Orobanchaceae), witchweeds (*Striga* spp., Scrophulariaceae) and dodder (*Cuscuta* spp.) (Press and Graves, 1995; Parker, 2009). These species infect the major world crops including maize, sorghum, rice, beans and a range of Solanaceae species. And they cause enormous yield losses in agriculture. Conventional control methods (resistant varieties, herbicides, crop rotation, etc.) are currently in use, but are only partially successful because of the limitation of effectiveness, costs or environment safety (Joel, 2000; Aly, 2007; Rector, 2008, Hearne, 2009). Just like developing new plant resistance against other pathogens, alternative methods are needed to control parasitic weeds.

From antiquity, parasitic plants have undergone various evolutionary events and evolved many forms of parasitism including facultative hemiparasite, obligate hemiparasite and holoparasite (Press and Graves, 1995), but the fundamental genetic mechanisms controlling fundamental parasitic processes are likely conserved in all species. Once chemical signals of host plants are recognized, parasitic weeds attach to and invade host tissues via specialized organ termed haustorium (Cook et al., 1966; Zwanenburg et al., 2009). This also functions as a physical and physiological bridge between the two species. Water, minerals, carbohydrates and other vital nutrients are translocated across haustoria from host to parasitic plants (Press and Graves, 1995; Haupt et al., 2001; Birschwilks et al., 2006).

Recent reports demonstrated that mobile mRNAs can also traffic between the two widely divergent species (Roney et al., 2007; David-Schwartz et al., 2008). To identify potential host transcripts in dodder grown on tomato, Affymetrix GeneChip Tomato Arrays analysis was performed to detect mRNA from dodder and tomato, RT-PCR was used to validate the putative positives identified. The result suggests 474 putatively mobile transcripts in the dodder parasitizing tomato (Roney et al., 2007). In another study, four genes mRNAs were found to move from host (tomato) to dodder by *in situ* RT-PCR amplified within parasitic tissue, molecules of up to 30 cm from the tomato-dodder connection were found in the growing dodder stem (David-Schwartz et al., 2008). Furthermore, RNAi signals have also been demonstrated to traffic between hosts and parasites. *GUS*-expressing *Triphysaria versicolor* roots were attached to hairpin *GUS*-expressing lettuce. Transcript quantification indicated a 10-95% reduction in the steady-state message level of *GUS* mRNA in *Triphysaria* attached to hpGUS lettuce and compared with control lettuce. *GUS* staining showed that *Triphysaria* roots parasitizing transgenic hpGUS lettuce lacked *GUS* activity in root tissues distal to the haustorium (Tomilov et al., 2008). These experiments demonstrate that hpRNA constructs engineered into host plants can silence the expression parasitic plants homology.

By identifying candidate targets that are crucial for growth, development or parasitic behaviors of parasitic plants, can engineer host plants harboring RNA silencing construct. This will degrade the targeted mRNA of parasitic weeds and lead to lethal or inhibited parasite development. This approach could be adapted for parasitic weed control. Preliminary evidences from two recent studies suggest that this approach may be available. The first well test of the strategy was on the research of *Orobanche* control. Mannose 6-phosphate reductase (*M6PR*) is a key enzyme which is involved with mannitol biosynthesis, it has been suggested that mannitol accumulation may be very important for *Orobanche* development. So the *Orobanche* M6PR gene was selected as a target to control this parasite. Transgenic tomato plants were produced bearing M6PR dsRNA-expression cassette, northern blot detection revealed that M6PR-siRNA was processed in transgenic tomato but was not detected in the parasite. Quantitative RT-PCR analysis showed that the level of endogenous M6PR mRNA in the tubercles and underground shoots of *Orobanche aegyptiaca* grown on transgenic host plants was reduced by 60-80%. Accordingly, there was a significant decrease in mannitol level and a significant increase in the percentage of dead *O. aegyptiaca* tubercles on the transgenic host plants (Aly et al., 2009). Another attempt of the same strategy was on transgenic maize for *Striga* resistance. Five *Striga asiatica* genes were used as targets, hairpin constructs were made and transformed into maize (*Zea mays* L.), subsequently challenged with germinating seeds of *Striga*. Unexpectedly, some differences in *Striga* growth rate were observed, *S. asiatica* parasitizing on transgenic hosts were not reproducibly compared with controls (de Framond et al., 2007). Although it is too early to draw conclusions because further analyses are still ongoing, two possibilities could be supposed; firstly, the selected genes were not perfect targets and secondly, the nature of connections between *S. asiatica* and maize is different with other participators.

However, further research is needed to explore the possibility of using RNAi to control the maize parasitic weed of *Striga*. Together with the viral studies, RNAi technology has potential for protecting crop plants from parasitic weeds. To achieve ideal efficiency, identification of optimizing targets for silencing is important.

Understanding the molecular basis of the interaction between hosts and parasitic weeds will facilitate identification of potential targets. Current effort focused on genetic factors involved in germination stimulant perception, haustorial formation and parasite development, and other reviews on the topic has provided detail discussion (Westwood et al., 2009; Yoder et al., 2009).

ENGINEERING FUNGUS RESISTANCE

Fungal attacks caused significant losses in most of the agricultural and horticultural plant species. Plant genetic engineering has been used to control fungal diseases. In addition to conventional strategies, most of the transgenic plants have generated fungus resistance by over-expressing antifungal molecules or constituting production of hypersensitive response (Punja, 2001; Grover and Gowthaman, 2003). These strategies were partly successful but were not desirable. Now, RNAi has been employed as novel alternative strategies and have exhibited a great potential for enhancing resistance to fungi.

In 1992, the phenomenon of fungi RNAi was first described in *Neurospora crass* by Romano and Macino (Romano and Macino, 1992), and later, much more examples were described in other fungal species. It seems that more fungus evolved from the form of gene suppression similar to higher organisms (Nakayashiki, 2005). With a relative simple organism structure, fungi transformation protocols had been developed and used for many years (Case et al., 1979; Fincham, 1989). Also Liu et al. demonstrated the first example of fungal RNAi by an hpRNA-expressing plasmid in the basidiomycetous yeast *Cryptococcus neoformans* (Liu et al., 2002). RNAi had been adopted to explore genes function in a wide range of fungal species and fungus-like organisms (Nakayashiki and Nguyen, 2008). These studies demonstrate that RNAi mechanism may be broadly existent, even though RNA silencing pathways appear to have diversified significantly (Nakayashiki et al, 2006).

To date, there is no existing published paper on RNAi-mediated gene suppression in fungi where the dsRNA molecules are taken up from artificial growth media or plant tissue. However, two U.S. patent applications (No. 20060247197 and No. 20080022423) elucidated that a strategy have been employed to combat plant-pathogenic fungus recently. In the first invention, numerous target genes of the rice blast fungus (*Magnaporthe grisea*) were selected for use in RNAi experiments. Using *in vitro* assay, germinating conidia were stained with dsRNA corresponding to target genes, the inhibition of germ tube and appressorium formation was observed under a microscope. It indicated that target gene expression was inhibited by RNAi due to uptake of dsRNA by the intact fungus. To further determined resistance to rice blast infection, the transgenic rice expressing specific dsRNA were produced and inoculated with *M. grisea* spores, the infection rate and lesion sizes were compared with control leaves (Van de Craen et al., 2006). In the second invention, three essential genes in *S. sclerotiorum* including tubulin, vATPase and Pac1, were chosen for analysis using *in vitro* experiments. *S. sclerotiorum* were inoculated to growth medium containing dsRNA molecules, the fungal growth and siRNA production was tested as measure of dsRNA-mediated gene suppression effects. Also in *in vivo* assay, the dsRNA-expressing cassettes were transformed into tobacco, Arabidopsis and soybean tissue. *S. sclerotiorum* was inoculated to transgenic plants and inhibition of fungal growth was evaluated (Roberts et al., 2008).

Although without detail research result, these patents indicated that the dsRNA outside intact fungal cell wall could be taken up by the fungal cells in sufficient amounts to specifically cause gene silencing, and RNAi was also achieved by transforming plants with a DNA construct encoding the dsRNA of fungal gene. The invention method would be used to alleviate plants from fungus pathogens, at least against obligately parasitic or semi-parasitic types.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The RNA silencing pathways seem to have diversified during the evolution of eukaryotes, but the phenomenon of RNAi that regulates gene expression appears to be common among eukaryotic organisms. Therefore, once exogenous elicitor (dsRNA or siRNA) production from transgenic plants is delivered into pest cells, the endogenous cognate transcripts would be degraded. RNAi-mediated plants resistance offer several advantages over conventional bio-engineering crops resistance. Firstly, in engineering broad-spectrum plant resistance even distinct lineages of plants pathogens have undergone convergent evolution and sharing homologues (Andersson, 2006). Therefore multiple pathogens resistance can be achieved by silencing conserved and essential genes. Secondly, the resistance has the potential to be more durable. The RNAi-mediated resistance is based on RNA hybridization rather than protein-protein interaction; the molecular hybridization cannot be inhibited by minority nucleic acid mutation (Escobar et al., 2001). Therefore, the possibility of the pests overcoming the resistance are likely difficult. In addition, this biotechnology represents a flexible means of developing pest resistant crops. In theory, all the pests' genes showing detrimental knockdown phenotype can be considered as potential targets. So this strategy would not be limited by the scarcity of resistance genes.

A key challenge and essential step for the RNAi-based crop-protection strategies is identification of the right targets. The optimal candidates of pathogenicity - related genes were involved in parasitism, detoxification or the essential genes knockout which lead to a lethal phenotype. One effective way to validate a potential target is to

introduce RNAi elicitor molecular into eukaryotic pest cells to evaluate its effect on the silencing phenotype. Another way is transformation of the model plant *Arabidopsis thaliana* for resistance investigation. However, understanding the mechanism underlying pests infection and damage will facilitate the development of the strategy for producing pests-resistant plants.

Another major concern is 'off-target' effects. RNAi has been considered to be highly gene specific, but cross-hybridization with transcripts containing partial identity to the introduced dsRNA sequence can induce knockdown of unintended genes, this may result in unexpected mutant phenotypes in addition to the target gene (Jackson et al., 2003; Ma et al., 2006).

Some improvements in the design rules have been developed to reduce 'off-target' effects, these include: 1) Several software and web-based tools have been developed that can identify the most common off-target sequences so that they can be excluded from RNAi constructs (Arziman et al., 2005; Jia et al., 2006; Koberle et al., 2006). 2) It might be best to avoid targeting a gene family that is highly conserved across the plant and animal kingdoms. 3) Sequences from 5' or 3' untranslated regions (UTR) are used as siRNA targets, since these are generally less conserved than those encoding open reading frames. Tuschl et al. recommend against selecting sequences within the 5' and 3' untranslated regions (UTRs) and regions near the start codon, as these may be richer in regulatory protein binding sites, UTR-binding proteins and/or translation initiation complexes, which may interfere with binding of the RISC (Tuschl, 2003). 4) Specific species targets can be screen through compa- rative genome, with the wealth of information emerging from genomics or ESTs projects on plant pathogens and their hosts, sequence information is now rapidly available for more species, and these resources facilitate RNAi- based biotechnological approaches for pests management. 5) Artificial microRNAs were used for specific gene silencing. The endogenous miRNAs from plant and animal usually have numbers of targets (Schwab et al., 2005; Lewis et al., 2005; Lim et al., 2005). However, artificial miRNAs (amiRNAs)-designed to target one or several genes provide a new and highly specific approach for effective gene silencing in plants. An alternative strategy has been recently employed to functional analysis which confers virus resistance in plants (Niu et al., 2006; Qu et al., 2007; Duan et al., 2008). It is also possible that transgenic plants expressing the designed amiRNAs could be used to protect a plant against invading or attacking organisms other than viruses. To date, four classes of harmful eukaryotic organisms of nematode, insect, weed and fungus are shown to be susceptible to the RNAi control strategy. Despite this, the resistance has been achieved using hpRNA constructs in plants, it remains to be seen whether this method is available for controlling a broad range of plant eukaryotes pathogens. The degradation of target mRNA occurs inside the pest cells and depends on pest RNAi system and this is not similar to RNAi-mediated resistance against viral viral or bacterial pathogens. Although the RNAi pathway seems to be an ancient mechanism that likely originated at a very early stage of eukaryotes' evolution, it seems to have diversified during the evolution of eukaryotes and are absent in certain eukaryotes (Shabalina and Koonin, 2008). In some fungal species, such as *Saccharomyces, Cerevisiae* and *Ustilago maydis*, the entire RNA silencing machinery appears to be lost (Nakayashiki and Nguyen, 2008). Therefore, it is possible that the silencing signal from host could not trigger 'inter-ference effect'. In addition, the aforementioned successful research focused on biotrophic plant pathogens, other species like grain moth pests, saprophytic nematodes and fungal, do not uptake nutrition from living tissue or cells of the host. Therefore, the host generated dsRNA or siRNA could not be delivered into cells of these pathogens smoothly.

Although significant progress on engineering plant resistance against harmful eukaryotes by RNAi has been made, most of the successful examples were developed either in a model plant (e.g. *A. thaliana*) or in greenhouse trial. Therefore further research is needed to evaluate whether this resistance can be kept in the target crop plant in the field. However, recent advances have brought high expectations for the future role of RNA-mediated resistance in crops. Once the novel resistance performance are in line with these expectations, this technology will create a new era in eukayotic pest management, and its application will be extended to the commercial product in agronomic crops.

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