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Innovation for Exploiting RNAi as RNA Based Herbicidal Compound for Invasive Weeds Management: Strategies, Challenges and Future Prospects

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ABSTRACT

The invasive weed plant poses a significant threat to the stability and biodiversity of ecosystems. However, an effective and economical method to control weeds is still lacking. Current control measures involve chemicals such as herbicides which pose challenges like chemical resistance and longer withholding periods. To enable more sustainable agriculture practices, it is important to develop technologies that combine targeted effectiveness with minimal environmental footprint. RNA interference (RNAi) is a eukaryotic process in which transcript expression is reduced in a sequence-specific manner. This makes it a perfect tool for developing efficient and effective biological control against weeds. RNA interference (RNAi) has been widely studied and applied in agriculture for trait improvement. Furthermore, spray-induced gene silencing (SIGS), in which the foliar application of dsRNA is used to silence the weed genes, holds great potential as an alternative to host-induced gene silencing (HIGS). Recently, SIGS approaches have attracted research interest, owing to their ability to mitigate weeds. Spray-induced gene silencing (SIGS) can produce RNAi silencing effects without introducing heritable modifications to the plant genome and is becoming a novel non-transformation strategy for plant protection. It is also suggested that parasitic weeds for example striga, dodder and mistletoe can also be controlled by stably transforming plants with a silencing construct homologous to a gene of the parasitic plant that is essential for survival or development. In this review, we discuss key advances made using RNAi technology and how they can be applied to control weeds. We will also discuss limitation and improvement for commercialization.

Keywords: Invasive Weeds/RNAi Bioherbicide/ Spray induced gene silencing bioherbicide/ siRNA/ dsRNA/ delivery

1. INTRODUCTION

The impact of invasive weed species has been called an “invisible tax” on our environment and economy. With ever increasing global transportation and travel has come an unprecedented spread of invasive and noxious weed species throughout the world. These weeds adapt quickly to new environments and go largely unchallenged by local flora and fauna. Many are unreachable by or have developed resistance to conventional control techniques. Invasive species cause direct economic losses in sectors such as forestry, ranching, and agriculture. The chemical pesticides such as herbicides are commonly utilised to control and combat weeds because of their low prices, high availability, fast mode of action, and perceived reliability. However, the extreme dependence on the use of these compounds has resulted in ubiquitous, low-level exposure, which is potentially harmful to both human health and ecosystems (Worrall et al., 2018). Besides risks to human health, widespread and repeated insecticide application can also result in environmental concentration build-up in soils (Chagnon et al 2014). Furthermore, direct application of these chemical pesticides have other important limitations like, poor specificity, short duration of protection, poor water-solubility, difficulties with site-specific uptake by the targeted pest, and inducing chemical-resistant pathogen strains (Worrall et al., 2018, Bai & Shaner 2004).

Biocontrol and physical removal are costly and labour intensive requiring large investments and again, often resulting in collateral damage to other organisms. Some invasive pest plants are now so well established that they are widely considered impossible to remove by any available technique. Thereby developing safe, environmentally friendly, effective, and sustainable weed management strategies has become a much-needed requirement to improve the productivity and sustainability of global agriculture. RNA interference (RNAi) has emerged as a promising candidate for the development of biological based control strategies, offering excellent target species specificity and sustainability management of weed affecting agriculture. Unlike conventional pesticides used to control weeds, RNAi based control uses the target pests own molecular mechanisms to initiate silencing of essential genes. Ideally, the RNAi approach drives mRNA degradation to block targeted protein production and inhibits weed growth, depending on the developmental stage of the weed gene of interest targeted. The RNAi mechanism is a conserved, nucleic acid metabolism, which can be initiated by exogenously applied or endogenously expressed double-stranded RNAs (dsRNA) in many species (Kim et al, 2015, Jain et al., 2020). In this review, the potential advantages that RNAi technology offers are detailed, along with the scope and significance of using RNAi to control weeds impacting the crop health. Additionally, key attributes of RNAi target identification, effector molecule design and different delivery methods used for the delivery of dsRNA are reviewed. In addition, the challenges and future perspective associated with the use of this technology in field are also discussed.

RNAi APPLICATION IN WEEDS: STRATEGIES

RNA interference (RNAi) is a next-generation technology for weed management. Weeds would be sprayed with small RNAs (sRNAs) capable of inducing gene silencing (referred to as spray-induced gene silencing, SIGS) without involving the use of transgenes, reaching traditional targets in chemical control, targets that are not today more sensitive to herbicides and even new targets. As a novel and efficient nontransformative strategy, the SIGS technique has been widely applied in agricultural and mammalian research. Mai et al 2021 studies *M. micrantha* genes encoding the chlorophyll a/b-binding proteins and examined the silencing efficiency by spraying the corresponding RNAi molecules on the leaves of *M. micrantha*.

The development of SIGS in weed science has been slower compared to other crop protection areas such as entomology and plant pathology due to the difficulty of obtaining stable molecules that easily enter the plant, without off-target risks to crops, and that in small amounts guarantee systemic silencing with effectiveness. For successful application, it is necessary to achieve the synthesis of sRNAs on a large scale, making the field application practical and economical, develop formulations that protect sRNAs inside and outside the plant, and substantially increase the genomic and transcriptomic information available for weeds. Once these barriers have been overcome, SIGS technology could be similarly used in the field as herbicides are used today, spraying directly on the crop and selectively controlling weeds. It will provide a new tool for weed management, herbicide resistance management, and potentially exploration of new plant enzyme targets never before achieved by chemical control.

RNA-Interference (RNAi)

RNAi is an endogenous, post-transcriptional gene regulation mechanism which has been identified in almost all eukaryotes; from plants, fungi, algae, protozoans, invertebrates, to vertebrates (Schuster et al., 2019; Chen et al.,2021). It was first reported by Napoli and Jorgensen in 1990 when they observed endogenous gene co-suppression while studying pigmented petunias (Napoli et al., 1990; Sen & Blau, 2006). In animals, RNAi was first documented in the nematode Caenorhabditis elegans (Guo & Kemphues 1995; Fire & Xu 1998). While the intracellular components of RNAi are similar across species, it was initially known as quelling in fungi, and post transcriptional gene silencing in plants (Wytinck et al., 2020). It is a highly conserved mechanism, which is highly sequence specific and selective in its activity (Bramlett et al., 2020).

RNAi Mechanism

While a comprehensive review on RNAi mechanism discussing all the different facets of this technology is outside the scope of this review, the basic mechanism of RNAi when initiated via delivered exogenously applied dsRNA are well described and are illustrated in Figure 1. RNAi-mediated post-transcriptional gene silencing is triggered by the processing of a dsRNA precursor into short single stranded RNA effector molecules (Schuster et al., 2019). There are three RNAi pathways, depending on the RNA class of effector involved, small-interfering RNA (siRNA), piwi-interacting RNA (piRNA), and microRNA (miRNA) (Schuster et al., 2019; Bramlett et al., 2020; Vogel et al., 2019). Of these, siRNA is considered to be the 'classical' pathway, with dsRNA being the trigger molecule for gene silencing in plant and other species (Vogel et al., 2019]. In the siRNA pathway, once taken up by cells dsRNA is cleaved by an endonuclease, Dicer, into pre-siRNA duplexes (Wytinck et al., 2020). The pre-siRNA is a 21–23 nucleotide (nt) long duplex with 2 nt overhangs at each of the 3′ termini. The pre-siRNA duplex is bound by the RNA-induced silencing complex (RISC), with one strand, the guide strand, being retained within the complex, while the complementary or passenger strand of the pre-siRNA duplex is degraded (Worrall et al., 2018, Nunes & Dean, 2012). The guide siRNA strand with the Argonaut proteins (Ago) within the activated RISC, identify matching mRNA in a sequence dependent manner, resulting in suppression of translation or mRNA degradation (You et al., 2020). This results in loss of protein function which may lead to lethality or stunted growth of the target organism (Worrall et al., 2018; Vogel et al., 2019).

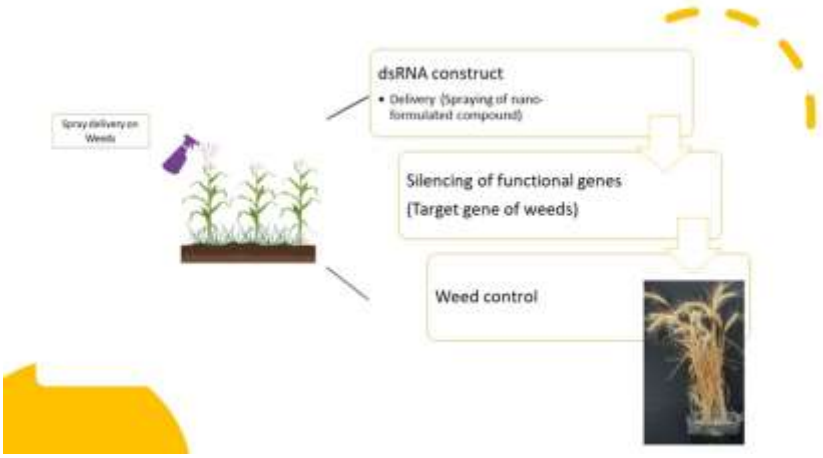

Figure 1. Schematic example of RNA interference (RNAi) delivery via spray/topical application on weeds. After application over the plant surface, the dsRNA would enter the cells of the weeds. dsRNA is cleaved by dicer-2 into pre-siRNA duplexes, that are further processed into small-interfering RNA

(siRNAs, 21–24 nt) effector molecules. The RNA-induced silencing complex (RISC) binds to the siRNAs and guides sequence-dependent degradation or translational inhibition of homologous mRNAs, which results in RNAi-mediated gene silencing (Image source: Google image)

RNAi Targets

Target gene selection depends on the class of RNA effector molecule to be used. While both siRNA and piRNA are generated from long, complementary dsRNAs, miRNA is endogenous in nature, usually processed from stem-loops, and siRNA are exogenous and are directly generated from cleaved dsRNAs (Zotti et al., 2018). The first step in developing an RNAi product is to identify potential target genes and design dsRNA targeting them. There are five factors which play a key role in influencing the efficiency of RNAi as biocontrol, namely, the concentration, nucleotide sequence, length of the dsRNA, effective period of the dsRNA induced silencing, and life stage of the target (Huvenne et al., 2010). The perfect RNAi target gene should be transcribed into an mRNA with a high turn overrate, that is translated into a protein with a short half-life, and is transcribed at all stages of life cycle, with the loss of function leading to mortality or severe impairment in the pest or pathogen of interest. In addition, the target transcript should be poorly conserved across species to maintain maximum specificity of mRNA for minimal environmental effects on non-target species (Vogel et al., 2019). This is a challenging criterion to meet, as those genes that are essential to cell viability tend to be more conserved between species. It has reported that the efficiency of dsRNA uptake is length dependent, requiring an optimal length and dosage to induce RNAi successfully (Saleh et al., 2006).

This ideal dosage changes according to the organism's susceptibility to RNAi and their potential for systemic RNAi, formulation for delivery, gene expression abundance, life and development stage for gene expression (Vogel et al., 2019). Various selection methods can be used to develop efficient RNAi targets to minimize side effects and enhance expected silencing. Since RNAi relies on high gene specificity to the selected target species based on sequence divergence, dsRNA needs to be screened and designed to be specific to a target gene or to target genes on a broad spectrum, closer to related species (Fletcher et al., 2020). To increase knockdown or have higher than the expected RNAi result, multiple targets can be selected for a single transcript to ensure variation within the target species is accounted for. A common application that can be used to evaluate off-target genes is the Basic Local Alignment Search Tool (BLAST), and it has been used to find contiguous matching sections of 17 nt or more in the genomes of interesting organisms (Fletcher et al., 2020). BLAST is a common approach for finding regions of identity, and for identifying the functionality of siRNA. Thereafter, a wide range of specialised programs can be used to design the sequences. These include siRNA-Finder (si-Fi), siDirect, dsCheck, and RNAi Designer by ThermoFisher (Luck et al., 2019).

dsRNA Uptake Mechanism

The use of RNAi to protect plants by suppressing essential gene function in pest species has been well documented in invasive weed *Mikania micrantha* (Mai et al. 2021). In this study, the genes encoding chlorophyll a/b-binding proteins were selected as targets of RNAi, based on high-throughput sequencing of *M. micrantha* transcriptome and bioinformatic analyses of sequence specificity. Three types of RNAi molecules, double-stranded RNA, RNAi nanomicrosphere, and short hairpin RNA (shRNA), with their corresponding short interfering RNA sequences were designed and synthesized for SIGS vector construction, from which each RNAi molecule was transcribed and extracted to be sprayed on *M. micrantha* leaves. Whereas water-treated control leaves remained green, leaves treated with RNAi molecules turned yellow and eventually wilted. Quantitative real-time PCR showed that the expression levels of target genes were significantly reduced in the RNAi-treated groups compared with those of the control, suggesting that all three types of RNAi herbicides effectively silenced the endogenous target genes, which are essential for the growth of *M. micrantha*. It identified a gene family encoding chlorophyll a/b-binding proteins that is important for the growth and development of *M. micrantha* and could serve as potential targets for controlling the spread of *M. micrantha.*

Delivery method

RNAi efficacy after cellular uptake had been a concern since the immune system and gut enzymes, mainly intestinal nucleases, digest dsRNA. Thereby affecting dsRNA activity as a pest control (Zhu & Palli 2020; Wei et al., 2022). This identified the need for a carrier system to protect the dsRNA from degradation and thus, the delivery system for dsRNA can play an important role in the success of RNAi activity. There is various delivery methods but spraying is better option for weed control. The spray-induced gene silencing (SIGS) delivery system which involves spraying of dsRNA that has been loaded onto plant surfaces (Zhang et al., 2022). A researchers reported that applying high-pressure spraying of GFP gene to plant can result in locally silencing of GFP gene (Dalakouras et al., 2016). These studies supported the idea that a specifically designed application system to different host or targeted gene could be required in some situations.

CHALLENGES AND FUTURE PROSPECTS

The excitement surrounding the development of RNAi for different agricultural applications has been building for years, with the full breadth of these potential benefits across many areas being thrown into sharp focus. Specifically, RNAi has the potential to replace conventional chemical pesticides with the ability to target genes selectively, and therefore only induce mortality in the target organism. As an emerging agricultural technology, RNAi has already shown its appealing capabilities in multiple areas. RNAi for developing sustainable agriculture has growing number of publications and patents (according to a search in the World Intellectual Property Organization database using the terms 'RNAi' and 'agriculture', more than 1000 patent applications have been lodged), yet very few agricultural applications have made it to market. Despite the potential of RNAi there are still many

challenges to be addressed to enable its application in the field. One important limitation in the development of regulatory framework for RNAi include difference in the efficacy of each dsRNA under environmental conditions. RNAi based products can be applied via some of the delivery methods described above. The exogenous spray application of RNAi as SIGS to improve plant health has the potential to become a fast- growing market. Despite existing framework available for regulatory assessment and approval of genetically modified plants, appropriate safety evaluations, and authorization procedures for SIGS-based products are less clear (Schutter et al., 2022). The degradation profile of each target dsRNA entering the environment needs to be extensively studied to assess the risk of unanticipated persistence (Bachman et al., 2020). The three environmental and highly researched microcosms of interest in this context are soil, surface water and sediment. Within soil foliar applied dsRNA degradation rate for DT50 (Time to 50% degradation) and DT90 (Time to 90% degradation) was <30 h and <35 h, respectively (Bachman et al., 2020). A researchers demonstrated that dsRNA remained stable in sterile water for three days. While this information lays a good foundation of dsRNA degradation in the ecosystem suggesting low risk of persistence (Fischer et al., 2017). As RNAi is a rapidly evolving field with most research focused on model organisms it is still not fully understood, making the design and implementation of regulations to be in constant flux. Safe consumption of dsRNAs by humans and other vertebrates holds a long history; as dsRNA and its processed products are natural components of food and feed (Davalos et al., 2019, OECD, 2020). Oral uptake of dsRNA or siRNA by humans or farm animals has been reported to have negligible impact due to degradation and multiple barriers in the gastrointestinal tract of mammals (Petrick et al., 2013, O Neill et al., 2011). The regulatory status of RNAi technology is different for each country. European Union (EU) regulations have been initially set on chemicals as active substances, no specific guidance documents defining the data requirements for the authorization of dsRNA-based application for plant protection. The Environmental Protection Authority (EPA) in New Zealand regulates RNAi under the Hazardous Substances and New Organisms (HSNO) Act. The New Zealand EPA and Decision-Making Committee decided that due to topical dsRNA lacking the ability to alter the genetic code, it is out of the scope of HSNO, and instead may be classed as a hazardous substance. However, EPA databases of hazardous chemical databases does not list dsRNA, making the regulations on dsRNA almost non-existent (Heinemann & Should 2019). In the United States, pesticide goods must be registered with the US Environmental Protection Agency (EPA) before they can be manufactured, transported, or sold (Leahy et al., 2014). In 2017, the EPA approved the first RNAi pesticide (Shaffer et al., 2020). SmartStax Pro is a GM maize seed that will use both transgenic insecticidal proteins and RNAi to combat western and northern corn rootworm (Head et al, 2017, Shaffer et al., 2020). In Australia, as of the 8 October 2019, the Gene Technology Regulations 2001 legislation was amended, to include a provision that explained that gene technology does not include approaches involving the introduction of RNA into an organism, if the RNA cannot be translated into a polypeptide, an infectious agent cannot be produced, or the organisms' genomic sequence is not altered as a result. If these conditions are met, the organism treated with dsRNA is not classified as a GMO under the Gene Technology Act of 2000 and is thus not regulated by the OTGR (Fletcher et al., 2020).

This existing data on RNAi research available to regulators to identify, assess, manage, and mitigate risks can help expand on traditional laws, biosafety regulations, market-based or economic regulatory schemes to create a specific niche assessment for non-GMO RNAi-products globally. The legal regulatory framework surrounding RNAi is looking more promising than ever due to the completed assessment to date and the regulatory framework already in places such as Australia regarding SIGS-products. While these outcomes have been focused on the plant/crop sector, they will have a profound impact on our knowledge of gene silencing and inform the way we apply this technology to control invasive weeds.

CONCLUSIONS

RNAi-based biological controls can offer high target specificity, non-GM, environmentally friendly strategy against weeds, insect and plant pathogens. As described above, the RNA-mediated management for weed is still in its infancy and substantial research focus will be required for it to reach its full potential. Additionally, RNAi applications also need to be explored and adopted from the regulatory and community acceptance aspects of this technology. If research is conducted diligently and thoroughly, encompassing bioinformatic identification, in silico best-design of RNAi effectors, and laboratory- and field-based RNAi toxicity studies, RNAi-based bioherbicides have the potential to revolutionize weed management in a safe, specific, and effective manner. For successful development of RNAi strategies to manage weed this issue must be addressed by understanding the movement of the dsRNA within to improve the penetration and persistence in the environment.

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