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SPECIAL ISSUE

OPINION

Seventy-five years of synthetic herbicide use in agriculture: Will there be 100?

RNAi as a tool for weed management: challenges and opportunities

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Abstract: 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. Here we present the main challenges and opportunities for using SIGS as a practical tool for weed management. 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. To overcome the challenges, 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. This 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.

Keywords: Crop protection; dsRNA; exogenous RNA, gene expression; siRNA.

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RNA interference (RNAi) is a powerful gene-silencing tool widely used in functional genomics and expression studies. In agriculture, the areas of crop protection and plant breeding are the most promising for practical use of this technology. So far, insect management has had the most significant advances in successfully utilizing RNAi as a tool in pest management (Zotti et al., 2018). In contrast, in weed science, the development has been slow, despite its potential use in controlling herbicide-resistant weeds and as an alternative tool to add to the integrated weed management programs.

RNAi action in a cell is a natural cellular process in eukaryotes, through which interfering RNAs can induce transcriptional and post-transcriptional gene silencing. This phenomenon was discovered in the 1990s when the overexpression of chalcone synthase in petals of transgenic *Petunia* spp. plants resulted in endogenous co-suppression of gene and transgene (Napoli et al., 1990). In 1998 with a *Caenorhabditis elegans* experiment, post-transcriptional silencing was found after injection of double-stranded RNAs (dsRNAs) in the cytosol (Fire et al., 1998).

Silencing is triggered by dsRNA presence in the cytoplasm (Bramlett et al., 2020). The dsRNAs are processed into small interfering RNAs (siRNAs) by DICER-type ribonucleases (DICER). siRNAs are unfolded and a strand is incorporated into a protein complex called RISC (RNA-induced silencing complex), where it serves as a guide to direct cleavage or translational repression of complementary mRNA (Dubrovina, Kiselev, 2019).

RNAi in crop protection has great potential. The demonstration that exogenously supplied RNA kills pests and pathogens, established the possibility that RNAi could maintain crop health (Koch, Kogel, 2017). Sprayable RNAi, known as spray-induced gene silencing (SIGS), has been proposed as a next-generation weed management method (Duke, Heap, 2017). The concept is to spray weeds with small RNAs (sRNA) that target mRNA of genes coding for 1) herbicide targets, 2) lethal phenotypes when transcription is reduced or eliminated by RNAi, and 3) normal growth and development (Figure 1) (Yoder et al., 2009; Duke, Heap, 2017). sRNAs would be designed to selectively target a weed or a group of related species (Westwood et al., 2018).

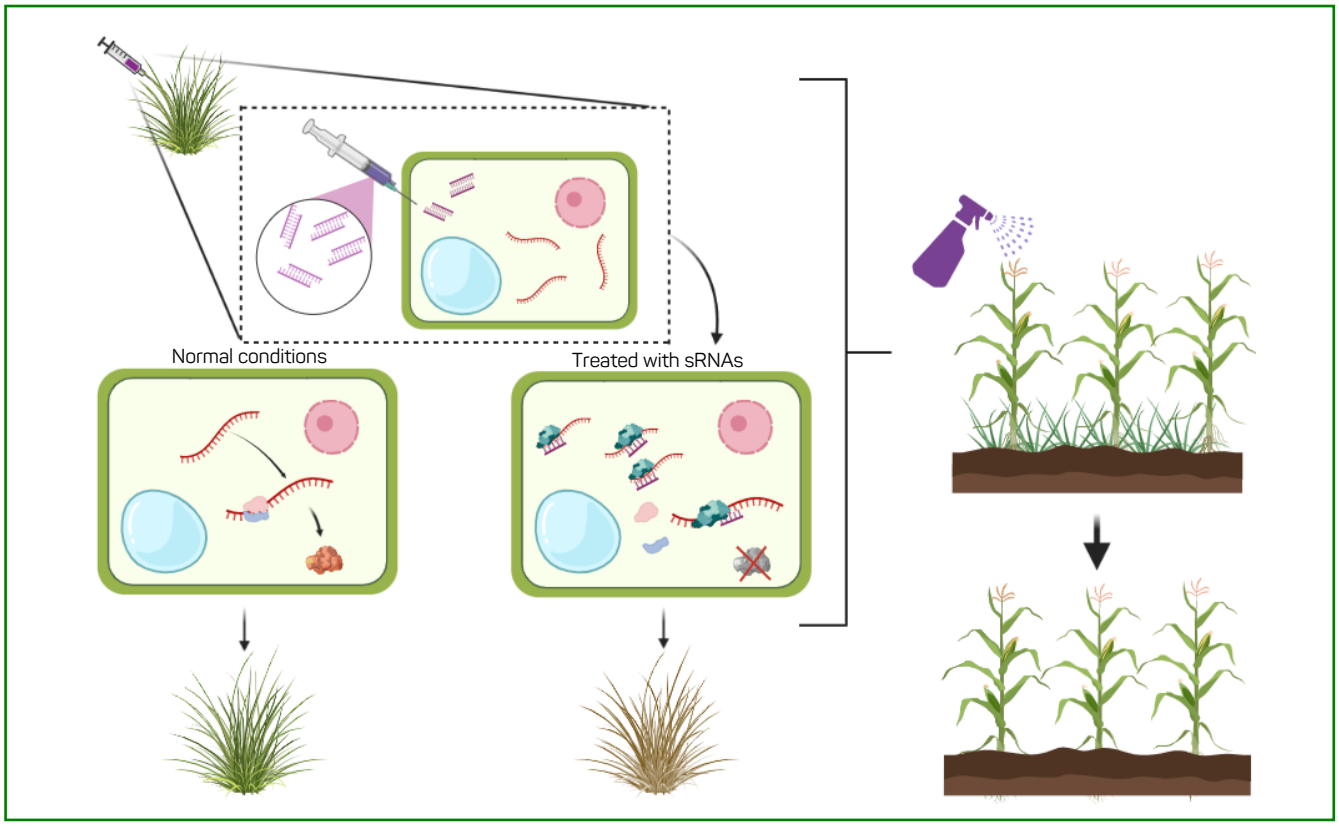

Figure 1 - Sprayable RNAi is a spray-induced gene silencing (SIGS) technology proposed for weed management. Weeds are sprayed with small RNAs (sRNA) that target mRNA of genes that are essential for plant survival. The mRNA targets and sRNAs must be specific to the weeds so as not to damage the crop. Created with BioRender.com

This new technology would broaden the spectrum beyond what has been achieved so far with chemical control (Figure 2). Besides silencing known herbicide targets, potential targets for herbicides that have not yet been developed due to various factors such as cost of goods or regulatory concerns could also be silenced. In resistance management, genes conferring resistance through increased gene expression and even those causing metabolic detoxification in non-target-site resistance would be additional objectives. New targets can also be explored, related to flowering, sterility, embryo formation, dormancy, and shattering. For parasitic plants, host-induced gene silencing (HIGS) has been discussed, which involves sRNAs synthesis in the host, directed to the genes from parasitic plants that interact with the crop (Wang, Jin, 2017; Tomilov et al., 2008). However, this would be beyond the transgenic-free approach enabled by SIGS.

Although RNAi has had important advances in plant pathology and entomology, its development in weed science has been limited. In 2011, Sammons et al. published a patent using SIGS to reverse glyphosate resistance in *Amaranthus palmeri* by silencing the increased expression of *EPSPS*, thereby restoring glyphosate as a control tool, in this and other weeds. The research included different targets, sRNAs sizes, and delivery methods. More recent SIGS publications have quantified endogenous and systemic silencing of reporter transgenes such as Green Fluorescent Protein (GFP) and Neomycin Phosphotransferase II (NPTII) (Hendrix et al., 2021; Dubrovina et al., 2020). Nevertheless, the current literature lacks studies that demonstrate the silencing of highly expressed endogenous genes in weeds.

In our opinion, there are four main reasons why RNAi technology for weed control has not advanced at the same speed as other crop protection areas: 1. The stability and delivery of sRNAs inside plants; 2. The close relatedness of many weeds with crops, at the genus or even species level, creates challenges to identify weed-species specific regions of genes to target; 3. Availability of sequenced and annotated genomes for weeds; and 4. sRNAs production costs.

The development of SIGS formulation should guarantee sRNAs delivery into the cytoplasm, overcoming foliar barriers (cuticle, cell wall), avoiding nuclease attack, and preserving RNA duplex structure, key in RNAi activation. Nanostructures for delivery of siRNAs have been widely studied in medicine and mammals and could be an alternative for delivery in weeds. Experiments with clay nanosheets and carbon dots protected nucleic acids from nucleases attack and favored siRNA stability and delivery in plants (Mitter et al., 2017; Schwartz et al., 2020; Bennett et al., 2020). In subsequent investigations,

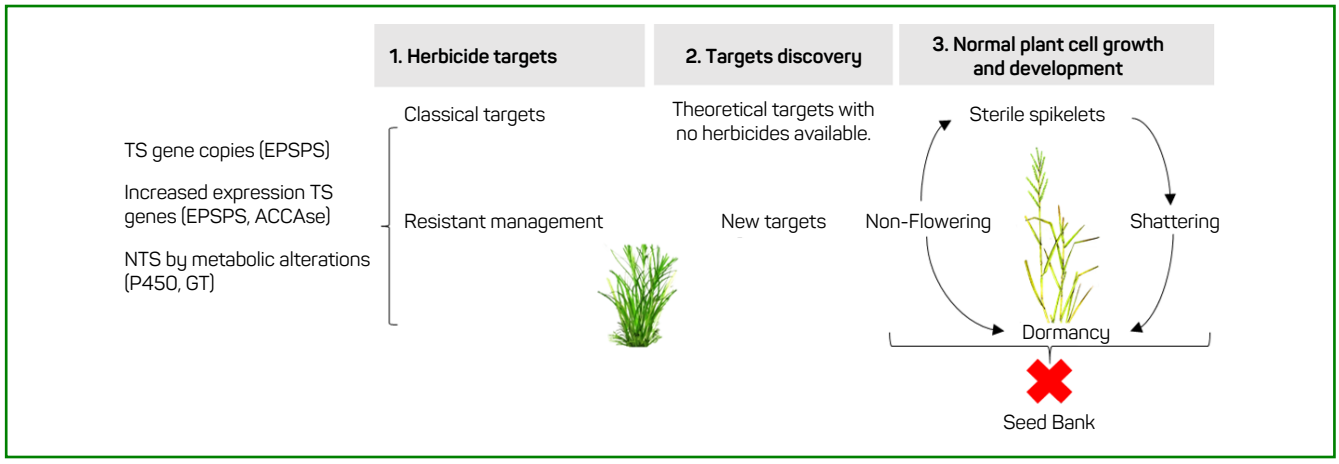

Figure 2 - RNAi targets for weed management. TS: Target site. NTS: Non-target site.

DNA-nanostructures were developed to hybridize with nucleic acids (DNA, ssDNA, and siRNAs). The shape, size, compactness, and stiffness allow siRNAs entry into the cytosol and improve silencing efficiency (Zhang et al., 2019). Even nanostructure geometry determines whether silencing occurs mainly due to mRNA cleavage or only translation inhibition.

Due to weeds and crops co-evolution, genetic similarity is crucial to sRNA design. dsRNAs can silence target genes but also paralogous genes if sequence homology is high (Zotti et al., 2018). On weeds, this could amplify silencing efficiency, because more than one gene could be silenced, but also cause orthologous genes silencing in closely related species, including crops. Hence, siRNAs are a more feasible alternative in weeds. They have more specificity, perfect complementarity, and substantially reduce off-target risk. This also means less stability and efficiency than dsRNAs. siRNA size is also critical to induce transitivity. In transitivity, a group of primary siRNAs induces a secondary siRNAs production as a self-reinforcing feedback loop amplifying the initial silencing signal (Vaistij et al., 2002; Dadami et al., 2014). Transitivity is necessary to achieve a systemic effect (Dalakouras, Papadopoulou, 2020).

For accurate sRNA design, genomes, transcriptomes, and software are required. siRNAs require a careful design that includes chemical modifications, such as methylation and phosphorylation, the key to protection against nucleases until they reach the cytosol. Likewise, transcriptome databases are required to evaluate off-target risk, not only for target species but also in non-target species. In the past due to sequencing costs, the investment in model plants and crops was prioritized over weeds. Fortunately, today, the International Weed Genomics Consortium (IWGC) is looking to cover the gap, grouping globally resources and researchers to develop genomic tools and resources for weed science research (Ravet et al., 2018).

Finally, historically the RNAi technology has had high producing costs ($12,500/g of dsRNA, a decade ago). In recent years, dsRNAs production in bacteria, yeast, and cell-free mass systems has reduced costs (Mezzetti et al., 2020). Indeed, start-up companies, like GreenLight Biosciences, RNAgri, and AgroRNA (Genolution) have developed cell-free bioprocessing platforms capable of producing sRNAs, in large-scale, at a cost of less than $0.50/g; compared to fermentation ($1/g), *in vitro* transcription ($1,000/g), and chemical synthesis ($100,000/g) (Taning et al., 2020). However, high throughput technologies for sRNA synthesis still need to be improved to make SIGS applications possible in the field. We do not have knowledge of whether major crop protection companies are actively working on SIGS for weed management. Several startup companies have made public indications of their work on SIGS in agriculture for pest management, such as RNAissance, NanoSUR, Greenlight Biosciences, and Trillium Ag. The primary focus for these companies appears to be SIGS for insect and disease management, but their approaches will also be applicable for weed management.

SIGS in weed science has many more barriers to overcome than other crop protection areas. For example, insects feed on RNAi triggers sprayed on plants, reducing the complexity of cellular uptake relative to plants. Sprayable sRNAs have to be in a stable formulation that enables delivery across leaf cuticle and cell walls to the target. Genetic differentiation between insects and plants is much higher than between weed species and crops, providing a much greater opportunity for selectivity in SIGS. However, SIGS still promises to be a next-generation technology for weed control (Figure 3). This tool is completely new and used in combination with other management practices that could support integrated weed management. To achieve the practical use of SIGS we will need to use the developments made in other areas even beyond plant science such as nanoparticle chemistry, formulations, and design tools developed in medicine, and strengthen the research and development of molecular tools in weed science.

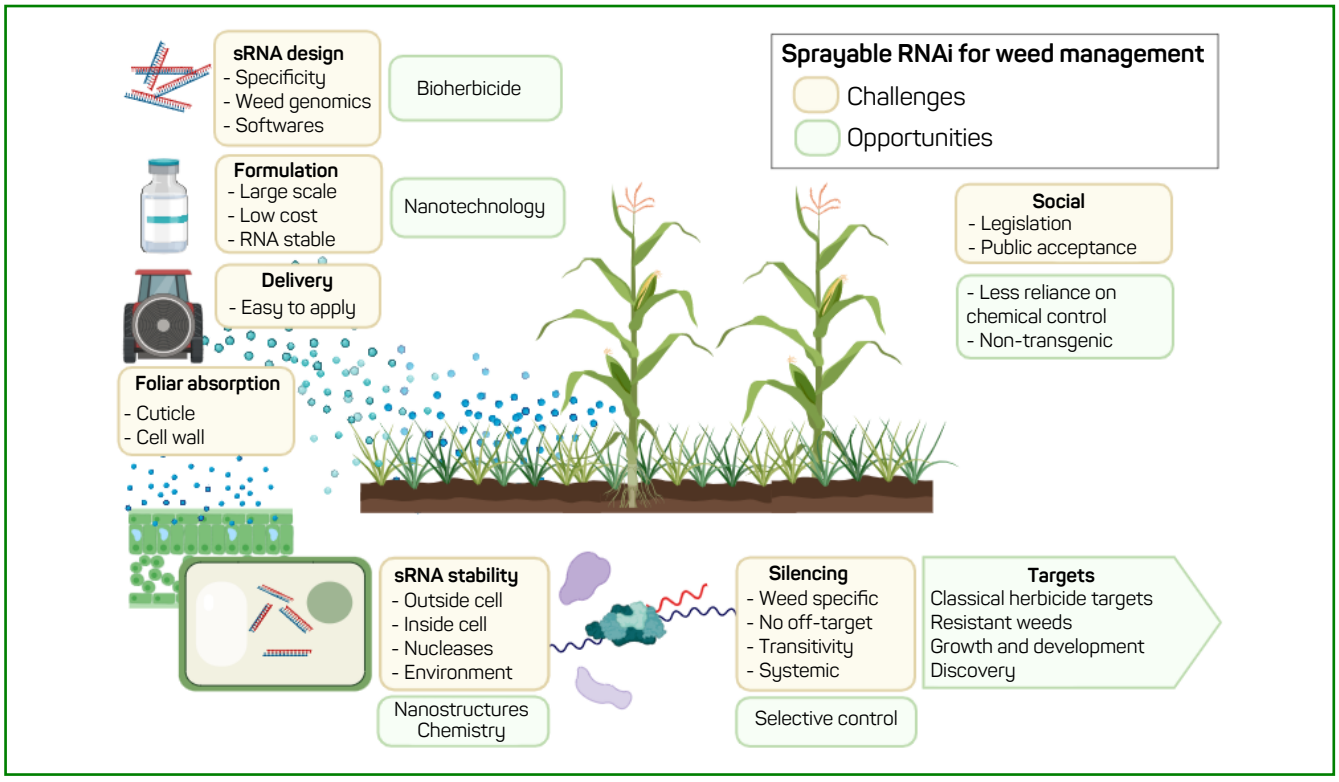

Figure 3 - Main challenges and opportunities of RNAi technology, to make feasible its use as a sprayable tool for weed management. Created with BioRender.com

Conflict of Interest

The authors have no conflict of interest to declare regarding the review.

Author's contributions

All authors read and agreed to the published version of the manuscript. LAA, TG, FPL, and DZ: conceptualization of the manuscript. DZ, LAA, FPL, and TG: writing the original draft of the manuscript. DZ, FPL, LAA, and TG: writing, review, and editing.

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