Non-destructive chemical control of *Microstegium vimineum* using RNAi technology

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The invasive species *Microstegium vimineum*, known colloquially as Japanese stiltgrass, is prevalent in many regions across the United States and has been found all along the East Coast. This grass was introduced to Tennessee around 1918 as a result of its frequent use as packaging for porcelain imports from China. *M. vimineum* is a monocotyledon (or monocot) in the Poaceae family, and an annual, shade-tolerant plant with a mixed mating system that produces 100–1,000 seeds. These remain viable in the soil for more than five years, and the species aggressively expands into forest land, crowding out native grasses and damaging the ecosystem. Currently, removal of invasive *M. vimineum* is only possible through chemical control or manual extraction, both of which negatively impact the surrounding plant life. A possible alternative, RNA interference (RNAi) technology, has already been proven effective in amplifying and silencing genes in other monocots such as corn, asparagus, and onions. In this project, the RNAi approach will be used to silence the cytochrome b6f complex—a critical component of the photosynthetic pathway—in M. vimineum, resulting in irreparable damage to the plant. Because of the selective nature of this technology, it will be possible to significantly reduce *M. vimineum* populations without causing excessive damage to the surrounding ecosystem.

Keywords RNA interference, *Microstegium vimineum*, plasmid, cytochrome b6f complex, invasive species

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Background

The invasive species *Microstegium vimineum*, also known as Japanese stiltgrass, is characterized by a single stalk with several offshooting leaves. It grows to a typical height of three feet, but can reach up to six feet. The leaves are thin, 3–4 inches long, and spaced apart on the stalk with an off-center silver rib. The roots are thin, so the plant can be uprooted easily. *M. vimineum* is a monocotyledon (or monocot) in the Poaceae family, and an annual, shade-tolerant plant with a mixed mating system that produces 100–1,000 seeds which remain viable in the soil for more than five years. It prefers damp, shaded environments near stream banks that are prone to regular flooding, but it can grow in almost any environment and is sometimes found on the edges of forests and roads. *M. vimineum* is widely prevalent in many regions across the United States, especially along the East Coast. The grass was historically used as packaging material for a variety of imports from Asia, and was first identified in the wild in Tennessee around 1918 (Swearingen & Bargeron, 2016).

Japanese stiltgrass aggressively expands into forest land, crowding out native grasses and damaging the existing ecosystem. This is due to its rapid growth cycle, which impairs the photosynthesis of smaller and slower-growing native plant species. Currently, removal of invasive *M. vimineum* is only possible through chemical control or manual extraction, both of which negatively impact the surrounding plant life. However, RNA interference (RNAi) is known to be effective in amplifying and silencing genes in other monocots such as corn, asparagus, and onions. In this project, RNAi technology will be used to silence the cytochrome b6f complex, which mediates electron transfer between photosystem II and photosystem I in *M. vimineum.* This is a key component of the photosynthetic pathway, such that its inhibition will result in irreparable damage to the plant by blocking photosynthesis and effectively shutting down its growth. Because of the selective nature of this technology, it will be possible to significantly reduce *M. vimineum* populations without causing excessive damage to the ecosystem.

Systems level

We postulated that a gene affecting photosynthesis would be the most effective target for controlling the spread of Japanese stiltgrass. The complete genome of *M. vimineum* is available in the well-known National Center for Biotechnology Information (NCBI) database ("Nucleotide," 2021). The decision to focus on depleting proteins involved in photosynthesis narrowed our search for possible gene targets to those associated with photosynthetic pathways. After analyzing the genome, we identified the *petN* gene, which codes for subunit 8 of the cytochrome b6f complex, as a suitable candidate.

A BLAST analysis was run on *petN* in order to determine the similarity between the *M. vimineum* gene and that of other plants in the surrounding ecosystem (Zhang et al., 2000). It was found that the gene was moderately conserved, meaning the genetic sequence varied by a single base pair among monocotyledon species. However, the species which had 100% alignment of this gene are located in Asia and Africa, easing concern over potential off-target inhibition within U.S. ecosystems.

Using the gene sequence for *petN,* target sequences for RNAi were identified. The targeting oligonucleotide (oligo) sequences necessary to produce the designed amiRNA (vide infra) will be synthesized commercially by IDT. These oligo sequences will then be inserted into a plasmid containing a T7 promoter. The resulting plasmid construct will be amplified using the HiScribe T7 High Yield RNA Synthesis Kit (New England BioLabs) to synthesize the desired RNAi construct by in vitro transcription. This RNA will then be inserted into Japanese stiltgrass through pinpricks created on the leaf's surface. Following this, the Japanese stiltgrass will be observed to determine the effectiveness of the technology.

Device level

Once the gene *petN* was chosen as our target, the FASTA sequence was identified using the NCBI website, as outlined above. This sequence was used as an input for the amiRNA Designer, a part of the Plant Small RNA Maker Suite (P-SAMS) website. This program can design

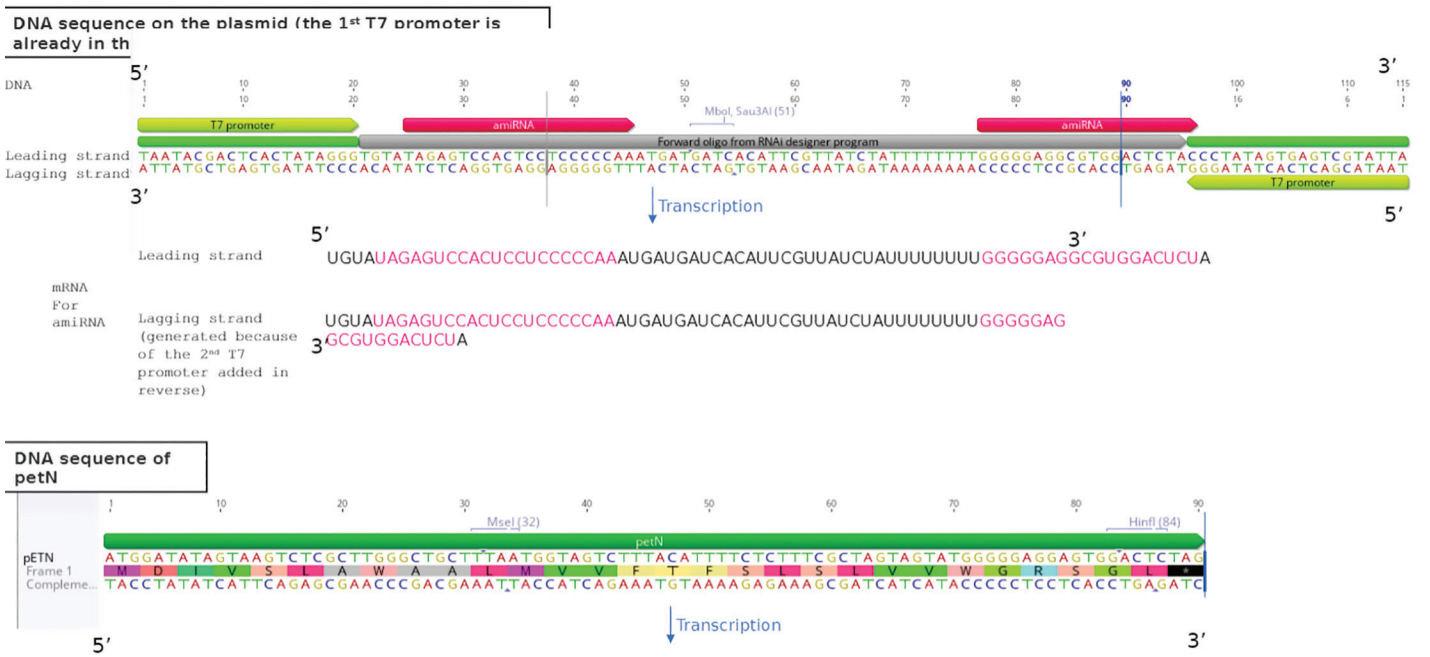

Figure 1. *Target sequence generated by the P-SAMS amiRNA Designer.*

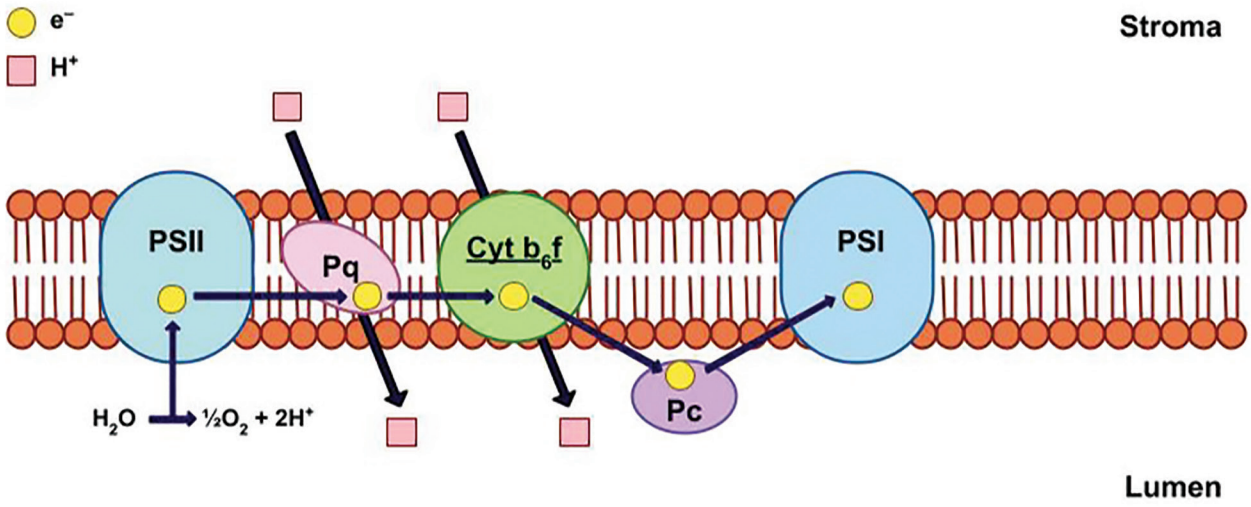

Figure 2. *Schematic illustrating the function of the cytochrome b6f complex in the process of photosynthesis.*

artificial microRNA (amiRNA) constructs for targeted gene knockdown in plants. It identifies possible target sites suitable for the use of RNAi, filters the sites based on a variety of factors, and produces a list of the genetic sequence of three predicted optimal target sites within the chosen gene (in this case, *petN*) to use for constructing the amiRNA. Following this process, the amiRNA Optimal Result 1 was chosen from the suggested options. This target sequence, shown in Figure 1, was then used to construct oligo sequences using IDT Oligo Builder.

In order to amplify a sufficient quantity of RNA molecules to insert into the plant, the HiScribe T7 High Yield RNA Synthesis Kit will be used, as mentioned above. In order to use this kit, the target sequence must first be inserted into a plasmid under the control of a T7 promoter, because the kit uses T7 RNA polymerase to transcribe

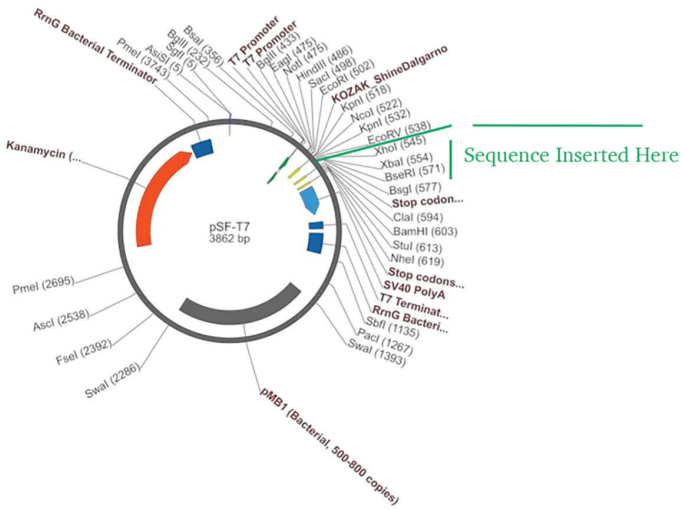

Figure 3. *Vector map of the pSF-T7 plasmid, showing the location in which the generated sequence will be inserted.*

RNA. The expression vector chosen was the pSF-T7 plasmid from Sigma-Aldrich, which contains the required T7 promoter.

After the plasmid and synthesized oligo sequences are in hand, the two oligos will be annealed to create the required double-stranded insert. Upon completion of this step, a restriction digest and ligation will be performed to insert the target sequence into the expression vector. Further details of this step are explained below. Following this, the in vitro transcription kit will be used to synthesize RNA sequences complementary to the template strand of the target insert.

It is unknown whether the inserted RNAi sequence will bind to complementary mRNA within the stiltgrass and simply block translation, or alternatively, if the insertion of this exogenous RNA may activate an RNA-induced silencing complex (RISC) within the targeted region. Both mechanisms are possible. RISC is a ribonucleoprotein complex that silences target mRNA sequences by activating various members of the complex—such as Dicer, transactivating response RNA-binding protein (TRBP), and Argonaute proteins—to interfere with mRNA function. The RISC member interacting with the inserted amiRNA is Dicer, an RNase III endonuclease that processes pre-microRNA (pre-miRNA) into mature miRNAs (Zhang et al., 2018). If the insertion of exogenous RNA into *M. vimineum* induces RISC, Dicer will react, causing enzymatic cleavage of the stiltgrass *petN* mRNA and inhibiting translation of the protein.

The most efficient method of implementation of the designed RNAi technology is currently under investigation. One method under consideration is particle bombardment with siRNA/transgenics, which has been used on plants in both the monocotyledon and dicotyledon families (Agrawal et al., 2003).

Particle bombardment is a process in which metal microparticles, coated with nucleic acids or a solution containing RNAi, are forcefully propelled at the target plants (Lacroix & Citovsky, 2020). Although this method has been proven effective, it is not realistic for use in our context and is better suited for larger implementations such as farming and agriculture. One alternative approach, which is similar in concept to the particle bombardment method, is the pinprick method. This involves using a pin to prick small holes into the target plant, creating a pathway for the RNAi solution to enter the cell. The RNAi solution is then dropped atop the holes with a micropipette. This method is a smaller-scale application of the particle bombardment method; however, it separates the destructive aspect of particle bombardment, the pinprick, from the application of the RNAi solution by micropipette.

Parts level

The plastoquinol-cytochrome c553/plastocyanin oxidoreductase, or the cytochrome b6f complex, is extremely important for light-dependent reactions in oxygenic photosynthesis. When an electron leaves photosystem II (PSII), it is transferred to plastoquinone (Pq), a mobile electron carrier, then to the cytochrome b6f complex, as shown in Figure 2 (OpenStax, 2015). At the Q0-site of the b6f complex, Pq is oxidized into plastoquinol (Tikhonov, 2014). Following this, the electron moves to another protein, plastocyanin (Pc), which transfers it to photosystem I (PSI). As electrons move from protein to protein between PSII and PSI, they lose energy. That energy is used to move hydrogen atoms into the thylakoid lumen that will later be used in the chemiosmotic synthesis of ATP.

A previous study conducted by Hager et al. (1999) reported that knockout of petN leads to the complete loss of function of the cytochrome b6f complex's role in photosynthesis, abrogating all photosynthetic activity. The exact reason for this is not currently understood. One theory is that shutdown of the cytochrome b6f complex in the electron transport chain causes a buildup of free radicals. These free radicals are highly reactive molecules with an unpaired and high-energy electron, which can damage the cell by breaking the bonds of other molecules (Wu et al., 2013).

As explained already, the FASTA sequence of the *petN* gene was retrieved from the NCBI database, then used to generate a targeting RNAi sequence by employing the amiRNA Designer on the P-SAMS website. This program generated three optimal target sites in the *petN* gene for RNAi, of which the first result was chosen. The target sequence is shown in Figure 1.

When designing a DNA oligo from which to transcribe the desired RNA, it was important to include blunt-end sequences for an appropriate restriction enzyme. The EcoRV enzyme was chosen due to its short recognition motif and high specificity for the target sequence (Pingoud & Jeltsch, 2001). It was also important to select a plasmid that already contained both a forward and reverse T7 promoter, as this would allow for the intended in vitro transcription protocol to generate the desired RNA without adding additional cost to the DNA oligo. The plasmid chosen was sourced from Sigma–Aldrich, and is shown in Figure 3 along with the cut location (EcoRV restriction site). By ensuring that additions to the code were minimal, the length of the oligo was kept under 100 bp, which improved the stability of the molecule and allowed costly purification processes to be omitted.

Safety

There exists a valid concern about the potential for the inserted RNA construct to exhibit the same inhibitory effect on other plant species, especially those with sequences which are highly conserved with *M. vimineum.* However, because the target species is invasive and not genetically influenced by the local ecosystem, a system designed specifically for its genome is unlikely to match local species as well. When BLAST analysis was conducted on the *petN* gene, the most genetically similar species were found to be uniquely present in Northeast Asia. Additionally, our targeting RNA strands were designed with no protection from environmental damage, so the risk of spread is minimal. While the possibility of 'freak' mutations and other unforeseen effects must be taken into account with any real-world genetic manipulation, similar RNAi modifications have been made to wild and domestic plant species, often at significantly larger scale than the process described in this paper, without any adverse effects on the ecosystem in which they were implemented (Vélez et al., 2020).

Discussions

After it was determined that the gene *petN* would be the most effective target for controlling the spread of Japanese stiltgrass, research was done to ensure that there would be minimal harm done to the environment if the proposed RNAi technology was released. Using the gene sequence for *petN*, target RNAi sequences were designed and used to construct oligo sequences with flanking EcoRV sites to enable cloning into the chosen destination vector. The plasmid, synthesized oligos, and in vitro transcription kit were all sourced commercially as detailed above.

To begin the process of RNA synthesis, a sufficient quantity of vector ligated with the synthesized oligo

insert was needed. The forward and reverse sequences were received from the supplier (IDT) with yields of 0.11 mg and 0.13 mg respectively. The sequences were annealed overnight, resulting in a theoretical 0.22 mg of DNA duplex. Unfortunately, our team did not have sufficient technical resources to properly verify the yield of this step, due to the prohibitive cost of suitable spectrophotometers and other relevant equipment.

A restriction digest with plasmid pSF-T7 was done to cut the DNA, allowing the insert (annealed oligos) to be added later. The digest was completed by pipetting 1 µL of plasmid DNA, 5 µL of 10× reaction buffer, 1 µL of EcoRV, and 43 µL of nuclease-free water into a PCR tube for a total volume of 50 µL. This process was completed four times in total. Once mixed, the tubes were incubated in a 37 °C water bath for 15 min. They were then placed in an 80 °C water bath for 20 min to heat inactivate the EcoRV enzyme. Another restriction digest was performed using the same procedure with the annealed DNA, and this was also completed a total of four times.

Following the restriction digests, gel electrophoresis was performed using the four reaction products and the undigested plasmid. In theory, the undigested plasmid should travel farther through the gel than the other samples. The results, pictured below (Figure 4), seem to show that the digest was not successful. Shortly after dying, the bands of color dissipated, making it impossible to analyze the gel. It is not currently known why this occurred, but it is possible that the loading dye used was too old. Another gel was run prior to this, but the results were not able to be photographed before the bands disappeared. A further gel was not run for the digested DNA due to limited resources.

Nevertheless, following completion of the DNA restriction digest, the digested plasmid vector and digested DNA insert were ligated together. The ligation reaction was performed by combining 2 µL NTP buffer, 50 ng vector DNA, 2.5 µL annealed DNA insert, 20 µL nuclease-free water, and 1 µL ligase in a microcentrifuge tube, then incubating at room temperature for 2 h. Following incubation, the ligase was heat inactivated in a 65 °C water bath for 10 min. Although the steps clearly outlined in the procedure were directly followed, it is unclear if the ligation was successful. Confirmation of ligation through gel electrophoresis or usage of a spectrophotometer was not possible due to a lack of the highly specific resources required, and a limited amount of funding.

After completion of the ligation process, it was necessary to use the HiScribe High Yield RNA Synthesis Kit, provided by NEB, to amplify the RNA required for *petN* gene targeting in *M. vimineum*. The kit protocol specifies a quantity of 1 µg of template sequence in approximately 2.5 µL of solution for peak yield (0.4 µg/µL); however, the theoretical concentration of DNA derived from the process described above was estimated as 0.0025 µg/µL. This made it impossible to maintain the prescribed concentration of other components in the reaction.

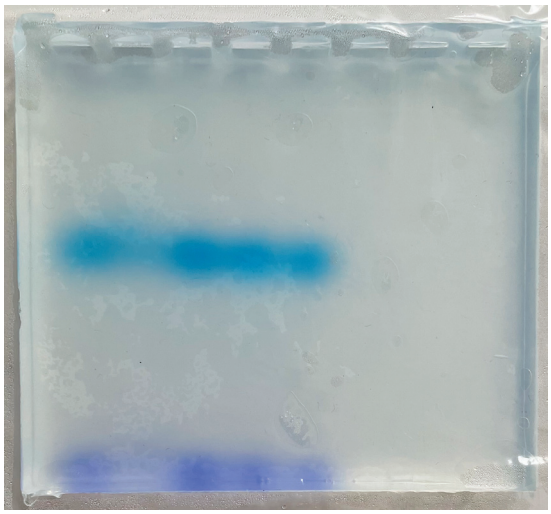

Figure 4. *An image of the completed gel. Wells 1–4 are the digested plasmid samples and well 5 is the undigested plasmid.*

In the end, the in vitro transcription reaction had a total volume of approximately 90 µL, and we decided to attempt the procedure using 0.2 µg of DNA with the standard quantities of T7 RNA polymerase and NTP buffer. There was a concern that the low concentration of template DNA available would make it difficult to achieve a high RNA yield. In addition, there was difficulty in deciding the most efficient transformation method to transfer the plasmid into Japanese stiltgrass (Figure 5).

Figure 5. *Image of the stiltgrass that will be used to test the RNA interference procedure.*

In general, limited resources proved to be a problem the team encountered when working on this project. There are several improvements that could be made to this procedure with additional time and resources. One significant concern throughout was the likelihood of excess DNA and other byproducts accumulating throughout the process and interfering with later steps. Choosing a plasmid with fewer base pairs between the T7 promoter and terminator, increasing the concentration of desired DNA, and removing unwanted fragments and/or byproducts via suitable purification procedures, are all measures which could have been taken to minimize this risk.

Next steps

While RNAi technology has already been extensively proven effective in similar applications (Mai et al., 2021), there are a variety of variables to be tested in the lab for this project. The cytochrome b6f complex has a significant role in productive photosynthesis, but there could be more potent targets yet to be discovered, and there will always be a measure of trial and error in locating the ideal gene to disrupt. Additionally, there is insufficient research regarding the method of physical delivery of RNAi sequences, especially in plants; several mechanisms of delivery, such as spraying RNA onto the leaves of *M. vimineum* or introducing it through the soil, are yet to be explored.

An efficient continuation of the research within this paper would begin with real-world testing of the proposed design using live *M. vimineum* specimens. Various methods of delivery could be tested consecutively, and should these transformations prove largely ineffective, an alternative gene could be chosen. For example, *psbA*, which codes for photosystem II protein D1, was also considered as a target in this work. Should the proposed system be effective as theorized, researchers could focus on probing the limits of delivery: Can the sequence be inaugurated into the soil before the plant sprouts to inhibit growth? How often must it be reapplied to remain functional? As the ultimate use case of the system is for large-scale invasive species control, some consideration must also be given to the ease of use and commercial viability of RNAi as a herbicide, including storage and transport stability as well as efficient methods of production.

Author contributions

All authors collaborated to determine the focus of the paper and set goals for research and project scope. T.G. researched the history of RNAi in plants and pre-existing methods for control of *M. vimineum*, as well as designing the plasmid and DNA oligonucleotide. H.G. determined target sites for RNAi using the website P-SAMS, researched the cellular response of *M. vimineum* to RNAi insertion, and investigated potential delivery methods. K.T. identified the target gene, *petN*, by genome searching to ensure selectivity for the target species, and researched relevant aspects of the cytochrome b6f complex. S.L. conducted extensive background research on *M. vimineum* (invasion region, characteristics, life cycle, preferred biosphere, and drawbacks of traditional extermination methods).

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