Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 12 Article 9

Fall 2011

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A novel gene silencing vector for plant genomics and biotechnology

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ABSTRACT

Gene silencing is a process of suppressing activity of specific genes by producing "interfering" RNA encoded by foreign genes. This process serves as the principle of genetic modification in plants and animals, which is an important tool in genomics and biotechnology, allowing scientists to manipulate organisms to better meet human demands. New approaches of gene silencing may enable improvements on current practices of genetic modification, and broaden the application and impact of gene silencing in biotechnology. Recently, a novel vector design consisting of the transcription of short gene fragments lacking transcription termination signals was demonstrated to be effective in partial silencing of two separate genes in the model plant, *Arabidopsis thaliana*. To test the efficacy of this unterminated transgene technique on a broader range of genes in *A. thaliana*, a DNA vector to clone gene fragments was required. The objective of the present study was to design a silencing vector for rapid cloning of gene fragments and test its utility on new genes. Here, we report the successful construction of a simple transgene vector, pSJN15A, for cloning gene fragments, then plant transformation upon *Agrobacterium* infection. The pSJN15A vector was designed for direct cloning of gene fragments obtained by polymerase chain reaction. Transcription of gene fragments is directed by read-through activity of a hygromycin resistance gene promoter. The pSJN15A vector was used to develop silencing vectors against four new *Arabidopsis* genes. Thus, pSJN15A serves as an important DNA resource for testing the efficacy of silencing mediated by the transcription of gene fragments in various dicotyledonous plant species.

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MEET THE STUDENT-AUTHOR

Dustin Hoover

I grew up in Conway, Ark., and graduated from Conway High School in 2007. That fall, I began to pursue a biology degree at the University of Arkansas, where I soon became a member of Alpha Epsilon Delta and Sigma Alpha Epsilon Fraternity. Over the duration of my attendance here, I have enjoyed attending football and baseball games and have developed an appreciation of the U of A student on-campus community.

With the tremendous help of Dr. Vibha Srivastava and Scott Nicholson (department of crop, soil and environmental sciences) I conducted an undergraduate honors research project funded by the University of Arkansas Honors College. I have also worked in Dr. Srivastava's lab as a technician. After earning my bachelor's degree, I will be attending medical school at the University of Arkansas for Medical Sciences. I would like to thank all my family and friends for their continued support.

INTRODUCTION

The field of biotechnology holds new importance for the growing human population in a global economy. Biotech applications will allow for the improvement of crops dedicated for food, feed, and bioenergy. One example of such applications is gene silencing. The cellular process of gene silencing has been used to develop molecular tools necessary to change the appearances and functions of organisms that humans depend upon for food, energy, and natural resources. By selectively eliminating or suppressing the expression of even one gene, scientists can produce favorable changes within an organism to better suit the diverse demands of societies around the world (Wang and Waterhouse, 2002). Gene silencing is also considered an upcoming platform for drug development and medical applications (Bernards, 2006). The research of this project investigates the possibility of partially silencing the expression of a gene, instead of completely abolishing expression. This new application would serve as a useful alternative tool to engineer new traits in plants for the future and current demands of food and energy.

Some of the first observations of successful gene silencing occurred when scientists introduced copies of a native gene sequence into petunias using a powerful promoter. What the scientists did not know at the time was that they were observing the effects of gene silencing, later termed as RNA interference (RNAi). The RNAi process begins with double-stranded RNA (dsRNA), which is processed into 21-24 nucleotide short interfering RNA duplexes (siRNA). These siRNA help guide a RNA-Induced Silencing Complex (RISC) to direct the degradation of homologous mRNA sequences (Baulcombe and Hamilton, 1999). Once these mRNA sequences are eliminated, the genetic information cannot be translated or expressed (Meister and Tuschl, 2004). Since the RNAi process occurs after the endogenous DNA has been transcribed, RNAi is also referred to as post transcriptional gene silencing (PTGS) (Vaucheret et al. 2001). Post transcriptional gene silencing via RNAi has previously been initiated through various methods. Studies have shown that injection of dsRNA in the form of sense and antisense RNA strands can lead to successful silencing in insects and nematodes (Fire et al., 1998). The most common silencing methods utilize a transgene coding for the expression of a "hair-pin loop" of DNA, a self-complementary strand that is processed into siRNA (Meister and Tuschl, 2004). These loop sequences arise after their sequences are incorporated into the organism's genome, along with a strong promoter sequence. The promoter ensures expression of the sequence and its consequential effects. The silencing demonstrated by hair-pin loops is long-term, as the vector continues to express the fold-back structure generating dsRNA (Fire et al., 1998). Expression of hairpin DNA structures (also called an inverted-repeat structure) is the most popular approach for inducing gene silencing in plants. However, such transgene constructs usually generate extreme levels of gene silencing leading to complete elimination of the target mRNA (knock-out).

For some applications, partial silencing (gene knockdown) of the target gene is a more desirable outcome. Therefore, alternative approaches must be explored. Recent research demonstrates the effectiveness of a single transgene construct, which lacks a transcription termination signal, as a partial silencer (Nicholson and Srivastava, 2006). This transgene construct is referred to as an "unterminated" construct hereafter. The study reported effective silencing of the Phytochrome A (*PHYA*) and Phytochrome B (*PHYB*) genes in *Arabidopsis* lines transformed with the unterminated *PHYA* or *PHYB* constructs. The silenced lines exhibited characteristic mutant phenotypes indicating successful modification of the target gene activity (Nicholson and Srivastava, 2009). Additionally, the findings indicated that unterminated transgenes do not produce a complete knockout of the targeted gene, but instead produce moderate to severe phenotypic suppression among primary transformants (Srivastava, pers. comm.).

The objective of our research was to develop a reliable model with which to apply this new transgene construct and then test the efficiency of the approach on several genes in *Arabidopsis*. The development and testing of an original model entailed: 1) construction of plasmids containing truncated *Arabidopsis* genes of interest; 2) verification of correct plasmid sequence; and 3) analysis of the silencing efficiency of the plasmid through introduction to Arabidopsis thaliana individuals.

MATERIALS AND METHODS

The DNA Vector Construction. The plasmid pPZP200 was used to construct the silencing vectors (Hajdukiewicz et al., 1994). This plasmid includes sequences for spectinomycin resistance and a T-DNA region. The T-DNA region is the section of DNA that would be incorporated into the plant's genome upon *Agrobacterium* infection. The pPZP200 plasmid was modified to include, within its T-DNA region, a hygromycin resistance gene driven by a 35S promoter and terminated by a *nos3*' transcription terminator. The pPZP200 plasmid was cut with restriction enzyme *XbaI* while suspended in autoclaved deionized water and the appropriate buffer in a 1.5-mL eppendorf tube. Incubation time was 1.5 hours. Temperature was set at 37 °C. The tubes were mechanically rotated while incubation took place. The 35S promoter sequence was extracted from pHPT using the same *XbaI* enzyme, then ligated into pPZP200 at the *XbaI* site. DNA fragments were ligated in a 1.5-mL eppendorf tube overnight at 16 °C using T4 DNA Ligase in autoclaved deionized water and the appropriate buffer. The resulting plasmid was named "pSJN15A" (Fig. 1).

To ensure ligation occurred as desired, pSJN15A was checked for correctness twice using restriction enzymes. In the first test, *EcoRI* was used to cut the plasmid in two places. These cuts would produce fragments of 0.75 and 8.0-kilobases in a correct ligation, while an incorrect ligation would yield fragments of 1.25 and 7.5-kilobases. The second trial test was based on cutting pSJN15A with *HindIII* and *Eco*RV. The expected fragments for correct ligation here were 1.65 and 7.1-kilobases, with 0.35 and 8.4-kilobase fragments representing an incorrect ligation. Directly downstream of the *XbaI* site within this plasmid, there is a *HindIII* restriction site where transgene fragments lacking a polyadenylated termination sequence were inserted to complete the plasmid as a silencing vector.

Truncated sequences of *Arabidopsis* genes were created for the purpose of testing the silencing efficiency of unterminated transgenes. DNA primer pairs were designed for the PCR amplification of 500-bp fragments of genes *Var*2, *ClV3*, *BRI1*, and *TTG* from native *Arabidopsis* genomic DNA, excluding the polyadenylation termination sequence in each case. These particular genes were chosen as viable targets to test the transgene due to their observable phenotypic influence on development. In designing the primers, a *Hind*III restriction site was added to both forward (5') and reverse (3') primer sequences. These sites ensured proper ligation of each transgene into pSJN15A at the *Hind*III site. For successful vector construction, no transgene sequence could contain additional *Hind*III restriction sites, as both pSJN15A and the transgene were each cut with *Hind*III prior to ligation.

Escherichia Coli Transformation. The synthesized vectors were used to transform separate cultures of *E. coli* (DH5α). Successfully transformed bacteria were selected on LB broth with 100 mg/L spectinomycin (Sigma-Aldrich, St. Louis, Mo.). The individual colonies were cultured in LB broth. The plasmids were then extracted from *E. coli* using the standard procedure. Briefly, the bacteria were centrifuged in microcentrifuge at full speed and the supernatant was drained off. The bacteria were then resuspended in a suspension buffer, utilizing a vortex to homogenize. A lysis buffer was added to break bacterial cells and release plasmid molecules, and then a high salt solution was added to precipitate cell debris. The resulting mixture was centrifuged and the supernatant was transferred to a new tube, leaving the debris behind. The plasmid was precipitated using 95% ethanol, inverted several times, and centrifuged. The ethanol was poured off and the DNA pellet was washed with 70% ethanol and air dried before adding 50 µL of autoclaved deionized water to dissolve the DNA pellet.

Arabidopsis Transformation. Vectors were then transferred to *Agrobacterium tumefaciens* strain GV3101 through electroporation. After spectinomycin selection of successful transformants, these bacteria were used in a floral dip transformation of *Arabidopsis* (Clough and Bent, 1998). Cultures (500 mL) of transformed *Agrobacterium*

were made for each plasmid, which were centrifuged at 6000 rpm for 20 minutes to pellet bacterial cells. The supernatant was discarded. The pellet was resuspended in 5% sucrose solution. Silwet (0.2 mL) of was mixed into each solution. One pot containing several *Arabidopsis* plants, with flowers having recently opened, was dipped for a few seconds into each solution. After dipping, each pot was placed on its side, covered with aluminum foil, and left for 24 hours. The next day, the plants were moved back to the light. Once the dipped plants were mature, seeds were collected. Demonstration of hygromycin resistance served as the selection marker for the successful *Arabidopsis* transformation. Therefore, primary transformant (T1) seeds were selected on Murashige Skoog (MS) media containing hygromycin (25 mg/L). The hygromycin resistant individuals were transferred to soil and allowed to mature.

Effectiveness of the construct was determined through evaluation of the rate of mutant phenotypes among the transformed T1 lines. Phenotypes of transformants containing the transgene were compared to phenotypes of wild type *Arabidopsis* individuals, which served as the control. In the ongoing project, RT-PCR and qRT-PCR will be done to analyze the rate of mRNA degradation among the putative silenced lines.

RESULTS AND DISCUSSION

Molecular Strategy. Previously, researchers reported that transcription of gene fragments lacking proper transcription termination signal (*3*' untranslated region), induces silencing of complementary genes within *Arabidopsis* genome (Luo and Chen, 2007; Nicholson and Srivastava, 2009). The production of truncated transcripts lacking polyadenylation signal (polyA) was the basis of silencing observed in these studies. Such polyA(-) transcripts are converted to double-stranded RNA (dsRNA) mediated by plant RNA Dependent RNA Polymerase 6 (RDR6) (Luo and Chen, 2007), which is degraded by plant RNA silencing machinery to generate short-interferring RNA (siRNA). The siRNA anneal with complementary mRNA to divert it from translation machinery to RNA degradation machinery. These two studies also found that transcription initiated by cauliflower mosaic virus 35S RNA promoter (35S promoter) is not completely terminated by the termination signal of the nopaline synthase gene (*nos3*'). This read-through activity of the 35S promoter generates RNA encoded by DNA sequence downstream of *nos3*'. Based on this, we designed a simple silencing vector in which 35S promoter of the selectable marker gene (hygromycin resistance), an essential component of the vector, drove read-through transcription of gene fragments cloned into unique restriction sites of the vector. As no *nos3*' or other transcription terminator sequences would be present downstream of the cloned fragments, the transcription would be improperly terminated. As a result, the unpolyadenylated RNA that would be synthesized are potent inducers of gene silencing.

Development of Silencing Vector. The pSJN15A cloning vector was successfully created and used to generate silencing vectors against 4 new *Arabidopsis* genes. The pSJN15A vector contains the following properties:

(a) A plasmid backbone of pPZP200 that contains a spectinomycin resistance gene for selecting bacterial strains transformed with this plasmid. This gene is important for mobilizing pSJN15A into *Agrobacterium tumefaciens* for subsequent plant transformations.

(b) A T-DNA region containing a hygromycin resistance gene. The T-DNA is transferred from bacteria into plant genome, thus hygromycin resistance is utilized for selecting transformed plant cells/seedlings.

(c) A unique restriction site, *Hind*III, downstream of the hygromycin resistance gene. Thus, a PCR fragment flanked by *Hind*III sites can be cloned into pSJN15A, and transcribed by the read-through activity of the 35S promoter of the hygromycin resistance gene.

It should be noted that the direction of the inserted transgene proved to be irrelevant, as transcription of both sense and antisense sequences would result in efficient silencing in transformed individuals. We recommend that at least 400 bps of gene fragment be cloned for efficient silencing by pSJN15A, as fragments smaller than 400 bps display significantly lower rate of silencing (Nicholson and Srivastava, pers. comm.).

Induction of Gene Silencing in Arabidopsis. The silencing vectors containing fragments of following genes were developed: *VARIEGATED 2 (Var2)*, *CLAVATA 3 (ClV3)*, *BRASSINOSTEROID INSENSITIVE 1 (BRI1)*, and *TRANSPARENT TESTA GLABRA 1 (TTG)*. These vectors were used to transform *Arabidopsis* through floral dip method. The resulting transgenic lines (T1 lines) were identified by plating seeds on hygromycin containing MS media. The seedlings were either directly evaluated or transferred to soil for phenotypic evaluation at the later stages of plant development. Loss of function (null mutation) of *Var2* generates white sectors on cotyledons and leaves of the seedlings, *CLV3* null mutation generates increased number of stamens and carpel in flowers, *BRI1* null mutation generates a range of phenotypes including reduced fertility, and *TTG* null mutation abolishes trichomes on stem base and leaves (TAIR, Arabidopsis Information Resource, www.Arabidopsis.org). Efficiency of silencing in T1 lines was assessed by the appearance of the respective mutant phenotype in the transgenic lines (Table 1). Appearance of white sectors on 77% of hygromycin resistant *Var2* seedlings grown on germination media, indicated gene silencing of *Var2* gene. Similarly, *BRI1* plants

displayed curled leaves and very low fertility phenotype. Approximately 50% of *BRI1* plants displayed severe phenotype characterized by high rate of sterility in plants. Silencing phenotypes in *CLV3* and *TTG* transformants were not apparent (Table 1), indicating that manifestation of *CLV3* and *TTG* mutant phenotypes requires severe suppression of these genes.

In the ongoing work, real-time PCR approach will be taken to study the level of steady-state mRNA in these transgenic lines. This analysis will reveal the quantitative strength of silencing induced by "unterminated" gene fragments.

CONCLUSIONS

The pSJN15A plasmid that was successfully constructed in this research demonstrated the potential of a new model, "unterminated" transgene, as a competent tool in future gene silencing applications. The model allows for simple insertion of a truncated gene sequence at a specific site, with no need for an additional promoter nor any regard for sequence direction, to form a complete vector. This design has application in gene silencing in various plant species.

ACKNOWLEDGEMENTS

I would like to thank Drs. Vibha Srivastava and Scott Nicholson for allowing me to assist them in the study of gene silencing. I would also like to thank Aydin Akbubak, Souman Nandy, Gulab Rangani, and Jamie Thomas for all of their assistance and patience throughout the duration of my research. Financial support for this project was provided through a University of Arkansas Honors College Research Grant.

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Table 1. Efficiency of unterminated transgene mediated silencing.

Gene[a]	Mutant phenotype[b]	Total number of transformed lines analyzed	Number of lines found to display mutant phenotype	Silencing Efficiency[c] (%)
Var2	Bleached cotyledons	35	27	77
CLV3	Altered floral organs	15	1	0.06
BRI1	Reduced fertility	9	5	55
TTG	No trichomes	18	0	0

[a] *Var2* = VARIEGATED 2; *CLV3* = CLAVATA 3; *BRI1* = BRASSINOSTEROID INSENSITIVE 1; *TTG* = TRANSPARENT TESTA GLABRA 1.
[b] From www.Arabidopsis.org.
[c] Percent lines displaying mutant phenotype.

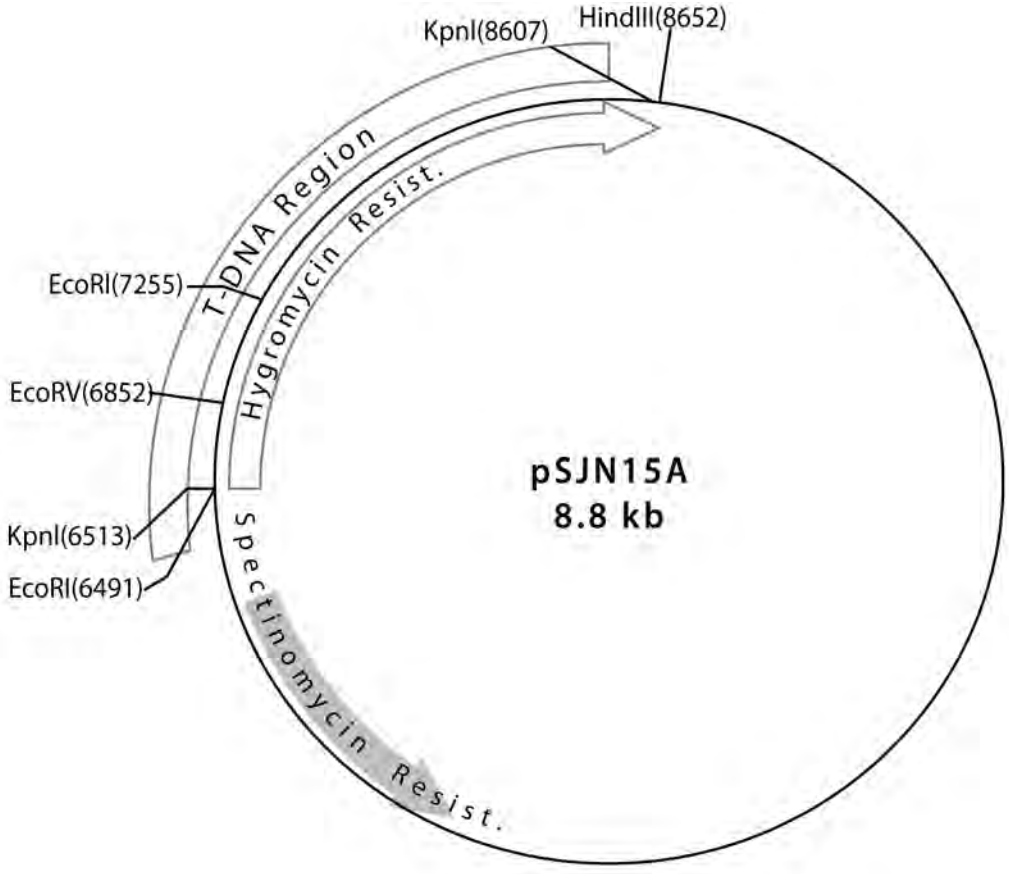

Fig. 1. Design of pSJN15A. The T-DNA region contains a hygromycin resistance gene, which consists of a 35S promoter driving hygromycin phosphotransferase gene (*HPT*), terminated by *nos3'* terminator. The *HindIII* site is the unique cloning site for introducing gene fragments. Note that the absence of *nos3'* after the cloning sites allows for the unterminated transcription of gene fragments. The plasmid contains a spectinomycin resistance gene in the backbone, which serves as a bacterial transformation selection gene.