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Elliot E. Pruett
*University of Arkansas, Fayetteville*

Soumen Nandy
*University of Arkansas, Fayetteville*

Vibha Srivastava
*University of Arkansas, Fayetteville*

# Efficiency of the nuclease I-*Sce*I in excising selectable marker genes from the plant genome

***Eliott E. Pruett*[*], *Soumen Nandy*[†], *and Vibha Srivastava*[§]**

## Abstract

Gene stacking is a method used in biotechnology by which multiple genes can be placed at a single genomic site, thereby simplifying plant breeding. In this approach, DNA nucleases are used for excising selectable marker genes (SMG), which are the unneeded components of transgenic plants. The goal of this project is to evaluate the effectiveness of the nuclease I-*Sce*I in excising DNA in plants. Specifically, this study tests heat-inducible I-*Sce*I through the use of a heat-shock promoter (HS) in order to control SMG excision by heat application. The DNA plasmid containing a visual marker gene flanked by I-*Sce*I target sites and the heat-inducible I-*Sce*I gene has been created and confirmed. *Arabidopsis thaliana* plants have been transformed with the plasmid, which will be used for testing the efficiency of HS:I-*Sce*I in excising DNA from plant genomes.

* Eliott E. Pruett is a May 2016 honors program graduate with a major in Crop Science and minors in Crop Biotechnology and Agricultural Business.

† Soumen Nandy is a post-doctoral associate in the Department of Crop, Soil, and Environmental Sciences.

§ Vibha Srivastava is the faculty mentor and a professor in the Department of Crop, Soil, and Environmental Sciences.

## Meet the Student-Author

***Eliott Pruett***

I grew up in Summers, Arkansas and graduated as salutatorian from Haas Hall Academy in Fayetteville, Arkansas in 2012. I graduated from the University of Arkansas in May 2016 with a B.S. in Crop Science with minors in Agribusiness and Crop Biotechnology. This project was done to fulfill the research requirement of the Crop Biotechnology minor. In the spring of 2016 I presented this research at the University of Arkansas Gamma Sigma Delta and the Southern Section of the American Society of Plant Biologists undergraduate poster competitions and won first place in both. In 2014 I was an intern at Kansas State University in the Sustainable Bioenergy Research Experience for Undergraduates program; in 2015 I participated in the Adair Plant Pathology Internship at the University of Arkansas; and I was the 2016 Department of Crop, Soil and Environmental Science Outstanding Senior. I will be pursuing a graduate degree at the University of Arkansas starting in the fall of 2016, continuing my research with my faculty mentor, Vibha Srivastava. I would like to thank Vibha Srivastava and Soumen Nandy for this opportunity and for their instruction.

## Introduction

Selectable marker genes (SMG) are an invaluable tool for generating transgenic plants; however, their presence in the transgenic crops is highly undesirable, and complicates the biotechnology risk assessment and regulatory procedure. Furthermore, due to the limited number of SMGs available to carry out plant transformation, they have to be reused when attempting to stack traits. This requires the use of DNA recombinases or nucleases to remove the selectable marker gene once it is no longer needed. Recombinases have been used both to add traits and remove SMGs; however, this can only be done once with each recombinase due to the reversibility of the process (Dale and Ow, 1991; Kumar and Fladung, 2001; Nandy et al., 2015). The alternative to using a recombinase is to use nucleases, which carry out irreversible forward reactions.

This alternative method is studied here using I-*Sce*I: a nuclease that causes double-stranded DNA breaks at a specific 18 base pair site (5'-TAGGGATAA^CAGGGTAAT-3'). When double-stranded breaks occur on both sides of a gene, the gene is deleted from the chromosome and the cell repairs the break. During this process, insertions and/or deletions (indels) can occur at the repair site (D'Halluin et al., 2013; Puchta and Fauser, 2014; Voytas 2013) making the site resistant to the nuclease and rendering the process irreversible. It is essential to know how frequently indels occur since large indels may alter the function of adjacent genes while small indels are acceptable. The promoter regulating I-*Sce*I is the soybean heat-shock protein 17.5E gene promoter (HS). It is inactive at 25 °C (room temperature) but highly active at 42 °C (Czarnecka et al., 1992). This heat-inducible I-*Sce*I will eliminate the need to retransform or cross plants for introducing I-*Sce*I activity, making the process much more efficient.

The overall goal of this project is to evaluate the efficiency of heat-inducible I-*Sce*I in excising a SMG from the genome of the model plant, *Arabidopsis thaliana*. The green fluorescence protein (GFP) gene was targeted for excision. The GFP expression is easily monitored as it emits green fluorescence under blue light. For testing I-*Sce*I efficiency in DNA excision in a plant genome, it is a prerequisite to develop transgenic plants containing a gene (GFP) flanked by I-*Sce*I sites, and the heat-inducible I-*Sce*I gene (HS:I-*Sce*I). These genes must be inserted into a DNA plasmid, which can then be inserted into the plant genome. This study developed these essential genetic resources, which will be used for evaluating I-*Sce*I efficiency in excising DNA from *Arabidopsis* genome.

The specific objectives of this project are to 1) build and confirm a DNA construct containing HS:I-*Sce*I, and 35S:GFP flanked by I-*Sce*I sites, in binary vector backbone; and 2) introduce the construct into *Arabidopsis* by *Agrobacterium*-mediated transformation. Completing these objectives is this first step towards testing HS:I-*Sce*I efficiency.

**Molecular Strategy**

In order to test heat-inducible I-*Sce*I, it is necessary to have a plasmid containing HS:I-*Sce*I and I-*Sce*I sites flanking the portion of DNA that is to be excised. The portion of DNA chosen was 35S:GFP, because it allows visual confirmation that the gene is active when inserted into plants. Additionally, the neomycin phosphotransferase (NPT) gene was inserted in order to confer resistance to the antibiotic kanamycin, which allows selection of transformed plants containing the plasmid. The HS:I-*Sce*I gene, 35S:GFP gene, 35S:NPT gene, and an I-*Sce*I site were already available but they needed to be combined to develop a structure (pEP4b) shown in Fig. 1, and cloned into a *Agrobacterium* binary vector (i.e. plasmid) for plant transformation. The 35S and HS are constitutive and heat-shock promoters, respectively, and the transcription terminator used in each of the genes is that of the nopaline synthase gene. When the plants carrying this construct are heat-shocked, I-*Sce*I will be activated, which will create double-stranded breaks (DSB) at I-*Sce*I sites leading to the deletion of GFP gene. The broken ends will be repaired by the cell incorporating indels at the repaired site (see Fig. 2). The deletion of GFP gene can be monitored by the disappearance of green fluorescence. Additionally, a primer pair can be used to determine excision via polymerase chain reaction (PCR; see Figures 1 and 2).

## Materials and Methods

**DNA Construction and *Agrobacterium* Transformation**

The first objective in this study was to create a DNA construct containing HS:I-*Sce*I and the GFP gene flanked by I-*Sce*I sites. The GFP gene is regulated by the 35S promoter from the cauliflower mosaic virus's 35S RNA gene. Cloning was done using standard molecular biology techniques using *Escherichia coli* cells. The *Agrobacterium* binary vector pPZP200 was used to construct the plasmid that contains a gene to confer resistance in bacteria to the antibiotic spectinomycin. Each gene was individually in-

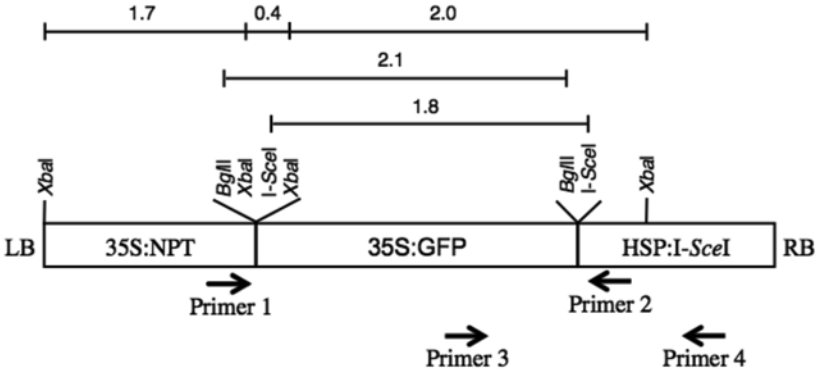


**Fig. 1.** pEP4b DNA construction in the binary vector pPZP200 (*Agrobacterium tumefaciens* vector) used to generate transgenic *Arabidopsis* lines. The vector contains 35S:NPT, 35S:GFP, and HSP:I-*Sce*I. LB and RB refer to the left and right borders of the *Agrobacterium* T-DNA. The sequence between these borders will be inserted into the *Arabidopsis* plants by the *Agrobacterium*. The location of polymerase chain reaction primers and restriction fragment sizes are shown.

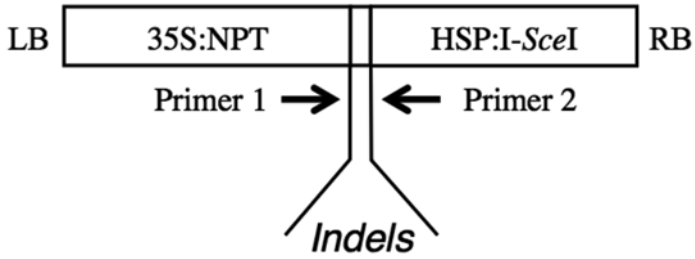


**Fig. 2.** Activation of heat-shock promoter leads to I-*Sce*I expression, which creates double-stranded breaks at the target I-*Sce*I sites. As a result, 35S:GFP gene is deleted and the broken ends are repaired by cellular DNA recombination process generating insertion-deletion (indels) at the cut site.

serted into the plasmid. This was done by digesting the plasmid and the gene by the same restriction enzymes, combining them in a single tube, and joining them together by DNA ligase. Following ligation, the new plasmid was inserted into a solution containing *E. coli* cells. This solution was then placed in a hot water bath, during which the *E. coli* cells would take up the new plasmid. The transformed *E. coli* was grown on media containing spectinomycin so that only the cells containing the plasmid would be able to grow. The plasmid was multiplied by the *E. coli* and then isolated. This process was repeated with each gene until they were all inserted into the pPZP200 vector. The final plasmid, pEP4b, was verified by restriction digestions, PCR and DNA sequencing.

**Plant Transformation**

The final plasmid, pEP4b, was introduced into *Agrobacterium tumefaciens* (strain GV3101) by electroporation using the Bio-Rad Gene Pulser, which was then used to transform *Arabidopsis thaliana* (Col-0 ecotype) plants. Plant transformation was done using the floral-dip method described by Clough and Bent (1998) to obtain transgenic seeds (T1). Seeds were collected from the dipped plants and grown on media containing the antibiotic kanamycin so that only plants containing pEP4b were able to grow.

The plasmid pEP4b was also inserted into rice callus to verify that GFP expression would occur in plant cells. Callus from the cultivar Nipponbare was grown on media, and pEP4b DNA was introduced by biolistic delivery method. The callus was incubated in a growth chamber for 48 hours before observing with a stereo-microscope for GFP fluorescence under blue light.

## Results and Discussion

The plasmid, pEP4b, has been developed for testing the efficiency of I-*Sce*I in excising DNA in plant genome. It contains a DNA construct consisting of 35S:GFP flanked by I-*Sce*I sites, a selectable marker gene, 35S:NPT, for isolating transformed *Arabidopsis* lines, and the heat-inducible I-*Sce*I. The construct and the order of the genes is shown in Fig. 1. This construct was cloned into the binary vector, pPZP200 backbone, to generate pEP4b of approximately 12,000 base-pairs (12 Kb) size. The structure of pEP4b was verified by restriction digestion, which is a standard molecular biology technique that uses restriction enzymes to cut DNA at specific sites. The enzymes used were *Bgl*II, *Xba*I and I-*Sce*I. The sites where they cut the DNA construct are shown in Fig. 1. Digesting with *Bgl*II should result in two DNA fragments, with lengths of 2.1 Kb (construct) and 9.9 Kb (backbone). Digesting with I-*Sce*I should result in two DNA fragments as well, with

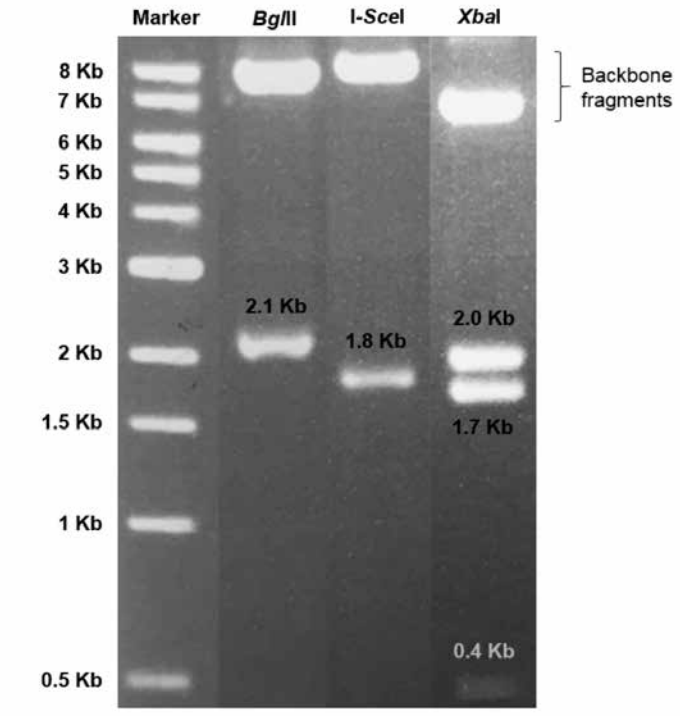


**Fig. 3.** Confirmation of pEP4b by restriction-digestion and gel electrophoresis. DNA marker lanes indicate DNA size standards, restriction enzymes are indicated on each lane, and the backbone fragments or the fragment sizes expected from the pEP4b construct (see Fig. 1) are given.

lengths of 1.8 Kb (construct) and 10.3 Kb (backbone). Digesting with *Xba*I should result in four DNA fragments, with lengths of 0.4 Kb (construct), 1.7 Kb (construct), 2.0 Kb (construct) and 7.8 Kb (backbone). As shown in Fig. 3, all of these fragments were obtained from the digestion of pEP4b with these restriction enzymes.

Note that digesting with I-*Sce*I is effectively what should happen when HS:I-*Sce*I is activated in plant cells. This confirms that the I-*Sce*I sites are functional. In order to test the functionality of the 35S:GFP gene, rice callus was bombarded with pEP4b using a gene gun and observed for GFP expression. The callus showed GFP expression, indicated as bright green spots, confirming the activity of GFP in plant cells (see Fig. 4a). This completes the first objective.

The plasmid was subsequently introduced into the *Agrobacterium tumefaciens* strain GV3101, which was used to transform *A. thaliana* by floral-dip method. The seeds (T1) from dipped plants were collected, and plated on seed germination media supplemented with kanamycin (50 mg/L). Seventeen T1 seedlings were able to grow on media and were confirmed by PCR to contain pEP4b. Figure 4b shows PCR amplification of pEP4b region spanning Primer 3 and Primer 4 (see Fig. 1) in the selected T1 plants. All 17 T1 plants have been transferred to pots containing a growing medium for seed production. This completes both objectives of this study. These plants will be further characterized by molecular techniques for the structure of pEP4b inserts and heat-induced activity of I-*Sce*I in each T1 line to select 2 -3 lines for further studies.


## Acknowledgements

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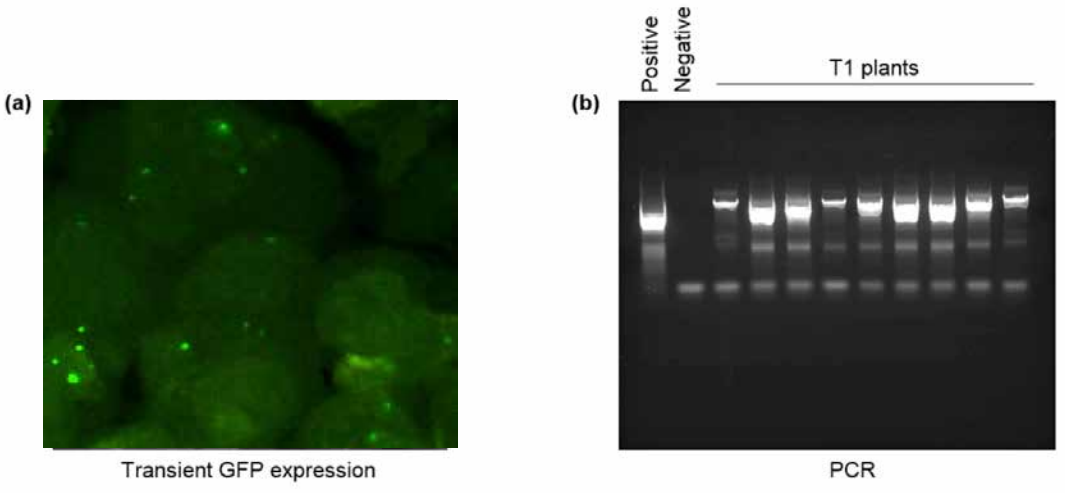


**Fig. 4.** Transient GFP expression in rice callus bombarded with pEP4b. The cells expressing GFP are seen as bright green spots. (b) Polymerase chain reaction (PCR) confirmation of *Arabidopsis* T1 plants transformed with pEP4b. Positive and negative controls refer to pEP4b and water controls, the presence of ~1 kb amplicons in 9 representative T1 lines is shown.

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