Spring 2015

Using Golden Gate Assembly to Rapidly Create Genetic Constructs for Transgenic Plants and Promoter Analysis

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Using Golden Gate Assembly to Rapidly Create Genetic Constructs for Transgenic Plant and Promoter Analysis"

written by

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and submitted in partial fulfillment of
the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

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4/20/2015

Using Golden Gate Assembly to Rapidly Create Genetic Constructs for Transgenic Plant and Promoter Analysis

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May 2015

Abstract:

Golden Gate Assembly, a genetic assembly method in which the ligation and digestion of oligonucleotides occurs in a single step, can be used for an array of purposes [2]. In order to test and take advantage of this assembly method our lab selected three experiments in which it could be used. These included an oxidative stress sensitive green fluorescent protein [4], a plant specific form of Golden Gate Assembly coined "Green Gate Assembly," [3] and a viral promoter verification system called "pClone Red." [1]

Arabidopsis (*Arabidopsis thaliana*) plants were analyzed for the presence of a redox-sensing green fluorescent protein (reduction-oxidation-sensitive green fluorescent protein [roGFP]). Blue LED lights with a filter were used to screen plants for GFP. Plants were screened using a non-sterile sand and antibiotic method in order to increase yield of plants containing gene of interest.

Two constructs were created to test the "Green Gate" method, an adaption of the "Golden Gate" method specifically for plant use. This method utilizes highly specific 4 base pair overhangs and a common type IIs restriction site in order to anneal multiple parts into one vector at once. A construct to transform future plants was created using the "Green Gate" method [2-3].

A bacteriophage named "Corndog" was analyzed at the 2014 HHMI conference for possible promoters. One of the suspected promoters was created for testing [5]. The promoter was created with specific overhangs so it could be placed in vectors with the "Golden Gate" method. After annealing, the promoter sequence was put into a pClone Red destination vector. The plasmid was then transformed into *Escherichia coli* cells and analyzed to confirm a working promoter. [1]

Introduction:

Golden Gate Assembly allows multiple parts to be digested and ligated in one highly efficient step. A part, is a term used to describe a portion of DNA that encodes for part of a gene but not the gene in its entirety needed to produces the desired protein. This method allows our lab to design and create genetic constructs rapidly. Each part has two specific and unique overhangs to ensure correct ligation. By taking advantage of type IIs restriction enzymes, such as BsaI, constructs are assembled seamlessly. The restriction site and unique cloning sequence are eliminated from the ligated product allowing digestion and ligation to occur in one step. [2]

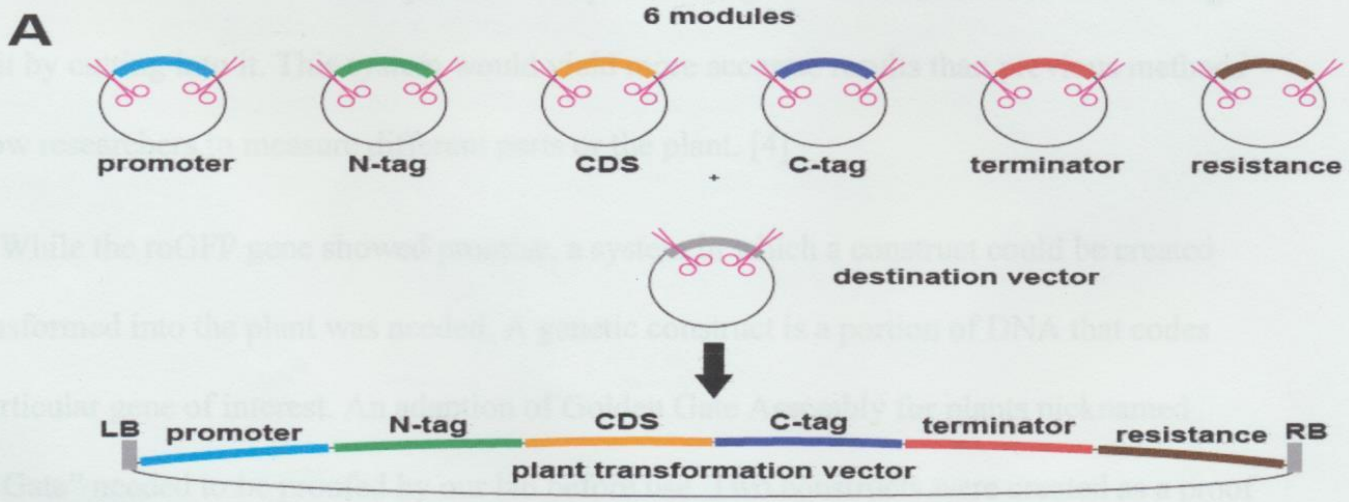

Fig 1

Fig 1 displays the way Golden Gate and Green Gate Assembly work conceptually. They go together in a brick like fashion. [2]

Research centered on oxidative stress defense pathways is important due to its link to programmed cell death induced by reactive oxygen species. The similarities in the way plants and humans handle oxidative stress makes plants a good model species for testing. Previous experiments involved stressing the plant then cutting discs from leaves or removing whole leaves and analyzing the stress. The problem with this technique is that injuring the plant stresses the plant in addition to the stress the researcher is testing. While the two stresses are different, it can be difficult to distinguish which is which. With the goal of finding a better method to study oxidative stress the roGFP gene showed promise. This gene produces a green fluorescent protein that is sensitive to reactive oxygen species and in turn to oxidative stress. Inserting this gene would allow researchers to measure plant stress by measuring the florescence without stressing the plant by cutting into it. This system would yield more accurate results than previous methods and allow researchers to measure different parts or the plant. [4]

While the roGFP gene showed promise, a system in which a construct could be created and transformed into the plant was needed. A genetic construct is a portion of DNA that codes for a particular gene of interest. An adaption of Golden Gate Assembly for plants nicknamed "Green Gate" needed to be proofed by our lab before use. Two constructs were created as a proof of concept. The first construct was made up of an ethanol inducible promoter (ALCA), a B-dummy, 3X GFP, a D-dummy, RBCS terminator, and a kanamycin resistant cassette. A dummy insert is a sequence that has no function but still allows for other parts with function to attach. These six parts were all inserted into an empty destination vector with gentamycin resistance. The second construct was made up of a constitutive 35s promoter, a B-dummy, Blue Fluorescent Protein, linker mCherry, RBCS terminator, and a kanamycin resistant cassette. These six parts were also inserted into an empty destination vector with gentamycin resistance. [3]

The final aspect of our research involves mycobacteriophage promoter verification using Golden Gate Assembly. A mycobacteriophage "Corndog" had its genome analyzed by a group of faculty members at an HHMI conference. Many putative promoters were found and our lab chose the six most likely putative promoters based on their sigma 70 scores. Sigma 70 scores use an algorithm to determine the likelihood the promoter will be functional in *Escherichia coli* cells [5]. These promoters were then created with specific four base pair overhangs for use in Golden Gate Assembly. The promoters were then inserted into a pClone Red vector. This vector has a GFP, RBS, and Bsa I cut site at its left border and an RFP, RBS, and Bsa I cut site at its right border. This vector will confirm the functionality and direction of the promoter sequence. The original left facing promoter in the pClone system has the ability to be ligated back into the original vector after digestion. If the original promoter is ligated back into the vector then the cells will fluoresce green, but if one or our designed promoters is ligated into the vector and is functional in *E. coli* cells then the cells will fluoresce red. Due to this, a positive result will have both green and red colonies. [1]

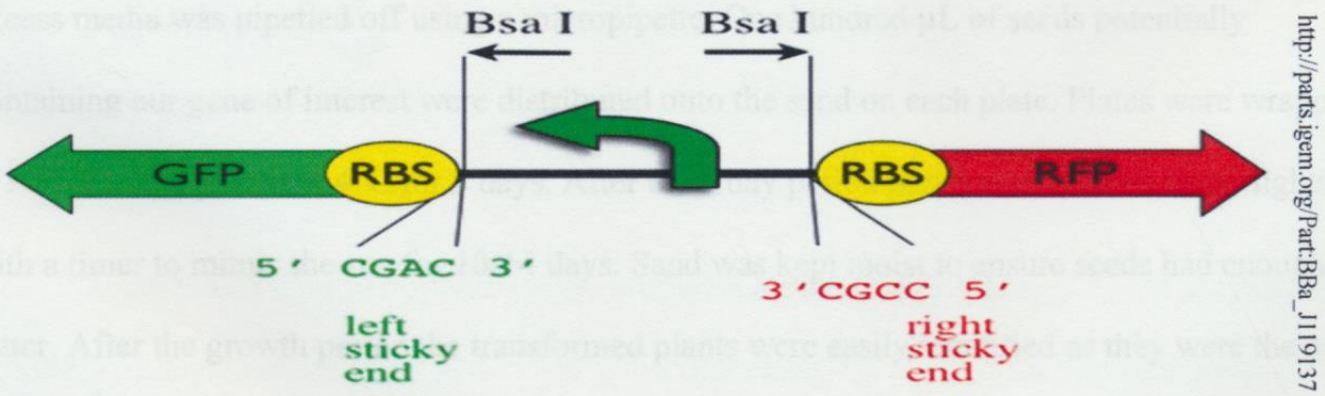

Fig 2

Fig 2 diagrams how the pClone Red vector is set up. A left facing promoter is bordered by an RBS and GFP on the left, and RBS and RFP on the right. There are Bsa I cut sites with sticky ends designed for the parts we are going to put in. [1]

Materials and Methods:

roGFP verification:

Arabidopsis plants from a line containing the roGFP gene were ordered and shipped to our lab. The plants came from a line demonstrated to have the roGFP gene along with a resistance to kanamycin, but not every plant shipped will contain our gene of interest. In order to ensure that the plants we grew contained our gene of interest we grew the seeds using a non-sterile sand method. Kanamycin was added to the sand so only the plants with the kanamycin resistance will survive the non-sterile sand selection. The seeds were grown in 35 mL of dry quartz sand in 4 100x15 mm Petri dishes. In order for the plants to be selected the sand was saturated with approximately 10 mL of ¼ Basal Salt medium with kanamycin added. The recipe for the Basal Salt medium was 0.8g of MS media, 0.1g of MES, 200 µL of 100 ug/mL Kanamycin, into 200 mL of pure water. After the sand was saturated the dish was gently tapped to distribute the sand evenly and remove air bubbles. After the sand was evenly distributed excess media was pipetted off using a micropipette. One hundred µL of seeds potentially containing our gene of interest were distributed onto the sand on each plate. Plates were wrapped in Parifilm and placed in 4°C for 3 days. After the 3 day period plates were placed under lights with a timer to mimic the sun for 10-14 days. Sand was kept moist to ensure seeds had enough water. After the growth period the transformed plants were easily identified as they were the only ones growing. The plants with our gene of interest were then potted in threes. Leaves from the plants with the roGFP gene were then analyzed and compared to plants without the gene using a fluorescent microscope and blue LED light with a red filter. This light and filter allows the user to visualize fluorescence. [4]

"Green Gate Assembly"

Two separate constructs to test "Green Gate" were designed. The first construct was made up of an ethanol inducible promoter (ALCA), a B-dummy, a 3X GFP, a D-dummy, an RBCS terminator, and a kanamycin resistant cassette. These six parts were all inserted into an empty destination vector with gentamycin resistance at the left border. The second construct was made up of a constitutive 35s promoter, a B-dummy, Blue Fluorescent Protein, linker mCherry, RBCS terminator, and a kanamycin resistant cassette. These six parts will be inserted into an empty destination vector with gentamycin resistance at the left border.

After the constructs were designed parts were selected from a kit supplied by Addgene, a company that supplies materials for genetics based research. Addgene ships its parts in frozen cells containing our plasmid of interest so they must be grown up to ensure enough cells to get DNA. The parts were located and using a 1000 µL pipette tip they were spread on plates with antibiotics specific to the part. All parts required ampicillin except for the empty destination vector which required gentamycin. After parts were allowed to grow in a 37°C incubator colonies were picked and LB broths with antibiotics added were inoculated and allowed to grow in a 37°C shaker for 24 hours.

When the 24 hour period was up the broths were miniprepped using a Zymo kit in order to collect concentrated plasmid. A 15 µl mixture containing 1.5 µL of each miniprepped part, 1 µL of the miniprepped destination vector, 1.5 µL of 10x CutSmart Buffer, 1.5 µL of 10x ATP, 1 µL of HF Bsa I enzyme, and 1 µL of T4 DNA Ligase, was collected in a PCR tube. The mixture was then placed into a thermocylcer as digestion and ligation occur at different temperatures. The cycle consisted of 50 cycles of 37°C for 5 min and 16°C for 5 min, then 50°C for 5 min, then 80°C for 5 min. After the cycle was done the mixture was frozen at 20°C until it could be

transformed into competent *E. coli* cells. Competent cells have been heat treated to allow the plasmid to enter with less resistance.

To transform the construct into the cells 5 µL of the thermocycled mixture was added to 50 µL of competent cells. The mixture was then put in the 37°C shaker for 30 min to allow for an outgrowth period. After the outgrowth period 10 µL of the cells were spread onto agar plates with kanamycin and gentamycin. If the process worked correctly and all of the parts went into the vector the bacteria would be resistant to both kanamycin and gentamycin so they would grow on the plates. [3]

pClone Promoter Verification:

Six promoters were selected from the mycobacteriophage "Corndog" based on their sigma 70 scores. These chosen sequences were sent off to be built as oligos with specific 4 base pair overhangs for Golden Gate Assembly. The sequences were as follows: [5]

PL7526	5' CGACCTTGACATTAGCCCTTGTCAAGAGTAGAAT
	3' GAACTGTAATCGGGAACAGTTCTCATCTTACGCC
PL1465	5' CGACTTGACATAAGCCTTCAACAAGAGTAAAAT
	3' AACTGTATTCGGAAGTTGTTCTCATTTTACGCC
PL7494	5' CGACTTGACATTAGCCCCTGTCAAGAGTAGAAT
	3' AACTGTAATCGGGGACAGTTCTCATCTTACGCC
PR440	5' CGACTTGACAGCCGTCGGAGGGTTGGTGCAGAAT
	3' AACTGTCGGCAGCCTCCCAACCACGTCTTACGCC
PL7404	5' CGACTTGACATTCCCGACAGGGCGGCGCATACT
	3' AACTGTAAGGGCTGTCCCGCCGCGTATGACGCC
PR7526	5' CGACTTGACAATGGCCATATTGGGCATAGGAT
	3' AACTGTTACCGGTATAACCCGTATCCTACGCC

Sequences from a known left facing promoter, right facing promoter, and non-functional promoter were also used as positive and negative controls. A pTac and p7 promoter were also sent to our lab from Todd Ekdhal, the developer of pClone, to be used as positive controls.

The left facing promoters, those beginning in "PL," would not work in the pClone vector as is. These sequences had to be flipped in order to make them right facing promoters. This way the pClone vector would function as intended. If this would not have been done then it would be impossible to distinguish a positive result from a negative result as they would both fluoresce green.

These 11 sequences were shipped as freeze dried single stranded DNA. This required the DNA to be rehydrated and then annealed together. A 20 μL annealing reaction was prepared using 16 μL of ultrapure water, 2 μL of 10X annealing buffer (1 M NaCl, 100 mM Tris-HCl pH 7.4), 1 μL of the top strand, and 1 μL of the bottom strand. This mixture was placed in 400 mL of boiling water for 4 minutes and then allowed to cool overnight. This ensured that the reaction reached the optimal temperature for annealing.

The pClone Red vector was sent to our lab by Todd Ekdhal. The vector was sent within *E. coli* cells on a plate so broths had to be inoculated and grown and miniprepped so purified plasmid could be worked with. This plasmid was nanodropped in order to verify the purity of the plasmid as well as the concentration.

Golden Gate Assembly was then performed. A 10 μL mixture containing 6 μL of water, 1 μL 10X T4 DNA Ligase Buffer with 10 mM ATP, 1 μL of 40 nM pClone destination vector, 1 μL of the desired annealed promoters, 0.5 μL of HF Bsa I, and 0.5 μL T4 DNA Ligase. A negative control replacing the promoter with water was also made. The reaction was then placed

into a thermocylcer set for 20 cycles of 37°C for 1 min and 16°C for 1 min, followed by a 15 min period at 37°C to cleave any remaining Bsa I sites. [5]

An alternate protocol was developed by New England Biolabs, NEB, and was used by our lab. NEB developed a buffer and enzyme mix that contained everything needed for Golden Gate Assembly. This protocol claimed to increase the efficiency of Golden Gate. Unlike the standard protocol this mixture was 20 uL. It contained 2 µL of 10x Golden Gate Buffer, 1 µL of destination plasmid (pClone Red in this case), 2 µL of insert, 1 µL of Golden Gate Assembly Mix, and 14 µL of water. This NEB protocol required the same PCR setting as the standard protocol.

To transform the 5 µL of the thermocycled mixture was added to 50 µL of competent cells. The transforming cells were put in the 37°C shaker for 30 min to allow for an outgrowth period. After the outgrowth period 10 µL of the cells were spread onto agar plates with ampicillin to ensure only cells with the pClone vector would grow.

Results:

roGFP verification:

With the fluorescent microscope in our lab it was not possible to distinguish Arabidopsis plants with our gene of interest and those without. Results not shown.

"Green Gate":

After many attempts we have not been able to obtain positive results for Green Gate as of publication. As of publication there was an issue with the empty destination vector. Addgene was contacted and they shipped a new vector which will be tested post publication.

pClone Promoter Verification:

After many attempts the p7 promoter showed one red colony and the pTac promoter showed two red colonies. These two promoters function as a positive control and proof of concept. With these functioning we moved onto testing promoters from "Corndog." The procedure was repeated with the PR440 and PR7526 putative promoters from "Corndog." The PR440 promoter showed 2 red colonies using the NEB protocol but did not show red colonies using the standard protocol. The PR7526 promoter showed 4 red colonies on the standard protocol but showed over a hundred on the NEB protocol. The four PL promoters were all left facing and would not work in this system because if they were ligated into the pClone red vector they would not be able to be distinguished from the original promoter within the pClone red vector. To solve this problem the left facing promoters were flipped to mimic right facing promoters. Once flipped the same protocol was followed. After flipping the left facing promoters we received positive results from the PL7404 promoter. Using the NEB protocol we observed 6 red colonies. We can confirm that PR7526, PR440, and PL7404 are functional right facing promoters in *E. coli* cells.

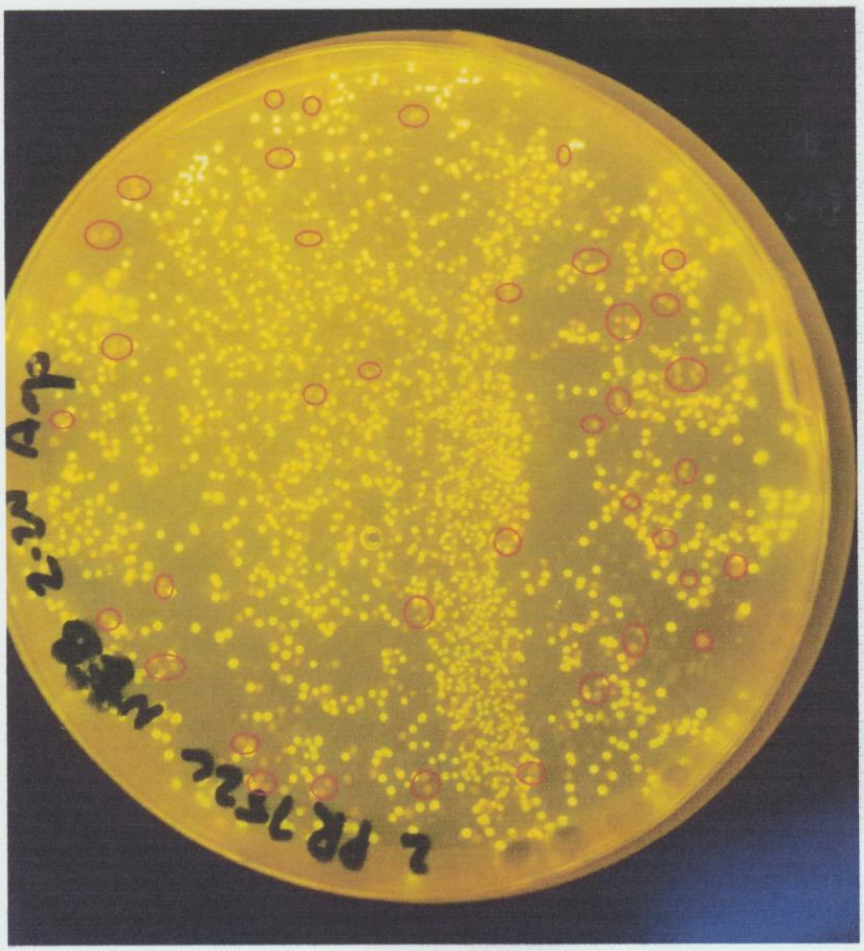

Fig 3

Figure 1 displays the results of the pClone promoter system with the PR7526 promoter taken from "Corndog" using the NEB protocol. There is a mixture of both red and green fluorescent colonies indicating a positive result. Many red colonies were circled in red for easier visualization.

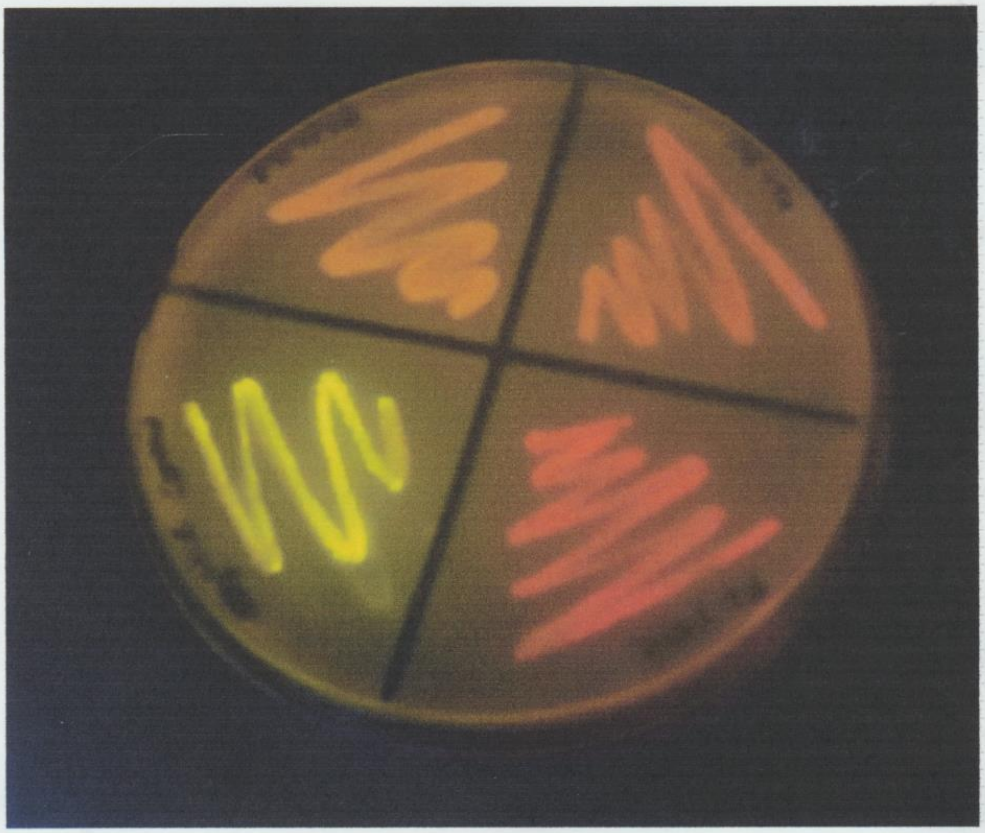

Fig 4

Red colonies from all three positive results, PL7404, PR7526, PR440, were streaked on to a plate with ampicillin for easily visualization and confirmation.

Discussion:

While the roGFP was not distinguishable in our lab, we have hope for this gene as it would help further the oxidative stress research as presence of the gene is variable. In previous studies using the gene, researchers grew hundreds of seeds before selecting their plants. Due to a limited number of seeds, we were not able to grow the amount of plants that previous researchers needed to get good data. With some different technology and more seeds, we believe this gene could work in our tobacco GOX system. The GOX gene converts cellular glucose into hydrogen

peroxide and glucono delta-lactone. Hydrogen peroxide can break down into a superoxide molecule which has the ability to cause oxidative stress. Leaf disc assays were used to determine hydrogen peroxide production in leaves at different developmental stages. Also, Western blots were done to determine protein expression levels. [4]

In the beginning, we misunderstood the pClone verification system. When we chose the six promoters, we were not aware that the left facing PL promoters would not work. If the left facing promoters ligated into the pClone red vector both positive and negative results would fluoresce green. That meant that four of the six promoters we began studying would not work. To solve this problem we flipped the left facing promoter's sequences so they would now mimic the right facing promoters. After many attempts we obtained three new promoters we knew worked. After tinkering with the protocol we were able to get two of the known promoters, pTac and p7, to work. The PR7526 promoter worked under both the standard and NEB protocol, while the PR440 promoter only worked under the NEB protocol. After flipping the PL7404 promoter worked using the NEB protocol. This showed that the PR7526, PR440, and PL7404 promoters are functional in *E. coli*. While PR7526 worked under both protocols, the NEB was significantly more efficient.

References:

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Supplemental Materials:

Non sterile selection of transgenic plants

1. Place ~35 mL dry quartz sand (Sigma #00653) or dry Silicon dioxide (Fluka #84880) in a 100x15 mm Petri dish. NOTE:*Alternative chromatography sands can work, but the specific type of sand matters a great deal, as some sands were found to be entirely unsuitable for certain antibiotic-selection regimes. The above two sands are our favorites, but by no means do these limit potential substrate choices. A given sand must be tested empirically within any given lab for suitability of use under the given selective agent. Generally, we found that the more 'white' the sand, the greater the number of selective agents capable of supporting transformant selection.*

Note: Both sand and media were autoclaved in order to keep everything as sterile as possible.

2. Saturate the sand by pipetting ~10 mL 1/4 MS Basal Salt media (without sucrose) that is buffered and pH-adjusted containing the selecting antibiotic. NOTE:*the range of antibiotic/herbicide added to the MS solution before saturating sand can often be up to twice that usually added for standard agar selection, but this must be empirically tested within the lab.*

(Ph of initial test was 5.8.

Recipe for MS media for initial test was:
0.8g of MS Media
0.1g of MES
And 200 mL of water)

3. Evenly disperse the wet sand by gently tapping the Petri dish against the lab bench. This distributes the muddy sand mixture and releases trapped air bubbles. Then pipette or decant off excess liquid media such that the wet sand is no longer muddy.

4. Carefully tap up to 100µL dry seed onto the wet sand. NOTE:*too much seed can result in unwanted fungal contamination, and furthermore, identification of transformants can be a problematic when within a dense seedling canopy.*

5. Stratify plates at 4°C for approximately two days, depending on genotype. Parafilm.

6. Move plates to a growth cabinet, as typical for agar selection. NOTE: *surgical tape can be wrapped around the Petri dish to slow evaporation; do not use parafilm as the lack of sucrose in the sand requires that the plants are dependent on CO_2 as a carbon source for growth.*

7. Approximately every 3 d, open the lid of the Petri dish and add 1 to 5 mL 1/4 MS Basal Salt media, or water, such that the plate is adequately wet. Do not over-water. NOTE:*under some selection conditions it might be necessary to add a second round of antibiotic treatment. This must be established empirically.*

8. After 10–14 d, transformed plants should be easily identifiable. NOTE:*failure to successfully identify transgenics means that the concentration of the selective agent needs to be modified and/or a different sand substrate needs to be tested.*

9. To remove selected transgenics, with their now sand-embedded roots, gently pipette ~10 mL water to the sand to mildly flood the plate. The selected transgenic seedlings can now be easily removed with forceps and transferred to soil for further growth. NOTE: *we additionally use the sand-selection method for generations after the T1 selection. As one example, we select transgenic F1 seeds on sand.*

Synthetic Promoter Design and Synthesis

The DNA sequence for the promoter wanted to study was converted into oligonucleotides that can be ordered from a DNA synthesis company and used in Golden Gate Assembly (GGA). The top strand oligonucleotide starts with CGAC sticky end and continues with a given promoter sequence, resulting in 5' CGACNNNNN…3', where the N's are the promoter sequence. The bottom strand begins CCGC and continues with the promoter sequence, resulting in 5' CCGCMMMMM…3', where the M's are the reverse complement of the promoter sequence. Production of both oligonucleotide sequences for promoters is automated by a convenient online tool called Oligator, developed by Dr. Laurie Heyer and her undergraduate students at Davidson College (21). Once our students generated their sequences, we placed an order for synthesis of the top and bottom strand oligonucleotides at Integrated DNA Technologies. Oligos that are less than 70 bp are delivered in about 3 business days. We ordered the oligos as "Lab Ready" which means that they arrive in solution with a concentration of 100 µM, instead of a dry pellet. If oligos arrive dry, they should be resuspended in water to 100 µM and stored frozen. [1]

Annealing Oligonucleotides

The top and bottom strand oligos were annealed prior to GGA. A 20 µL annealing reaction was prepared using 16 µL of H2O, 2 µL of 10X annealing buffer (1 M NaCl, 100 mM Tris-HCl pH 7.4), 1 µL top strand promoter oligonucleotide, and 1 µL bottom strand promoter oligonucleotide. The annealing reaction was boiled in 400 mL of water for 4 minutes and allowed to slowly cool for at least two hours (preferably overnight) in the same water bath. In preparation for GGA, the annealed oligonucleotides were diluted with water to the same concentration as the destination vector. This provides a 1:1 molar ratio of promoter insert to pClone vector in the GGA reaction. For GGA, we use a vector concentration of 40 nM (60 ng/µL of pClone basic, 73 ng/µL of pClone Green, or 73 ng/µL of pClone Blue). We diluted the annealed oligonucleotides to 40 nM as well. The Lab Ready 100 µM oligonucleotides were diluted to 5 µM (5000 nM) in the annealing reaction. After annealing, we further diluted the annealed oligos 125-fold (5000 nM/40 nM) by adding 124 µL of H2O to 1 µL of the cooled oligonucleotides. [1]

Golden Gate Assembly

GGA reactions were performed in a total volume of 10 µL containing 6 µL H2O, 1 µL 10X T4 DNA Ligase Buffer (supplied by the company as 300mM Tris-HCl (pH 7.8 at 25°C), 100 mM MgCl2, 100 mM DTT and 10 mM ATP), 1 µL of 40 nM pClone destination vector, 1 µL of 40 nM annealed promoter oligonucleotides, 0.5 µL HF (high fidelity; New England BioLabs) Bsa I (10 units), 0.5 µL T4 DNA Ligase (New England BioLabs; 50 Weiss units). GGA reactions were placed in a thermal cycler set for 20 cycles of 1 minute at 37° C followed by 1 minute at 16°C.

The 37°C temperature favors digestion by BsaIHF while the 16°C favors ligation by T4 DNA ligase. GGA terminates with a 15 minute incubation at 37° C to cleave any remaining BsaI sites. Negative controls were prepared in which the annealed oligos were replaced by water.

Reflection:

Coming into research I thought I would be primarily learning about genetics but more than that I ended up learning how to think critically and problem solve. Don't get me wrong, I certainly learned more than my fair share about genetics, but it is the problem solving skills that will stay with me for the rest of my life. Doing this research for almost a year has been one of the most impactful learning experiences of my life to date.

In the summer of 2014 I began my research under Dr. Nathan Reyna at Ouachita Baptist University. I worked alongside Jordan Burt and Jesse Kitchens, but they were researching a different topic. We helped each other accomplish tasks, but we were all researching our own subjects. I feel like this team mentality gave me a different experience that other research students at OBU and other institutions. I was able to learn a lot of techniques that I would not have been able to learn otherwise, such as PCR and Western Blot. Learning these techniques and assisting the other students also allowed me to learn about interesting topics outside of my field. Jesse Kitchen and Jordan Burt's research was centered on oxidative stress signaling pathways induced by reactive oxygen species. These pathways are what link programmed cell death with multiple human diseases. There is a strong conservation of the signaling pathway between both plants and animals allow us to use plants to safely and accurately study this pathway without using animals. Tabaco plants transformed with a gene expressing insect glucose oxidase (GOX) were used as the sample for the experiments. PCR was used to ensure the plants had the GOX gene. The GOX gene converts cellular glucose into hydrogen peroxide and glucono delta-lactone. Hydrogen peroxide can break down into a superoxide molecule which has the ability to cause oxidative stress. Leaf disc assays were used to determine hydrogen peroxide production in leaves at different developmental stages. Also, Western blots were done to determine protein

expression levels. Where the two studies differed is that Kitchens used plants with a constitutive promoter (CAM 35s promoter) while Burt used an estradiol inducible promoter (XVE-estradiol promoter). While I did my own research this summer, our lab was set up in way in which I was able to learn about so many different things. I was able to put my mark into multiple areas and really grow into a well-rounded lab researcher. Not everyone can have their name on three studies after one summer of research.

Another interesting twist to doing research at Ouachita during the summer is the community that OBU creates for its research students. There were only a handful of us all living near each other working together in the same building. We were forced to become like a family. I personally was only close to three of the people doing summer research at the start of the summer but I left with significantly more friends. The faculty went out of their way to create an environment in which we could form relationships together. All of us, faculty included, went on two trips together. One day we all took the day off and floated down the river. Another day the faculty got canoes and kayaks and went down the river. Both of these trips and the family like environment helped make doing research at Ouachita a completely different experience than anywhere else.

I could not have asked for a better faculty advisor than Dr. Reyna. In the many problems I faced during my research Dr. Reyna could have easily solved the problem for me, but instead he would allow me to come up with possible solutions and pointed me in the right direction. An example of this occurred when it came time to select our Arabidopsis plants containing the roGFP gene. Normally plants must be sterilized using a long procedure that takes a lot of time and is inconvenient for our purposes. Instead of forcing me to use the usual technique, Dr. Reyna allowed me to research a new non-sterile sand selection method that greatly decreased the time

of the experiment. This technique was so successful it is still be used in his Cell Biology lab for plant selection. Another example of this was when dealing with a faulty empty destination vector for Green Gate Assembly. We were not sure what to do so I called the company, Addgene, and they were able to determine that our empty destination vector was not working and sent us another one. This guided independent form of learning was a great experience and really helped further my learning.

While working in the lab, I developed a passion for the critical thinking skills that working in the lab required on a constant basis. One summer was not enough so I continued my project through the Fall 2014 semester with Luke Fruchey. Luke came in to help me with a project I had been working on for an entire summer so I was ahead of him in this field. Teaching someone how to do my project and having them work alongside me forced me to know every little detail about the project. I had to be able to answer Luke's questions or he would not be able to help with the project to the best of his ability. If I did not know something Dr. Reyna was always there to help us but like my dad always says "If I have to tell you to do something I could have done it myself." Anything Luke and I could figure out on our own allowed more time for Dr. Reyna to do other work he needed to get done. Luke and I focused on the pClone Red promoter verification system project instead of the roGFP or Green Gate projects. We believed that this project was the closest to working after the summer and it had the most immediate benefits due to its possible use in the bioinformatics lab offered to freshmen at OBU. The pClone system could greatly further the learning of students in this lab by allowing them to find putative promoters and test their functionality experimentally.

In the spring of 2015 I continued my work with Dr. Reyna. This time I was no longer with Luke Fruchey but I now had Logan Kuhn as my partner. Luke continued his work on the

pClone system but I was on to a new project. Logan and I continued our project focusing on the Green Gate Assembly system. Perfecting this system would allow us to rapidly transform plants in our GOX oxidative stress system. Unfortunately as of writing this the Green Gate system has not been successful.

The largest obstacle I had to overcome during my research experience was dealing with failure. When you are doing an experiment that has never been done in your lab or never been done at all the desired results are far from guaranteed. In our lab that was definitely the case. The positive results I fought so hard to see were never shown. Consistently seeing negative results is very discouraging and it is hard not to be down on the project. Eventually I was able to see that negative results are not always bad. When something does not work you are forced to analyze the procedures and properties of said procedures to find the error and fix it. This careful analysis is the basic property of research. Just because the experiment does not go as well as you want it to does not mean it is a failure.

After eight months of research we finally obtained positive results in the pClone system. When Logan and I looked under the blue LED light and saw those red colonies we acted like kids on Christmas morning. We jumped up and down and screamed like children. It was not a moment I expected during research, but a great one. It was just awesome to work so hard on something and just when you think nothing is going to work, you get the results you wanted.

This project gave me the opportunity to present my research twice at large research conferences. I created a poster to visually represent my work and presented my research to many people. To make sure I was prepared Dr. Reyna helped me with the layout and design of my poster. Since I had never done a poster or presented research this preparation was essential to preparing me and calming my nerves. The faculty also set up a time where we presented our

posters to other faculty members. This gave us a taste of the environment and helped prepare us. The first place I presented was in Little Rock, AR at UAMS. At the end of the summer we traveled to UAMS and presented. Doing this for the first time, and doing it well, gave me confidence. People were genuinely interested in my project and that is hard to find. Not many people on this planet care about genetics based research but the people at UAMS did. It was nice to be acknowledged for my efforts. The second time we traveled was to the IMBRE conference at the University of Arkansas in Fayetteville, AR. This conference was much larger than the UAMS conference. There were many more researchers, both undergrad and graduate. I was much more confident presenting my second time around. After realizing that I am the expert concerning my research gave me confidence even when presenting to the genetics professors from Hendrix and University of Arkansas.