MCDB 201L: Molecular Biology Laboratory
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By submitting this essay, I attest that it is my own work, completed in accordance with University regulations. –Sarah McAlister

The Construction of a Universal Entry Vector to Facilitate Genetic Modification of Rhizobia

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Abstract

Rhizobia are soil bacteria that fix nitrogen for legume plants, and legume plants in turn provide rhizobia with carbohydrates for metabolism. This symbiotic relationship eliminates the need for urea fertilizers, thereby reducing environmental toxicity. In order to expand the benefits of rhizobia to other crops, the bacteria must be genetically modified.

To genetically modify rhizobia, a high-throughput cloning platform was successfully created in this study using a pYU2585 vector—known to be viable within rhizobia. Using ligation-independent cloning (LIC), a PCR-synthesized DNA fragment was inserted into the vector to make a universal entry vector. The recombinant plasmid was transformed into *E. coli* cells, successful recombinants were selected, and a reporter gene was inserted into the recombinant plasmid to measure gene expression.

In future studies, this created universal entry vector can help to facilitate Multiplex Automated Genomic Engineering (MAGE) in rhizobia, with the aim of improving rhizobia's ability to establish symbiotic relationships with different plant species.

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Introduction

Rhizobia are soil bacteria that have a symbiotic relationship with legume plants. In this symbiosis, rhizobia fix nitrogen for the legume plants to use in their metabolism, and the legume plants provide rhizobia with life-sustaining carbohydrates. [1] Although this symbiotic relationship is well established between rhizobia and legume plants, rhizobia have not been found to naturally interact with other crops in the same manner. Therefore, in order to supply crops with sufficient nitrogenous compounds for metabolism, urea fertilizer is used in large quantities despite known toxic environmental effects [2]. In accord with the motivations of the proposed Unified Microbiome Initiative [3], recent emphasis has been placed on creating a way in which rhizobia bacteria could symbiotically interact with other crops, as to avoid the need for toxic, synthetically-produced fertilizers. This biological approach to crop fertilization could be further enhanced with the genetic introduction of other plant growth promoters (PGPs) within the rhizobia bacteria; not only would crops have access to fixed nitrogen, but they could also have access to other beneficial traits such as pest and pathogen resistance [4]. Beyond providing fixed nitrogen and other PGPs to crops, rhizobia also have the unique ability to remediate contaminated soil via degradative processes and enhancement of other advantageous bacteria [5], making them an ideal focus for genetic modification for agriculture.

In order for crops—other than legumes—to take advantage of rhizobia's multifaceted agricultural benefits, rhizobia's structure needs to be genetically modified to allow for symbiosis with other crops. To do so, a high throughput-cloning platform should be used to allow for highly efficient systematic testing of different gene edits to rhizobia that would facilitate symbiosis with other crops. Along with symbiosis, edits could also consider familiar and novel PGPs; many have been experimented with to date [6-8]. A genome editing protocol has not yet

been optimized in rhizobia, so work will need to go into understanding the key ways in which genetic modification will be needed. For example, genomic self-repair mechanisms would need to be silenced to allow for incorporation of DNA modifications.

The aim of this project is to broaden the functionality of rhizobia via genetic modification so they can form symbiotic relationships with crops other than legume plants. Doing so would increase rhizobia's potential as a biofertilizer, thus reducing environmental toxicity caused by synthetic agricultural fertilizers. To accomplish this aim, three main steps are necessary, two of which were completed in this experiment. First, a high throughput, universal vector was created so that various gene edits could be tested. This final vector was assembled from a series of steps: polymerase chain reaction (PCR) created a ligation independent cloning (LIC) cassette for the vector, which enabled the efficient binding of different gene edits to the universal vector [9]. The final linear template was the product of two separate "bricks" annealed together following several PCR cycles: one brick included a promoter and LIC sequence, while the other brick included a T1 terminator. Different promoters were tested (Anderson strong, medium and weak) to modulate the expression level of the inserted genes in order to optimize conditions of rhizobia for different crops. Second, a citrine reporter gene was inserted to measure activity/expression level for the given gene edits. Finally, different gene edits were tested to determine the conditions required for rhizobia to form optimal symbiotic relationships with other crop species. This final step was not completed in this experiment but is discussed at the end of the paper as a future direction.

Method

Genetic stock

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One Shot TOP10 *E. coli* competent cells were used as the microorganism subject of this study. The genotype of the strain used is F^- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

PCR amplification of Astr_LIC

In order to modulate gene expression in varying levels, three different promoters were amplified during this process: Anderson strong, Anderson medium, and Anderson weak. A ligation independent cloning (LIC) cassette was inserted as the gene for this amplified sequence, and a T1 terminator was used at the end. This synthetic gene sequence was separated into two different "bricks"—the first included the genes for the Anderson promoter and the LIC cassette, and the second included the T1 terminator. Using PCR, this individual study amplified the first brick using the Anderson strong promoter.

PCR was conducted using eight different test samples of the Anderson strong promoter and the LIC cassette. The different test samples included Phusion® High-Fidelity PCR Master Mix with HF Buffer or Phusion® High-Fidelity PCR Master Mix with GC Buffer, 2.4% DMSO or no DMSO, and either the DNA template or dH_20 in place of the DNA template, so as to have controls. 10µM primers (Fw P5264 and Rv P5267) and template DNA at ~50ng/µl concentration were used. The final volume of the sample was 25 µl. This procedure was used for all PCR reactions in this study unless otherwise noted, and the DNA sequences of all primers used in this experiment can be found in **Table 1.**

PCR was then run using the following conditions:

	Temperature (°C)	**Time**	**# of Cycles**
Initial denaturation	98	30 s	1
Denaturation	98	10 s	30
Annealing	61	30 s	30
Extension	72	2 min	30
Final extension	72	5 min	1

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Hold	4	Indefinitely	1

The PCR products were analyzed using agarose gel electrophoresis (AGE). In each well of a pre-cast agarose gel, 5µl PCR product, 5X Loading Dye, and dH_20 were added to yield a final volume of 20µl. An Invitrogen 1KB PLUS DNA Ladder was added to a separate well. SYBR Safe DNA Gel Stain was used for visualization of the DNA in the agarose. This procedure was used for all AGE visualizations in this study unless otherwise noted. The sample was run for 60 minutes at 110V.

Review of the gel revealed ghost bands, suggesting some impurities (e.g. primers and dimers) in the mix. To remove impurities, the Anderson strong promoter and LIC cassette gene sequence was purified using Kleen Spin Columns. The procedure for using Kleen Spin Columns can be found on p. 2 of the Quantum Prep® PCR Kleen Spin Columns manual, in the section entitled "Technical Information". An AGE of the resulting product (*Astr_LIC)* was run according to standard procedure and no impurities were detected.

Quantification of Astr_LIC

The concentration of the PCR product (*Astr_LIC)* was determined using spectrophotometry. A 2µl sample of the product was placed on a ThermoScientific nanodrop spectrometer and its concentration was recorded. A260/A280 and A260/A230 ratios were also recorded using the nanodrop spectrophotometer to identify potential sources of impurity.

Binding Astr_LIC to T1 terminator

The *Astr_LIC* sample of concentration 53 ng/µl was annealed to the T1 terminator sequence 'brick', a DNA sequence amplified separately via PCR. The T1 terminator of concentration 72ng/µl (Yameli, MCDB 201L, 2016) was diluted with dH_20 to a concentration of ~50 ng/µl. PCR was then run using the two 'bricks'. The first cycle was run in absence of

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primers in order to create the necessary template strand following annealing. In all following cycles, forward and reverse primers were used (Fw P5270 and Rv P5273) to amplify the fragment.

Standard PCR procedure was conducted using eight different test samples of the *Astr_LIC* and *T1* terminator samples. 10µM primers (Fw P5270 and Rv P5273) and template DNA at ~50ng/µl concentration (for both the *Astr_LIC* and *T1* terminator fragments) were used. The final volume of the sample was 25µl.

PCR was then run using the following conditions:

	Temperature (°C)	**Time**	**# of Cycles**
Initial denaturation	95	2 min	1
Denaturation	98	30 s	35
Annealing	70	15 s	35
Extension	72	2 min, 13 s	35
Final extension	72	2 min	1
Hold	4	Indefinitely	1

The PCR products were analyzed using standard AGE procedure. The samples were run for 40 minutes at 150V, and review of the gel revealed significant impurities. To remove impurities, the *Astr_LIC_T1* PCR product was run in a larger well of an agarose gel. In the gel, a 60µl sample was added, containing dH_20, 40 µl PCR product, and 5x Loading Dye. Once the gel was run, DNA was extracted from the gel using the QIAquick® Gel Extraction Kit protocol. The excised gel mass was 308mg, so protocol calculations were made based on that amount.

Standard AGE procedure was again run. The samples were run for 40 minutes at 150V, and the analyzed gel revealed a pure sample so no further purification was needed. A nanodrop DNA analysis was done to assess the concentration of the product.

Ligation independent cloning of Astr_LIC_T1 with pYU2585 vector

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The pYU2585 vector was used for this project, created by Dr. Maria Moreno at Yale University; according to other nomenclature, the vector was named pPZP200bΔT, but it will be referenced as pYU2585 for the duration of this paper. In order to prepare the *Astr_LIC_T1* DNA fragment for insertion into pYU2585, the following were combined to yield a final reaction volume of 10 µl: *Astr_LIC_T1* product at 39.0 ng/µl, dH_20, 10X T4 Buffer (NEB 2), dCTP at 25 mM, 100X BSA (NEB), 1M DTT, and T4 DNA polymerase. After being incubated at 22°C for 30 minutes and 70°C for 20 minutes in a thermocycler, the *Astr_LIC_T1* product was combined with Bsal-T4 treated vector in a 2:1 ratio. This vector was obtained from the following reagents of final volume 100µl: Bsal-digested vector, 10X T4 Buffer (NEB 2), dGP at 25mM, 100X BSA (NEB), 1M DTT, T4 DNA polymerase, and dH_20. The product was incubated at 22°C for 30 minutes and 70°C for 20 minutes in a thermocycler.

The reaction of the T4-treated vector and *Astr_LIC_T1* DNA fragment was incubated for 15 minutes at 22°C and then placed on ice.

Bacterial transformation of pYU2585::Astr_LIC_T1 into E. coli

50µl of *E. coli* Invitrogen TOP 10 competent cells were added to 3µl pYU2585::*Astr_LIC_T1* and incubated on ice for 15 minutes. The mixture was then heat-shocked via incubation at 42°C for 30 seconds before being returned to ice. 100µl of SOC (Super Optimal broth with Catabolite repression) medium was added to the mixture, and it was then incubated at 37°C for 1 hour. Following incubation, 100µl of cells and 50µl of SOC medium were plated on a lysogeny broth (LB) plate containing spectinomycin at 100mg/L. Spectinomycin resistance gene was present within the pYU2585 vector. On a separate LB plate, 50µl of cells and 100µl SOC medium were plated. For both plates, 3 mm Fisher Scientific glass

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beads were used to evenly spread the cells and SOC medium on the plate surface. Following plating, both plates were incubated at 37°C for 16 hours before being stored in 4°C storage.

Following incubation, replica plating was carried out to confirm the presence of *Astr_LIC_T1* in the plasmids. Fifteen isolated colonies from the cells plated with SOC medium were streaked onto two fresh LB plates (one containing 100 mg/L spectinomycin and one containing 100 mg/L spectinomycin + 5% sucrose) and incubated at 37°C for 16 hours before being stored in 4°C storage. Bacterial cells from each of the 15 colonies were also used to make PCR reaction mixtures. Standard procedure for the PCR reactions and subsequent AGE visualization was used. 10µM primers (Fw P5268 and Rv P5269) were used in the PCR reaction, and the final volume of the sample was 25 µl. AGE of the PCR product was run for 40 minutes at 150 V.

PCR was run using the following conditions:

	Temperature (°C)	**Time**	**# of Cycles**
Initial denaturation	95	5 min	1
Denaturation	98	30 s	35
Annealing	67	15 s	35
Extension	72	30 s	35
Final extension	72	2 min	1
Hold	4	Indefinitely	1

Isolation of pYU2585::Astr_LIC_T1 DNA

Plasmid DNA was isolated from the *E. coli* genomic DNA using the QIAGEN® Plasmid Midi Kit (Qiagen, Inc.). Instructions for this procedure can be found in the 2012 *QIAGEN® Plasmid Purification Handbook.*

Construction and insertion of citrine reporter gene

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PCR amplification of citrine was conducted. Standard procedure was followed. 10µM primers (Fw P3743 and Rv 3745) and template DNA (pYU3034-2 at 5ng/µl) were used. The final volume of the sample was 25 µl.

PCR was run under the following conditions:

	Temperature (°C)	**Time**	**# of Cycles**
Initial denaturation	98	2 min	1
Denaturation	98	30 s	35
Annealing	71.8	15 s	35
Extension	72	42 s	35
Final extension	72	2 min	1
Hold	4	Indefinitely	1

AGE of the PCR products was run at 150V for 40 minutes using standard procedure. To remove impurities, the citrine DNA fragment was purified using Kleen Spin Columns and, to confirm successful purification, another AGE was run using standard procedure.

The purified citrine was prepared as an insert via the same process used to prepare the *Astr_LIC_T1* DNA fragment for entry into the pYU2585 vector. Ligation-independent cloning and transformation into *E. coli* bacterial cells were conducted following the same steps taken for the pYU2585::*Astr_LIC_T1* recombinant plasmid. When the *Astr_LIC_T1* DNA fragment was inserted into the pYU2585 vector, it took the place of the LIC cassette.

PCR amplification and AGE visualization of citrine were conducted to ensure that the aforementioned procedures were successful. Standard procedure was followed. 10µM primers (Fw P3743 and Rv P3745) and transformed *E. coli* bacterial cells were used. The final volume of the sample was 25 µl, and PCR was run under the following conditions:

	Temperature (°C)	**Time**	**# of Cycles**
Initial denaturation	98	5 min	1
Denaturation	98	30 s	35
Annealing	71.8	15 s	35
Extension	72	42 s	35

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Final extension	72	2 min	1
Hold	4	Indefinitely	1

AGE was run at 150V for 40 minutes. Plasmid DNA was isolated from the *E. coli* genomic DNA using the QIAGEN® Plasmid Midi Kit (Qiagen, Inc.).

DNA sequencing

The final step to this experiment involved the DNA sequencing of pYU2585::*Astr_LIC_T1* and pYU2585::*Astr_Cit_T1*. The W. M. Keck Foundation Biotechnology Resource Center at Yale University performed DNA sequencing. DNA samples were submitted for sequencing according to Premixed Tube Submission Policy Protocol (*see http://keck.med.yale.edu/dna/protocols/tube/).* Sequence analysis was carried out using the Lasergene SeqMan Pro software (DNASTAR version 12.3.1).

Results

Design of universal entry vector

The aim of this project was to develop a universal entry vector that is viable within rhizobia and allows for the insertion of different genes of interest. **Figure 1** shows an overview of this process. pYU2585 was chosen as the vector in this project given its proved viability within rhizobia, and a synthetic gene was constructed that contained an Anderson strong promoter (Astr), LIC cassette (LIC), and T1 terminator (T1).

Once the *Astr_LIC_T1* synthetic gene was inserted into pYU2585 via ligation independent cloning, the vector was transformed into *E. coli* bacterial cells and recombinant plasmids were selected. A citrine reporter gene was added to test gene expression levels, with the future goal of swapping it for different genes of interest.

Design of Astr_LIC_T1 synthetic gene

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The synthetic gene *Astr_LIC_T1* contained an Anderson strong promoter (Astr), LIC cassette (LIC), and T1 terminator (T1). Anderson promoters were used because they can successfully express proteins in *E. coli* at varying levels (strong, medium, and weak). A T1 terminator was chosen due to its ability to successfully function in *E. coli.* A LIC cassette was chosen to facilitate ligation independent cloning and to aid in negative selection processes due to its *SacB* gene (the *SacB* gene produces lethal metabolites in the presence of sucrose). In order to anneal these DNA fragments needed for the synthetic gene, primers were designed to create homology between the pieces; the pieces were then annealed/extended via PCR.

Construction of Astr_LIC_T1 synthetic gene

In order to construct the *Astr_LIC_T1* synthetic gene, the LIC cassette was amplified from template DNA via PCR, and primers were added to create the Anderson strong promoter and homology with the T1 terminator. This resulted in an *Astr_LIC* fragment of length 1994 bp (**Figure 2B-1**).

Separately, the T1 terminator (T1) was amplified from template DNA via PCR, and primers were added to create homology with the LIC cassette. This resulted in a fragment of length 240 bp (**Figure 2B-2**). Via PCR, the T1 terminator DNA fragment was annealed to the *Astr_LIC* fragment, and LIC 3' and 5' extensions were added with primers to result in a final synthetic *Astr_LIC_T1* gene of length 2246 bp (**Figure 2B-3**). **Figure 2A** shows an overview of this full process.

Generation of pYU2585::Astr_LIC_T1 and bacterial transformation

Following the successful construction of the *Astr_LIC_T1* synthetic gene, the *Astr_LIC_T1* DNA fragment was inserted into the pYU2585 vector via ligation independent cloning. Then, using the heat-shock method, the pYU2585::*Astr_LIC_T1* plasmid was

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transformed into *E. coli* bacterial cells. Recombinant plasmids were selected using positive and negative selection (**Figure 3**); the positive selection was conducted on an agar plate with 100 mg/L spectinomycin, while the negative selection was conducted on an agar plate with 100 mg/L spectinomycin and with 5% sucrose.

The pYU2585::*Astr_LIC_T1* vector was then sequenced. There was 99% overall alignment between pYU2585::*Astr_LIC_T1* and the reference sequence, and there was 100% sequence alignment between pYU2585::*Astr_LIC_T1* and the reference sequence in 5 regions of interest: LIC 5' and 3' Extensions, Anderson strong promoter, T1 Terminator, and BSA restriction sites. See **Figure 4** for the DNA sequences of these 5 key regions of interest. These are the key regions of interest for sequencing because other relevant regions of the plasmid (e.g. origin of replication, spectinomycin resistance gene) exhibited functionality in previous portions of this experiment.

Construction of Astr_Cit_T1 synthetic gene

As presented by **Figure 5A**, there were several steps involved in the construction of the pYU2585::*Astr_Cit_T1* recombinant plasmid. **Figure 5B** illustrates the results of these steps: **Figure 5B-1** shows that the citrine gene was amplified via PCR and resulted in a final length of 720 bp, and **Figure 5B-2** shows the citrine inserted within the pYU2585::*Astr_Cit_T1* recombinant plasmid. **Figure 5C** reveals the DNA sequence of the citrine gene. DNA sequencing has not yet been completed for citrine, but it is expected to reveal that the citrine reference sequence and constructed gene have 100% alignment.

Discussion

The aim of this project was to take initial steps in broadening the functionality of rhizobia via genetic modification to allow symbiosis with additional plant species and to increase the

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benefits offered by rhizobia to other plants. To carry out this aim, a universal entry vector was designed to enable insertion of genes of interest via ligation independent cloning; the entry vector could then be placed within rhizobia (**see Figure 1**). Results from this project indicate that this universal entry vector was successfully developed.

Preliminary analyses confirmed that the pYU2585 vector was compatible with rhizobia, making it useful for this project. Following confirmation of compatibility with rhizobia, the *Astr_LIC_T1* DNA fragment was constructed and inserted into the pYU2585 vector. The agarose gel electrophoresis (AGE) visualizations in **Figure 2B** indicate the successful construction of this fragment through three iterations of PCR; the starting materials of the LIC Cassette and T1 terminator were converted via primers and PCR into a single *Astr_LIC_T1* DNA fragments with 5' and 3' LIC extensions for insertion into the pYU2585 vector (see **Figure 2A**).

Astr_LIC_T1 was successfully inserted into the pYU2585 vector, and pYU2585::*Astr_LIC_T1* was transformed into *E. coli* bacterial cells. The success of these procedures was verified through the following: (1) positive and negative selection yielded bacterial colonies containing pYU2585::*Astr_LIC_T1* (see **Figure 3A-B**), (2) AGE visualization highlighted the presence of the T1 terminator within the recombinant plasmid (see **Figure 3C**) and (3) sequencing the recombinant plasmid revealed a 99% match between intended and actual sequence of pYU2585::*Astr_LIC_T1* (see **Figure 4**). This high similarity (99%) indicates that few mutations occurred during PCR.

The construction of this recombinant plasmid marks the successful creation of a universal entry vector that can be inserted into rhizobia and used to express genes of interest. In the final stages of the experiment, the LIC cassette was replaced with the Citrine (Cit) reporter gene (see

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Figure 5A). The band visualized in **Figure 5B-2** reveals that this replacement was successful, a verification of the plasmid's capacity to interchange genes via ligation independent cloning.

Limitations and Future Directions

The project had no unexpected results, but there are several limitations that must be considered. First, although sequencing analysis reported a 99% match between the intended and actual gene sequence of the constructed recombinant plasmid, there was evidence of point mutations in several locations; these mutations were likely introduced during the PCR cycles run during this project. Although small in number and not found in any key regions in the plasmid, the mutations' potential impact on the functioning of the plasmid should be recognized.

Another significant limitation in this project is the uncertain viability and functionality of the plasmid and its components in different organisms—most importantly, in rhizobia. Anderson promoters have not been used previously in rhizobia, so their functionality and expression levels remain unknown. Even if Anderson promoters serve as effective promoters in rhizobia, their expression levels may be considerably different than expected. The same is true for the T1 terminator; although known to function well in *E. coli*, it has never been tested for compatibility with rhizobia. If one of the recombinant plasmid's components do not work within rhizobia, other options would need to be tested in its place.

The most salient future direction of this research is to use the pYU2585::*Astr_LIC_T1* recombinant plasmid to facilitate Multiplex Automated Genome Engineering. Multiplex Automated Genome Engineering, or MAGE, is a method of using oligonucleotides to generate many mutations across a genome. To make MAGE optimally efficient, a beta protein is needed to facilitate recombination. This protein—beta recombinase—increases efficiency from 1:100,000 recombination successes to 1:3 recombination successes [10]. *E. coli* lambda phages

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create one variant of this beta protein, but different beta homologs to the protein must be evaluated to find one that is functional within rhizobia. This will require various homologs to be inserted into the pYU2585::*Astr_LIC_T1* entry vector and tested within rhizobia.

Before MAGE can be introduced to rhizobia, mismatch repair must be muted to prohibit polymerases from editing newly introduced mutations. This requires the silencing of the *MutS* gene, which is normally responsible for correcting genomic coding errors [11]. The exact homolog to the *MutS* gene has not been identified within rhizobia, but sequence comparisons between known *MutS* gene sequences and the rhizobium genome should aid in its identification. Once identified, it will be necessary to devise a method of muting this gene, potentially via processes like RNA interference [12].

Only after these aforementioned steps can MAGE be employed in rhizobia in attempt to create mutations that confer benefits to rhizobia and/or its symbionts. This could include mutations that facilitate the interaction of rhizobia with a wide variety of different plants (other than just legume plants); such would allow the rhizobia to provide these plants with fixed nitrogen [1], replacing the need for toxic urea fertilizers [2]. Beneficial mutations would also include those that that confer additional benefits to plant symbionts, such as pest and pathogen resistance [4]. This could provide a natural alternative to fertilizers and pesticides and could reduce overall environmental toxicity.

In summary, the pYU2585::*Astr_LIC_T1* entry vector was successfully created in this project and provides the opportunity to expand the host range of rhizobium to plants other than legumes. Its ligation independent cloning platform allows easy replacement of different genes of interest, and these different genes will be essential to rhizobia's successful relationship with legumes and (potentially) other plants.

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Tables and Figures

Table-1
DNA sequences for primers used during PCR reactions.

Primer Name	Sequence
P5264	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCAGGATACTTCCAATCCATGGAGACC
P5267	TCCCCCATCAAGCTTTATCCACCTTTACTGGAGACCG
P5268	CAGTAAAGGTGGATAAAGCTTGATGGGGGATCC
P5269	CCTGCGTTATCCCCTGATT
P5270	TACTTCCAATCCTTGACGGCTAGCTCAGTCCTAGGTA
P5273	TATCCACCTTTACTGCCTGCGTTATCCCCTGATT
P3743 GGAGCT	TACTTCCAATCCATGGTGAGCAAGGGCGA
P3745	TATCCACCTTTACTTCACTTGTACAGCTCGTCCATGC

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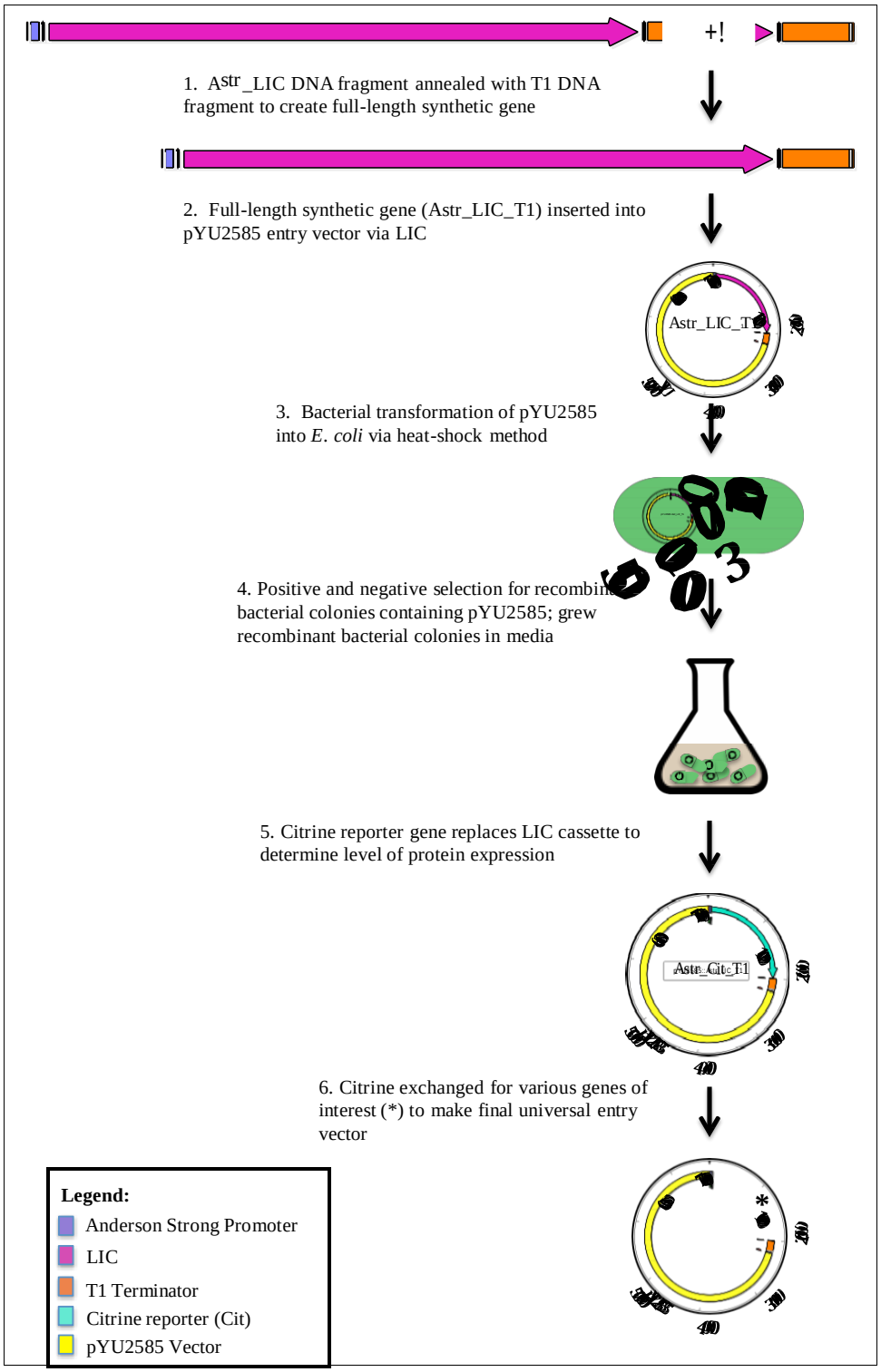

Figure-1. Designing a universal entry vector for rhizobia.
This figure broadly details the design process for the creation of the pYU2585::Astr_LIC_T1 universal entry vector.

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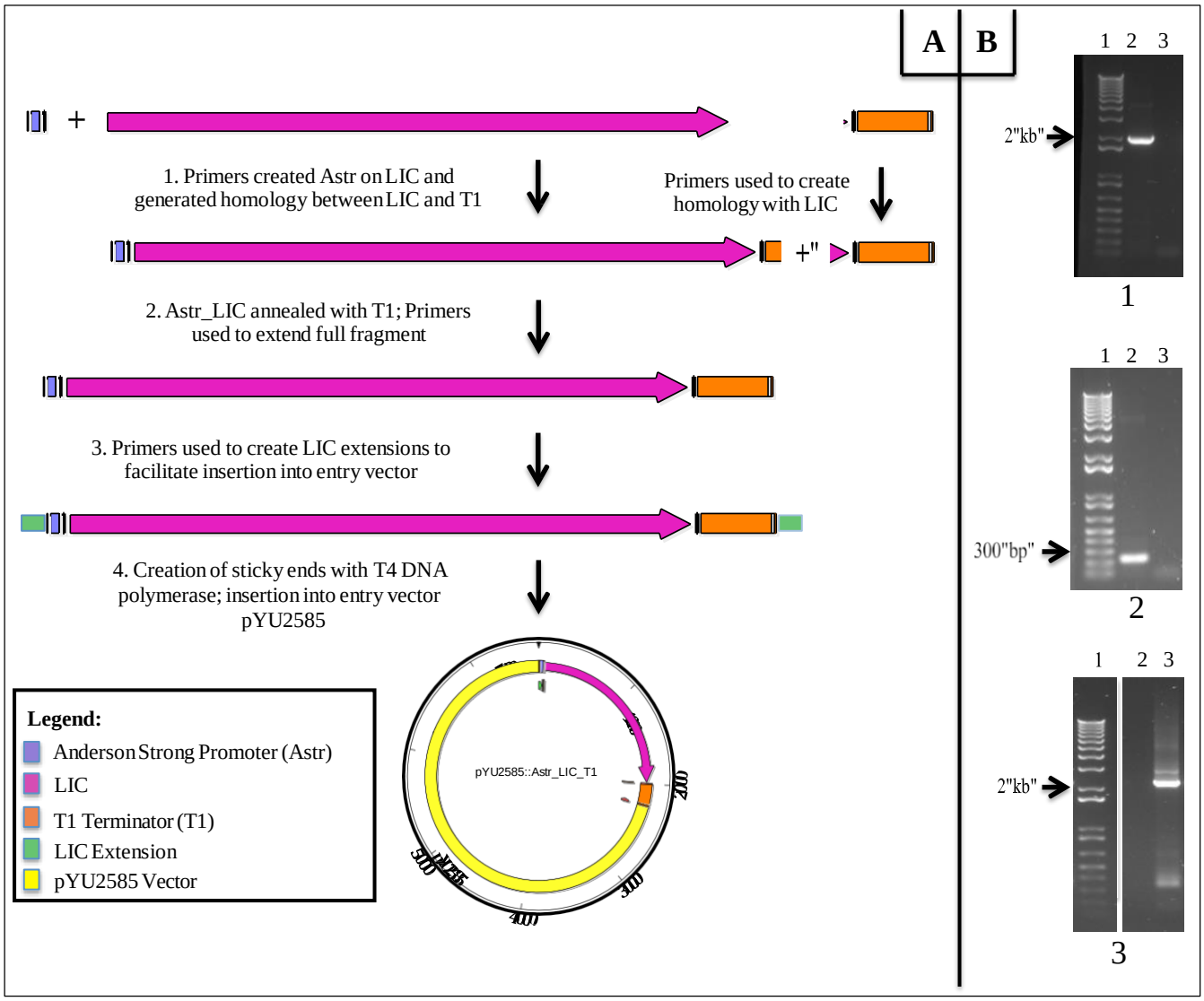

Figure-2. Creation of pYU2585::Astr_LIC_T1.
(A) Details of the construction of the pYU2585::Astr_LIC_T1 recombinant plasmid. **(B)** A display of the gel electrophoresis images of the intermediary and final products of the process. All gels were run in 1% agarose with 1X Rapid Run Buffer. For Image 1, Lane 1=1kb PLUS DNA Ladder, 2=sample of interest, 3=control; Image 1 shows the successful construction of the Astr_LIC DNA fragment, size=1994 bp. For Image 2, Lane 1=1kb PLUS DNA ladder, 2=sample of interest, 3=control; Image 2 shows the successful construction of the T1 terminator with LIC homology, size=240 bp. For Image 3, Lane 1=1kb PLUS DNA Ladder, Lane 2=control, Lane 3=sample of interest; Image 3 shows the successful construction of the Astr_LIC_T1 synthetic gene with annealed 3' and 5' LIC extensions, size=2246 bp.
Note: Image 2B-2 came from Yameli, MCDB 201L, Spring 2016.

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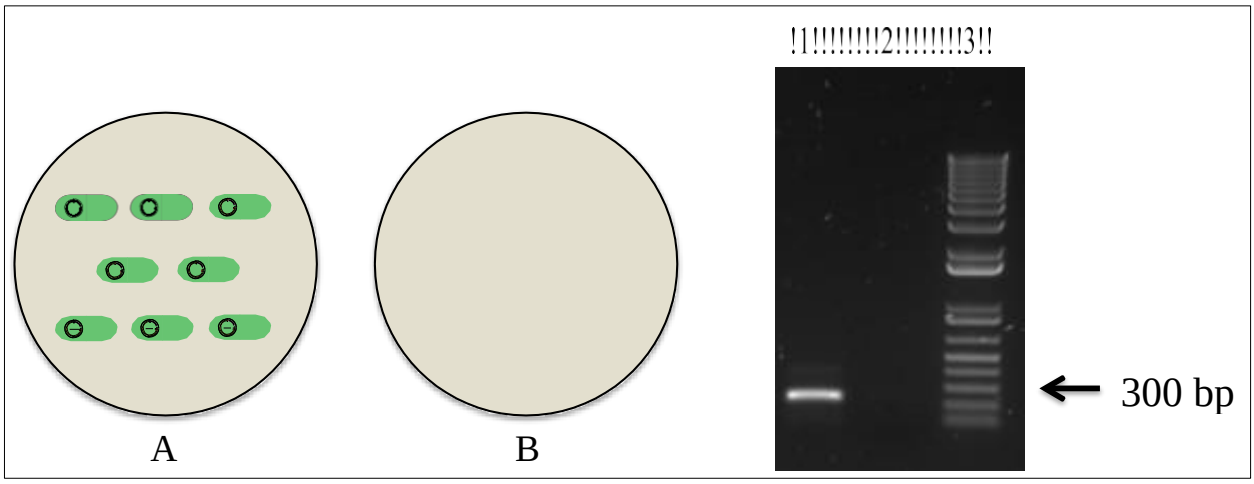

Figure-3. Selection of recombinant bacterial colonies with pYU2585::Astr_LIC_T1. **(A)** Positive selection for the recombinant *E. coli* colonies containing pYU2585::Astr_LIC_T1. Plate A contained 100 mg/L spectinomycin and facilitated positive selection; the pYU2585 vector contains a spectinomycin antibiotic resistance gene, so all bacterial colonies containing this vector of interest survived. **(B)** Negative selection for the recombinant *E. coli* colonies containing pYU2585::Astr_LIC_T1. Plate B contained 100 mg/L spectinomycin and 5% sucrose and facilitated negative selection; Astr_LIC_T1 contains the SacB gene within the LIC cassette, which produces lethal metabolites in the presence of sucrose. All bacterial colonies containing the Astr_LIC_T1 synthetic gene died. **(C)** The gel electrophoresis image shown above was run using 1% agarose gel and 1X Rapid Run Buffer. Lane 1=sample of interest, Lane 2=control, Lane 3=1kb PLUS DNA Ladder. The gel was run to detect the presence of the T1 terminator in the recombinant bacterial cells. T1 terminator size=240 bp, so the image verified that the T1 terminator was present in the selected bacterial cells.

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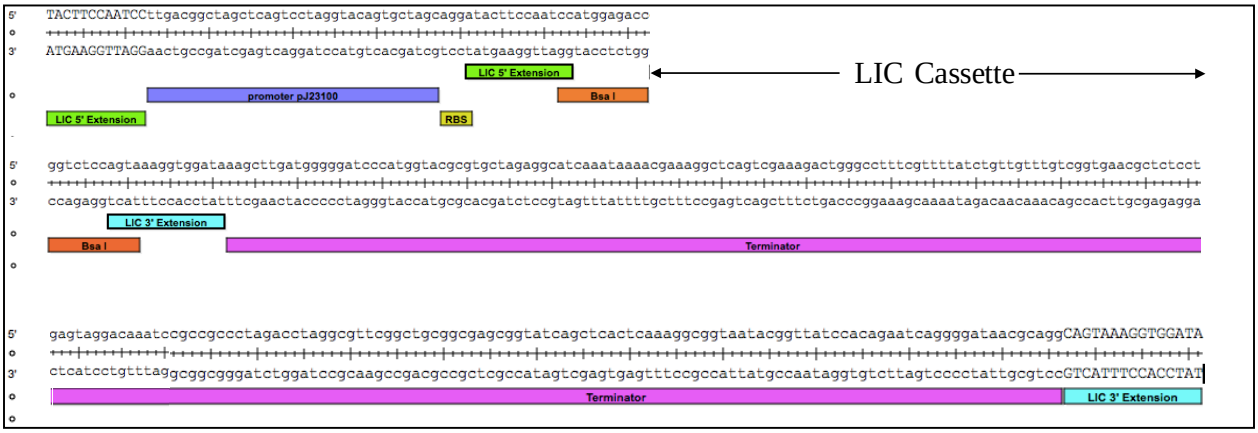

Figure-4. DNA sequence for pYU2585::*Astr_LIC_T1*. The image above shows 5 labelled key regions of the sequence: LIC 5' and 3' Extensions, Anderson strong promoter, T1 Terminator, and BSA restriction sites. Through DNA sequencing, these regions were found to align completely (100%) with the reference sequence. Capitalized letters in the sequence represent DNA sequences homologous to the pYU2585 entry vector; this homology facilitates insertion into the vector.

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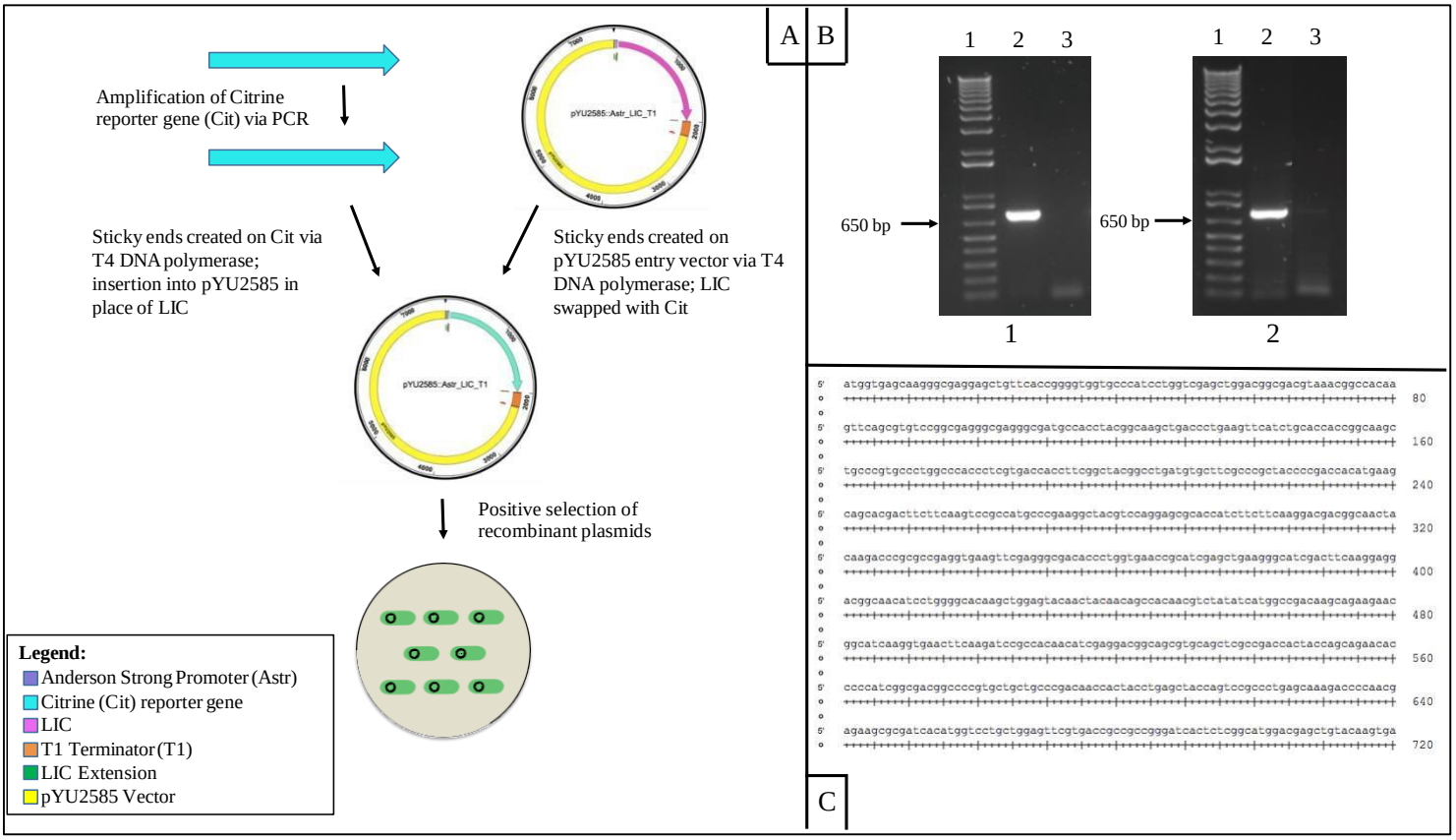

Figure-5. Construction of pYU2585::*Astr_Cit_T1*.
(A) Steps for generating the pYU2585::*Astr_Cit_T1* recombinant plasmid. **(B)** A display of the gel electrophoresis images of the intermediary and final product of the process. Gels were run in 1% agarose with 1X Rapid Run Buffer. For Image 1, Lane 1=1kb PLUS DNA Ladder, 2=sample of interest, 3=control; Image 1 shows the successful PCR amplification of citrine, size=720 bp. For Image 2, Lane 1=1kb PLUS DNA ladder, 2=sample of interest, 3=control; Image 2 shows the successful insertion of *Astr_Cit_T1* within the pYU2585 vector, size=720 bp. **(C)** The DNA sequence of the citrine reporter gene; the constructed citrine gene is expected to align completely (100%) with the reference sequence.

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