Function of TOPLESS in *Oryza sativa*

Discovering Science, Culture, and Myself

Report by Joshua Lee

Peking University | 5 Yiheyuan Rd, Haidian | Beijing, China | english.PKU.edu.cn/

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Abstract

The TOPLESS gene family, which is composed of the TOPLESS (TPL) gene and four TOPLESS-related (TPR) genes, functions as a transcriptional corepressor that plays vital roles in plant development. The TPL and TPR genes play an especially significant role in the morphology of leaves, one of the most important organs of a plant. The leaves of plants are responsible for carrying out photosynthesis, the mechanism that utilizes sunlight to synthesize sugars, which plants use as food, from carbon dioxide and water, creating oxygen, the air we breathe, as a byproduct. These TPL and TPR genes have been studied in *Arabidopsis thaliana* and have also been proven to play a major role in auxin and jasmonate pathways. Auxin is a plant growth hormone that promotes cell growth, plant elongation, and plays a role in root development. Jasmonate hormones regulates pollen development, fruit ripening, and other developmental processes, and it is also responsible for responding to biotic and abiotic stresses.

Although these genes have been extensively studied in the *Arabidopsis thaliana*, an organism widely used in plant biology due to its fast life cycle and small genome, only preliminary studies have been conducted about the TPL and TPR genes on rice. The goal of this project is to discover the role of these 2 genes through the creation of loss-of-function mutants.

To do so, I obtained the nucleotide sequence for the TPL gene in the *Arabidopsis thaliana* from an online database and used it as a query sequence to run it through the basic local alignment search tool (BLAST) tyto find similar sequences in the Oryza sativa, or rice. I selected 4 genes to silence because of the phenomenon of functional redundancy. Often times, genotypic alterations do not affect the phenotype of an organism because different genes may carry out similar functions. To make sure that a phenotypic mutation occurs, I will knock out four different genes, each located on different chromosomes but with similar sequencing, simultaneously.

Traditional methods for inducing gene mutations were the use of ethyl methanesulfonate and T-DNA. Ethyl methanesulfonate (EMS) creates point mutations in order to silence genes; however, it's target site for mutation is unspecific and random. Using this method to silence genes would require gene mapping in order to find where the mutation took place — a process that is very time consuming. Manipulating T-DNA to cause insertional mutagenesis not only prevents gene transcription, but unlike EMS, can mark where the mutation took place. However, T-DNA insertion is ineffective in causing null mutations in smaller genes because of the large range of its effect; it may disrupt the transcription of neighboring genes.

In order for specific mutations to occur, other methods were created. These methods intentionally cause site-specific double stranded breaks (DSBs) in an organism's DNA to utilize the organism's DNA repair mechanisms. The systems that can induce DSBs are Zinc finger nucleases (ZFN), Transcription activator-like effector nucleases (TALENs), and now, CRISPR/Cas. The new CRISPR/Cas system allows for simultaneous gene silencing and is more efficient, more inexpensive, and less time consuming than ZFNs and TALENs, both of which having greater potential to create off target mutations. The CRISPR/Cas system's applications are boundless, but in the context of my experiment, has the potential to create a greater quality and greater quantity rice by understanding the roles of the TPL and TPR genes — genes known to play vital roles in the morphology of leaves, auxin pathways, jasmonate and signalling — specifically in rice. Greater quality and greater quantity rice will have direct impact on food security as rice is cultivated on eleven percent of the world's arable land.

1.1 CRISPR Cas System

In 1972, Paul Berg's creation of recombinant DNA allowed for technologies to drive genetic clone engineering, which radically affected many areas of biology. It's invention led to innovations that prompted greater yields and nutritional value in crops, and fostered many medicinal advancements as well. This groundbreaking discovery caused a great excitement in the science world — an excitement that spread even to the public after the successful cloning of Dolly, the sheep.

Similarly, a new discovery in the world of science is causing great excitement. This new technology, targeted genome editing via RNA guided Cas9 endonucleases, or the CRISPR/Cas system, has the ability to directly impact many aspects of our society. From increasing farm yields to targeting the genes responsible for pandemic diseases, the CRISPR-Cas9 has boundless applications. Its potential to further the increase of food production, food nutrition, and food security can not be neglected.

1.2 History of CRISPR Research

Clustered regularly interspaced short palindromic repeats (CRISPRs) were first found in 1987 with the discovery of the *iap* gene in *Escholoria coli* when scientists observed homologous nucleotide sequences separated by unique spacers. In 2005, scientists hypothesized that the CRISPRs played a role in microbial immune systems due to the similarities between spacers on the CRISPR array and phage DNA.

This hypothesis was proven true when scientists learned that these CRISPRs, along with CRISPR associated genes (Cas genes) played a role in bacterial adaptive immunity against phages and viruses in the *Streptococcus thermophilus* through the experimentation and observation of phage-bacteria interactions. A phage sensitive *S. thermophilus* strain was introduced to a bacteriophage, and the *S. thermophilus'* CRISPR loci was then analyzed. It was shown that the *S. thermophilus* integrated additional spacers into its CRISPR loci identical to the bacteriophage DNA, becoming resistant to the bacteriophage. The deletion of these acquired spacers caused the *S. thermophilus* to be resensitized to the bacteriophage.

Then in 2012, a mechanism to manipulate the CRISPR/Cas systems for RNA mediated targeted genome editing was designed using the Cas9 endonuclease, CRISPR RNAs (crRNAs), and trans-activating crRNAs (tracrRNAs) to guide double stranded breakages for subsequent genome editing.

Prior to the usage of the CRISPR/Cas system to induce double stranded breakages, Transcription activator-like effector nucleases (TALENs) and Zinc finger nucleases (ZFNs) were used as mechanisms for targeted genome editing but are more expensive, less efficient, less accurate, and more prone to off-target effects and mutations than the CRISPR/Cas system is.

1.3 CRISPR/Cas in Nature

The CRISPR/Cas system in bacteria and archaea plays a major role in bacterial and archaeal defense against invasive organisms and is so far the only adaptive immunity system discovered in archaea and

bacteria. It is based on the self-non-self discrimination principle but is unique in its integration of genetic material from invasive viruses and plasmids to protect itself from reinfection. This system of adaptive immunity has three phases — acquisition, expression, and interference.

In the acquisition phase, also referred to as the vaccination or adaptive phase, bacteria or archaea with CRISPR loci add new spacers into its CRISPR array by including pieces of foreign sequences into its own bacterial or archaeal chromosome. The short fragments of the invasive organism's DNA are called protospacers, and the addition of these spacers into the CRISPR array are polarized to the end closest to the Cas genes.

Next is the expression phase, or the CRISPR RNA biogenesis phase, in which the CRISPR array is transcribed as a precursor CRISPR RNA (pre-crRNA), which is subsequently cleaved into smaller CRISPR RNAs (crRNAs) by endoribonucleases. Each of these crRNAs are made up of a spacer as well as a CRISPR repeat.

Lastly, during the interference stage, the mature crRNAs along with Cas endonucleases recognize specific sequences of the invasive organism and will silence the foreign sequences.

In summary, short fragments homologous to infectious agents' DNA are adapted into a CRISPR array and are used in the form of guide CRISPR RNAs (crRNAs) to protect itself from reinfection from previously encountered invasive organisms.

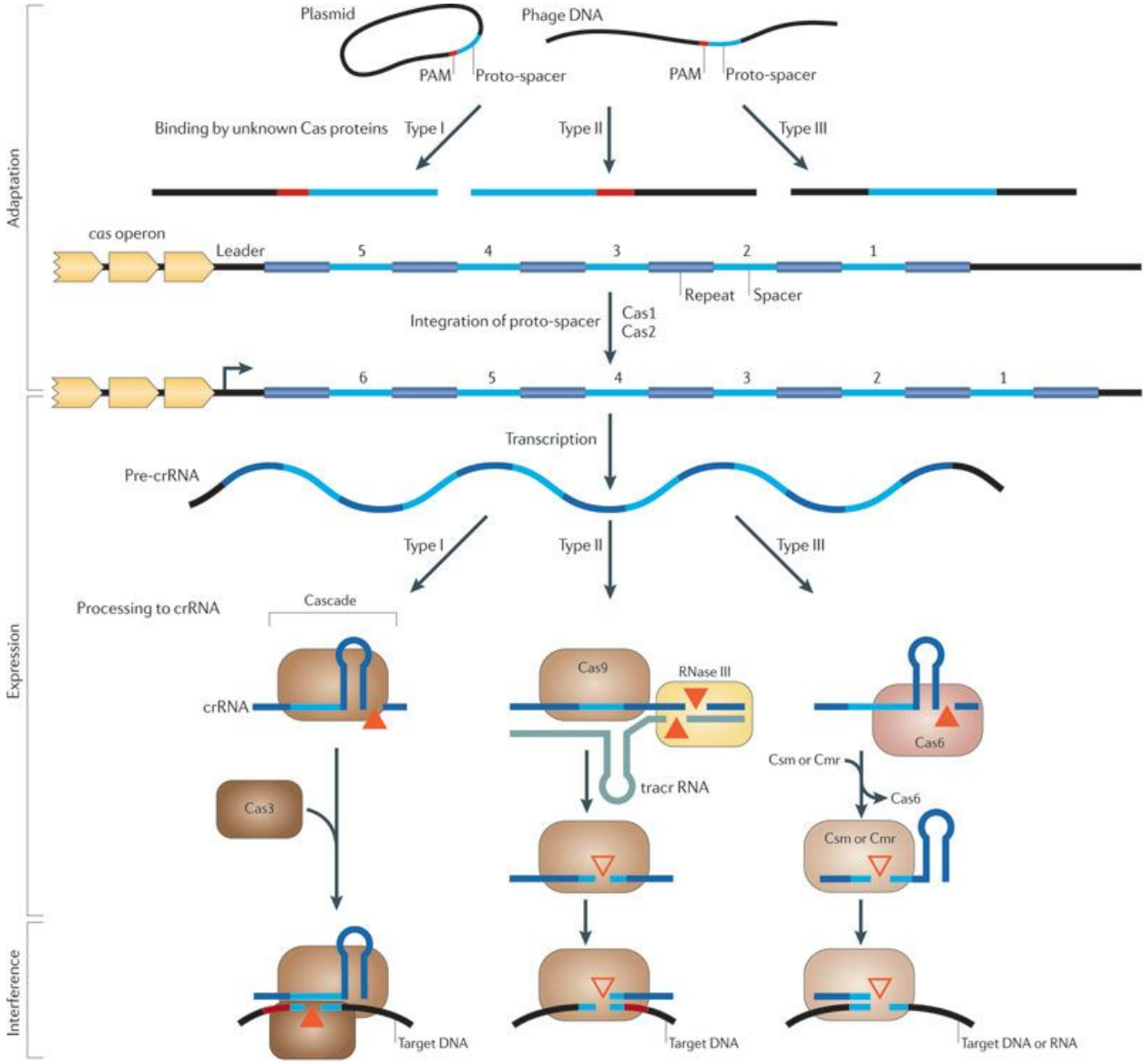
1.4 Types I,II, and III

The CRISPR-Cas system in adaptive immunity can be classified into three distinct types based on the CRISPR-associated (*cas*) genes used, the placement of *cas* operons, and the arrangements of spacer-repeats in the CRISPR array.

The similarities between the CRISPR complexes of Type I and Type III CRISPR-Cas systems suggest that they share a common ancestor while the Type II system's origin is still obscure.

Type I and Type II systems both require a protospacer adjacent motif (PAM) that is located immediately downstream of the protospacer on the non-target DNA strand.

Type II CRISPR-Cas is the minimal CRISPR-Cas system because it only utilizes 3 to 4 *cas* genes: the pair of *cas1* and *cas2* genes that are required for spacer integration. Type I and III often have the *cas1* and *cas2* genes as well. Type II systems are especially unique due to the signature *cas9* gene which is a protein that can target and cleave invasive DNA on its own unlike the Type I and Type III systems which require multiple genes for DNA targeting and subsequent DNA cleavage.



(Source: Nature Journal)

1.5 Applications of the CRISPR/Cas System

The CRISPR array in bacteria and archaea can be used as a phylogenetic tree due to the way spacers are acquired — chronologically— spacer integration is polarized to one end of the CRISPR array. The function of the array as a genetic record can give insight into events that occurred over time, such as the evolution of different strains of bacteria and archaea and what organisms the bacteria and archaea have interacted with.

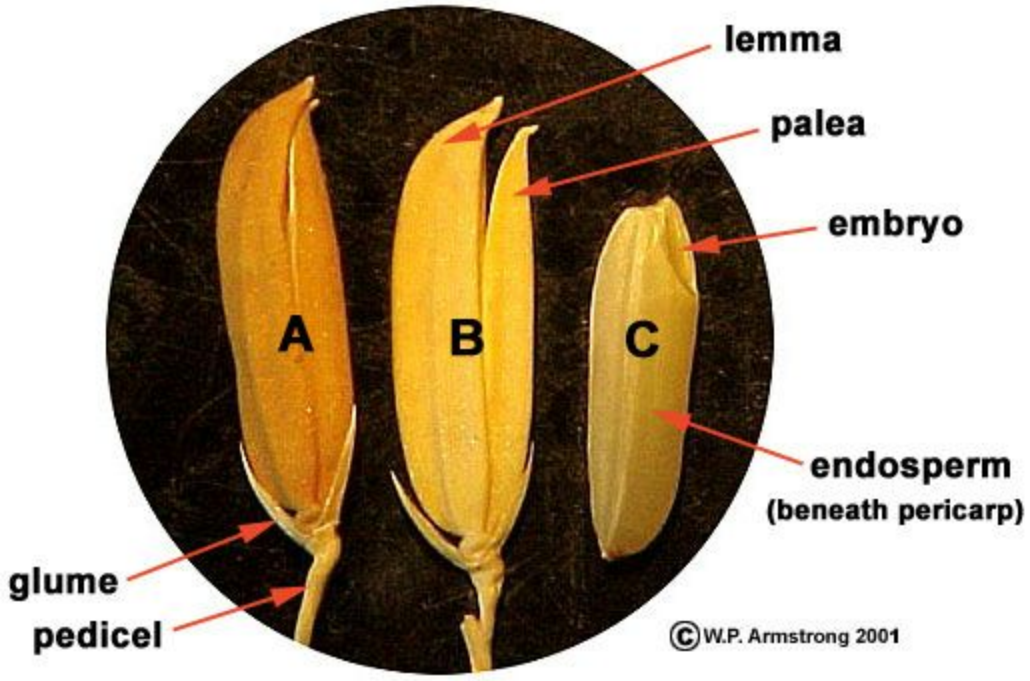
Scientists have also exploited its natural immune function to vaccinate different diseases. Such applications have already been put into use in the food industry, more specifically in the yogurt and cheese producing industries. More recently, scientists have found that the Cas9 gene could be

reengineered for genome editing in plant organisms which is what my experiment is based on: repurposing the Cas9 gene to silence the TOPLESS (TPL) and TOPLESS-related (TPR) genes.

2.1 Oryza Sativa

Oryza sativa, or asian rice, is a cereal grain that is considered a staple food to a large population of the world. Rice is a monocot that is grown in places with an annual rainfall of at least 1500 mm. *Oryza sativa* naturally has little drought tolerance.

In my project I manipulated rice seedlings to grow and integrate foreign DNA via RNA mediated CAS endonucleases to knock out 4 different genes simultaneously.



I shed the lemma and the palea to expose the endosperm and the embryo— inserting the embryo into a cell growth medium to induce the callus. The callus, which is a group of undifferentiated cells, is really important.

3.1 The TPL (TOPLESS) Gene

The function of TPL and TPL-related proteins are known in *Arabidopsis thaliana* but not *Oryza sativa*. TIE 1 is a transcriptional repressor that, when overexpressed in *Arabidopsis,* is known to curve the leaves of the plant upwards instead of being flat. The underexpression of TIE1 leads to a downward curved leaf.

TIE 1 proteins interact with the transcription factors TCP. On a molecular level, the overexpression of TIE 1 leads to a "fatter" cell. TCP binds with the promoter region of the gene. Cofactors like TIE1 help TCP.

4.1 The Experiment

4.2 Procedures and Materials

Tuesday June 16th, 2015

Today I found the sequence of the *TOPLESS (TPL)* gene in the *Arabidopsis thaliana* from an online database using the gene identification At1g15750.

Wednesday June 17th, 2015

Today I used the protein sequence of the At1g15750 to find similar matches in the *Oryza sativa* and found 3 matches that were similar.

Then I used those 3 matches (gene ID: Os03g0254700, Os08g0162100, Os01g0254100) to create a new parameter for the second round of finding potential protein sequence matches, in the *Oryza sativa,* to the *Arabidopsis thaliana*. I found 1 with the gene ID, Os05g0240200.

I found the Complementary DNA Sequence (CDS) as well as the complete genomic sequence for all four protein sequences: Os03g0254700, Os08g0162100, Os01g0254100, Os05g0240200. I found the CDS sequence in addition to the genomic sequence, because the CDS sequence only contains exons while the genomic contains exons and introns. When I create the target site, I do not want to include any part of an intron by accident.

Thursday June 18th, 2015

Today I used the local vector NTI database bioinformatics system to find the target sites on the four genes I selected.I aligned the genomic sequence and the CDS of each gene to eliminate any untranscribed regions (UTRs); I also did this to find the exons of each gene as well.

1) I aligned the genomic sequence and the CDS of each gene to eliminate any untranscribed regions (UTRs); I also did this to find the exons of each gene as well.
2) I labeled the UTRs "Deletion" and aligned the "Deletion" with the genomic sequence to find the exons' exact location.
3) I labeled 4 exons on all the genes as potential target sites
4) Then I used a program Dr. Huang made to find potential spacers for all the four *Oryza sativa TOPLESS* genes.
5) I used spacers corresponding to the exons I labeled and made sure that the spacers were a good distance from each other. For each spacer I chose, I found its complementary sequence.
6) Then I sent the materials to a partner institution so that I could get the spacers!

I selected 2 target sites on each of the four genes to create 2 entry vectors that would be able to silence all four genes. I did this as a precautionary measure just in case 1 of the entry vectors fails to work or be

constructed as planned. Therefore, I ordered 16 spacers: 8 target sites with the sequences for both strands of the DNA.

Spacers 5'-3'

GGCAGCACTTTGAAGACCTGGTGC	TPL1-Spacer F
aaacgcaccaggtcttcaaagtgc	TPL1-Spacer R
GGCAggcggtggagatactcgtga	TPL1-Spacer F2
aaactcacgagtatctccaccgcc	TPL1-Spacer R2
GGCAcaaccttggtaaagccaccg	TPL2-Spacer F
aaaccggtggctttaccaaggttg	TPL2-Spacer R
GGCAtgaagcttgtcacggaacaa	TPL2-Spacer F2
aaacttgttccgtgacaagcttca	TPL2-Spacer R2
GGCATTGAAGAGAAGGTCCATGCT	TPL3-Spacer F
aaacagcatggaccttctcttcaa	TPL3-Spacer R
GGCAATGAACAGCTGTCTAAGTAC	TPL3-Spacer F2
aaacgtacttagacagctgttcat	TPL3-Spacer R2
GGCACTGCGCCTCCGGAAGAAGTT	TPL4-Spacer F
aaacaacttcttccggaggcgcag	TPL4-Spacer R
GGCATCTCATAAACAAGCTATCCC	TPL4-Spacer F2
aaacgggatagcttgtttatgaga	TPL4-Spacer R2

Dr. Huang's program

1) Spacers are 20 base pairs (bp) long
2) He found all potential candidates by first scanning genome for a PAM sequence (NGG) and the 20 bp before it
3) He aligned all the putative sequences to the whole rice genome to make sure there would be no off target effects unless there was no PAM sequence before it.

Friday June 19th, 2015

Today we received the spacers (16) I engineered and they were in powdered form. I added distilled water (ddH_20) in each spacer epitube to make the concentration of the spacer 10 micromolars. Then I put the complimentary spacers together in tubes to anneal them using the polymerase chain reaction (PCR) machine.

PCR procedure and information

Important materials

- Template (Usually the vector)
- Primers
- DNA Polymerase

- DNTPs
- Buffer
- Mineral oil
- 2 Controls (- and +)

Steps

1) Denaturation
2) Anealation
3) Extension

Settings

1) 96º for 2 minutes
2) 94º for 30 seconds
3) 56º for 30 seconds
4) 72º for 90 seconds
5) repeat 35 times
6) 72º for 1 second
7) 15º for 1 second

Total per PCR tube: 11 microliters

2 taq mix	55 microliters
2 spacers (reverse/forward)	2.75/2.75 microliters
ddH_2O	49.5 microliters

While the PCR machine was going, I made sure that there was enough BSA1 restriction enzymes by running it through the gel electrophoresis; there was enough. Then I used the BSA1 to linearize the entry vector for the TOPLESS spacers. I used the fragments from the PCR machine and inserted them into the entry vector.

Entry Vector	1 microliter
Fragments	6 microliters
5xT4 Buffer	2 microliters
T4 Ligase	1 microliter

Figures in Page 30 of Notebook

I left the 8 epitubes in the 22ºC fridge because that is the best temperature T4 Ligase works in, and left them in there for 3 hours.

I got 8 epitubes of *Escholoria coli* competent cells: treated with dimethyl sulfoxide (DMSO) so that the *E. coli* more easily accepts exogenous DNA by weakening the cell membrane. Then I proceeded with the *E. coli* transformation.

Procedure for *E. coli* transformation

1) put the entry vector into the tube of *E. coli* competent cells
2) leave it in for at least 10 minutes on ice
3) Do a heat shock at 42ºC for 90 seconds
4) Put it back in ice for 5 minutes
5) Put 300 microliters of LB growth medium in the tube to culture the bacteria
6) Put it in a shaking bed at 37ºC (best temperature for *E. coli* to grow) for one hour

Take out the 8 epitubes of transformed *E. coli* solution and spread 200 microliters of it onto 8 petri dishes with the LB + antibiotic Kana on it so that only the *E. coli* with the successfully inserted entry vector will grow on the plate. The entry vector possesses a Kana resistant gene.

Store the 8 plates into a 37ºC chamber and wait overnight.

Saturday June 20th, 2015

I took out all 8 plates and the plates successfully grew the transformed *E. coli*. Then I got 2 plates and created a scratch plate.

Diagram on page 32 of notebook

I put the 8 plates I used to screen for the positive integration of the entry vector into a 4ºC chamber.
I put the scratch plates into the growth chamber at 37ºC and waited for 8 hours for them to grow.

Sunday June 21st, 2015

I took out the scratch plates and made a PCR mastermix

	per PCR tube (μL)	all 8 tubes + 1 (μL)
2xGCL Buffer	5	45
dNTPs	.8	7.2
M13R Primer/Reverse Spacers	.2/.2	1.8/1.8
Ex taq	.06	.54
ddH_2O	3.74	33.66
Total	10	90

Add mineral oil for every tube

Then run the gel electrophoresis on the products of the PCR (add loading buffer) and wait for 10-15 minutes. We succeeded in producing positive clones. I then marked which sample gave the best indication of positive clones and referred back to my scratch plate. I then scraped a part of the scratch plates from

each column (1/RF, 1/R2F2, ..., 4/R2F2) in the hood and placed the scraped bacteria into 10mL bottles of liquid LB+Kana. I put it in the shaking bed at 37ºC and waited overnight.

Monday June 22nd, 2015

I took out the bacterial solution from the 37ºC shaking bed. Then I extracted the plasmid.

Procedure for plasmid extraction

1) Fill the epitube with the bacterial culture, then centrifuge it at 9000 rpm so the bacteria is at the bottom of the tube while the LB+Kana solution is on the top
2) Dispose of the LB+Kana solution and refill the epitube again with the bacterial solution and centrifuge it again to get more bacteria at the bottom of the epitube
3) Dispose of the liquid around the bacteria
4) Use 250µL of the resuspension liquid to resuspend the bacteria by using the vortex machine
5) Add in 250 µL of the cell membrane softening solution and mix it carefully
6) Add 350µL of the protein and cell membrane eliminating solution and mix it carefully
7) Centrifuge the epitube at 15000 rpm for 10 minutes.
8) Collect the solution and add it into the column tube
9) Wait for 1 minute for the white part of the column tube to collect the plasmid
10) Centrifuge for 1 minute at 10000 rpm
11) Dispose of the liquid at the bottom of the column tube
12) Add in 700 µL of the washing buffer and centrifuge for 1 minute at 13000 rpm
13) Dispose of the liquid at the bottom of the column tube
14) Centrifuge the epitube again for 2 minutes
15) For 10 minutes, let the top part of the column tube sit on a new epitube so that the remaining washing buffer can evaporate
16) Add 40 µL of water
17) Centrifuge for 1 minute at 15000 rpm and throw away the column tube top.
18) Test the concentration of the plasmid with the Gel Electrophoresis

We then sent 6 µL of the plasmid solution to get sequenced to make sure it was correct.

Tuesday June 23rd, 2015

The sequencing results came back; here are the results.

TPL2 FR, TPL2 F2R2, and TPL4 FR had point mutations. TPL3 F2R2 and TPL 4 F2R2 were good. I will need to re-extract the plasmids from TPL2 FR, TPL2 F2R2, and TPL4 FR.

Wednesday June 24th, 2015

I extracted the plasmids from the TPL2 FR, TPL2 F2R2, and TPL4 FR scratch plates.

Thursday June 25rd, 2015

We added restriction enzymes to cut our entry vectors at specific sites so that we could try and make a single entry vector with 4 spacers.

The restriction enzymes we used were ECORI, SALI, and XHOI.

The System

Plasmid	20 µL
ECORI / XHOI or SALI	1 µL / 1 µL
H 10x Buffer	5 µL
ddH_2O	23 µL

Saturday June 27th, 2015

We extracted the entry vector fragments that were cut by the restriction enzymes using gel electrophoresis.

Procedure

1) Put the entry vector fragments into the gel electrophoresis and wait for the gel to run
2) Cut the pieces of gel with the desired fragment size
3) Put the pieces into an epitube and add 200 µL of membrane binding solution
4) Incubate the epitubes at 65°C until the gel is melted
5) Put the solution into a minicolumn assembly and let it sit for 1 minute
6) Centrifuge at 16000 x g for 1 minute
7) Discard the flow through
8) Add 500 µL of membrane washing solution and centrifuge it at 16000 x g for 5 minutes
9) Discard the flow through
10) Centrifuge again for 1 minute
11) Transfer the microcolumn to an epitube
12) add 50 µL of water
13) Centrifuge for 1 minute at 16000 x g
14) Discard the microcolumn

I then ran a gel to test my products. They were good.
Then I did a ligation reaction to make 2 entry vectors with 2 spacers each
-TPL1 FR cut by ECORI and XHOI combined with TPL3 FR cut by ECORI and SALI
-TPL1 F2R2 cut by ECORI and XHOI combined with TPL3 F2R2 cut by ECORI and SALI

The system

ECORI and XHOI product	1 µL
ECORI and SALI product	6 µL
5x Buffer	2 µL
T4 Ligase	1 µL

Put it into a 22ºC chamber

Sunday June 28th, 2015

Today I transformed *E. coli* with the TPL 1+3 FR, TPL 1+3 F2R2, TPL 2 FR, TPL 2 F2R2

Monday June 29th, 2015

I PCR'd my products from yesterday's bacterial transformation.

The system

Entry Vector	Reverse Primer	Forward Primer
TPL2 F2R2	M13R	TPL 2 Spacer R2
TPL2 FR	M13R	TPL 2 Spacer R
TPL4 FR	M13R	TPL 4 Spacer R
TPL 1+3 FR	TPL1 Spacer R2	TPL 3 Spacer F2
TPL 1+3 F2R2	TPL1 Spacer R	TPL 3 Spacer F

I then ran a gel electrophoresis and only TPL2 F2R2, TPL2 FR, and TPL4 FR had positive clones. I made scratch plates and put them into the LB grows solution to put into the shaking bed. I used scratch #6 and #7 to put into the growth solution.

Tuesday June 30th, 2015

Today I collected the plasmids from the bacteria I grew yesterday: TPL2 F2R2, TPL2 FR, TPL4 FR, TPL 1+3 FR, TPL 1+3 F2R2. Then I sent them to get sequenced.

Wednesday July 1st, 2015

We got the sequencing results back and I seemed to have mixed up some spacers. The results were TPL1 FR + TPL4 F2R2 and TPL1 F2R2 + TPL3 FR instead of TPL1 FR + TPL3 FR and TPL1 F2R2 + TPL3 F2R2, respectively.

However, it is okay. Our new plan is this: combine TPL1 FR + TPL4 F2R2 + TPL2 FR + TPL3 F2R2 and combine TPL1 F2R2 + TPL3 FR + TPL2 F2R2 + TPL4 FR.

Thursday July 2nd, 2015

I Cut the plasmids TPL2 FR, TPL2 F2R2, TPL4 FR, TPL3 F2R2 with the restriction enzymes ECOR I, SAL I, and XHO I.

The Enzymes

Plasmids	Restriction Enzyme 1	Restriction Enzyme 2
TPL2 FR	ECOR I	XHO I
TPL2 F2R2	ECOR I	XHO I
TPL4 FR	ECOR I	SAL I
TPL3 F2R2	ECOR I	SAL I

The system (50 µL)

Plasmid	20 µL
H 10X Buffer	5 µL
ECOR I + SAL I/XHO I	1 µL / 1µL
ddH_2O	23 µL

Friday July 3rd, 2015

Today I collected the plasmids using gel electrophoresis. Then I did a ligation reaction between TPL3 F2R2 and TPL2 FR as well as between TPL4 FR and TPL2 F2R2.

Saturday July 4th, 2015

Today I PCR'd my products from yesterday

The System

2 x Taq Mix	50 µL
Primers	2.5 µL / 2.5 µL
ddH_2O	40.5 µL

The Plasmids	The Primers
TPL 2 FR / TPL 3 F2R2	TPL 2 Spacer R + TPL 3 Spacer F2
TPL 2 F2R2 / TPL 4 FR	TPL 2 Spacer R2 + TPL 4 Spacer F

I made scratch plates and put the tips I used to create the scratches in the plates into the LB grows solution to put into the shaking bed. I put the scratch plates in the incubator.

Tuesday July 7th, 2015

I extracted the plasmids from the LB growth solution bottles and got the plasmids sequenced.

Thursday July 16th, 2015

Today I cloned the rice TPL genes using 2 different polymerases

Cloning *in situ* with PFU		Cloning *in situ* with KOD Plus	
5x Fast PFU Buffer	10 µL	10x Fast KOD Buffer	5 µL
2.5 mM DNTPs	5 µL	2 mM DNTPs	5 µL
50 mM MgSo4	1 µL	25 mM MgSo4	2 µL
Primers	1/1 µL	Primers	1.5/1.5 µL
Fast PFU	1 µL	KOD Plus	1 µL
Template DNA	4 µL	Template DNA	4 µL
ddH_2O	27 µL	ddH_2O	30 µL

Sunday July 19th, 2015

Today I used the TPL 1/3 and 2/4 to do the LR reaction to be put into the destination vector

The 5 µL system

CRISPR-Dest 1	.5 µL
Entry Vector	1 µL
LR Clonase	1 µL
ddH_2O	2.5 µL

Monday July 20th, 2015

Today I got 300 Rice (Nipponbare) seeds and took of its outer layer. Then I made the Q1 growth medium. It must be done in the hood because the rice is easily contaminated. Pour the solution into plates and after it cools down. Put the seeds into the solidified solution so that half the embryo is submerged.

Q01 solution: The Induction Medium

N6 Salos (ions)	1.99 g
Vitamin solution	500 µL
L-Proline	1.439 g
Casamino acids	.15 g
Sucrose (energy for plants)	15 g
2, 4D (Auxin and other plant hormones)	1000 µL
Gelrite	2 g
ddH_2O	500 mL

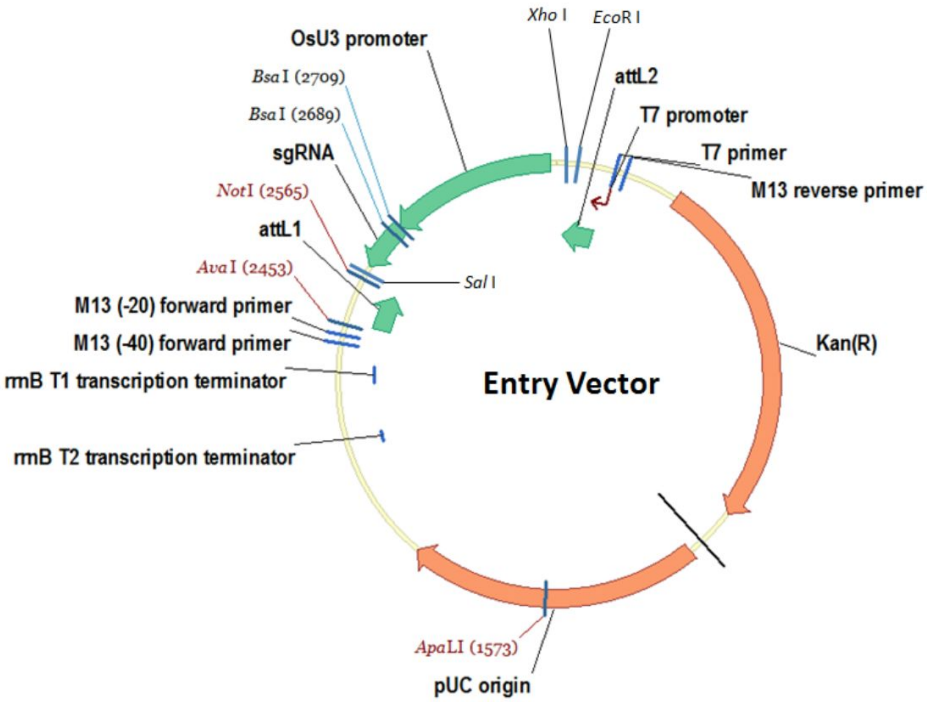
Friday July 24th, 2015

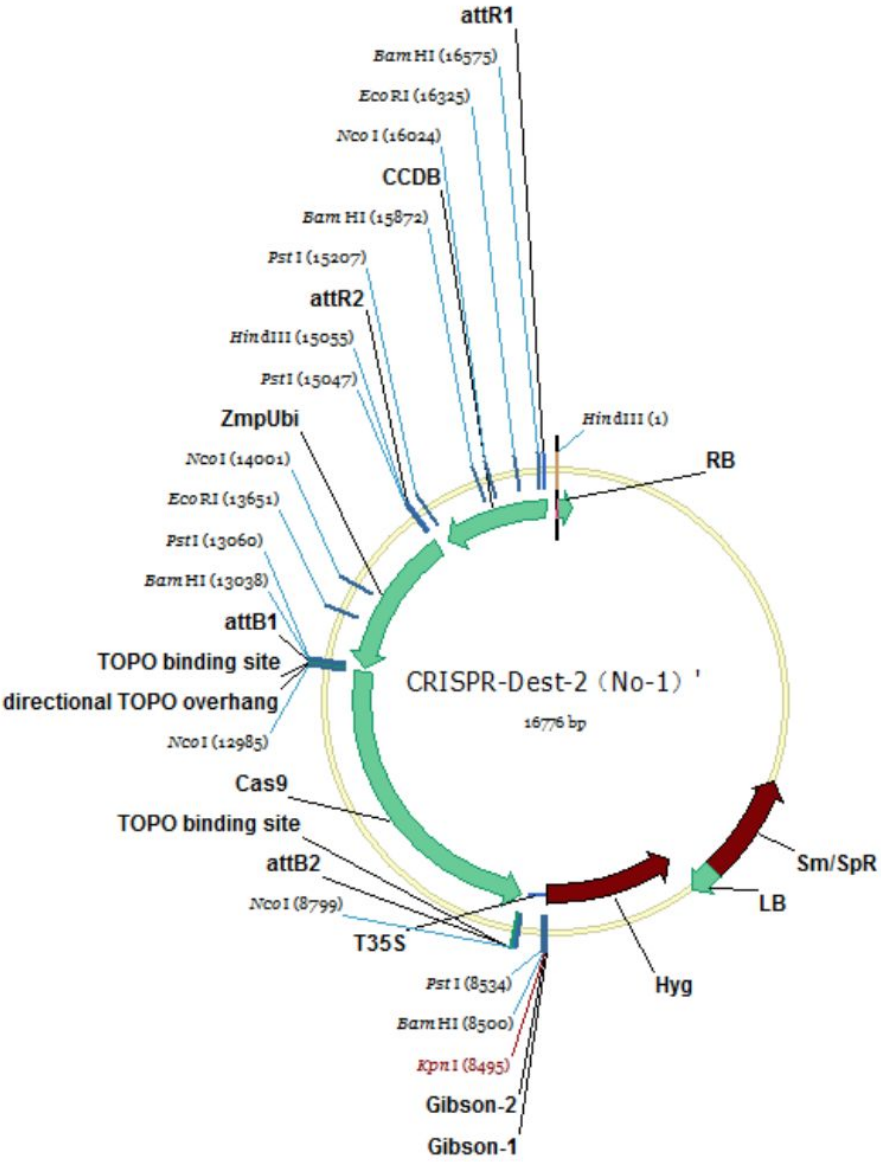
Extract the plasmid of the LR Reaction, and transform the agrobacterium using the plasmids from the LR reaction.

1) Get the Agro competent cells but dilute them with 500 µL ddH_2O
2) Add 50 µL of the diluted competent cell solution to 100 µL of the plasmid
3) Put the 150 µL solution into a special cup to break the cell membrane for the plasmid to enter the agrobacterium
4) Add LB solution and culture it at 28°C for 2 hours
5) Spread it onto a medium with RIF and SPE antibiotic. Wait for 1.5 days with it in a 22°C room.

4.3 Results

So far, we have integrated the paired spacers into two different entry vectors, with which we then put into the destination vector. I put seeds into the callus inducing medium with 24D+auxin. Then I put the callus into the co-culture medium with hygromycin after introducing the *Agrobacterium tumefaciens* with the vector to the callus.The selection medium with a





higher concentration of hygromycin selects callus that correctly integrated the entry vector.

The calluses are currently in the differentiation medium with Auxin (for elongation of shoots and to regulate plant growth) and cytokinin (hormone needed for cell division). These hormones differentiate the callus cells into specific tissue.

4.4 Moving Forward

After its turn in the differentiating medium (should be 4 to 5 inches long), I will add it to a "basic nutrition" medium with only basic nutritional content (sugar, nitrogen, etc.). Then I'll place it in the soil to observe the phenotype, comparing it to the wild type *Oryza sativa*.

I'm continuing the project in collaboration with Dongshu Guo.

5.1 The Experience of a Lifetime

I learned so much about molecular and plant biology, but I also learned so much about the beautiful and rich Chinese culture. I've made so many friends that will definitely last a lifetime.

I did a lot of things for the first time — from trying new foods, to celebrating my first birthday away from home. Every experience was a learning experience, and they have definitely shaped me to be a better me.

6.1 Work Cited

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