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National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University
农业农村部农业基因组学重点实验室（武汉）

基因编辑
操作手册
Tad-CBE

华中农业大学作物遗传改良全国重点实验室
湖北洪山实验室
棉花遗传改良团队

二〇二五年六月·武汉

基因编辑平台

GENOME EDITING PLATFORM

目录

棉花遗传改良团队：https://cotton.hzau.edu.cn/

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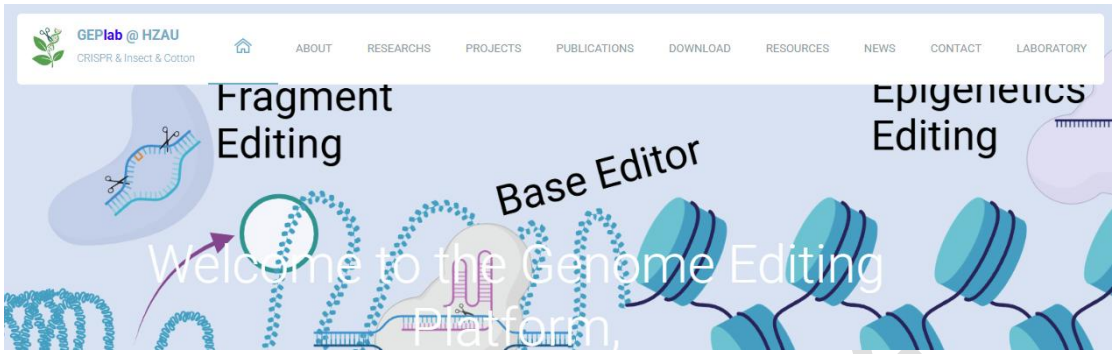
基因编辑平台：http://jinlab.hzau.edu.cn/GenomeEditingPlatform/

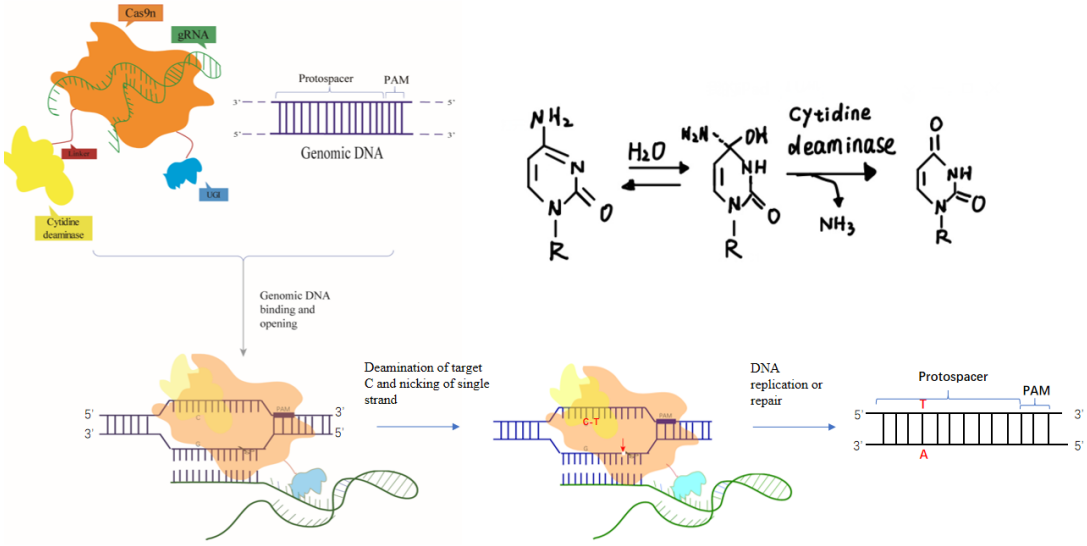


Tad-CBE protocol（中文版）

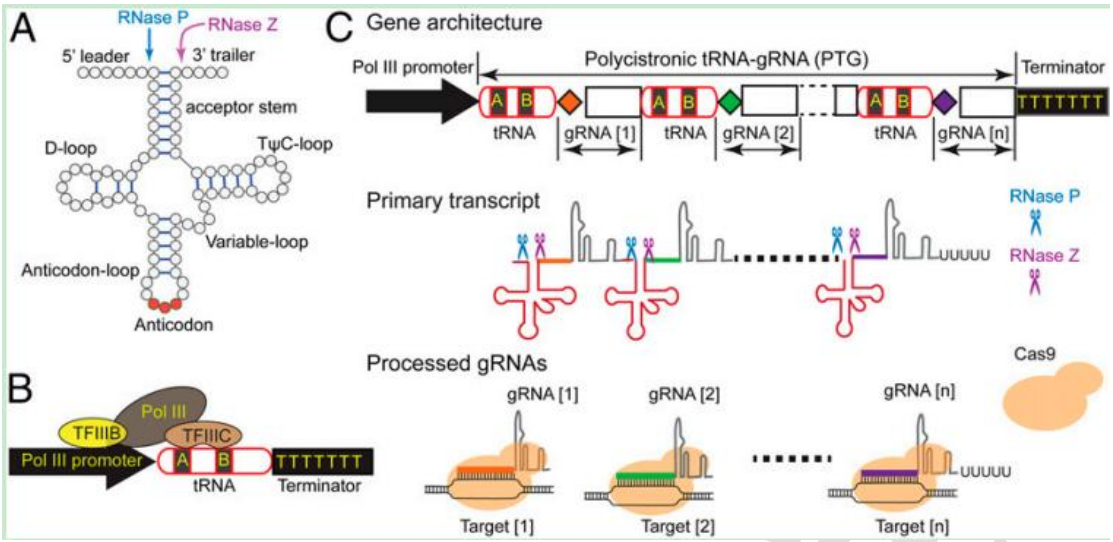
——以 CLA 为例

1.基本原理

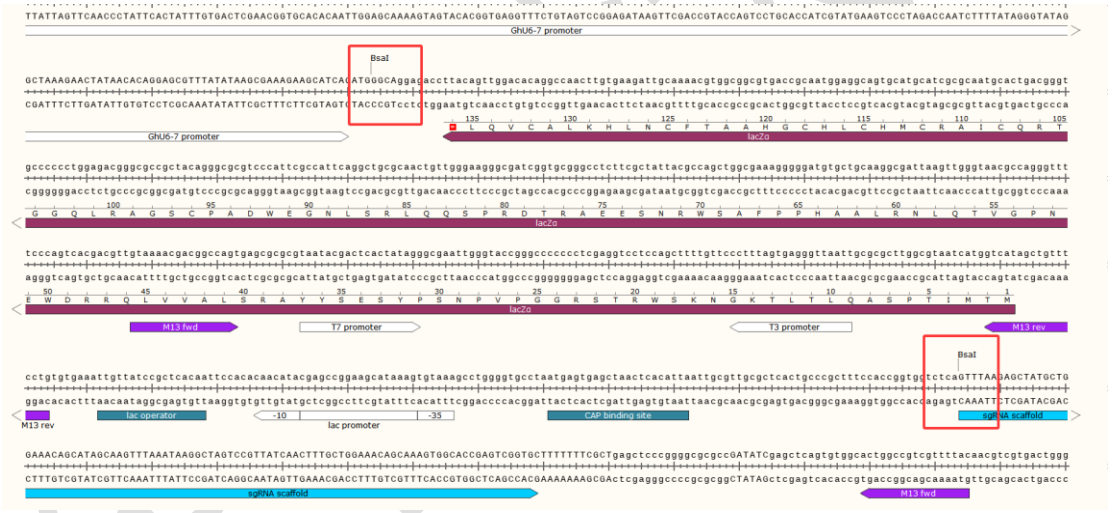
Tad-CBE 是一种经过优化的胞嘧啶碱基编辑系统，能够在不造成 DNA 双链断裂（DSB）的情况下，将 DNA 中的 C•G 碱基对精准地转换为 T•A 碱基对。Tad-CBE 主要由以下三个功能模块组成：(1) Tad 脱氨酶：将靶点 DNA 链上的胞嘧啶（C）脱氨为胸腺嘧啶（T）；(2) Cas9 nickase（D10A）：一个只在非编辑链上产生单链裂口的 Cas9 酶突变体，配合 sgRNA 实现靶向；(3) sgRNA：指导 Cas9 准确识别靶点序列，使脱氨酶作用于目标 C 碱基所在窗口。



我们采用的载体可以串联多个 tRNA+靶标+gRNA（下图 C），从而提高突变效率，植物体自身的 RNase P 和 RNase Z 分别切割 tRNA 的 5’端和 3’端（如下图 A），可以将多个靶标（引导序列）的 gRNA 释放，与目标序列互补后，Cas9/gRNA 会在 PAM 前 3bp 左右的位置精确的切割靶 DNA 双链。



目前我们实验室 Tad-CBE 为卡纳抗性，以及引导 gRNA 转录的棉花内源 U6 启动子（编号为 U6-7），但驱动 Cas9 表达的依然是水稻 Ubiquitin2 启动子，原核中的抗性均为 kan。以下主要以 U6-7 启动子载体连接两个靶标位点，针对同一基因为例介绍载体构建过程。



Tad-CBE

2. 设计靶标以及设计引物

CLA 序列及靶标：可以进行手动搜索， Blast 后，具有特异性的序列可以作为靶标。最好在 PAM 前 3bp 左右的位置有唯一的酶切位点，方便后续的验证。负链为 G-A 突变，正链为 C-T 突变。突变的碱基最好在远离 PAM 的第 4-8 的窗口内。

CTCGACGGACCTATACCACCTGTGGGAGCTTTGAGCAGTGCTCTCAGCAGGCTGCAAT
CAAACAGGCCTCTTAGAGAACTGAGAGAGGTTGCAAAGGTAAGTAGAAATGAATAGA
ACCCAGAAAACTAATATAATTTTTACTAGAAACTAAAACTTACTAAGACTGGATGTGGT

TTGATTGCAGGGAGTTACCAAGCAAATCGGTGGGCCTATGCATGAACTGGCTG

CAAAAGTTGATGAGTATGCTCGTGGAATGATCAGTGGTTCTGGATCTACACTTTTCGAA
GAACTTGGACTATATTATATTGGACCTGTTGATGGCCACAACATCGATGATTTAGTTTCT
ATTCTCAAAGAGGTTAAGACTACTAAAACAACGGGTCCGGTCTTGATCCATGTTGTCAC
TGAGAAAGGCCGAGGTTATCCATATGCAGAGAGAGCTGCTGACAAGTATCACGGTAAC
ATACAGAATAAGCCTTTTTAGAAGAGAGATCATTTCATATGATTTAATTTACTGGTGCC

TCGATATCTGACCTTAGTTTAGGGAATAAAAGAATAAACTGTACCTGGATTTCTTTTCTC
AGGAGTGGTGAAGTTCGATCCGGCAACTGGAAAGCAATTCAAAGGCAA
TTCTGCTACCCAGTCTTACACTACATATTTTGCTGAGGCTTTGATTGCGGAAGCTGAGG
CAGACAAAAATATTGTTGCCATCCATGCTGCAATGGGAGGTGGAACCGGATTAAACCT
CTTCCTCCGCCGGTTCCCACAAAGATGTTTCGATGTGGGGATAGCTGAACAACATGCTG
TCACCTTTGCTGCAGGCTTGGCCTGTGAAGGCTTGAAACCTTTTTGTGCAATCTACTCA
TCATTCATGCAAAGGGCTTATGACCAG

插入目标序列连接在载体图方框所示 BasI 酶切位点之间，最终连接方法为一步克隆法。

*aagcatcagatgggca**AACAAA*GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGC
CACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA*CATAGGCCCACCGA*
*TTTGCT**GTTTTAGAGCTAGAAATA***GCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGT
CTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCG
GCTGGTGCA*TTTCATATGATTTAATTTAC**gttttagagctagaa*……*（载体 gRNA）*

注：小写部分是载体序列；下划线部分是 tRNA;加粗部分是 gRNA；斜体部分是靶标位置。

需要用到的引物：

CLA1 as: 5’ AGCAAATCGGTGGGCCTATGtgcaccagccgggaat3’（靶标 1 互补序列）
CLA 2 s:5’ CATAGGCCCACCGATTTGCTgttttagagctagaaata 3’（靶标 1 序列）
CLA 2 as: 5’ GTAAATTAAATCATATGAAAtgcaccagccgggaat3’（靶标 2 互补序列）
inf CLA as: 5’ ttctagctctaaaacGTAAATTAAATCATATGAAA3’（靶标 2 互补序列）
pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAAGCACCAGTGGTCTAG3’
inf pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAA 3’

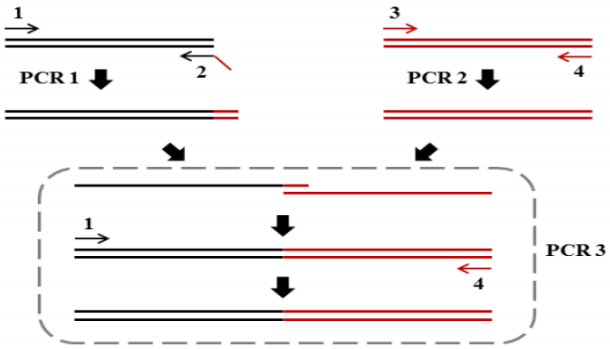
小写部分是固定序列，引物设计只需修改引物中的下划线标示的靶标序列即可。

其中，**inf pRGEB32-7 s、pRGEB32-7 s、u6-7 s** 为通用引物无须更改。pRGEB32-7 s 含有载体一部分序列，方便 In-fusion 连接。因插入序列中有多个 tRNA，inf pRGEB32-7 s 为增强扩增特异性，序列长度比 pRGEB32-7 s 短。Inf CLA as 含有载体序列接头，方便后续 In-fusion 连接。

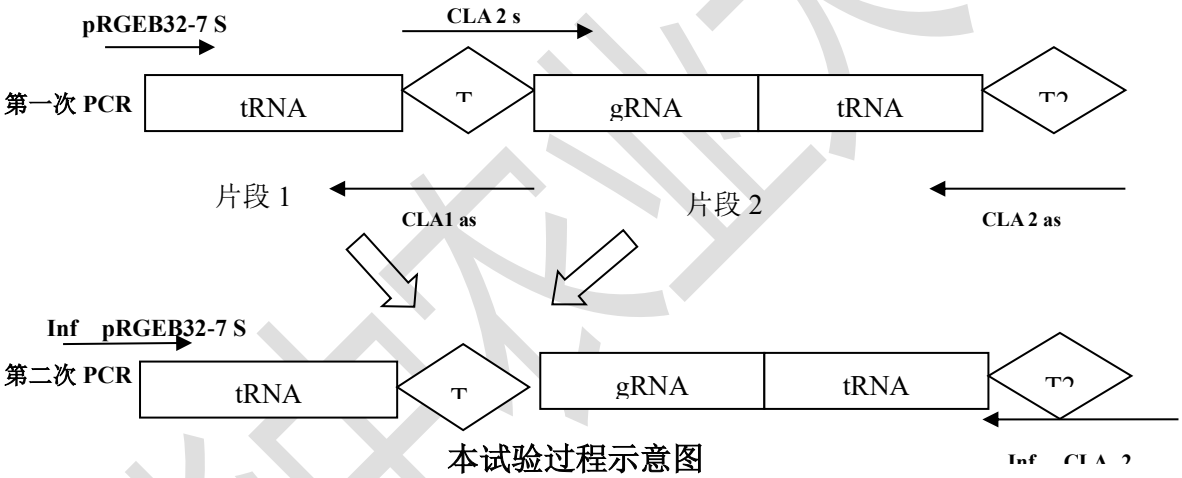
3. 第一次 PCR 扩增

重叠延伸 PCR：

PCR 循环中，扩增产生的新链是引物和延伸产物共同组成的，而且新链可以作为下一循环的模版。如果在引物的 5‘端加入接头，下一轮循环过后产生的链便会带有接头互补的序列。如此循环，最后的产物中，几乎所有的产物都含有接头序列。如果将接头序列设计得和待融合片段的引物互补，那么两个独立 PCR 的产物便可以借助这一区域产生配对，经 Taq 酶延伸成为融合片段。



重叠延伸示意图



本试验过程示意图

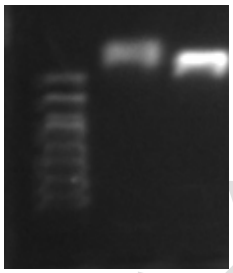
gRNA+tRNA 组合序列可自行合成序列连接到公共载体（如用 T 载体），第一次 PCR:1、2 两个片段分别从含 gRNA+tRNA 载体的菌液或者质粒中扩增。（相关序列可见附件）

20μl 体系：

	1	2
ddH_2O	**16.1**	**16.1**
Buffer	**2**	**2**
dNTP	**0.3**	**0.3**
S primer	**pRGEB32-7 s 0.2**	**CLA 2 s 0.2**
AS primer	**CLA1 as 0.2**	**CLA 2 as 0.2**
Taq	**0.2**	**0.2**
模板	**1**	**1**

PCR 条件：

预变性：95℃ 4min
变性：95℃ 30S
退火：55℃ 30S
延伸：72℃ 20S
循环：3C
变性：95℃ 30S
退火：60℃ 30S
延伸：72℃ 20S
循环：27C
最后延伸 72℃ 5min
电泳检测



5K 1

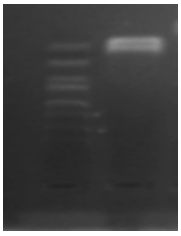
4.第二次 PCR 及纯化：

使用重叠延伸 PCR 将两个小片段进行连接，

		100μl 体系
ddH_2O		**83.5μl**
Buffer		**10μl**
dNTP		**1.5μl**
S primer	**Inf pRGEB32-7 s**	**1μl**
AS primer	**Inf CLA as**	**1μl**
Taq		**1μl**
片段 1		**1μl**
片段 2		**1μl**

PCR 条件：

预变性：95℃ 4min
变性：95℃ 30S
退火：59℃ 30S
延伸：72℃ 20S
循环：28C
最后延伸 72℃ 5min



5k 拼接

电泳检测
使用凝胶回收的试剂盒对 PCR 产物进行纯化、测浓度

5.载体 酶切：

	100μl 体系：
Tad-CBE	10μg
10X cut smart Buffer	10μl
BsaI	4μl
ddH_2O	up to 100μl

37℃ 酶切 5.5h

可**与第四步同时做**，电泳检测酶切效果、使用凝胶回收的试剂盒对酶切产物进行纯化、测浓度。

6.In-fusion 连接:

目的片段	100ng
线性化表达载体	100ng
Exnase	0.5μl
CE Buffer	1μl

37℃水浴 30min，冰上放置 5min，可-20℃保存备用

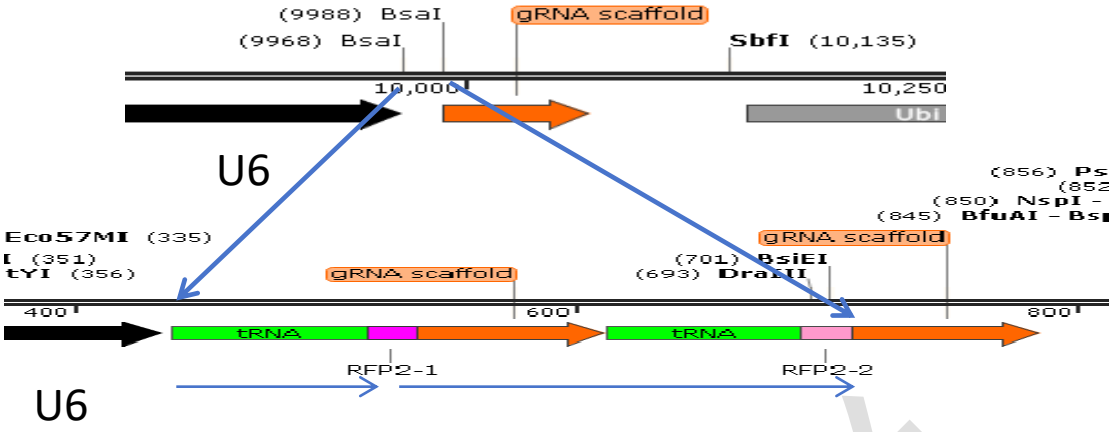
7.转化感受态

连接产物与感受态混合，冰上放置 30min，42℃热激 90S，冰上静置 5min。取 300μlSOC 加入新的 2ml 离心管，将热激产物加入 SOC 中，37℃震荡 45min，涂于 KAN 皿，37℃培养过夜，挑单克隆于 500μl kan 抗性液体 LB,37℃震荡 3h,使用 Inf pRGEB32-7 s Inf CLA as 引物阳性检测，电泳跑胶，挑阳性送测序（**GC rich**）.
阳性检测引物/测序引物
u6-7 s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

阳性检测电泳图：

5K 阴 阳

构建成功载体图：



8. 电转

测序成功后，电转农杆菌
阳性检测引物
u6-7 s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

9.效果验证

（1）提取愈伤/单株 DNA，PCR 扩增含有靶标位置的一段序列，TA 克隆测序或 barcode 高通量测序。

Tad-CBE protocol（英文版）

——Using CLA as an Example

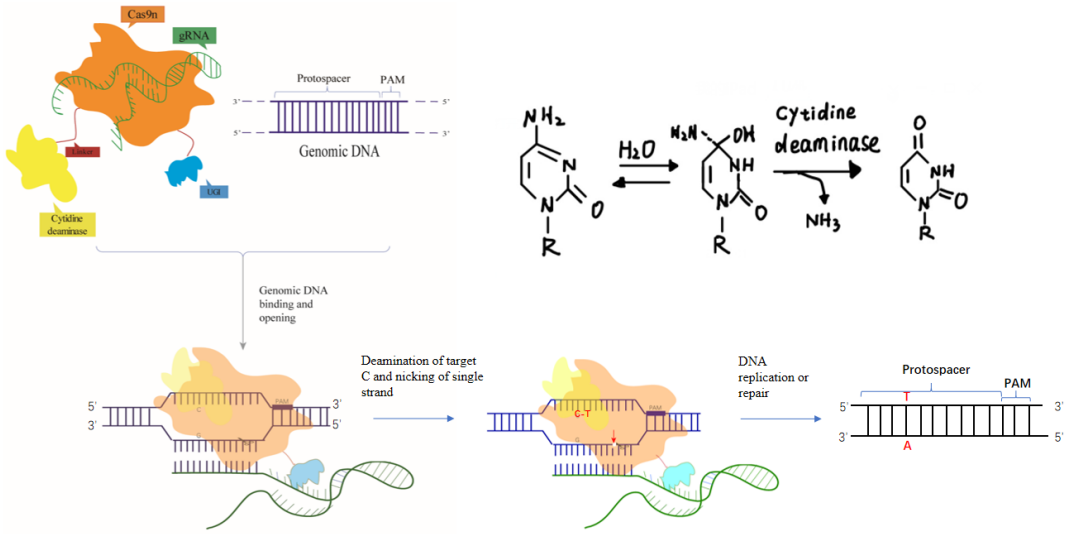
1. Basic Principles

Tad-CBE is an optimized cytosine base editing system capable of precisely converting C•G base pairs into T•A base pairs without introducing DNA double-strand breaks (DSBs). Tad-CBE consists of the following three functional modules:

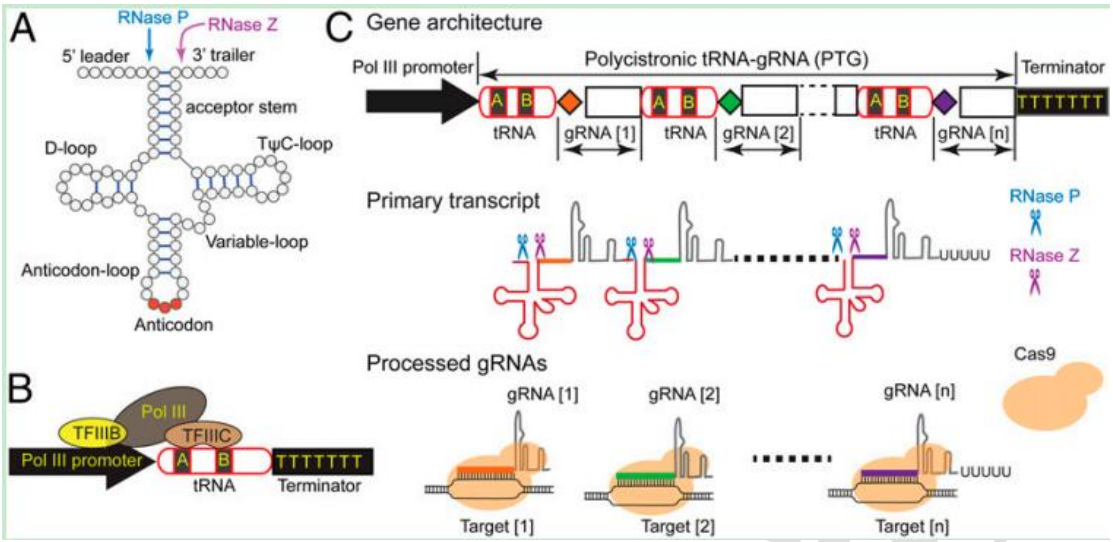
(1) **Tad deaminase**: deaminates the target cytosine (C) on the DNA strand, converting it into thymine (T);

(2) **Cas9 nickase (D10A)**: a mutated Cas9 enzyme that generates a single-strand nick on the non-edited strand, guided by the sgRNA;

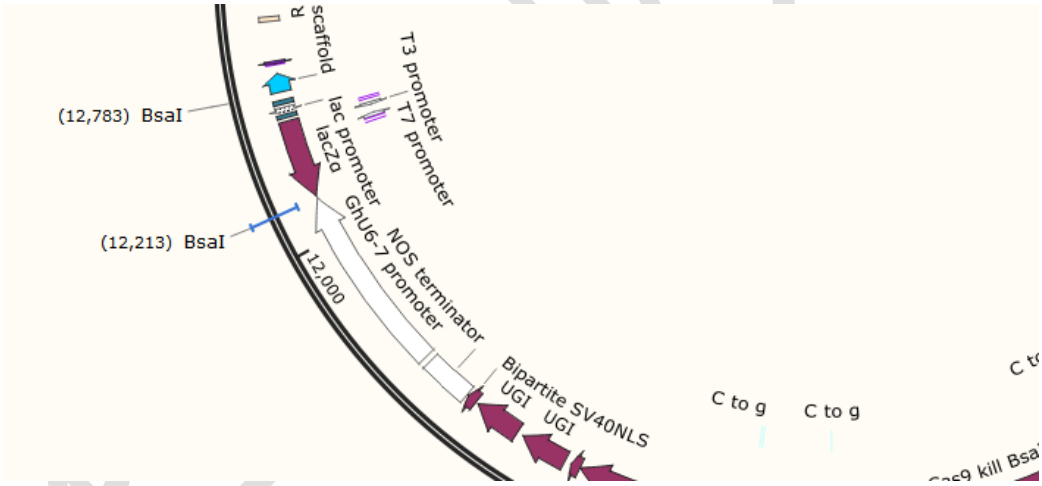
(3) **sgRNA**: guides Cas9 to accurately recognize the target sequence, enabling the deaminase to act on the cytosine base within the editing window.



Our vector system allows tandem integration of multiple **tRNA + target + gRNA** units (Fig. C), enhancing mutation efficiency. Plant endonucleases RNase P and RNase Z cleave the 5' and 3' ends of tRNA (Fig. A), releasing gRNAs that guide Cas9/gRNA to cleave the target DNA at the specified site.



In our lab, we modified the Tad-CBE vector by replacing the kanamycin resistance marker with hygromycin and incorporating the cotton endogenous U6 promoters (U6-7) for gRNA transcription. The protocol below focuses on constructing a vector with two target sites under the U6-7 promoter using rice Ubiquitin2 as the Cas9 promtor.



2. Target Search and Primer Design

CLA Sequence and Targets: Use the CRISPR-P website (http://cbi.hzau.edu.cn/crispr/) to search for targets (limited to *Gossypium raimondii* data). Alternatively, manually BLAST sequences for specificity. Prefer targets with unique restriction sites near the PAM (3 bp upstream) for downstream validation. G-to-A mutation on the negative strand corresponds to an C-to-T mutation on the positive strand. The target base is ideally located within positions 4 to 8 distal from the PAM site.

CTCGACGGACCTATACCACCTGTGGGAGCTTTGAGCAGTGCTCTCAGCAGGCTGCAAT

CAAACAGGCCTCTTAGAGAACTGAGAGAGGTTGCAAAGGTAAGTAGAAATGAATAGA
ACCCAGAAAACTAATATAATTTTTACTAGAAACTAAAACTTACTAAGACTGGATGTGGT
TTGATTGCAGGGAGTTACCA**AGCAAATCGGTGGGCCTATG**CATGAACTGGCTG

CAAAAGTTGATGAGTATGCTCGTGGAATGATCAGTGGTTCTGGATCTACACTTTTCGAA
GAACTTGGACTATATTATATTGGACCTGTTGATGGCCACAACATCGATGATTTAGTTTCT
ATTCTCAAAGAGGTTAAGACTACTAAAACAACGGGTCCGGTCTTGATCCATGTTGTCAC
TGAGAAAGGCCGAGGTTATCCATATGCAGAGAGAGCTGCTGACAAGTATCACGGTAAC
ATACAGAATAAGCCTTTTTAGAAGAGAGATCA**TTTCATATGATTTAATTTAC**TGGTGCC

TCGATATCTGACCTTAGTTTAGGGAATAAAAGAATAAACTGTACCTGGATTTCTTTTCTC
AGGAGTGGTGAAGTTCGATCCGGCAACTGGAAAGCAATTCAAAGGCAA
TTCTGCTACCCAGTCTTACACTACATATTTTGCTGAGGCTTTGATTGCGGAAGCTGAGG
CAGACAAAAATATTGTTGCCATCCATGCTGCAATGGGAGGTGGAACCGGATTAAACCT
CTTCCTCCGCCGGTTCCCACAAAGATGTTTCGATGTGGGGATAGCTGAACAACATGCTG
TCACCTTTGCTGCAGGCTTGGCCTGTGAAGGCTTGAAACCTTTTTGTGCAATCTACTCA
TCATTCATGCAAAGGGCTTATGACCAG

Insert the target sequence and ligate between the MfeI digestion sites shown in the box of the vector diagram. The final ligation method is the one-step cloning method.

*aagcatcagatgggca**AACAAA***GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGC
CACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA*CATAGGCCCACCGA*
*TTTGCT**GTTTTAGAGCTAGAAATA***GCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGT
CTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCG
GCTGGTGCA*TTTCATATGATTTAATTTAC**gttttagagctagaa*……***（载体 gRNA）***

notes：Note: Lowercase = vector backbone; underscores = tRNA; bold = gRNA; italics = target site.

Primers:：

CLA1 as: 5’ AGCAAATCGGTGGGCCTATGtgcaccagccgggaat3’（靶标 1 互补序列）
CLA 2 s:5’ CATAGGCCCACCGATTTGCTgttttagagctagaaata 3’（靶标 1 序列）
CLA 2 as: 5’ GTAAATTAAATCATATGAAAtgcaccagccgggaat3’（靶标 2 互补序列）
inf CLA as: 5’ ttctagctctaaaacGTAAATTAAATCATATGAAA3’（靶标 2 互补序列）
pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAAGCACCAGTGGTCTAG3’
inf pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAA 3’

The lowercase part is a fixed sequence. Primer design only requires modifying the target sequence indicated by the underline in the primer.

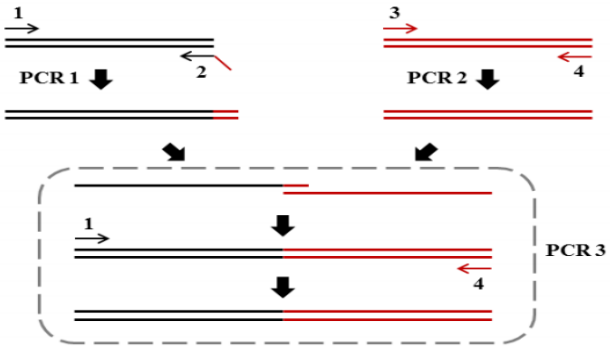
Among them, inf pRGEB32-7 s, pRGEB32-7 s, u6-7 s are universal primers and do not need to be changed. pRGEB32-7s contain a part of the vector sequence, facilitating In-fusion ligation. Because there are multiple trnas in the inserted sequence, inf pRGEB32-7 s, in order to enhance amplification specificity, have shorter sequence lengths than pRGEB32-7 s. Inf CLA as contains carrier sequence

connectors for convenient subsequent In-fusion connections.

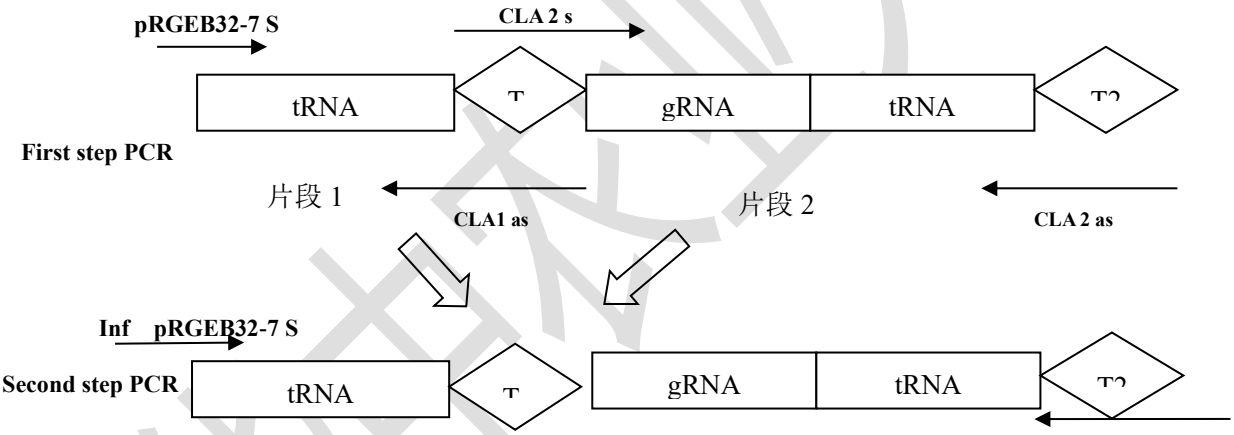
3. First PCR Amplification

Overlap Extension PCR:

This method generates fusion fragments by designing primers with complementary overhangs. Two overlapping PCR products are combined and extended to form a single construct.



Overlapping extension schematic diagram



This is a schematic diagram of the test process

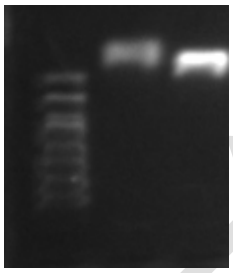
The gRNA+tRNA combination sequence can be synthesized by itself and ligated to a common vector (such as using the T vector). In the first PCR: Fragments 1 and 2 were amplified respectively from the bacterial liquid or plasmid containing the gRNA+tRNA vector. (The relevant sequence can be found in the attachment.)

Reaction Mixture (20 μL):

	1	2
ddH_2O	**16.1**	**16.1**
Buffer	**2**	**2**
dNTP	**0.3**	**0.3**
S primer	**pRGEB32-7 s 0.2**	**CLA 2 s 0.2**
AS primer	**CLA1 as 0.2**	**CLA 2 as 0.2**

Taq	**0.2**	**0.2**
Template DNA	**1**	**1**

PCR Conditions:



1. Initial denaturation: 95° C for 4 min
2. Denaturation: 95° C for 30 s
3. Annealing: 55° C for 30 s
4. Extension: 72° C for 20 s
    - Repeat steps 2 – 4 for 3 cycles
5. Denaturation: 95° C for 30 s
6. Annealing: 60° C for 30 s
7. Extension: 72° C for 20 s
    - Repeat steps 5 – 7 for 27 cycles
8. Final extension: 72° C for 5 min

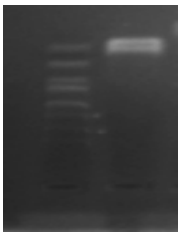
Electrophoresis: Verify amplification.

4. Second PCR and Purification

Combine the two PCR products from Step 3 using overlap extension，

		Reaction Mixture (100 μL)
ddH_2O		**83.5μl**
Buffer		**10μl**
dNTP		**1.5μl**
S primer	**Inf pRGEB32-7 s**	**1μl**
AS primer	**Inf CLA as**	**1μl**
Taq		**1μl**
Fragment 1		**1μl**
Fragment 2		**1μl**

PCR Conditions:



1. Initial denaturation: 95° C for 4 min
2. Denaturation: 95° C for 30 s
3. Annealing: 59° C for 30 s
4. Extension: 72° C for 20 s
    - Repeat steps 2 – 4 for 28 cycles
5. Final extension: 72° C for 5 min

Purification: Use a gel extraction kit to purify the product and measure concentration.

5. Vector Digestion

Digest the vector with BsaI.

Reaction Mixture (100 µL):

	Volume (µL):
Tad-CBE	10 µg
CutSmart Buffer	10 µL
BsaI	1 µL
ddH_2O	Up to 100 µL

Digestion: 37°C for 5.5 h.

6. In-Fusion Cloning

Assemble the digested vector and insert using In-Fusion technology.

Components:

- **Insert (100 ng)**
- **Linearized vector (100 ng)**
- **Exnase (0.5 µL)**
- **CE Buffer (1 µL)**

Incubation: 37°C for 30 min, then ice for 5 min. Store at -20°C.

7. Transformation

Transform competent cells with the ligation product.

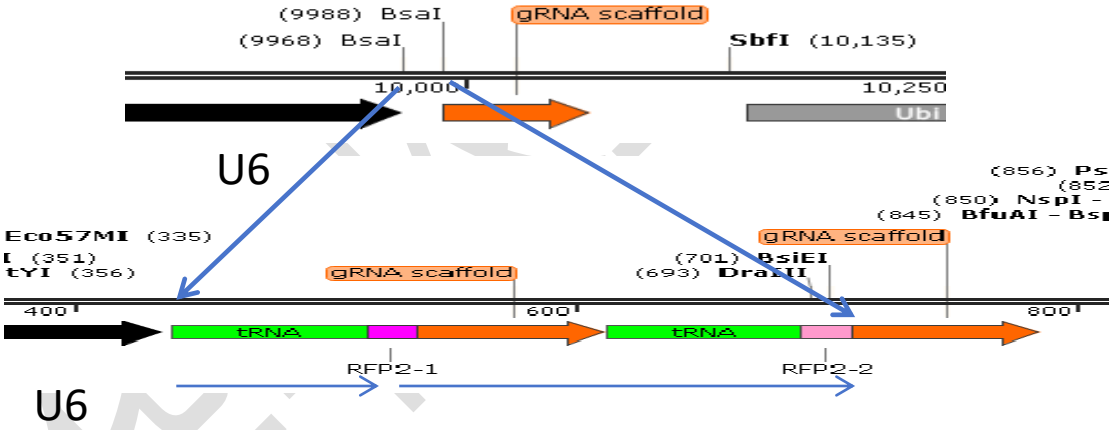
Steps:

Mix with SOC medium and incubate at 37°C for 45 min.
Plate on kanamycin agar plates and incubate overnight.
Pick colonies for PCR validation using primers:
u6-7s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

Positive clones are sequenced (GC-rich regions).
Electrophoresis: Confirm positive clones.

Positive detection electrophoresis pattern：

Construct a successful carrier diagram：



8. Agrobacterium Electroporation

Electroporate the verified construct into *Agrobacterium*.

u6-7 s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

9. Validation

Genomic DNA Extraction: PCR amplify the target region, clone, and sequence.