**实验方案—pBUE411-2gR PCR鉴定及测序确认**

1. 登录到网站<http://www.genome.arizona.edu/crispr/CRISPRsearch.html>，筛选靶点。靶点最好带酶切位点【Cas9切割点（离PAM/NGG 3bp）位于酶切位点中】。也可以手动设计靶点，然后到<http://www.rgenome.net/cas-offinder/>网站评估脱靶情况。
2. 设计引物，引物结构如下：

MT1T2-F: AATAATGGTCTCAGGCGNNNNNNNNNNNNNNNNNNN

MT1T2-F0: GNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC

MT1T2-R0: NNNNNNNNNNNNNNNNNNNCGCTTCTTGGTGCC

MT1T2-R: ATTATTGGTCTCTAAACNNNNNNNNNNNNNNNNNNN

将1个19-nt靶点序列替换引物F0/BsF中的19-nt N；另一个19-nt靶点的倒转互补序列替换-R0/BsR中的19-nt N。

1. PCR扩增：以稀释100倍的pCBC-MT1T2为模板进行四引物PCR扩增。-BsF/-BsR为正常引物浓度；-F0/-R0稀释20倍。
2. 纯化回收PCR产物，建立如下酶切-连接体系（restriction-ligation）：

方法一：

|  |  |  |
| --- | --- | --- |
| 成分 | 体积 | 反应条件 |
| 1. PCR片段 (964-bp) | 2 | 5 hours at 37°C  5 min at 50°C  10 min at 80°C |
| 1. pBUE411 | 2 |
| 1. 10xNEB T4 Buffer | 1.5 |
| 1. 10xBSA | 1.5 |
| 1. BsaI (NEB) | 1 |
| 1. T4 Ligase (NEB)/高浓度 | 1 |
| 1. ddH2O | 6 |
| 1. Total | 15 |

方法二：【自文献Analytical Biochemistry 437 (2013) 172–177】

**Restriction-ligation reaction**

Purified PCR products each 1-3 μl (20-50 ng)

10x ligase buffer (Promega) 1 μl

T4 DNA ligase (Promega, HC, 20 u/μl) 0.5 μl (10U)

BsaI (NEB, 10 u/μl) 0.5 μl (5U)

Sterile water to 10 μl

Incubate at 37°C for 2 minutes and 16°C for 5 minutes, both steps are repeated 50 times, followed by incubation for 5 minutes at 80°C (heat inactivation).

1. 取5ul转化大肠杆菌感受态。Kan板筛选。OsU3-FD3+TaU3-RD=831bp菌落PCR鉴定，OsU3-FD3和TaU3-FD2测序确认。

注1：菌落PCR及测序引物：

OsU3-FD3 GACAGGCGTCTTCTACTGGTGCTAC

TaU3-RD: CTCACAAATTATCAGCACGCTAGTC [rc: GACTAGCGTGCTGATAATTTGTGAG]

TaU3-FD: TTAGTCCCACCTCGCCAGTTTACAG

TaU3-FD2: TTGACTAGCGTGCTGATAATTTGTG

注2：pBUE411问王志平要；pCBC-MT1T2问邢慧丽要。

**Sequence of MT1T2-PCR with Targets 1 and 2 for monocots**



(Target-1)-(gRNA-Sc)-(OsU3t)-(TaU3p)-(Target-2)

ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCGTCGCATGTTTGCAGTTTTATTTTCCGTTTTGCATTGAAATTTCTCCGTCTCATGTTTGCAGCGTGTTCAAAAAGTACGCAGCTGTATTTCACTTATTTACGGCGCCACATTTTCATGCCGTTTGTGCCAACTATCCCGAGCTAGTGAATACAGCTTGGCTTCACACAACACTGGTGACCCGCTGACCTGCTCGTACCTCGTACCGTCGTACGGCACAGCATTTGGAATTAAAGGGTGTGATCGATACTGCTTGCTGCTCATGAATCCAAACCACACGGAGTTCAAATTCCCACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTGTGAATATTATATCTGTCGTGCAAAATTGCCTGGCCTGCACAATTGCTGTTATAGTTGGCGGCAGGGAGAGTTTTAACATTGACTAGCGTGCTGATAATTTGTGAGAAATAATAATTGACAAGTAGATACTGACATTTGAGAAGAGCTTCTGAACTGTTATTAGTAACAAAAATGGAAAGCTGATGCACGGAAAAAGGAAAGAAAAAGCCATACTTTTTTTTAGGTAGGAAAAGAAAAAGCCATACGAGACTGATGTCTCTCAGATGGGCCGGGATCTGTCTATCTAGCAGGCAGCAGCCCACCAACCTCACGGGCCAGCAATTACGAGTCCTTCTAAAAGCTCCCGCCGAGGGGCGCTGGCGCTGCTGTGCAGCAGCACGTCTAACATTAGTCCCACCTCGCCAGTTTACAGGGAGCAGAACCAGCTTATAAGCGGAGGCGCGGCACCAAGAAGCGNNNNNNNNNNNNNNNNNNNGTTTAGAGACCAATAAT

**Primers:**

MT1-BsF: ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNGTT

MT1-F0: TGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC

MT2-R0: AACNNNNNNNNNNNNNNNNNNNCGCTTCTTGGTGCC

MT2-BsR: ATTATTGGTCTCTAAACNNNNNNNNNNNNNNNNNNNC

**Template:** pCBC-MT1T2

**Length:** 964-bp

**Notes:**

1. The 19-nt N in primers represents any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers).
2. For the assembly of two gRNA expression cassettes, use MT1-BsF/MT1-F0/MT2-R0/MT2-BsR four-primer mixture with MT1-F0/MT2-R0 diluted to 20 times of MT1-BsF or MT2-BsR, resulting in MT1T2-PCR.

pBUE411-2gR (pBUE411+MT1T2-PCR) 【构建2个gRNA载体】

AGTAATTCATCCAGGTCTCCAAGTTCTAGGATTTTCAGAACTGCAACTTATTTTATCAAGGAATCTTTAAACATACGAACAGATCACTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCATAGCACAAGACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCACGTGATGTGAAGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGC---G**NNNNNNNNNNNNNNNNNNNgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttttttttcgttttgcattgagttttctccgtcgcatgtttgcagttttattttccgttttgcattgaaatttctccgtctcatgtttgcagcgtgttcaaaaagtacgcagctgtatttcacttatttacggcgccacattttcatgccgtttgtgccaactatcccgagctagtgaatacagcttggcttcacacaacactggtgacccgctgacctgctcgtacctcgtaccgtcgtacggcacagcatttggaattaaagggtgtgatcgatactgcttgctgct---catgaatccaaaccacacggagttcaaattcccacagattaaggctcgtccgtcgcacaaggtaatgtgtgaatattatatctgtcgtgcaaaattgcctggcctgcacaattgctgttatagttggcggcagggagagttttaacattgactagcgtgctgataatttgtgagaaataataattgacaagtagatactgacatttgagaagagcttctgaactgttattagtaacaaaaatggaaagctgatgcacggaaaaaggaaagaaaaagccatacttttttttaggtaggaaaagaaaaagccatacgagactgatgtctctcagatgggccgggatctgtctatctagcaggcagcagcccaccaacctcacgggccagcaattacgagtccttctaaaagctcccgccgaggggcgctggcgctgctgtgcagcagcacgtctaacattagtcccacctcgccagtttacagggagcagaaccagcttataagcggaggcgcggcaccaagaagcgNNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC---TTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCGTCGCATGTTTGCAGTTTTATTTTCCGTTTTGCATTGAAATTTCTCCGTCTCATGTTTGCAGCGTGTTCAAAAAGTACGCAGCTGTATTTCACTTATTTACGGCGCCACATTTTCATGCCGTTTGTGCCAACTATCCCGAGCTAGTGAATACAGCTTGGCTTCACACAACACTGGTGACCCGCTGACCTGCTCGTACCTCGTACCGTCGTACGGCACAGCATTTGGAATTAAAGGGTGTGATCGATACTGCTTGCTGCT

TaU3p

gRNA

gRNA

OsU3p

TaU3-FD2

TaU3-RD

OsU3-FD3

OsU3-FD3+TaU3-RD=831bp菌落PCR鉴定。OsU3-FD3和TaU3-FD2测序确认。与上述序列进行比对即可。也可以根据上述序列重新设计PCR及测序引物。终止子T10后面的序列正确与否关系不大。

~~OsU3-FD: 5' AGTAATTCATCCAGGTCTCCAAGTT 3'~~

OsU3-FD2: 5' AGTAATTCATCCAGGTCACCAAGTT 3' （与OsU3-FD相比有1个碱基差异）

注意：pBUE411的OsU3p在OsU3-FD处有一BsaI酶切位点，但因为受dcm甲基化影响，不影响预期酶切，PCR扩增产物中的OsU3p中的BsaI已突变去除。

OU3-RD: 5' TGGTCCCTGACTGCACATCTGATTC 3'

OsU3-FD3 GACAGGCGTCTTCTACTGGTGCTAC

TaU3-RD: CTCACAAATTATCAGCACGCTAGTC [rc: GACTAGCGTGCTGATAATTTGTGAG]

~~TaU3-FD: TTAGTCCCACCTCGCCAGTTTACAG~~

TaU3-FD2: TTGACTAGCGTGCTGATAATTTGTG