

Additional file 3

Methods S2

Golden Gate cloning method for the assembly of one or two gRNAs

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Simplified protocol

1. Manually search for 23-bp target sites (5'-N₂₀NGG-3') within exons of genomic DNA sequences of genes of interest, and then evaluate target specificities on the website of potential off-target finder (<http://www.rgenome.net/cas-offfinder/>). Users can also search for target sites on the website of genome-wide prediction of plant CRISPR/Cas9 target sites (<http://www.genome.arizona.edu/crispr/CRISPRsearch.html>).
2. Design primers:
 - a) Find names of inserts/oligos (for one gRNA) or PCR fragments/primers (for two gRNAs) according to plant species (monocots or dicots) and gRNA numbers in Table S2-1.
 - b) Find the sequences of the oligos/primers according to the names.
 - c) Replace 19-nt N in the forward primers with your 19-nt target sequences in front of PAM (NGG), and 19-nt N in the reverse primers with reverse complement sequences of your 19-nt target sequences in front of PAM (NGG).
3. Carry out PCR reactions according to information provided under the sequences of the PCR fragments. As an example, the reaction mixture and reaction conditions are as follows for construction of pHSE401-2gR-CHLI:

Component	Volume	Cycling conditions
10× KOD plus Buffer	5 µl	
MgSO ₄ (25mM)	3 µl	
dNTPs (2mM, Toyobo)	4 µl	
KOD plus (Toyobo)	1 µl	
pCBC-DT1T2 (diluted to 200 times)	1 µl	1. One cycle: 94 °C, 2 min.
DT1-BsF (20 µM)	1 µl	2. 30 cycles: 94 °C, 15 sec;
DT1-F0 (1 µM)	1 µl	60 °C, 30 sec; 68 °C, 1 min.
DT2-RO (1 µM)	1 µl	3. One cycle: 68 °C, 5 min
DT2-BsR (20 µM)	1 µl	
ddH ₂ O	32 µl	
Total volume	50 µl	

4. Set up Golden Gate reactions as follows:

Component	Volume	Reaction conditions
Purified PCR fragments (~100 ng/µl)	2 µl	5 hours at 37°C
pHSE401 or others (~100 ng/µl)	2 µl	5 min at 50°C
10× T4 DNA Ligase Buffer (NEB)	1.5 µl	10 min at 80°C
10× BSA	1.5 µl	
<i>Bsa</i> I (NEB)	1 µl	
T4 DNA Ligase (HC, NEB)	1 µl	NOTE: It is essential to use a
ddH ₂ O	6 µl	High Concentration (HC) Ligase
Total volume	15 µl	(2 million units/ml, NEB)

5. Transform *E.coli* competent cells with 5 µl of reaction mixture, and select positive clones on kanamycin LB agar plates.
6. Identify correct clones by colony PCR and verify them by sequencing.

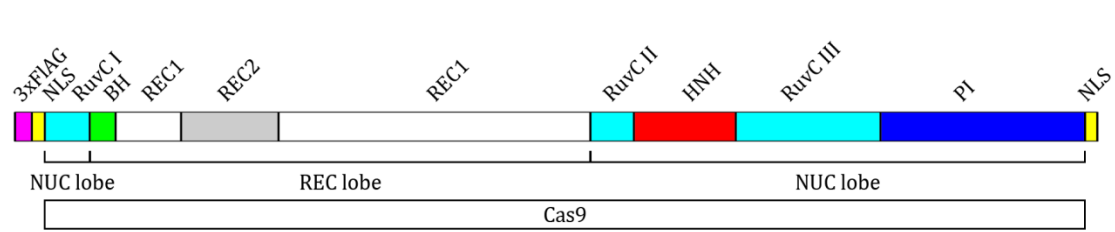
Table S2-1 Nomenclature of inserts/oligos and PCR products/primers

For dicots		
Insert or PCR product	Oligos or Primers	Number of gRNAs
01-insert	Oligo-01-F Oligo-R	1
DT1T2-PCR	DT1-BsF/DT1-F0 DT2-R0/DT2-BsR	2
For monocots		
Insert or PCR product	Oligos or Primers	Number of gRNAs
11-insert	Oligo-11-F Oligo-R	1
MT1T2-PCR	MT1-BsF/MT1-F0 MT2-R0/MT2-BsR	2

Notes:

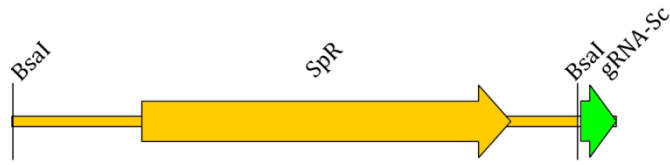
1. Dilute the F0/R0 primers to 20 times of the F/R primers.

Sequence of maize codon-optimized Cas9 (zCas9)



ATG GATTACAAGGACCACGACGGGGATTACAAGGACCACGACATTGATTACAAGGATGATGACAAGATGGCTCCGAAGAAGAAGAGGAAGGTTGGC
 ATCCACGGGGTCCAGCTGCTGACAAGAAGTACTCGATCGGCCCTCGATATTGGGACTAACTCTGTTGGCTGGGCCGTGATCACCGACGAGTACAAGGTGC
 CCTCAAAGAAGTTCAAGGTCCTGGGCAACCCGATCGGCATTCCATCAAGAAGAATCTATTGGCGCTCTCTGTTCCGACAGCGGGCAGACGGGCTGAGG
 CTACGCGGCTCAAGCGCACCGCCCGCAGGCGGTACACGCGCAGGAAGAATCGCATCTGTACTCTGACGAGATTTTCTCCAACGAGATGGCGAAGGTTG
 AC GATTCTTTTCCACAGGCTGGAGGAGTCTTCTCTGAGGAGGATAAGAAGCAGCAGCGGCATCCAATCTTCGGCAACATTGTGACGAGGTTGC
 CTACCAGAGAAGTACCTACGATCTACCATCTCGGGAAGAAGCTCTGGACTCCACAGATAAAGCGGACCTCCGCTGATCTACCTCGCTGCGCCACA
 TGATTAAGTTCAGGGGCCATTTCTGATCGAGGGGATCTCAACCCGACAATAGCGATGTTGACAAGCTGTTACATCCAGCTCGTGACAGCTACAACCA
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 GTGCCAAGCTGCAGCTCTCCAAGGACACATACGACGATGACCTGGATAAACCCTCGGCCAGATCGCGGATCAGTACGCGGACTCTGCTCGGTGCCA
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 CACCATCAGGATCTGACCTCTGAAGGCGCTGTCAGGCGACGCTCCCGAGAAGTACAAGGAGATCTTCTCGATCAGTCAAGAAGCGCTACGCTG
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 GAGGATGACGAATTTGACAAGAACCTGCCAATGAGAAGGTCTCCCTAAGCACTCGCTCTGTACGAGTACTTCAAGTCTACAACGAGCTGACTAAG
 GTGAAGTATGTGACGAGGCGATGAGGAAGCGGCTTTCTGCTGTTGGGAGCAGAAGAAGGCCATCGTGGACCTCTGTTCAAGACCAACCGGAAGGT
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 ACCTACCAGATCTCTGAAGATCATTAAAGGATAAGGACTTCTGACAAACGAGGAGAAAGGATATCTCGAGGACATTGTGCTGACACTCACTCTGTT
 CGAGGACCGGGAGATGATCAGGAGCGCTGAAGACTTACGCCATCTCTCGATGACAAGGTATGAAAGCAGCTCAAGAGGAGGAGGTACACCGGCT
 GGGGGAGGCTGAGCAGGAAGCTCATCAACGGCATTCCGGACAAGCAGTCCGGGAAGACGATCTCGACTTCTGAAGAGCGATGGCTTCGCGAACCCG
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 CGCCAAGTCGGAGCAGGAGATTGGCAAGGCTACCGCCAAGTACTTCTACTCTAACATTATGAATTTCTTCAAGACAGAGATCACTCTGCGCAATGGCG
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 ATGCCACAAGTGAATATCGTCAAGAAGACAGAGGTCCAGACTGGCGGGTCTCTAAGGAGTCAATTCTGCCTAAGCGGAACAGGCAAGCTATCGCC
 GCAAGAAGGACTGGATCCGAAGAAGTACGGCGGTTTCGACAGCCCACTGTGGCTACTCGGTCCTGTTGTGGCGAAGGTTGAGAAGGGCAAGTCC
 AAGAAGCTCAAGAGCTGAAGGAGCTGCTGGGGATCAGGATTATGAGGCGCTCCAGCTTCGAGAAGAACCAGTATCTCTGAGGCGGAAGGGCTA
 CAAGGAGGTGAAGAAGGACCTGATCATTAAAGTCCCAAGTACTCACTCTTCGAGCTGGAGAACGGCAGGAAGCGGATGCTGGCTCCCGTGGCGAGCT
 GCAGAAGGGGAACGAGCTGCTCTGCGCTCAAGTATGTGAATCTCTACTTCCGCTCCACTACGAGAAGCTCAAGGGCAGCCCGAGGACAACGA
 GCAGAAGCAGCTGTTCTGTCGAGCAGCACAAGCATTACCTCGACGAGATCATTGAGCAGATTTCGAGTTCCTCAAGCGCGTATCTGGCCGACGCGAAT
 CTGGATAAGGTTCTCTCCGCTACAACAAGCACCGGACAAGCCAATCAGGGAGCAGGCTGAGAATATCATTCATCTTCCACCTGACGAACCTCGGCC
 CCCCTGCTGTTTCAAGTACTTCGACACAATATCGATCGCAAGGATACACAAGCACTAAGGAGGTTCTGGACGGACCTCATCCACAGTCAAGTAC
 GGCTCTACGAGACGCGCATCGACTGTCTCAGCTCGGGGGCAGCAAGCGGCCAGCGGCGACGAAGAAGCGGGGCGAGGCGAAGAAGAAGAAGTGA

Sequence of SpR-gRNA-Scaffold



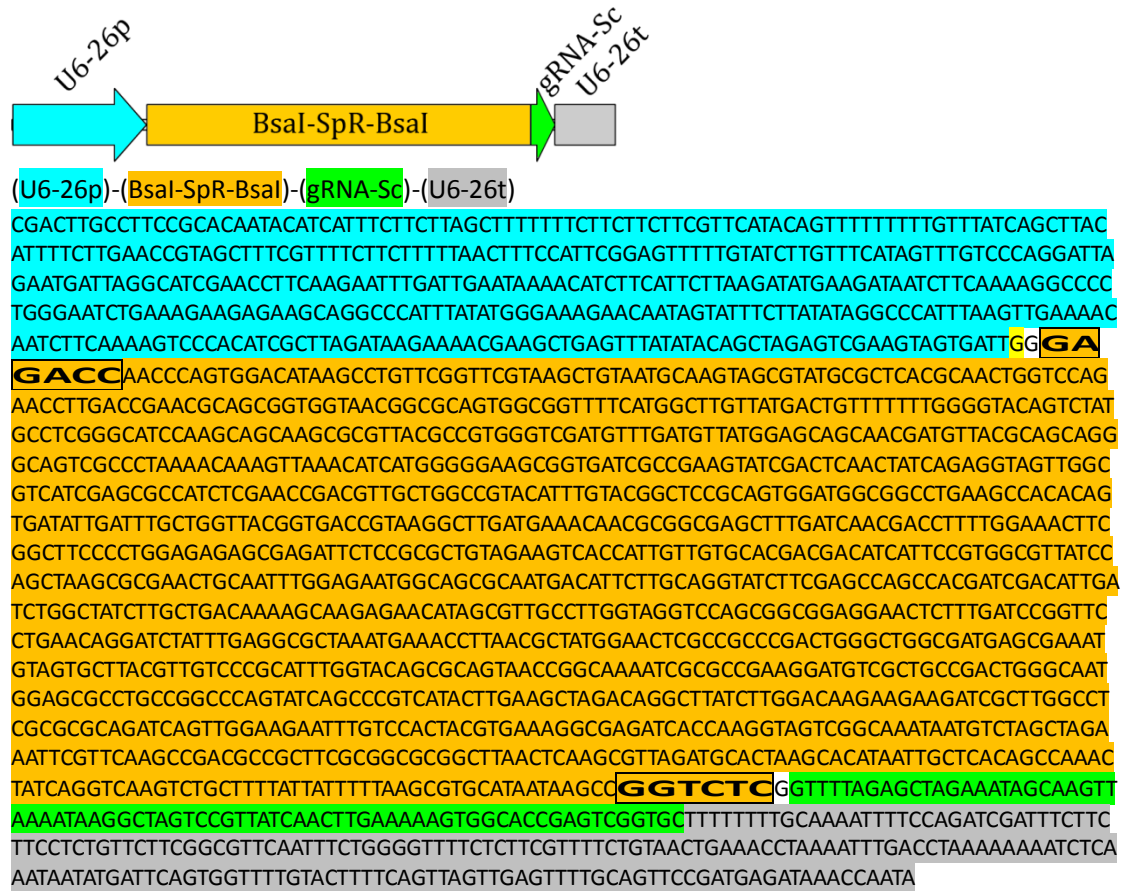
(**Bsal**)-(SpR)-(Bsal)-(gRNA-Sc)

GAGACCAACCCAGTGGACATAAGCCTGTTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCT
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TTATGACTGTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCG
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GGGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCG
AACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATT
GATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACCGCGCGAGCTTTGATCAACGACCTTTTGGGA
AACTTCGGCTTCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACAT
CATTCGTTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGG
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AACCTAACGCTATGGAACCTCGCCGCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCG
CATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGC
CTGCCGCCAGTATCAGCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTTG
GCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATACCAAGGTAGTCGGCAA
ATAATGTCTAGCTAGAAATTCGTTCAAGCCGACCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCA
CTAAGCACATAATTGCTCACAGCCAACTATCAGGTCAAGTCTGCTTTTATTATTTTAAAGCGTGCATAATAAG
CCGGTCTCGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAA
AGTGGCACCGAGTCGGTGC

Notes:

1. SpR, spectinomycin-resistance gene; gRNA-Sc, gRNA scaffold.
2. Enlarged and boxed letters indicate *Bsal* sites.

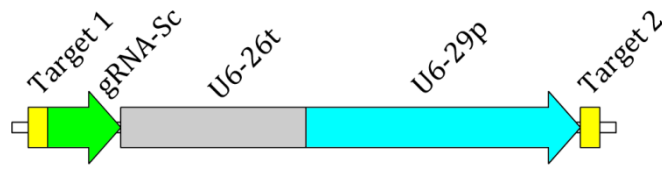
Sequence of U6-26p-driven gRNA expression cassette without target



Notes:

1. SpR, spectinomycin-resistance gene; gRNA-Sc, gRNA scaffold.
2. Enlarged and boxed letters indicate *Bsal* sites.

Sequence of DT1T2-PCR with Targets 1 and 2 for dicots



(Target-1)-(gRNA-Sc)-(U6-26t)-(U6-29p)-(Target-2)

ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
 GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCCTTTTTTTGCAAATTTCCAGATCGATT
 CTTCTCCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTCTCGTTTTCTGTAACCTGAAACCTAAAATTTG
 ACCTAAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTCCGAT
 GAGATAAACCAATAATTAATCCAACTACTGCAGCCTGACAGACAAATGAGGATGCAAACAATTTTAAAGTTT
 ATCTAACGCTAGCTGTTTTGTTCTCTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAGGCT
 TGTTTTACTTAAGGCCAATAATGTTGATGGATCGAAAGAAGAGGGCTTTAATAAACGAGCCCGTTTAAG
 CTGTAAACGATGTCAAAAACATCCACATCGTTGAGTTGAAAATAGAAGCTCTGTTTATATATTGGTAGAG
 TCGACTAAGAGATTGNNNNNNNNNNNNNNNNNNNNGTTTAGAGACCAATAAT

Primers:

- DT1-BsF: ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNNGTT
- DT1-F0: TGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC
- DT2-R0: AACNNNNNNNNNNNNNNNNNNNNCAATCTCTTAGTCGACTCTAC
- DT2-BsR: ATTATTGGTCTCGAAACNNNNNNNNNNNNNNNNNNNNCAA

Template: pCBC-DT1T2

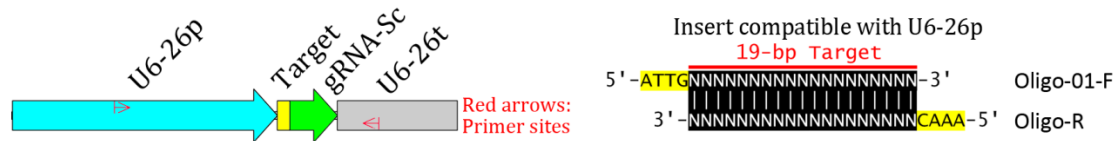
Length: 626-bp

Notes:

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. For the assembly of two gRNA expression cassettes, use DT1-BsF/DT1-F0/DT2-R0/DT2-BsR four-primer mixture with DT1-F0/DT2-R0 diluted to 20 times of DT1-BsF or DT2-BsR, resulting in DT1T2-PCR.

Sequence of one gRNA expression cassette for dicots

23-bp insert + pHSN401 et al.



(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)

CGACTTGCCTCCGCACAATACATCATTTCTTCTTAGCTTTTTTCTTCTTCTCGTTCATACAGTTTTTTTTGT
 TTATCAGCTTACATTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTCCATTCCGGAGTTTTGTATCTT
 GTTTCATAGTTTGTCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTGATTGAATAAAACATCTT
 CATTCTAAGATATGAAGATAATCTTCAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATA
 TGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAGTCCACATCGCTT
 AGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGNNNNNNNNNNNNNNNN
 NNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCGA
 GTCGGTGC TTTTTTTTGC AAAATTTCCAGATCGATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGG
 TTTTCTTCTCGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGTGTT
 TTGTACTTTTCAGTTAGTTGAGTTTGCAGTTCGGATGAGATAAACCAATA

Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- Red letters indicate primer sites.
- Primer sequences are as follows:

Colony PCR primers (5'→3'):

U6-26p-F: TGTCCAGGATTAGAATGATTAGGC

U6-26t-R: CCCAGAAAATTGAACGCCGAAGAAC

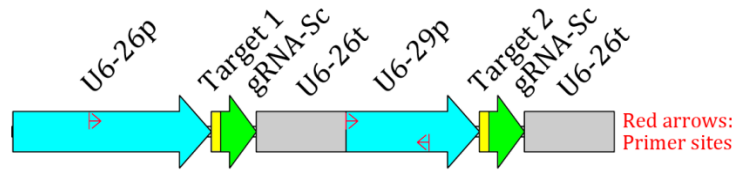
(U6-26p-F + U6-26t-R = 423 bp)

Sequencing primers (5'→3'):

U6-26p-F: TGTCCAGGATTAGAATGATTAGGC

Sequence of two gRNA expression cassettes for dicots

DT1DT2-PCR + pHSN401 et al.



(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)-(U6-29p)-(Target-2)-(gRNA-Sc)-(U6-26t)

CGACTTGCCTCCGCACAATACATCATTCTTCTTAGCTTTTTCTTCTTCTCGTTCATACAGTTTTTTTTTGT
 TTATCAGCTTACATTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTCCATTCGGAGTTTTGTATCTT
 GTTTCATAGTTTGTCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTGATTGAATAAAACATCTT
 CATTCTAAGATATGAAGATAATCTTCAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATA
 TGGGAAAGAACAATAGTATTCTTATATAGGCCATTAAAGTTGAAAACAATCTTCAAAGTCCACATCGCTT
 AGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCAAGTAGTGATTGNNNNNNNNNNNNNNNN
 NNNGTTTATAGAGCTAGAAATAGCAAGTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGA
 GTCGGTGCTTTTTTTTGCAAAATTTCCAGATCGATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGG
 TTTTCTTCTGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGTGTT
 TTGACTTTTTAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATA**TTAATCCAACTACTGCAGCCT**
GACAGACAAATGAGGATGCAACAATTTAAAGTTTATCTAACGCTAGCTGTTTTGTTTCTTCTCTGTTG
 CACCAACGACGGCGTTTTCTCAATCATAAAGAGGCTTGTTTTACTTAAGGCCAATAAT**GTTGATGGATCGA**
AAGAAGAGGCTTTTAAATAACGAGCCGTTAAGCTGTAAACGATGTCAAAAACATCCACATCGTTCA
 GTTAAAATAGAAGCTCTGTTTATATATTGGTAGAGTCGACTAAGAGATTGNNNNNNNNNNNNNNNNNN
 NN**GTTTATAGAGCTAGAAATAGCAAGTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT**
CGGTGCTTTTTTTTGCAAAATTTCCAGATCGATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGGTT
 TTCTTCTGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGTGTTTT
 GTACTTTTCAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATA

Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- Red letters indicate primer sites.
- Primer sequences are as follows:

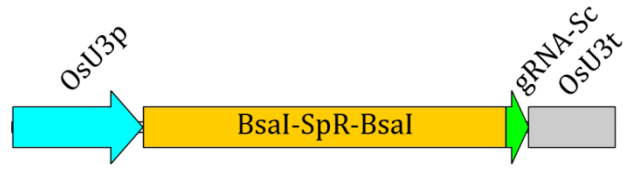
Colony PCR primers (5'→3'):

U6-26p-F: TGTCCAGGATTAGAATGATTAGGC
 U6-29p-R: AGCCCTTCTTTCGATCCATCAAC
 (U6-26p-F + U6-29p-R = 726 bp)

Sequencing primers (5'→3'):

U6-26p-F: TGTCCAGGATTAGAATGATTAGGC
 U6-29p-F: TTAATCCAACTACTGCAGCCTGAC

Sequence of OsU3p-driven gRNA expression cassette without target



(OsU3p)-(BsaI-SpR-BsaI)-(gRNA-Sc)-(OsU3t)

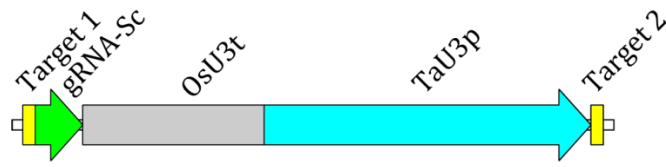
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AGTAATTCATCCAGGTCTCCAAGTTCTAGGATTTTCAGAAGCTGCAACTTATTTTATCAAGGAATCTTTAAACATACGAACAGATCA
CTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTGAGGAATCAGATGTGCAGTCAGGGACCATAGCA
CAAGACAGGCGTCTTCTACTGGTGTACCAGCAATGCTGGAAGCCGGGAACACTGGGTACGTTGAAACCACGTGATGTGA
AGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGC
CCATTACGCAATTGGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAGAGTTG
TGCAGATGATCCGTGGCGTTGAGACCAACCCAGTGGACATAAGCCTGTTGCGTTCGTAAGCTGTAATGCAAGTAGCGT
ATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTATGGCTTGTATGA
CTGTTTTTTGGGGTACAGTCTATGCCTCGGCATCCAAGCAGCAAGCGCTTACGCCGTGGGTTCGATGTTGATGTTATGGAG
CAGCAACGATGTACGAGCAGGGCAGTCGCCCTAAACAAAGTTAAACATCATGGGGGAAGCGGTGATCGCCGAAGTATCG
ACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTG
GATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTACGGTGACCGTAAGGCTTGATGAAACAACGGCGGAGCTTT
GATCAACGACCTTTTGAAACTTCGGCTTCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCAACATTGTTGTGCAGC
ACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTGCAGGTATCT
TCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCG
CGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACCTCGCCGCC
GACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAA
GGATGTCGCTGCCACTGGGCAATGGAGCGCCTGCCGGCCAGTATCAGCCGTCATACTTGAAGCTAGACAGGCTTATCTTG
GACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGCTCACTACGTGAAAGGCGAGATCACCAAGGT
AGTCGGCAAATAATGTCTAGCTAGAAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACCTAAGCGTTAGATGACTA
AGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTAAAGCGTGCATAATAAGCCGGTCTCG
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TGTTCAAAAAGTACGAGCTGTATTTCACTATTTACGGCGCCACATTTTCATGCCGTTTGTGCCAACTATCCCAGCTAGTGAAT
ACAGCTTGGCTTACACAACACTGGTGACCCGCTGACCTGCTCGTACCTCGTACCGTTCGTACGGCACAGCATTGGAATTAAG
GGTGTGATCGATACTGCTTGCTGCT
    
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Notes:

1. The OsU3p contains a *BsaI* site affected by Dcm methylation and therefore uncuttable when vectors are proliferated in commonly used *E. coli* strains (dcm+).
2. Enlarged and boxed letters indicate *BsaI* sites.

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



(Target-1)-(gRNA-Sc)-(OsU3t)-(TaU3p)-(Target-2)
 ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
 GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTCTTTTTTTTTTCGTTTTGCATTGAGTTTTCT
 CCGTCGCATGTTTGCAGTTTTATTTCCGTTTGCATTGAAATTTCTCCGTCTCATGTTTGCAGCGTGTCAAAA
 AAGTACGCAGCTGTATTTCACTTATTTACGGCGCCACATTTTCATGCCGTTTGTGCCAACTATCCCGAGCTAGT
 GAATACAGCTTGGCTTACACAACACTGGTGACCCGCTGACCTGCTCGTACCTCGTACCGTCTGACGGCACA
 GCATTTGGAATTAAGGGTGTGATCGATACTGCTTGCTGCTCATGAATCCAACCACACGGAGTTCAAATTCC
 CACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTGTGAATATTATATCTGTCGTGCAAATTCCTGGCCT
 GCACAATTGCTGTATAGTTGGCGGCAGGGAGAGTTTAACTTACTAGCTGCTGATAATTTGTGAGAAA
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 GATGCACGGAAAAAGGAAAAGAAAAGCCATACTTTTTTTAGGTAGGAAAAGAAAAGCCATACGAGACT
 GATGTCTCTCAGATGGCCGGGATCTGTCTATCTAGCAGGCAGCCCCAACCTCACGGGCCAGCAATT
 ACGAGTCTTCTAAAAGCTCCCGCCGAGGGGCGCTGGCGTCTGTGCAGCAGCACGTCTAACATTAGTCC
 CACCTCGCCAGTTTACAGGGAGCAGAACCAGCTATAAGCGGAGGCGCGGCACCAAGAAGCGNNNNNNNN
 NNNNNNNNNNNNNGTTTTAGAGACCAATAAT

Primers:

MT1-BsF: ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNNGTT
 MT1-F0: TNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC
 MT2-R0: AACNNNNNNNNNNNNNNNNNNNCGCTTCTTGGTGCC
 MT2-BsR: ATTATTGGTCTCTAAACNNNNNNNNNNNNNNNNNNNNC

Template: pCBC-MT1T2

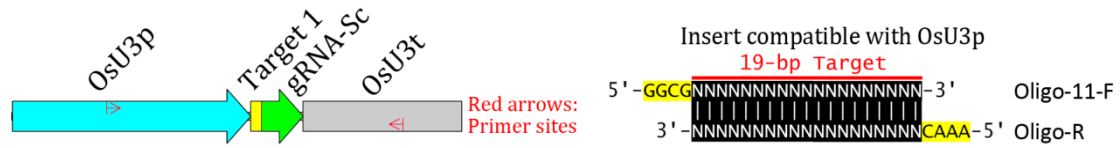
Length: 964-bp

Notes:

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
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.

Sequence of one gRNA expression cassette for monocots

23-bp insert + pBUN411 et al.



(OsU3p)-(Target-1)-(gRNA-Sc)-(OsU3t)

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AGTAATTCATCCAGGTCTCCAAGTTCAGGATTTTCAGAACTGCAACTATTTTATCAAGGAATCTTAAACATACGAACAGATCA
CTTAAAGTCTCTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCATAGCA
CAA GACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCACGTGATGTGA
AGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGCCGGC
CCATTACGCAATTGGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAGAGTTG
TGCAGATGATCCGTGGCGNNNNNNNNNNNNNNNNNNNNGTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGCCGTT
ATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTCGTTTTGCATTGAGTTTCTCCGTGCGCATGTTGCAGTTTTATT
TTCCGTTTTGCATTGAAATTTCTCCGTCTCATGTTGCAGCGTGTCAAAAAGTACGCAGCTGTATTTCACTTATTTACGGCGCCA
CATTTCATGCCGTTTGTGCCAACTATCCCGAGCTAGTGAATACAGCTTGGCTTACACAACACTGGTGACCCGCTGACCTGCT
CGTACCTCGTACCGTCGTACGGCACAGCATTGGAATTAAGGGTGTGATCGATACTGCTTGCTGCT
    
```

Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- The OsU3p from vectors contains a *Bsa*I site affected by Dcm methylation and therefore uncuttable when vectors are proliferated in commonly-used *E. coli* strains (dcm+).
- Red letters indicate primer sites.
- Primer sequences are as follows:

Colony PCR primers (5'→3'):

OsU3p-F3: GACAGGCGTCTTCTACTGGTGCTAC

OsU3t-R: TATCACTAGCTCGGGATAGTTGGC

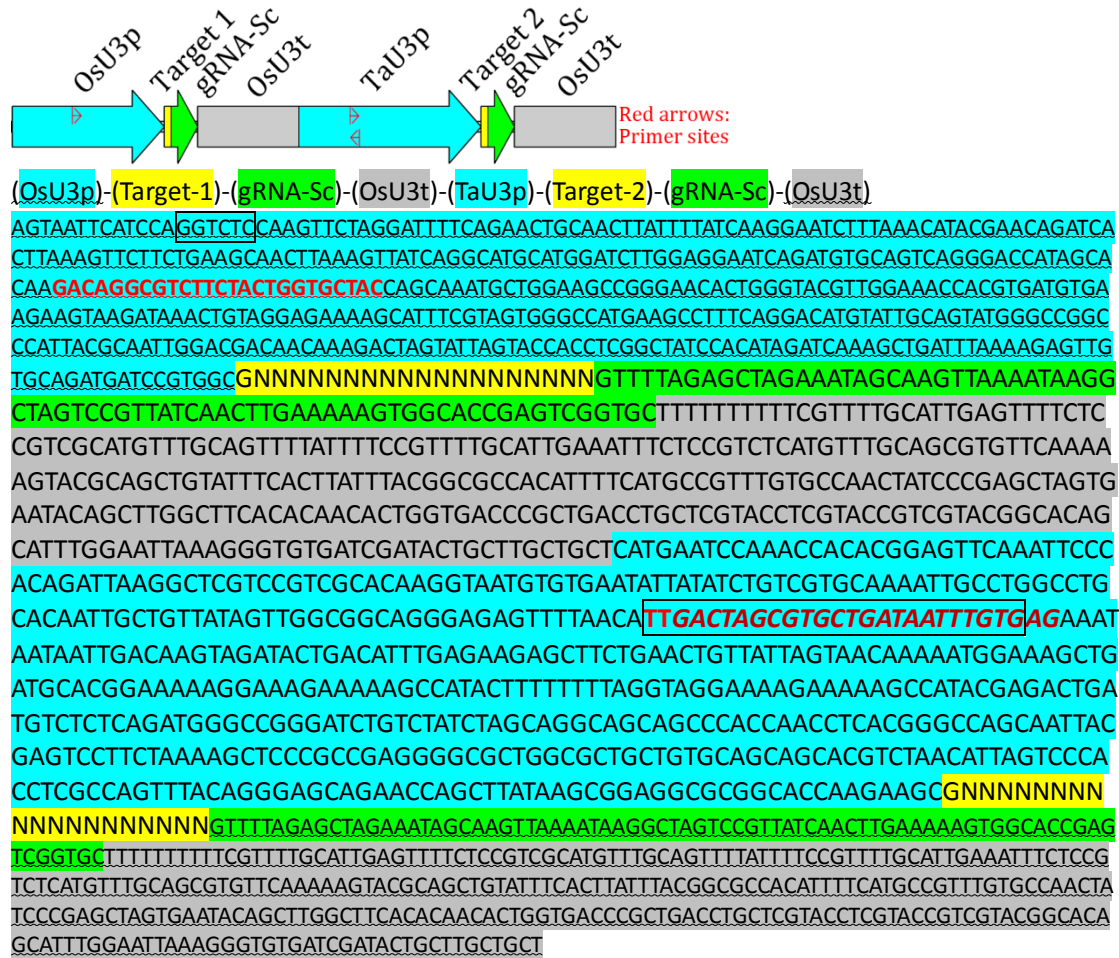
(OsU3p-F3 + OsU3t-R = 543 bp)

Sequencing primers (5'→3'):

OsU3p-F3: GACAGGCGTCTTCTACTGGTGCTAC

Sequence of two gRNA expression cassettes for monocots

MT1T2-PCR + pBUN411 et al.



Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- Red letters indicate primer sites.
- Primer sequences are as follows:

Colony PCR primers (5'→3'):

OsU3p-F3: GACAGGCGTCTTCTACTGGTGCTAC
 TaU3p-R: CTCACAATTATCAGCACGCTAGTC
 (OsU3p-F3 + TaU3p-R = 825 bp)

Sequencing primers (5'→3'):

OsU3p-F3: GACAGGCGTCTTCTACTGGTGCTAC
 TaU3p-F2: TTGACTAGCGTGCTGATAATTTGTG