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Supplementary information

Direct delivery and fast-treated Agrobacterium co-culture (Fast-TrACC) plant transformation methods for Nicotiana benthamiana

In the format provided by the authors and unedited

Supplementary Table 1.

sgRNA array position	Plasmid	Bacterial Selection	Cloning Marker	Type IIS Enzyme	Source	Addgene #	Description
1	pTAG1	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176863	Entry of sgRNA spacer at position 1 in multi-guide array
2	pTAG2	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176864	Entry of sgRNA spacer at position 2 in multi-guide array
2 + end	pTAG2E	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176865	Entry of sgRNA spacer at position 2 (end vector for 2 sgRNA assembly)
3	pTAG3	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176866	Entry of sgRNA spacer at position 3 in multi-guide array
4	pTAG4	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176867	Entry of sgRNA spacer at position 4 in multi-guide array
4 + end	pTAG4E	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176868	Entry of sgRNA spacer at position 4 (end vector for 4 sgRNA assembly)
5	pTAG5	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176869	Entry of sgRNA spacer at position 5 in multi-guide array
6 + end	pTAG6E	Spectinomycin	LacZ (Blue/White)	Esp3I	This manuscript	176870	Entry of sgRNA spacer at position 6 (end vector for 6 sgRNA assembly)

Supplementary Table 1. Step 6, Procedure 1: Vectors for assembly of gRNA spacer in positional tRNA backbone. sgRNAs are assembled into backbones with respect to the number of sgRNAs desired in the final array. Note that even numbers of sgRNAs are assembled (2, 4 or 6 sgRNAs). The final sgRNA in the array must be placed into a 'X + end' plasmid backbone to ensure proper tRNA array assembly. The blue highlighted vectors for position 1 and 2+End relate to Fig. 2ab where they are referenced for assembly of two sgRNAs into a B module. LacZ = beta-galactosidase for blue/white screening.

Supplementary Table 2.

Part	Contents	Plasmid	Bacterial Selection	Cloning Marker	Type IIS Enzyme	Source	Addgene #	Description
Promoter	AtUbi10	pMC-1-1AtUbi10-6	Spectinomycin	N//A	BsaI	This manuscript	176871	*A. thaliana* Ubiquitin 10 promoter
	CaMV 35S + TMV 5'UTR Ω	pICH51266	Spectinomycin	N//A	BsaI	Engler et al., 2014	50267*	*Cauliflower Mosaic Virus* 35S promoter + *Tobacco Mosaic Virus* 5' UTR Ω element
	CaMV 35S (2x) + TMV 5'UTR Ω	pICH51288	Spectinomycin	N//A	BsaI	Engler et al., 2014	50269*	Double *Cauliflower Mosaic Virus* 35S promoter + *Tobacco Mosaic Virus* 5' UTR Ω element
	CmYLCV	pMC-1-CmYLCV-6	Spectinomycin	N//A	BsaI	This manuscript	176872	*Cauliflower Mosaic Virus* 35S promoter
TAG vectors	***See Supplementary Table 1 for vectors assembled in Steps 1-14, Procedure 1***							
Terminator	CaMV 35S	pMC-9-35S-11	Spectinomycin	N//A	BsaI	This manuscript	176881	*Cauliflower Mosaic Virus* 35S terminator
	HSP	pMC-9-HSP-11	Spectinomycin	N//A	BsaI	This manuscript	176882	*A. thaliana* heat shock protein terminator
	Nos	pICH41421	Spectinomycin	N//A	BsaI	Engler et al., 2014	50339*	*A. tumefaciens* nopaline synthase terminator
Backbone	MoClo to pMOD B	pJC225	Carb/Amp	Cam/CcdB (lethal)	BsaI	This manuscript	176883	MoClo compatible B module backbone vector with CcdB bacterial lethal selection.

Supplementary Table 2. Step 15, Procedure 1: Vectors for assembly of individual gRNA-tRNAs into module B vectors. Individual sgRNA vectors assembled into positionally numbered backbones (tRNA assembled guide (TAG) vectors) may be assembled into a B module vector with a single Pol II promoter and terminator of choice (See Fig 2b for reference). The Voytas Lab has tested the promoter and terminator combinations that are listed here. Additional promoters and terminators may be used.[9] The colors in the 'Parts' column coordinates with Fig 2b as a reference for assembly into B module vectors. CcdB = toxin of Ccd operon for negative selection of vector backbone ligation products without insertions (vectors containing this gene must be propagated in CcdB resistant cell lines; * = available as a part of the MoClo Plants Part Kit from Addgene (Kit #1000000047). Cam = chloramphenicol resistance; Carb/Amp = carbenicillin/ampicillin resistance.

Supplementary Table 3.

Part	Contents	Plasmid	Bacterial Selection	Cloning Marker	Type IIS Enzyme	Source	Addgene #	Description
A Module	Empty	pMOD_A0000	Carb/Amp	N/A	AarI	Cermak et al., 2017	90997	Empty vector for with T-DNA assembly without A module contents
	35s:AtCas9	pMOD_A0101	Carb/Amp	N/A	AarI	Cermak et al., 2017	90998	*Cauliflower Mosaic Viru* s 35s promoter driving *A. thaliana* codon optimized Cas9
	35s:GFP	pMOD_A3001	Carb/Amp	N//A	AarI	Cermak et al., 2017	91042	*Cauliflower Mosaic Virus* 35s promoter driving green fluorescent protein
	CmYLCV:GFP	pMOD_A3003	Carb/Amp	N//A	AarI	Cermak et al., 2017	91043	*Cestrum yellow leaf curling virus* promoter driving green fluorescent protein
	ZmUbi:GFP	pMOD_A3010	Carb/Amp	N//A	AarI	Cermak et al., 2017	to be submitted	*Zea mays* Ubiquitin 10 promoter driving green fluorescent protein
	35s:Luciferase	pMOD_A5801	Carb/Amp	N//A	AarI	Maher et al., 2020	176877	*Cauliflower Mosaic Virus* 35s promoter driving optimized firefly luciferase (LUC+; PROMEGA)
	CmYLCV:Luciferase	pMOD_A5803	Carb/Amp	N//A	AarI	Maher et al., 2020	176878	*Cestrum yellow leaf curling virus* promoter driving optimized firefly luciferase (LUC+; PROMEGA)
B Module	Empty	pMOD_B0000	Carb/Amp	N/A	AarI	Cermak et al., 2017	91058	Empty vector for with T-DNA assembly without B module contents
	35S:ipt	pMOD_B7101	Carb/Amp	N/A	AarI	This manuscript	127227	*Cauliflower Mosaic Virus* 35s promoter driving *A. tumefaciens* isopentenyl transferase
	AtU6:gRNA (NbPDS)	pMM100	Carb/Amp	N/A	AarI	Maher et al., 2020	127225	sgRNA targeting *N. benthamiana* phytoene desaturase
	See Supplementary Table 1 for TAG array assembly into B module							
C' Module	Empty	pMOD_C'0000	Carb/Amp	N/A	AarI	Cermak et al., 2017	127226	Empty vector for with T-DNA assembly without C' module contents
	AtUbi10:Luciferase	pMOD_C'5802	Carb/Amp	N/A	AarI	Maher et al., 2020	176879	*A. thaliana* Ubiquitin 10 promoter driving optimized firefly luciferase (LUC+; PROMEGA)
	Nos:Wus2	pMOD_C'5014	Carb/Amp	N//A	AarI	Maher et al., 2020	127219	*A. tumefaciens* nopaline synthase promoter driving *Z. mays* WUSCHEL2
D Module	Empty	pMOD_D0000	Carb/Amp	N/A	AarI	Cermak et al., 2017	127230	Empty vector for with T-DNA assembly without D module contents
	AtUbi10:Luciferase	pMOD_D5802	Carb/Amp	N/A	AarI	Maher et al., 2020	176880	*A. thaliana* Ubiquitin 10 promoter driving optimized firefly luciferase (LUC+; PROMEGA)
	35s:ipt	pMOD_D7101	Carb/Amp	N//A	AarI	Maher et al., 2020	127229	*Cauliflower Mosaic Virus* 35s promoter driving *A. tumefaciens* isopentenyl transferase
Backbone	BeYDV replicon T-DNA	pTRANS_201	Kanamycin	Cam/CcdB (lethal)	AarI	Cermak et al., 2017	91105	*A. tumefaciens* pCambia transformation vector with *Bean Yellow Dwarf Virus* replicon
	BeYDV replicon T-DNA (KAN)	pTRANS_221	Kanamycin	Cam/CcdB (lethal)	AarI	Cermak et al., 2017	91115	*A. tumefaciens* pCambia transformation vector with *Bean Yellow Dwarf Virus* replicon and kanamycin plant selection

Supplementary Table 3. Step 19, Procedure 1: Vectors for assembly of module vectors into transformation vector backbones. A, B , C' and D modules may be assembled into a final T-DNA vector. If no insert at a given position is desired, an 'empty' module may be used in its place. The colors in the 'Parts' column coordinate with Fig. 1 and Fig. 2c as a reference for assembly into the final BeYDV Replicon T-DNA. CcdB = toxin of Ccd operon for lethal negative selection of vector backbone products without insertion (vectors containing this gene must be propagated in CcdB resistant cell lines); Cam = chloramphenicol resistance; Carb/Amp = carbenicillin/ampicillin resistance.

Supplementary Table 4.

Part	Contents	Plasmid	Bacterial Selection	Cloning Marker	Type IIS Enzyme	Source	Addgene #	Description
A Module	CcdB cloning entry site	pMOD_A4800EC	Carb/Amp	Cam/CcdB (lethal)	Esp3I	This manuscript	176873	Cloning entry site for inserting sequences of interest into module A
B Module	CcdB cloning entry site	pMOD_B4800EC	Carb/Amp	Cam/CcdB (lethal)	Esp3I	This manuscript	176874	Cloning entry site for inserting sequences of interest into module B
C' Module	CcdB cloning entry site	pMOD_C'4800EC	Carb/Amp	Cam/CcdB (lethal)	Esp3I	This manuscript	176875	Cloning entry site for inserting sequences of interest into module C'
D Module	CcdB cloning entry site	pMOD_D4800EC	Carb/Amp	Cam/CcdB (lethal)	Esp3I	This manuscript	176876	Cloning entry site for inserting sequences of interest into module D

Supplementary Table 4. Vectors for modular assembly of genes of interest. These vectors may be used to create new modules. For additional module options, see Cermak et al.[8] The colors in the 'Parts' column coordinate with Fig. 1 and Fig. 2c as a reference for assembly into the final BeYDV Replicon T-DNA. LacZ = beta-galactosidase for blue/white screening; CcdB = toxin of Ccd operon for lethal negative selection of vector backbone products without insertion (vectors containing this gene must be propagated in CcdB resistant cell lines); Cam = chloramphenicol resistance; Carb/Amp = carbenicillin/ampicillin resistance.

Supplementary Table 5.

Construct	A Module	B Module	C' Module	D Module	Bacterial Selection	Addgene #	Description
pMM114	CmYLCV:Luciferase	AtU6:sgRNA (NbPDS3)	Nos:ZmWUS2	35S:IPT	Kanamycin	127211	Expression cassette containing LUC+ reporter, sgRNA targeting *N. benthamiana* phytoene desaturase, *Z. mays* WUSCHEL2, *A. tumefaciens* isopentenyl transferase.
pMM117	35S:Luciferase	AtU6:sgRNA (NbPDS3)	Empty	Empty	Kanamycin	127212	Expression cassette containing LUC+ reporter, sgRNA targeting *N. benthamiana* phytoene desaturase and no developmental regulators; may be used as negative control.
pMM134	CmYLCV:Luciferase	AtU6:sgRNA (NbPDS3)	35S:IPT	Empty	Kanamycin	127215	Expression cassette containing LUC+ reporter, sgRNA targeting *N. benthamiana* phytoene desaturase, *A. tumefaciens* isopentenyl transferase.
pMM135	CmYLCV:Luciferase	AtU6:sgRNA (NbPDS3)	Nos:ZmWUS2	Empty	Kanamycin	127216	Expression cassette containing LUC+ reporter, sgRNA targeting *N. benthamiana* phytoene desaturase, *Z. mays* WUSCHEL2
pMKV057	Empty	AtUbi10:Luciferase	Nos:ZmWUS2	35S:IPT	Kanamycin	133312	Expression cassette containing LUC+ reporter, *Z. mays* WUSCHEL2, *A. tumefaciens* isopentenyl transferase.
pMKV058	Empty	AtUbi10:Luciferases	Nos:ZmWUS2	Empty	Kanamycin	133313	Expression cassette containing LUC+ reporter, *Z. mays* WUSCHEL2
pMKV059	Empty	AtUbi10:Luciferase	Empty	35S:IPT	Kanamycin	133314	Expression cassette containing LUC+ reporter, *A. tumefaciens* isopentenyl transferase.
pMKV060	Empty	AtUbi10:Luciferase	Empty	Empty	Kanamycin	133315	Expression cassette containing LUC+ reporter and no developmental regulators; may be used as negative control.

Supplementary Table 5. Select preassembled transformation vectors. Vectors from Maher et al. 2020 may used to test Fast-TrACC and Direct Delivery protocols prior to creating additional vectors of interest. Note that all expression cassettes described in this protocol are contained within a Bean Yellow Dwarf Virus (BeYDV) replicon, which itself is contained within the T-DNA borders of an *A. tumefaciens* pCambia transformation vector. The colors for A, B, C' and D modules coordinate with Fig. 1 and Fig. 2c as a reference for assembly into the final BeYDV Replicon T-DNA.

Supplementary Table 6.

Oligo Name	Sequence	Description
	Sequencing primers to verify integrity of TAG MoClo vectors (Step 14, Procedure 1)	
oCS837	GCCTTTGAGTGAGCTGATACC	Forward primer for sequencing sgRNA in tRNA entry vectors from step 14
oJC770	TGACGTCTAAGAAACCATTATTATCATGAC	Reverse primer for sequencing sgRNA in tRNA entry vectors from step 14
Spec_R	GTTCTGGACCAGTTGCGTGAG	Alternative reverse primer for sequencing sgRNA in tRNA entry vectors from step 14
	Sequencing primers to verify integrity of B module assembly (Step 18, Procedure 1)	
oCS837	GCCTTTGAGTGAGCTGATACC	Forward primer for sequencing B module assembly of sgRNA-tRNA array from step 24
oSC836	GCGACACGGAAATGTTGGATAC	Reverse primer for sequencing B module assembly of sgRNA-tRNA array from step 24
BlaA_R	CACCAGCGTTTCTGGGTGAG	Alterative reverse primer for sequencing B module assembly of sgRNA-tRNA array from step 24
Ori	GCCTTTGAGTGAGCTGATACC	Alternative forward primer for sequencing B module assembly of sgRNA-tRNA array from step 24
	sgRNAs described in Maher at al. [6]	
AtU6:sgRNA (NbPDS)	TTGGTAGTAGCGACTCCATG	sgRNA sequence targeting PDS2 homologs in *N. benthamiana* (Niben101Scf14708g00023.1 and Niben101Scf01283g02002.1)

Supplementary Table 6. Primers used to verify integrity of Golden Gate assemblies and sgRNAs arrays.