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Multiple T-DNA Delivery to Plants Using Novel Mini Binary Vectors with Compatible Replication Origins

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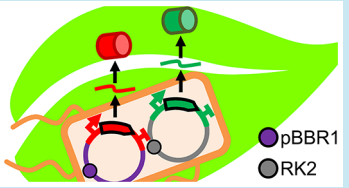
Supporting Information

ABSTRACT: Improved plants are necessary to meet human needs. *Agrobacterium*-mediated transformation is the most common method used to rewire plant capabilities. For plant gene delivery, DNA constructs are assembled into binary T-DNA vectors that rely on broad host range origins for bacterial replication. Here we present pLX vectors, a set of mini binary T-DNA plasmids suitable for Type IIS restriction endonuclease- and overlap-based assembly methods. pLX vectors include replicons from compatible broad host range plasmids. Simultaneous usage of pBBR1- and RK2-based pLX vectors in a two-plasmid/one-*Agrobacterium* strain strategy allowed multigene delivery to plants. Adoption of pLX vectors will facilitate routine plant transformations and targeted mutagenesis, as well as complex part and circuit characterization.



KEYWORDS: *binary T-DNA vector, pBBR1, Agrobacterium tumefaciens, plant biotechnology, multigene transfer, ethanol-inducible switch, Potyviridae, Ugandan cassava brown streak virus*

Plants are plastic organisms that sense and respond to environmental stimuli. These responses or specific plant features might not fit human needs, and their manipulation can be achieved by targeted use of plant-interacting microorganisms, or by plant genetic transformation.[1−3] Plant biotechnology uses advanced tools to generate plants with new functions, better agronomic traits, or to produce new products.[1] Synthetic biology applies engineering principles to facilitate the production of organisms with customized functions and for precise control of specific biological functions.[4] Genetic components of complex biological systems are reduced to DNA parts with modular and defined assignments. Once characterized with the aid of computational tools, parts libraries are assembled to yield pathways and networks with predictable outputs.[5] Methods to analyze dynamic molecular devices have been used to engineer plants with tunable functions.[6]

Plant parts libraries and assembly standards have been described, and are based on Type IIS restriction endonucleases (*e.g.*, BsaI) for one-step digestion-ligation reactions (Golden Gate).[7−9] Alternatively, overlapping DNA molecules can be joined into multicomponent constructs by one-step isothermal DNA assembly (Gibson assembly).[10]

Assembled DNA constructs are transferred directly to the plant, or introduced into disarmed-pTi *Agrobacterium tumefaciens* strains.[1] *Agrobacterium* thus serves as a shuttle chassis for plant delivery of constructs maintained into binary T-DNA vectors.[1] Multigene transfer is imperative in multiplex gene editing and to engineer complex traits, circuit designs, and metabolic pathways.[1,11−13] Conventional stacking methods require substantial breeding efforts that can be overcome by placing multiple genes within a single T-DNA, or simultaneous plant cell infections with multiple *Agrobacterium* strains, each harboring a different binary T-DNA vector.[11] Components of bacterial circuits are often distributed among several compatible plasmids,[5] although simultaneous use of compatible binary T-DNA vectors is a seldom-applied strategy in plant biotechnology[14]. Possible explanations include limited flexibility due to large plasmid sizes (>15 kb)[14] or lack of replication independence.[15]

To expand the plant synthetic biology toolbox, we focused on the pBBR1 plasmid[16] for design of novel binary T-DNA vectors that ease multigene construct assembly and delivery. pBBR1 replicates in *Escherichia coli* and *Agrobacterium*, its broad-host-range origin is small in size (≈ 1.5 kb) and belongs to an incompatibility group different from those of commonly used binary vectors.[16] Generated binary T-DNA vectors are suited for Golden Gate and GoldenBraid[8] cloning as well as for overlap-dependent methods (Gibson assembly; Figure 1A). Vectors were tested for transient and stable plant transformation, genome editing and for agro-inoculation of a new viral infectious clone. Finally, we inserted T-DNA vectors with compatible replication origins into *Agrobacterium* cells (Multiplexing; Figure 1A). We used a two-vector/one-strain approach in transient expression assays to deliver a simple buffer gate, and evaluated *in planta* gate responses to its chemical inducer.

RESULTS AND DISCUSSION

In the design of new binary T-DNA vectors, we chose basic principles: (i) reduced size, (ii) stability, (iii) broad host range

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Vector	Size (bp)	Origin	T-DNA*	Cloning features§					
				Bacterial selection	Cassette	Golden Gate	Golden Braid	Gibson assembly	Multiplexing
pLX-B2	3287	pBBR1	octopine	KAN	alpha1	■	■	■	■
pLX-B3	3349	pBBR1	octopine	SP	alpha1	■	□	■	n.t.
pLX-B4	3165	pBBR1	octopine	GENT	alpha1	■	□	■	n.t.
pLX-B2α2	3287	pBBR1	octopine	KAN	alpha2	■	■	■	n.t.
pLX-B3Ω1	3349	pBBR1	octopine	SP	omega1	□	■	■	n.t.
pLX-B3Ω2	3349	pBBR1	octopine	SP	omega2	□	■	■	n.t.
pLX-Z4	3740	RK2	succinamopine	GENT	alpha1	■	□	■	■

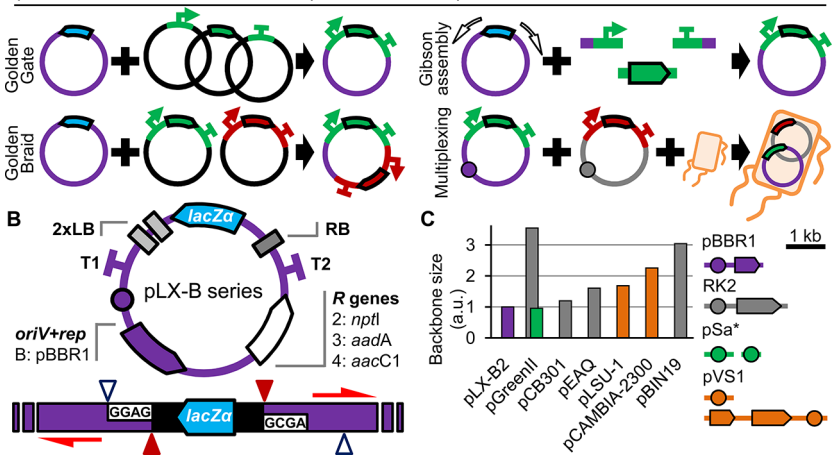

Figure 1. Novel binary T-DNA vectors of the pLX series and their features. (A) Short list of new binary T-DNA vectors generated in this study and their cloning features. *pTi type of right and left border source, all vectors include a second consensus left border sequence; §cloning cassette nomenclature according to GoldenBraid standards;[8] solid square, suitable; open square, not suitable; n.t., not tested. Bottom, cloning feature diagrams of pLX vectors (purple). Parts or transcriptional units can be assembled from plasmid libraries into pLX vectors using BsaI-based Golden Gate and GoldenBraid standards. Overlapping DNA fragments can be joined into linearized pLX vectors by Gibson assembly; open arrows, primer binding sites. pLX vectors with compatible replicons can be multiplexed into *Agrobacterium* cells for multi T-DNA delivery. (B) Organization of pBBR1-based pLX plasmids. Binary vectors are composed of three modules, (i) a T-DNA cassette that includes a right border, an *Escherichia coli* reporter gene, two left borders and is flanked by bacterial transcriptional terminators (T1 and T2); (ii) the broad host-range pBBR1 origin for plasmid replication in *E. coli* and *Agrobacterium* (*oriV* + *rep*); and (iii) antibiotic resistance (*R*) genes. Plasmid vectors are indicated by a letter that reflects their origin module (B, pBBR1-derived origin) and a digit according the *R* gene (2, *npt*I, gene that confers resistance to kanamycin; 3, *aad*A, to spectinomycin/streptomycin; 4, *aac*C1, to gentamicin). Bottom, cloning features of a pLX vector T-DNA cassette. The *lacZα* reporter is flanked by two divergent BsaI recognition sites (solid triangles), the nonpalindromic overhangs generated by BsaI digestion allow assembly of transcriptional units using one-step digestion-ligation Golden Gate cloning. Convergent BsmBI sites (open triangles) are included to build multiple transcriptional unit constructs by GoldenBraid assembly. Alternatively, pLX vectors can be linearized by inverse PCR using divergent primers (red arrows), DpnI-treated, and used to join one or several overlapping inserts by one-step isothermal DNA assembly (Gibson assembly). (C) Relative size comparison of pLX-B2 backbone and selected binary vectors (T-DNA cassette sequences were not considered). Graph bars are colored according plasmid replication origins shown on the right; pVS1- and pSa-based binary vectors include a narrow-host-range origin for maintenance in *E. coli*; *pSa origin in pGreen-based vectors is not autonomous, and size of the RK2-based pSoup plasmid required for pGreenII maintenance in *Agrobacterium* is also shown in the bar graph. Glyphs according to Synthetic Biology Open Language visual format.

replication origin for maintenance in *E. coli* and *Agrobacterium*, (iv) origin compatible with the most commonly used binary T-DNA vectors, (v) consistency with current plant synthetic biology standards, and (vi) the possibility to adopt overlap-dependent methods for construct assembly. The origin from the small broad-host-range pBBR1 plasmid[16] matches many of our criteria, and we used it in the pLX vector series. The pBBR1-based backbone of pLX vectors is substantially smaller than the widely used pBIN- and pCAMBIA-derived vectors, and equals to pGreen-derived vectors,[17,18] the smallest available binary plasmids (Figure 1B, C). Replication of pGreen vectors in *Agrobacterium* requires a coresident plasmid that supplies the pSa-*RepA* gene (*e.g.*, pSoup). In contrast, pLX vectors facilitate flexible experimental designs since their replication is autonomous in both *E. coli* and *Agrobacterium*. Additional features of pLX vectors include diverse antibiotic resistance genes, a T-DNA with borders from an octopine-type pTi and a second consensus left border sequence that was shown to reduce backbone transfer[15] (Figure 1B). Bacterial synthetic terminators based on different scaffolds (T1 and T2) were included to increase plasmid stability.[5,19]

For cloning purposes, the T-DNA cassette hosts the *E. coli* *lacZα* reporter gene flanked by Type IIS restriction endonuclease sites (Figure 1B). Sequences of BsaI- or BsmBI-produced overhangs agree with proposed syntax; pLX vectors are thus suitable for assembly of single and multiple eukaryotic transcriptional units from standard DNA parts libraries.[8,9] We further designed divergent primer annealing regions with no sequence similarity and secondary structures (arrows, Figure 1B; Table S1). These allow linearization of the small pLX backbones by inverse PCR, and subsequent use in multiple overlapping fragment cloning by Gibson assembly. Golden Gate is a robust system used by many plant scientists;[9] Gibson assembly is very versatile, since it requires no parts domestication steps, but has not been widely adopted for plant construct building.

To test both assembly approaches and the utility of pLX plasmids in plant T-DNA delivery, we generated constructs from diverse material sources, including plasmids, synthetic

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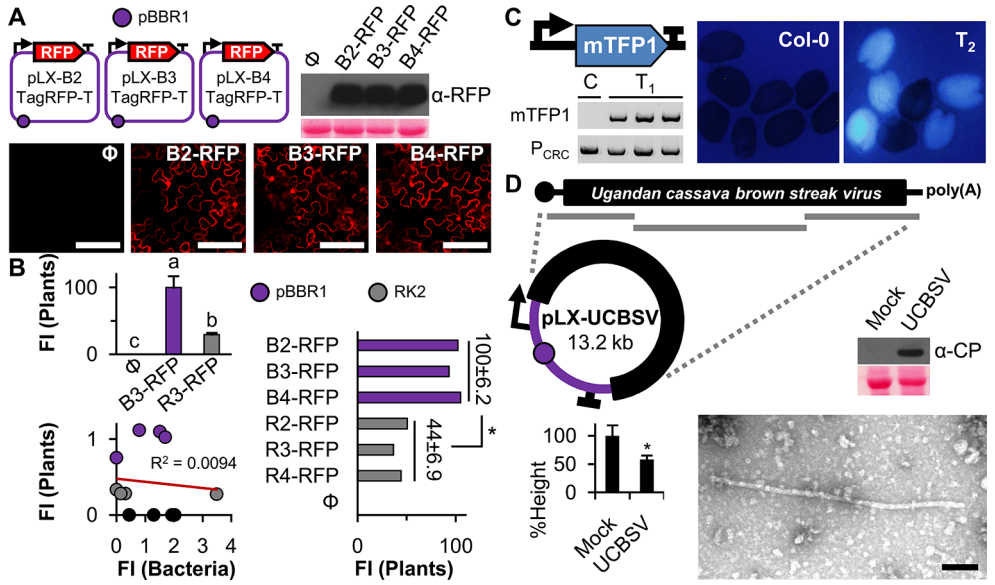

Figure 2. Plant transient and stable transgene expression, and viral agro-inoculation using pLX vector series. (A) The *Cauliflower mosaic virus* (CaMV) 35S promoter-driven TagRFP-T gene (RFP) was inserted into different pLX backbones with pBBR1 origin. Constructs were delivered to *Nicotiana benthamiana* plants by agro-infiltration, and data were collected at 6 days postagro-infiltration (dpa). Cell RFP fluorescence was imaged by confocal microscopy: ϕ, empty control; pLX-B2-TagRFP-T (B2-RFP), pLX-B3-TagRFP-T (B3-RFP) and pLX-B4-TagRFP-T (B4-RFP). Scale bars, 100 μm. RFP accumulation was assessed by immunoblot analysis; Ponceau red-stained blot as loading control. (B) In transient expression assays, RFP vectors from the previous panel (purple) were compared to RK2-based pLX vectors (gray): pLX-R2-TagRFP-T (R2-RFP), pLX-R3-TagRFP-T (R3-RFP) and pLX-R4-TagRFP-T (R4-RFP). RFP fluorescence intensity (FI) of bacterial suspensions and infiltrated plant samples (at 4 or 6 dpa) was measured in a plate reader. Bar graphs show plant FI values, mean ± SD ($n \geq 3$); ϕ, empty control; letters indicate $p < 0.05$, one-way ANOVA and Tukey's HSD test; *$p = 0.00047$, Student's *t*-test. Scatter plot shows linear regression analysis of plant and bacterial FI values; B3-RFP, R3-RFP, and empty control samples are colored in purple, gray and black, respectively. (C) Transgene construct assembled in pLX-B2-P_{CRC}:mTFP1 for stable transformation of *Arabidopsis*. A cyan fluorescent protein gene (mTFP1) is driven by the *A. thaliana* endogenous cruciferin C promoter, which is active in seeds (P_{CRC}). The construct was transformed into plants by floral dipping, and genomic DNA PCR was performed to confirm stable transgene integration, using transgene-specific (mTFP1; 765bp) or control primers (P_{CRC}; 1081bp). Each line represents a single plant sample; C, untransformed plant sample; T1, independent lines selected by cyan fluorescence of seed collected from *Agrobacterium*-treated plants. Fluorescence images are shown of seeds collected from a single T_1 (T_2) or untransformed plants (Col-0). (D) Use of a pLX vector to generate an RNA virus infectious cDNA clone. Three RT-PCR fragments (gray boxes) spanning the entire *Ugandan cassava brown streak virus* (UCBSV) genome were cloned into a linearized pLX vector by Gibson assembly. The pLX-UCBSV vector obtained was delivered to *N. benthamiana* plants by agro-infiltration and data collected at 12 dpa. Relative height of mock- or pLX-UCBSV-infiltrated plants are plotted, mean ± SD ($n = 4$); *$p = 0.0059$, Student's *t*-test. Transmission electron micrograph shows particles observed in infected plant samples; scale bar, 100 nm. Viral accumulation was assessed by anti-UCBSV coat protein (CP) immunoblot analysis of upper noninoculated leaf samples; Ponceau red-stained blot as loading control.

fragments, genomic DNA, and RNA from a plant-infecting virus. Standard transcriptional units were combined by GoldenBraid, a Golden Gate derivative that allows binary assembly of multipartite constructs.[8] Overlapping inserts were joined by Gibson assembly into pLX backbones previously linearized by inverse PCR, DpnI-treated and gel-purified (Figure 1A; Supporting Methods).

A transcription unit bearing a red fluorescent protein[20] (RFP; Figure 2A) as a reporter was assembled in pLX backbones with pBBR1 origin and different antibiotic resistance genes. Transient expression of RFP in *Nicotiana benthamiana* leaves was evaluated by *Agrobacterium*-mediated delivery. At 6 days post agro-infiltration, RFP was detected in pLX samples (B2-RFP, B3-RFP, B4-RFP; Figure 2A) and its subcellular localization matched previous reports.[21] RFP accumulation was confirmed by immunoblot analysis (Figure 2A). Although larger than pBBR1, the RK2 replicon is relatively small and has previously been used to generate autonomous mini binary vectors.[22] We replaced the pBBR1 replication module of pLX vectors by a minimal RK2 origin to build pLX-R2, -R3 and -R4 vectors (Figure 2B; Table S2). Compared to RK2, the use of pBBR1-based pLX vectors led to higher RFP accumulation in plant transient expression assays (Figure 2B). The result was independent of resistance genes used for plasmid selection, and did not correlate significantly with the *Agrobacterium* fluorescence that might derive from undesired RFP accumulation in bacteria (Figure 2B).

We assessed the suitability of pBBR1-based pLX vectors to mediate stable transgene integration into plant genomes. We placed a cyan fluorescent protein[20] (mTFP1) under the control of an *Arabidopsis thaliana* endogenous seed-specific promoter from the cruciferin C gene (AT4G28520; Figure 2C). The construct was transformed into *A. thaliana* plants by floral dipping, and bright fluorescence was detectable in T_1 seeds. Transgene integration was confirmed by PCR analysis of T_1 plants, and inspection of T_2 seed fluorescence (Figure 2C).

Plant viruses are important pathogens and sources of biotechnological tools. Agro-inoculation is the most efficient way of delivering viral vectors,[3] although they can present instability problems.[23] We transferred the whole cDNA sequence of an RNA virus from a previously described clone[24] into a pLX vector (Figure S1). The newly generated pLX-PPV plasmid showed no instability, and initiated viral infections following plant agro-inoculation (Figure S1). To further explore pLX vector capabilities, we focused on the potyvirid *Ugandan cassava brown streak virus* (UCBSV), a major

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threat to the staple food crop cassava.[25−27] We generated the new infectious pLX-UCBSV clone by one-step assembly of three RT-PCR fragments that span the entire 9.1-kb UCBSV genome (Figure 2D). *Nicotiana benthamiana* plants agro-inoculated with pLX-UCBSV showed reduced height. In upper noninoculated leaves, we detected filamentous particles typical of potyvirid virions, and confirmed UCBSV coat protein accumulation in immunoblot analysis (Figure 2D). pLX vectors are thus a means for large transcriptional unit assembly with no intermediate subcloning steps.

We incorporated into pLX vectors the alpha and omega level cloning cassettes from the GoldenBraid system,[8] which relies on BsaI and BsmBI sites for iterative transcriptional unit assemblies (Figure 1A, 3A). Standardized units for plant delivery of kanamycin resistance (NptII) and red fluorescent protein (DsRED) genes[28] were assembled into pCAMBIA- and pLX-derived vectors, with pVS1 and pBBR1 origins, respectively (Figure 3B; Table S2). Compared to pCAMBIA, the pLX backbone significantly enhanced DsRED accumulation in transient expression assay, and showed similar stable transformation efficiency (Figure 3B). Transient expression of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system in leaves allows targeted genome mutagenesis.[29] By agro-infiltration, we delivered pCAMBIA- and pLX-derived constructs that host a Cas gene (hCas9), and single-guide RNA targeting two *N. benthamiana* xylosyltransferase genes (sgRNA XT1 and XT2; Figure 3C). BsmBI and SpeI site loss was predicted to occur in edited XT1 and XT2 loci, respectively. In infiltrated samples, mutagenesis was confirmed by the appearance of cleavage-resistant bands in PCR/digestion assays (Figure 3C). Compared to pCAMBIA and consistent with DsRED transient expression results, the pBBR1-based pLX vector showed greater mutagenesis efficiency (Figure 3C).

pBBR1 shows no incompatibility with known plasmids.[16] We sought to use this feature for multiple T-DNA delivery from independent vectors hosted in the same *Agrobacterium* cell. To facilitate vector multiplexing, we characterized a disarmed octopine-type *Agrobacterium* strain (C58C1−313) that is sensitive to antibiotics commonly used in plasmid selection and stably retains its Ti plasmid (Figure S2A−C). We designed pLX-Z4, a novel T-DNA vector compatible with pBBR1-based pLX plasmids (Figure 1A, S2D). The pLX-Z4 backbone shows minimal sequence similarity with a reference pBBR1-based pLX vector, since it incorporates the RK2 replication origin, lambda phage terminators, and T-DNA border sequences from a succinamopine-type pTi (Figure S2E, Table S1). Sequence analyses predicted that pBBR1-based pLX vectors could be multiplexed with pLX-Z4 and a wide array of binary vectors commonly used by plant scientists (Figure S2F).

An ethanol-responsive buffer gate was built to evaluate the two-pLX vector/one-*Agrobacterium* strain plant transformation strategy (Figure 4A). Gate components were distributed into the gentamicin-selectable pLX-Z4-P_{mas}:RFP-AlcR, which codes for RFP (used as expression control) and *Aspergillus nidulans* AlcR transcription factor[30] under the mannopine synthase promoter (P_{mas}), which directs constitutive expression in plants, and the kanamycin-selectable pLX-B2-P_{EtOH}:NEON, which encodes a bright green fluorescent protein[31] (NEON, output) under P_{EtOH}, a synthetic promoter activated by AlcR in the presence of the inducer (Figure 4B, S3A). Plasmids were introduced sequentially into *Agrobacterium* C58C1−313 strains. Transformed cells that simultaneously harbored pLX-Z4-

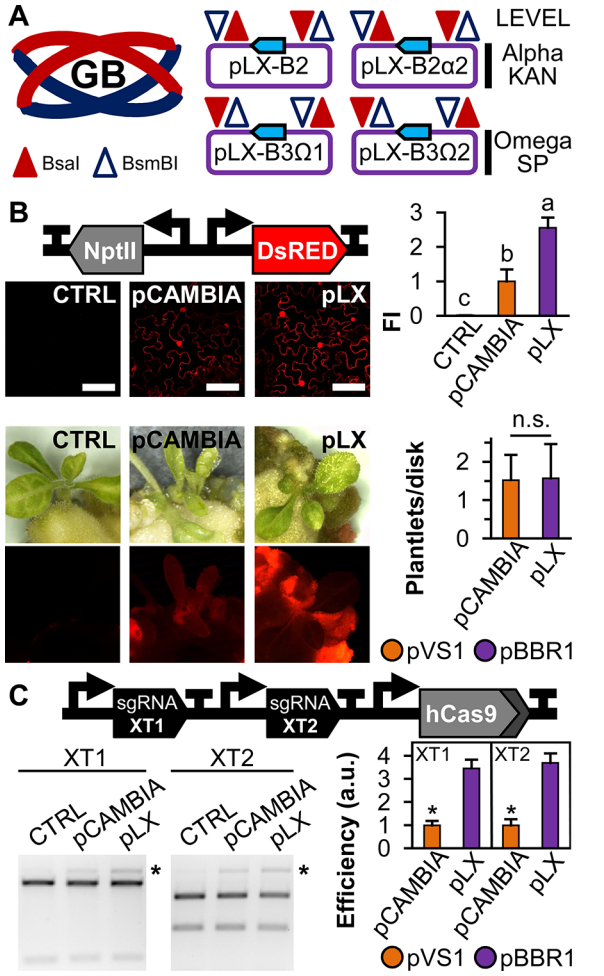

Figure 3. Transcriptional unit assembly into pLX vectors using the GoldenBraid standards. (A) Scheme of pLX vectors that incorporate cloning cassettes compatible with GoldenBraid iterative assembly. Alpha level kanamycin-resistant plasmids have divergent BsaI and convergent BsmBI sites; omega level spectinomycin-resistant plasmids have divergent BsmBI and convergent BsaI sites. All plasmids include the pBBR1 origin and the *lacZα* reporter. (B) pCAMBIA-derived and pLX vectors were used to assemble standardized units for kanamycin resistance (NptII) and red fluorescent protein (DsRED) expression in plants. Constructs were transiently or stably transformed into *N. benthamiana* plants, top and bottom panels, respectively. In agro-infiltrated leaf samples, cell DsRED fluorescence was imaged by confocal microscopy (scale bars, 100 μm) and quantified in a plate reader. FI values were plotted, mean ± SD (n = 4); letters indicate p < 0.05, one-way ANOVA and Tukey's HSD test. In stable transformation assays, leaf disks were cocultured with indicated *Agrobacterium* strains and transferred to kanamycin containing medium. Images show plantlets imaged under an epifluorescence microscope at 40 days post inoculation. Plot shows transformation efficiency defined as number of kanamycin-resistant plantlets that showed DsRED fluorescence per inoculated leaf disk, mean ± SD (n = 7); n.s., p = 0.91. CTRL, control; pCAMBIA, GB1686 vector; pLX, pLX-B2-NptII-DsRED. (C) Targeted mutagenesis using a GoldenBraid-based CRISPR/Cas system in transient expression assays. *Nicotiana benthamiana* plants were infiltrated with pCAMBIA- and pLX-derived plasmids that bear transcriptional units for human codon-optimized *Streptococcus pyogenes* Cas9 (hCas9), and sgRNA targeting the endogenous Niben101Scf04205Ctg025 (XT1) and Niben101Scf04551Ctg021 (XT2) genes. Gels show PCR/digestion assays; asterisks mark cleavage-resistant DNA bands. Mutagenesis efficiency was estimated by quantifying ratio of uncleaved/cleaved bands and plotted, mean ± SD (n = 4); *p < 0.001. CTRL, hCas9

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Figure 3. continued

delivered with no sgRNA sequences; pCAMBIA, GB1108 vector; pLX, pLX-B2-XT1-XT2-hCas9. Vector origins are indicated: pVS1, orange; pBBR1, purple.

P_{mas}:RFP-AlcR and pLX-B2-P_{EtOH}:NEON (RFP-AlcR + P_{EtOH}:NEON) showed resistance to gentamicin plus kanamycin (green, Figure 4C). The RFP-AlcR + P_{EtOH}:NEON strain was infiltrated into *N. benthamiana* leaves, and plants were treated with water or ethanol. As anticipated, while RFP fluorescence was visible in both conditions, NEON fluorescence was significantly higher in the presence of the gate inducer (Figure S3B). Circuit modeling requires quantitative characterization of genetic parts.[5,6] To test whether the two-vector/one-strain expression approach is compatible with medium-throughput analyses, we collected leaf disks from RFP-ALCR + P_{EtOH}:NEON-infiltrated leaves, placed them in 96-well plates and evaluated gate responses 24 h post-treatment (hpt). Gate function was maintained in leaf disks, since NEON fluorescence was detected only in the presence of gate input (Figure 4D). Output fluorescence intensity quantification in intact leaf disks showed appropriate gate responsiveness and sensitivity, since 0.1% ethanol was sufficient to trigger >200-fold induction (Figure 4E). NEON detection requires no lysis or substrate addition step, which allowed us to measure gate kinetics in a continuous-read assay. In the conditions tested, the NEON/RFP fluorescence intensity ratio was significantly higher than the water control at 1.5 hpt and reached a plateau at 15 hpt (Figure 4F).

The combination of the results highlights the flexibility of our vectors, which can be used (i) to assemble standard DNA parts by Golden Gate and GoldenBraid cloning, (ii) to join fragments by Gibson assembly when removal of internal restriction sites and parts domestication is unfeasible or not desirable (*e.g.*, construction of viral clones), and (iii) to deliver multi T-DNA cassettes by multiplexing vectors with compatible origins.

CONCLUSION

Production of "smart" plants that satisfy ever-growing and rapidly changing human needs requires advanced biotechnology tools and synthetic biology solutions. Multigene delivery to plant allows multitrait engineering by simultaneous heterologous transgene integration, edition of genome loci, or a combination of both.[1,11−13,32,33]

We show that novel pBBR1-based and RK2-based binary T-DNA vectors can be coupled to allow multi T-DNA delivery from *Agrobacterium* in a two-plasmid/one-strain approach. *Rhizobiaceae* can host multiple plasmids,[34,35] and we predict that use of alternative compatible replication origins will further expand this multigene delivery design to an "*N*-plasmid/one-strain" strategy. This strategy can be combined by coinfection with multiple *Agrobacterium* strains, to further increase the number of genes delivered.

Designed vectors follow recommended Type IIS genetic syntax,[9] which facilitates assembly of available DNA parts libraries. As an alternative, we applied overlap-dependent methods[10] for gene cloning. Use of modular overlap sequences was proposed,[36,37] and these vectors might ease adoption of Gibson assembly standards by the plant science community.

Rapid prototyping of DNA parts is needed to scale up synthetic pathway and circuit engineering.[4] We provide

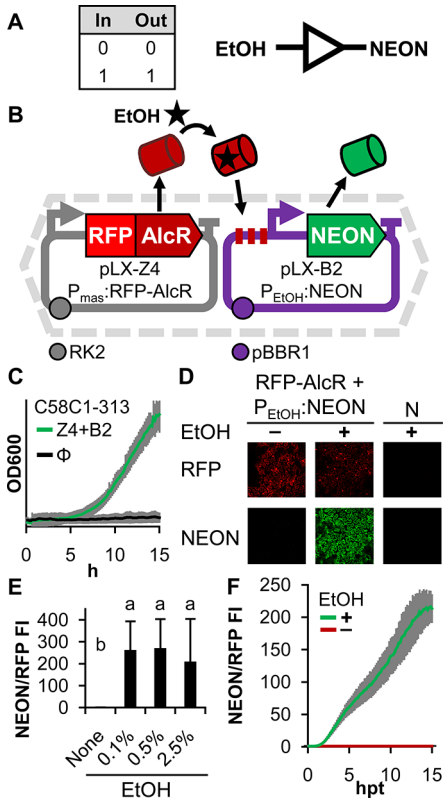

Figure 4. Use of the two-pLX vector/one-*Agrobacterium* strain strategy for multiple T-DNA delivery, and gate expression in plants. (A) Buffer gate truth table. Symbol of a buffer gate that uses ethanol (EtOH) as input and mNeonGreen (NEON) fluorescence as output. (B) Genetic circuit that implements the previous panel gate. The dashed hexagon represents a single *Agrobacterium* strain (RFP-AlcR + P_{EtOH}:NEON) that hosts two compatible binary T-DNA vectors, pLX-Z4-P_{mas}:RFP-AlcR and pLX-B2-P_{EtOH}:NEON, which confer gentamicin and kanamycin resistance, respectively. Once delivered to plants, the constitutive mannopine synthase promoter (P_{mas}) drives expression of RFP and AlcR. In the presence of EtOH (star), AlcR binds to, and activates an otherwise silent synthetic promoter (P_{EtOH}). NEON accumulation results from activation of the gate. (C) Growth curves of *Agrobacterium* C58C1−313 that harbors no binary T-DNA vectors (black, ϕ), or the RFP-AlcR + P_{EtOH}:NEON strain (green, Z4+B2). Gentamicin- and kanamycin-supplemented medium was inoculated with the indicated strains, and absorbance measured in a plate reader. The plot shows mean ± SD (n = 6); h, hours. (D) *Nicotiana benthamiana* leaves were untreated (N), or infiltrated with the *Agrobacterium* RFP-AlcR + P_{EtOH}:NEON strain. Leaf disks were collected, placed in a 96-well plate and supplied with or without EtOH. Cell RFP and NEON fluorescence was imaged by confocal microscopy at 24 h post-treatment (hpt). (E) Leaf disks from agro-infiltrated patches were placed in a 96-well plate and different amounts of inducer were added. Fluorescence intensities (FI) were measured in a plate reader at 22 hpt, and relative NEON/RFP FI value of the noninducer condition (None) was set to 1. Bar graph shows mean ± SD (n = 18). Letters indicate $p < 0.01$, one-way ANOVA and Tukey's HSD test. (F) Kinetics of the EtOH-responsive buffer gate. Leaf disks from agro-infiltrated patches were treated with water (red, minus) or 0.1% EtOH (green, plus), and fluorescence intensity measured in a plate reader. Relative NEON/RFP FI value of the water condition was set to 1. The plot shows mean ± SD (n = 5).

evidence that choice of binary vector origins can modulate transient expression outputs in plants. pBBR1 has higher copy number than RK2 and pVS1 origins,[16] used in pBIN and pCAMBIA series, respectively. Our results suggest that in

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Agrobacterium, use of pBBR1-based pLX vectors increases the number of T-DNA cassettes available for delivery to plants and transient expression. In turn, stable T-DNA integration into the host genome appears to be restrained by limiting factors that are independent of the vectors tested.[38]

We studied a simple buffer gate on a medium-throughput scale using a method that employs plant transient expression of fluorescent proteins *via* agro-infiltration, intact leaf disk analyses, and fluorescence plate readers. Quantitative protoplast-based methods were developed;[6] due to their small sizes, pLX plasmids are also likely to be suitable for direct delivery in transfection assays, and to help in part/circuit characterization.

In summary, we present binary T-DNA vectors based on compatible broad-host replication origins as a new tool for plant synthetic biology as well as a flexible framework for multigene transfer and DNA parts characterization.

MATERIALS AND METHODS

Plant Transformation and Agro-inoculation. DNA constructs were generated using chemically synthesized and available parts.[19,20,24,28,39,40] A UCBSV isolate was obtained from DSMZ (PV-0912). *Escherichia coli* DH10B strain was used for cloning and plasmid propagation. Binary T-DNA vectors were transformed into *Agrobacterium* cells by freeze−thawing or electroporation, and delivered to *N. benthamiana* and *A. thaliana* Col-0 plants as described.[20,41,42]

Protein Detection. Plant samples that express fluorescent proteins were visualized under an epifluorescence stereoscope, confocal microscope, or imaged in a laser scanner.[20] Fluorescence was measured by placing leaf disks in 96-well flat-bottom plates; in kinetics studies, plates were sealed with optical adhesive films (4311971, Applied Biosystems). The signal was acquired in a monochromator-based plate reader (Infinite M200, Tecan), as reported.[20] Total protein extracts were resolved by SDS-PAGE, and immunodetection was done using rabbit anti-tRFP (AB234, Evrogen), -UCBSV CP (AS-0912, DSMZ), and -PPV CP sera as primary antibodies. For electron microscopy, plant extracts were incubated with collodion-coated carbon-stabilized copper grids, and negative-stained with 2% uranyl acetate. Grids were observed in a transmission electron microscope (JEM 1011, Jeol).

Targeted Genome Mutagenesis. Transient expression of CRISPR/Cas constructs, and PCR/restriction enzyme assays were done as described.[40] Intensities of cleaved and cleavage-resistant bands were quantified using the ImageJ software (https://imagej.nih.gov/ij/).

Detailed methods are found in Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00354.

Stability, and usage of pLX-PPV; characterization of the *Agrobacterium* strain C58C1−313, the RK2-based pLX-Z4 vector and vector sequence analyses; sequence and *in planta* induction of the ethanol-responsive synthetic promoter used in the study (Figure S1−S3); detailed methods, lists of used synthetic T-DNA cassettes, plasmids and primers (Table S1−S3); supplementary references (PDF)

Data that were used in statistical analyses and to generate figure plots (XLS)

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Author Contributions

FP conceived and designed the experiments. FP, LCB, AG performed the experiments. FP, LCB, AG, JAG analyzed the data. FP, LCB, CSM, JAG contributed reagents/materials/analysis tools. DO and JMB-O designed and performed GoldenBraid-based experiments. FP wrote the paper. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

The *Ugandan cassava brown streak virus* genome and plasmid sequence information has been deposited at GenBank under accession numbers KY825166 and KY825137−KY825159, respectively.

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