GENE ASSEMBLY IN AGROBACTERIUM VIA NUCLEIC ACID TRANSFER USING RECOMBINASE TECHNOLOGY (GAANTRY)

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Abstract Plant biotechnology provides a means for the rapid genetic improvement of crops including the enhance ment of complex traits like yield and nutritional quality through the introduction and coordinated expression of multiple genes. GAANTRY (geneassembly in Agrobacterium bynucleic acidtransfer using recombinase technology) is a flexible and effective system for stably stacking multiple genes within an Agrobacterium virulence plasmid transfer DNA (T-DNA) region. The system provides a simple andefficient method for assembling and stably maintaining large stacked constructs within the GAANTRY ArPORT1 Agrobacterium rhizogenes strain. The assembly process utilizes unidirectional site-specific recombinases in vivo and an alternating bacterial selection scheme to sequentially assemble multiple genes into a single transformation construct. A detailed description of the procedures used for bacterial transformation, selection, counter selection, and genomic PCR validation with the GAANTRY system are presented. The methods described facilitate the efficient assembly and validation of large GAANTRY T-DNA constructs. This powerful, yet simple to use, technology will be a convenient tool for transgene stacking and plant genetic engineering of rice and other crop plants.

Key words Plant biotechnology, Transformation, Agrobacterium rhizogenes, Transfer DNA (T-DNA), Transgene assembly, Site-specific recombination, Gene stacking, Virulence plasmid, Genetic engineering

Introduction Plant research has utilized Agrobacterium-mediated plant transfor mation as a means of plant genetic engineering for more than 30 years. In most

instances one or a few genes are introduced into the plant genome, but recently the assembly of large transfor mation constructs that carry multiple genes has been desired. Unfortunately, it is typically challenging to construct and efficiently transform plants with large constructs carrying five or more genes. Since the in vitro manipulation of large constructs can be difficult and/or inefficient to perform using traditional cloning techniques, a variety of alternative approaches have been developed for stacking multiple genes together. Strategies employing rare-cutting homing endonucleases, commercial cloning systems (i.e., multisite Gate way), Gibson assembly, type IIS restriction enzymes, homologous recombination in yeast, the Cre site-specific recombinase, as well as combinations of these methods have been developed. Although these efforts have been shown to successfully pro duce large and complex constructs, these systems all utilize either a binary vector plasmid or a binary bacterial artificial chromosome plasmid vector as the transformation construct and have frequently exhibited inefficient assembly processes and/or instability problems in E. coli or Agrobacterium. Also, relatively few of these approaches have been shown todevelop large stacked constructs that efficiently generate low copy, high-quality stable transgenic plants with all of the expected functional phenotypes. Recently, we designed and constructed the gene assembly in Agrobacterium via nucleic acid transfer using recombinase technol ogy (GAANTRY) system and demonstrated its capacity to sequen tially stacking ten cargo sequences within the virulence plasmid T-DNA of the Agrobacterium rhizogenes strain ArPORT1 (Fig. 1) [14]. The GAANTRY assembly process utilizes three unidirec tional site-specific recombinases in vivo and an alternating bacterial selection scheme to iteratively build multiple genes into a transfer DNA (T-DNA). Below we provide a detailed description for a simplified protocol for GAANTRY assembly and strain validation. This powerful, yet simple to use, transgene stacking technology will be a valuable tool for plant genetic engineering of rice and other crop plants.

The GAANTRY system requires only a recipient Agrobacterium strain and four plasmid vectors for construct assembly [14]. 1. The ArPORT1 bacterial strain is a

kanamycin-resistant Agro bacterium rhizogenes GAANTRY recipient strain. It is a dis armed strain (where the native T-DNA has been removed from the pRi virulence plasmid) and is recA making it deficient in homologous recombination. The recA gene was inactivated using a tetracycline resistance marker and homologous recom bination. Although the strain is resistant to 5 mg/L tetracy cline, the antibiotic is not typically used for selection. The strain contains a 320 base pair (bp) sequence of the left border region of Agrobacterium tumefaciens strain C58 (including the 25 bp LB direct repeat), the 56 bp A118 attP recognition site, the nptIII gene conferring bacterial kanamycin resistance, and the 106 bp ParA single multimer resolution site (MRS) in place of the native T-DNA.

TheBandP “Donor”vectors carry attB (or attP) recom binase recognition sites respectively. In short, the A118 and TP901-1 recombinase recognition sites flank a clon ing region in each plasmid where cargo sequence(s) of interest can be inserted. The cloning region includes a large number of unique restriction recognition sites for traditional restriction enzyme-based cloning of sequences of interest. The donor plasmids also contain a kanamycin or gentamicin bacterial resistance marker, respectively, as well as the sacB negative selection marker (conferring sucrose sensitivity) and a ParA MRSrecognition sequence. Diagrams of the B and P Donor plasmids are shown in Fig. 2. The complete annotated plasmid sequences for the B Donor (MG687272) and P Donor (MG687284) plas mids are available from GenBank. Note, Donor vectors that enable Gateway or Golden Gate cloning of cargo sequences are also available [14]. (b) The B and P “Helper” plasmids confer ampicillin resis tance in E. coli and carry an operon expressing either the A118 and ParA or the TP901-1 and ParA recombinase enzymes, respectively. The annotated plasmid sequences for the BandPHelper vectorsareavailable fromGenBank (MG687274, MG687275).

2.2 Growth Medium, Culture Conditions, and Equipment

1. Low-salt Luria-Bertani (LB) medium: 10 g/L Bacto Tryptone, 5 g/L yeast extract, and 5 g/L NaCl. Adjust pH to 7.5 with sodium hydroxide (NaOH) and sterilize by autoclaving. For LB solid medium add 10 g of Bacto agar prior to autoclaving.

Supplement LB medium with appropriate antibiotics for plas mid selection in E. coli and strain selection in Agrobacterium (see item 3).

2. LB solid medium with 5% (w/v) sucrose: LB medium (as described above) with 50 g/L sucrose added and the appropriate antibiotic.

3. Antibiotics gentamicin, kanamycin, and carbenicillin. For plas mid selection in E. coli, 10 mg/L gentamicin is used, while 100 mg/L gentamicin is for selecting GAANTRYAgrobacter ium ArPORT1 strains. For both E. coli and Agrobacterium, 50 mg/L kanamycin is used for selection. Carbenicillin at 100 mg/L is the concentration used for selecting the helper plasmids in E. coli.

4. Incubator/shaker at 30 and 37 C.

5. Eppendorf ThermoMixer.

6. Microcentrifuge.

7. Electroporation cuvettes (2 mm gap).

8. Gene Pulser electroporation apparatus.

9. PCR thermocycler.

10. Agarose gel electrophoresis apparatus

Preparation of Donor and Helper Plasmids

1. To insert cargo DNA sequences within the Donor plasmids, manipulate P and B Donor vectors using standard cloning and E. coli microbiological techniques.

2. Toprepare the plasmids, streak desired clones for single colony selection from a 80 C glycerol stock onto LB plates with the appropriate antibiotic selection.

3. Transfer a single colony to 5 ml of LB liquid broth with appropriate selection in a 15 ml snap-cap tube and incubate overnight at 37 C under continuous shaking (250 rpm).

4. Harvest the cells by centrifugation at 10,000 g and follow a protocol of choice for mini plasmid preparation (e.g., the Zymo Research ZR Plasmid Miniprep protocol or a similar product from other vendors) (see Note 1). Prior to use, dilute the Donor and Helper plasmids in water to a 50 ng/μl concentration.

Preparation of the Electrocompetent ArPORT1 Recipient Strain

To prepare the electrocompetent cells, streak GAANTRY A. rhizogenes ArPORT1 recipient strain on LB medium to produce individual isolated colonies. Antibiotic selection will dependonthedesired recipient strain. For the original strain or strains carrying two or other even numbered stacks of cargo, the antibiotic selection will be 50 mg/L kanamycin. Alterna tively, if the desired strain contains odd numbered stacks of cargo, the appropriate selection will be 100 mg/L gentamicin. 2. Transfer a single colony to 5 ml LB medium with suitable selection in a 15 ml snap-cap tube and incubate at 30 C under continuous shaking (250 rpm) for 16–18 h (OD600 > 1.5). 3. Dilute the overnight culture by adding 150 μl of culture to 1.35 ml of nonselective LB medium in a 1.5 ml microcentri fuge tube. Make four tubes per transformation. 4. Place tubes in an Eppendorf ThermoMixer at 30 C and 900 rpm for 3–4 h until the density reaches OD600 ¼ 0.4–0.6 (see Note 3). 5. Whenthedesired optical density is reached, centrifuge the cells at 1000 gfor 5 min at room temperature. 6. Discard the supernatant and gently resuspend the cells in 100 μl of sterile water. Combine the four tubes. The cultures are kept at room temperature and all the procedures are per formed at room temperature (~22–24 C) from this point on. 7. Add 1 ml of sterile water to the combined cell suspension. 8. Centrifuge at 1000 g for 5 min, and then pour off the supernatant. 9. Wash the cells one more time by repeating steps 7 and 8. 10. Resuspend cells in 50 μl of sterile water and use for immediate electroporation.

It is important to use freshly prepared plasmid DNA, especially when the cargo size is >10 kilobase pairs, since large Donor constructs recombine less efficiently. Transformation efficiency is also significantly reduced when old or poor-quality plasmid samples containing predominantly non-supercoiled plasmid DNAare used. 2. Multiple different methods for the generation of electrocom petent cells can successfully be used with GAANTRYassembly; howeverit is recommendedthat theroomtemperaturemethod described by Tu and colleagues (see Subheading 3.2) be used for Donor plasmid constructs that are greater than 15 kilobases in size [16]. If electrocompetent cells are being premade for archiving at 80 C and later use with modestly sized donor vectors, then the method described by Collier and colleagues is

preferred. 3. The best transformation efficiency occurs when cells in log phase growth (OD600 ¼ 0.4–0.6 which typically takes 3–4 h of growth at 30 C after starting from a saturated culture) are used. The transformation efficiency drops if the bacterial cells grow beyond an OD600 ¼ 0.6.

For optimal results, the cells should be used immediately for electroporation. Tu and colleagues reported a 30% loss in transformation efficiency when the cells were stored at room temperature for 1 h, ~60% loss after 4 h, and ~80% loss after 24 h [16]. 5. Proper transformation occurs when no arcing within the elec troporation cuvette is observed and a time constant of 4–5 ms is obtained. If the sample arcs, there is likely too much salt in the bacterial mixture or the plasmid DNA. To avoid this prob lem, use water to elute the plasmid DNA from the purification column (not the elution buffer provided in the plasmid isola tion kit). If arcing persists, and the DNA was eluted in water, it is possible that the competent cells were not washed thor oughly enough; therefore, add an additional washing step (see Subheading 3.2, steps 7 and 8) to remove residual salts. Sam ples that arc within the electroporation cuvette typically will not produce colonies on the selection plate. 6. Plating the cells directly on medium with 5% w/v sucrose after transformation (skipping the initial selection on medium with out sucrose) is not recommended. This will significantly decrease the number of colonies recovered and can lead to the recovery of clones that have not undergone complete GAANTRY-mediated integration and excision.7.TheGAANTRYstackingsystemrelies on the iterative toggling between kanamycin and gentamicin resistance to insert addi tional sequences of interest; therefore, if a cargo sequence that expresses nptII in Agrobacterium is inserted into the T-DNA, it will block the ability to effectively use kanamycin selection for the insertion of additional cargo sequences. For example, inserting a CaMV35S promoter-nptII-nos terminator cargo sequence into ArPORT1 will confer kanamycin resistance, making the strain constitutively resistant to kanamycin. This will block the ability to use this strain for the insertion of additional cargo sequences carried in a P Donor plasmid. This phenomenon will be detected in the normal screening process by recovering colonies that are sucrose insensitive, but

perpet ually retain resistance to kanamycin. There are at least three ways to avoid this problem: (1) use promoter sequences on nptII cargo that do not express in Agrobacterium; (2) place a plant intron within the nptII coding sequence, blocking bacte rial translation of a functional kanamycin resistance protein; or (3) stack the nptII-containing cargo sequence into the gene assembly last (when no more additional cargo needs to be added). Note, a similar problem will exist if a cargo sequence conferring bacterial resistance to gentamicin is added to the ArPORT1 T-DNA.

Sample a colony using a 20 μl micropipette tip held vertically at a90 angle relative to the petri plate. Touch the colony lightly to ensure that only a small numberofcells are removed. Picking up too many bacteria can inhibit the PCR reaction or cause nonspecific amplification products to appear. 9. To allow the use of a single thermocycling program for screen ing with multiple PCR reactions, it is worthwhile to design primers with similar annealing temperatures and to make the amplicon sizes for each junction a unique size between 300 and 2000 base pairs. Choosing the size of the amplicon such that it is unique to each of the newly formed junctions enables the size of each amplicon to be diagnostic for a particular GAANTRY assembled sequence during agarose gel electrophoresis. In addition, it is also useful to design a pair of primers that can be used as a positive control for ArPORT1 strain genomic DNA. This primer pair can be used as a positive control PCR reaction from any ArPORT1 DNA sample. Since this simple genomic isolation procedure can fail by taking too many or too few bacterial cells (see Note 8), it is helpful to include a positive control PCR amplification reaction to validate that each geno mic DNA sample is of sufficient quantity and quality for amplification. 10. The PCR extension time can vary depending on the type of polymerase that is used and the size of the expected amplicon. Typically, 1 min of extension at 68 C is used per kilobase of amplicon for Taq polymerase but follow the polymerase man ufacturer’s instructions when designing the thermocycler amplification program.

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