



SacB-SacR Gene Cassette As the Negative Selection Marker to Suppress *Agrobacterium* Overgrowth in *Agrobacterium*-Mediated Plant Transformation

Yiming Liu^{1,2}, Jiamin Miao³, Sy Traore³, Danyu Kong³, Yi Liu³, Xunzhong Zhang¹, Zachary L. Nimchuk⁴, Zongrang Liu⁵ and Bingyu Zhao^{3*}

¹ Department of Crop and Soil Environmental Science, Virginia Tech, Blacksburg, VA, USA, ² Chinese Academy of Tropical Agricultural Sciences/Key Laboratory of Crop Gene Resources and Germplasm Enhancement in Southern China, Tropical Crops Genetic Resources Institute, Ministry of Agriculture, Danzhou, China, ³ Department of Horticulture, Virginia Tech, Blacksburg, VA, USA, ⁴ Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, ⁵ USDA-ARS-Appalachian Fruit Research Station, Kearneysville, WV, USA

OPEN ACCESS

Edited by:

Artur Burzyński,
Polish Academy of Sciences, Poland

Reviewed by:

Benoit Lacroix,
Stony Brook University, USA
Laura R. Jarboe,
Iowa State University, USA

*Correspondence:

Bingyu Zhao
bzha007@vt.edu

Specialty section:

This article was submitted to
Biomolecular Engineering,
a section of the journal
Frontiers in Molecular Biosciences

Received: 30 June 2016

Accepted: 07 October 2016

Published: 26 October 2016

Citation:

Liu Y, Miao J, Traore S, Kong D, Liu Y, Zhang X, Nimchuk ZL, Liu Z and Zhao B (2016) SacB-SacR Gene Cassette As the Negative Selection Marker to Suppress *Agrobacterium* Overgrowth in *Agrobacterium*-Mediated Plant Transformation. *Front. Mol. Biosci.* 3:70. doi: 10.3389/fmolb.2016.00070

Agrobacterium overgrowth is a common problem in *Agrobacterium*-mediated plant transformation. To suppress the *Agrobacterium* overgrowth, various antibiotics have been used during plant tissue culture steps. The antibiotics are expensive and may adversely affect plant cell differentiation and reduce plant transformation efficiency. The SacB-SacR proteins are toxic to most *Agrobacterium tumefaciens* strains when they are grown on culture medium supplemented with sucrose. Therefore, SacB-SacR genes can be used as negative selection markers to suppress the overgrowth of *A. tumefaciens* in the plant tissue culture process. We generated a mutant *A. tumefaciens* strain GV2260 (*recA-SacB/R*) that has the SacB-SacR cassette inserted into the bacterial genome at the *recA* gene locus. The mutant *Agrobacterium* strain is sensitive to sucrose but maintains its ability to transform plant cells in both transient and stable transformation assays. We demonstrated that the mutant strain GV2260 (*recA-SacB/R*) can be inhibited by sucrose that reduces the overgrowth of *Agrobacterium* and therefore improves the plant transformation efficiency. We employed GV2260 (*recA-SacB/R*) to generate stable transgenic *N. benthamiana* plants expressing a CRISPR-Cas9 for knocking out a WRKY transcription factor.

Keywords: plant transformation, *Agrobacterium* overgrowth, CRISPR-Cas9, *Nicotiana benthamiana*, SacB-SacR gene cassette

INTRODUCTION

Agrobacterium-mediated genetic transformation is one of the most popular techniques used for the generation of transgenic plants (Gelvin, 2000; Tzfira and Citovsky, 2006). Efficient *Agrobacterium*-mediated transformation protocols have been developed for various plant species (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Zhao et al., 2000). In general, the *Agrobacterium*-mediated transformation involves the generation of a sterile

explant that can be co-cultured with *Agrobacterium*. Subsequently, the infected *Agrobacterium* cells are eliminated or suppressed by using various antibiotics, and the transgenic plant cells are selected by using antibiotics or other chemicals (Jones et al., 2005; Tsuda et al., 2012). During the *Agrobacterium*-mediated transformation process, one major problem is the overgrowth of *Agrobacterium* that could significantly reduce the plant transformation efficiency. To eliminate or inhibit the *Agrobacterium* overgrowth, different antibiotics such as carbenicillin, TimentinTM, Augement, Clavamox, and Cefotaxime are used during the plant tissue culture selection steps (Bhau and Wakhlu, 2001; Tereso et al., 2006; Zang et al., 2009; Li and Qu, 2011; Ren et al., 2012). For example, carbenicillin is a semi-synthetic penicillin antibiotic that interferes with cell wall mucopeptide biosynthesis of gram-negative bacteria (Silva and Fukai, 2001). Under selection pressure, *Agrobacterium* cells could gain mutations that are resistant to the carbenicillin, which results in the overgrowth of *Agrobacterium*. Most antibiotics are generally expensive and may negatively affect plant cell differentiation (Ellis et al., 1989; Yu et al., 2001; Li and Qu, 2011). Therefore, a more reliable and cost-effective method to inhibit the overgrowth of *Agrobacterium tumefaciens* is highly desirable.

The *SacB-SacR* genes were originally isolated from *Bacillus subtilis* and encode levansucrase, an enzyme involved in both the hydrolysis of sucrose and the biosynthesis of levan (Chambert and Petitglatron, 1989; Quandt and Hynes, 1993; Traore and Zhao, 2011). Levan cannot be metabolized by most gram-negative bacteria including *A. tumefaciens* and is therefore toxic to this group of organisms (Gay et al., 1985; Schweizer, 1992). The *SacB-SacR* gene cassette, driven by its native promoter, has been used as a negative selectable marker for many gram-negative bacteria, and works by preventing the transformed bacterial cells from growing on culture medium supplemented with sucrose (Ried and Collmer, 1987). Our previous work demonstrated that the *SacB-SacR* genes can be used as negative selection markers to inhibit the growth of *Agrobacterium* strain GV2260 on Luria Broth agar medium supplemented with 5% sucrose (Traore and Zhao, 2011). Sucrose has been frequently used as the carbon source in synthetic plant tissue culture medium (Yaseen et al., 2013), although other sugars such as maltose, fructose, and sorbitol have also been used. It is interesting to test if growth of an *Agrobacterium* strain carrying the *SacB-SacR* gene cassette can be inhibited on plant tissue medium supplemented with sucrose.

The *recA* gene was originally identified as a conserved gene involved in homologous DNA recombination in various bacterial species (Clark and Margulies, 1965; Brendel et al., 1997; Song et al., 2003). *RecA*-dependent recombination was initially identified through analysis of conjugational recombination (Clark and Margulies, 1965; Bi and Liu, 1994), where *recA* can promote homologous pairing of DNA molecules and catalyzes the strand exchange reaction leading to the formation of hetero-duplex DNA *in vitro* (West, 1992; Bi and Liu, 1994). Deletion of the *recA* gene in the bacterial genome can reduce the rate of homologous recombination, and therefore increase plasmid DNA stability. Deletion of the *recA* gene in *E. coli*

has no obvious deleterious effect on bacterial growth (Kurnit, 1989; Lovett et al., 1993). Several *Agrobacterium* strains with deletion of the *recA* gene were also developed (Farrand et al., 1989).

In this study, we attempted to integrate the *SacB-SacR* gene cassette at the *recA* gene locus in the genome of *A. tumefaciens* strain GV2260. The derived mutant strain GV2260 (*recA-SacB/R*) was used to transform *Nicotiana benthamiana* (*N. benthamiana*) plant cells in both transient assays and stable transformation. We demonstrated that the mutant strain GV2260 (*recA-SacB/R*) maintains its capacity of transforming plant cells, and its growth can be efficiently inhibited by regular tobacco tissue culture medium supplemented with 3% sucrose. Stable transgenic plants carrying a CRISPR-*Cas9* construct for knocking out a WRKY transcription factor were successfully recovered after transformation with the mutant *Agrobacterium* strain. Therefore, the mutant *A. tumefaciens* strain should have great value for large scale, high-throughput plant transformation applications in the future.

MATERIALS AND METHODS

Bacteria Strains

Escherichia coli (*E. coli*) DH5 α [F⁻endA glnV44 thi-1 *recA1* relA1 *gyrA96* *deoR* nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, *hsdR17*(r_K⁻m_K⁺), λ^-], *A. tumefaciens* (GV2260) [C58 background, rifampicin-resistant with the Ti plasmid (pTiB6s3)], and *Escherichia coli* (*E. coli*) helper P600 (Traore and Zhao, 2011).

Plant Materials

N. benthamiana (PI 555478) plants were propagated in a growth chamber programmed for 16 h light (140 μ mol m⁻²s⁻¹ cool white fluorescent irradiance) at 28°C and 8 h dark at 24°C. *Agrobacterium*-mediated transient assays were conducted on three- to 4-week-old plants.

Cloning of the *Agrobacterium recA* Gene Fragment

A 780 bp DNA fragment with deletion of the N and C-terminus of the *recA* gene was amplified from the genomic DNA of GV2260 using primers: *recA* For, 5'-caccatcgatcatgaagctcgg-3' and *recA* Rev, 5'-gcccggacttctcagcat-3'. The PCR reaction as performed using the iProofTM high fidelity Taq DNA polymerase (Bio-Rad, Hercules, CA). The PCR program consisted of 1 cycle at 98°C (2 min), followed by 30 cycles at 98°C (30 s), 55°C (45 s), and 72°C (1 min), and finished with a 1 cycle extension at 72°C (7 min). The PCR product was separated on a 0.8% agarose gel, stained with 0.01% ethidium bromide solution, and visualized using the Gel-Document Image SystemTM under UV light (Bio-Rad).

The PCR product was purified using the AccuPrepTM Gel Purification Kit (Bioneer, Alameda, CA) and cloned into the TopoEntr/DTM vector (Invitrogen, Carlsbad, CA) following the instructions of the user manual. The derived plasmid vector was designated as TopoEntr-*recA* and has been sequenced at the core facility of the Virginia Bioinformatics Institute (Blacksburg, VA).

Development of an Integrational Construct Carrying *recA* Fragment and the *SacB-SacR* Gene Cassette

The suicide vector pLVC18L (Zhao et al., 2011) was modified by insertion of the *SacB/R* and the *ccdB* gene cassettes. The *Npt2* promoter-*SacB/R* fragment was amplified through overlap PCR from pEG101-*SacB/R* and pDSK519-GFP (Matthysse et al., 1996; Traore and Zhao, 2011) using primers: 1846pLvc18 XbaNpt2 Infusion For1, 5'-TGCCATTGCTGC AGGTCGACTCTAGAGATATCACATGGCGATAGCTAG ACTG-3'; 1777Npt2Pro_ *SacB/R* Rv Rev1, 5'-GTGATGGGT TAAAAAGGATCGATCCGCGCCATCAGATCCTTG-3'; 1778Npt2Pro_ *SacB/R* Rv For2, 5'-CAAGGATCTGATGGCGCG GATCGATCCTTTTTAACCCATCAC-3'; 1847pLvc18 XbaSacB Infusion Rev2, 5'-CTCGGTACCCGGGGATCCTCTAGAGAT ATCTTATTTGTTAACTGTTAATTGTCCT-3'.

The PCR product was cloned into the *XhoI* site of pLVC18L using a Gibson cloning kit (New England BioLabs Inc., Ipswich, MA). The derived construct was designated as pLVC18L-Npt2-*SacB/R*. A *ccdB* gene cassette (frame B) (Invitrogen) was further cloned into the *SmaI* site of pLVC18L-Npt2-*SacB/R* to generate pLVC18L-Npt2-*SacB/R*-DesB. The *recA* gene fragment from TopoEntr-*recA* was subcloned into pLVC18L-Npt2-*SacB/R*-DesB using a Gateway[®] LR cloning kit (Invitrogen) following the instructions of the user manual. The derived plasmid construct was named pLVC18L-Npt2-*SacB/R*-*recA*, and has been confirmed by sequencing at the core facility of the Virginia Bioinformatics Institute (Blacksburg, VA).

Integration of the pLVC18L-Npt2-*SacB/R*-*recA* Construct into the Genome of *Agrobacterium tumefaciens* Strain GV2260

The suicide vector pLVC18L-Npt2-*SacB/R*-*recA* was integrated to the genome of *A. tumefaciens* strain GV2260 by tri-parental conjugation and was selected on LB medium supplemented with tetracycline (10 µg/mL) as previously described (Traore and Zhao, 2011). A mutant GV2260 strain carrying the *Npt2-SacB/R* cassette was confirmed by PCR amplification of the tetracycline resistance gene and *SacB/R* gene using primers: tetracycline For, 5'-atgaaatctaacaatgcgctcat-3'; tetracycline Rev, 5'-tacgagttgcatgat aaagaagaca-3', and *SacB-SacR* For, 5'-cagcatatcatggcgtgtaatatg-3'; *SacB-SacR* Rev, 5'-ctcggataccgggatcctctagatattctattgttaac tgtaattgtcct-3'. The derived mutant strain was designated as GV2260-*SacB/R*.

Development of the Plasmid Vector pEarleygate101-YFP-HA

The YFP gene open reading frame plus the HA epitope tag was amplified from vector pEarleygate101 (Earley et al., 2006) with primers 2702pEG101-yfpHA For, 5'-ATTTGG AGAGGACACGctcggagAtgAGCAAGGGCGAGGAGCTGTTC ACCG-3'; 2703pEG101-yfpHA Rev, 5'-TCGACTGCAGAA TTCGAAGCTTGAGctcggagATCTGAG-3'. The PCR product was gel purified and cloned into pEarleygate101 that had been

digested with *XhoI*. The derived construct was designated as pEarleygate101-YFP-HA.

Development of a CRISPR-Cas9 Construct pgRNA-NbWRKY70 for Knocking Out Tobacco Transcription Factor WRKY70

A putative tobacco WRKY transcription factor NbWRKY70 was identified from GenBank (accession number AF421157). To knock out NbWRKY70, we identified a guiding RNA (GCA ATCGACGGGTTAATTTCGCGG) targeting the NbWRKY70 gene. An *Arabidopsis* U6 promoter, NbWRKY70 guiding RNA, and the PAM terminator were amplified through overlap-PCR using primers 2284AtU6gRNA common For1, CAGCAACTC ATTACAACCTGTTTaaagctttcgttgaacaacgga; 2285AtU6gRNA common Rev1, CGACTCTAGACACGGGGTGGTTTaaaaaaa gcaccgactcgggtgcc; 2846NbWRKY70 gRNA For, GCAATCGAC GGGTTAATTTCGgttttagagctagaataag; 2847NbWRKY70 gRNA Rev, CGAATTAACCCGTCGATTGCaatcactactctgcactca.

The PCR product was gel purified and cloned into the *PmeI* site of pM3UT-Cas9 using a Gibson cloning kit (New England BioLabs), where it contains a *Cas9* gene driven by the *Arabidopsis* Ubiquitin 10 promoter. The *Cas9* gene was originally codon optimized and synthesized based on the *Arabidopsis* genes (Zachary Nimchuk, unpublished data). The derived construct was designated as pgRNA-NbWRKY70.

Conjugation of pEarleygate101-YFP-HA and pgRNA-NbWRKY70 into *Agrobacterium tumefaciens* Strain GVV2260 and GV2260-*SacB/R*

The plasmid vectors pEarleygate101-YFP-HA and pgRNA-NbWRKY70 were conjugated into *A. tumefaciens* strain GVV2260 and GV2260-*SacB/R* using tri-parental conjugation, selected on LB medium supplemented with rifampin 100 µg/mL, and Kanamycin 50 µg/mL or Spectinomycin 50 µg/mL as previously described (Traore and Zhao, 2011).

Agrobacterium-Mediated Transient Assays in *N. benthamiana* Plants

Agrobacterium-mediated transient assays in *N. benthamiana* plants were performed as described previously (Wydro et al., 2006). In brief, the *Agrobacterium* strains were streaked on Yeast Extract Tryptone (YT) media supplemented with rifampicin 100 µg/mL, tetracycline 10 µg/mL, and kanamycin 50 µg/mL and incubated at 28°C for 2 days. Bacterial cells were harvested and re-suspended in induction buffer composed of 10 mM MgCl₂, 10 mM MES (pH 5.6), and 100 µM acetosyringone and incubated for 3 h at room temperature. The bacterial inoculums were adjusted to OD₆₀₀ nm = 0.6 and infiltrated into the stomata of the fully expanded *N. benthamiana* leaves using a 1-mL blunt-end syringe without a needle. The inoculated plants were incubated at room temperature under continuous light for 20–48 h before the detection of expressed proteins. The fluorescent signal of YFP-HA fusion protein was monitored 24 h after inoculation by fluorescent microscopy (Zeiss Axio Observer.A1, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Generation of Transgenic Tobacco Plants Using Either Wild Type or Mutant *Agrobacterium* Strains

Agrobacterium strain GV2260 or GV2260-*SacB/R* carrying plasmid pgRNA-*NbWRKY70* were used for tobacco transformation following a previously described protocol (Horsch et al., 1989). In brief, the fully expanded leaf from a 4-week-old *N. benthamiana* plant was collected and sterilized in 10% bleach for 20 min. The leaf was cut into 1 cm² leaf disks that were infected with *Agrobacterium* culture diluted to OD₆₀₀ = 0.1. The infected leaf disks were co-cultured on MS medium supplemented with 6-BA (1 mg/L) and NAA (0.1 mg/L) and 3% maltose at 25°C in the dark for 2 days. The infected leaf disks were soaked in liquid MS medium for 5 min and then rinsed one time with liquid MS medium. The leaf disks were then blotted dry and transferred to a selection medium (MS medium supplemented with kanamycin 300 mg/L, cefotaxime 150 mg/L and 3 or 5% sucrose). Each treatment has at least 100 leaf disks with three replicates.

The selection mediums were incubated at 25°C under continuous light for 25–30 days for shoot regeneration. The transgenic shoots were transferred to rooting medium (MS medium supplemented with Kanamycin 100 mg/L and 3% sucrose).

The putative transgenic tobacco plants were confirmed by PCR with primers: PMOA36-TBS *PmeI* For, 5'-TGATAGAGT AGTTCATATGGA-3' and PMOA36-TBS *PmeI* Rev 5'-GCTTCC CAACCTTACCAGAG-3'. The PCR products were gel purified and sequenced at the core facility at Virginia Bioinformatics Institute.

Bacterial Genomic DNA, Plasmid DNA, and Plant Genomic DNA Isolation

Bacterial genomic DNAs were isolated using a ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research Corporation, Irvine, CA). Plasmid DNAs were isolated using an AccuPrep™ Plasmid Extraction Kit (Bioneer Corporation, Alameda, CA). Plant genomic DNAs were isolated by using the CTAB method as previously described (Zhang et al., 2013).

RESULTS AND DISCUSSION

Development of a Sucrose-Sensitive Mutant *Agrobacterium* Strain GV2260-*SacB/R*

To generate a mutant *Agrobacterium* strain that is sensitive to sucrose, a suicide vector pLVC18L carrying the *SacB-SacR* gene cassette was integrated into the *recA* gene locus in the genome of *A. tumefaciens* strain GV2260 through marker-exchange mutagenesis. The mutant strain was named GV2260-*SacB/R*. The integration of the *SacB-SacR* gene cassette in GV2260-*SacB/R* was confirmed by PCR amplification. As shown in **Figure 1A**, the plasmid DNA of pLVC18L-*Npt2-SacB/R-recA* and the genomic DNA of GV2260-*SacB/R*, but not the wild type strain GV2260, can amplify the *SacB-SacR* gene and a tetracycline resistance gene located on the suicide vector pLVC18L-*Npt2-SacB/R-recA*. All

three DNAs can amplify the *recA* gene fragment. These results suggest that GV2260-*SacB/R* is carrying the *SacB-SacR* gene cassette. The mutant strain GV2260-*SacB/R* is expected to carry a non-functional *recA* gene (Lovett et al., 1993; Bi and Liu, 1994). In the future, it will be interesting to test the stability of plasmids maintained in GV2260-*SacB/R*.

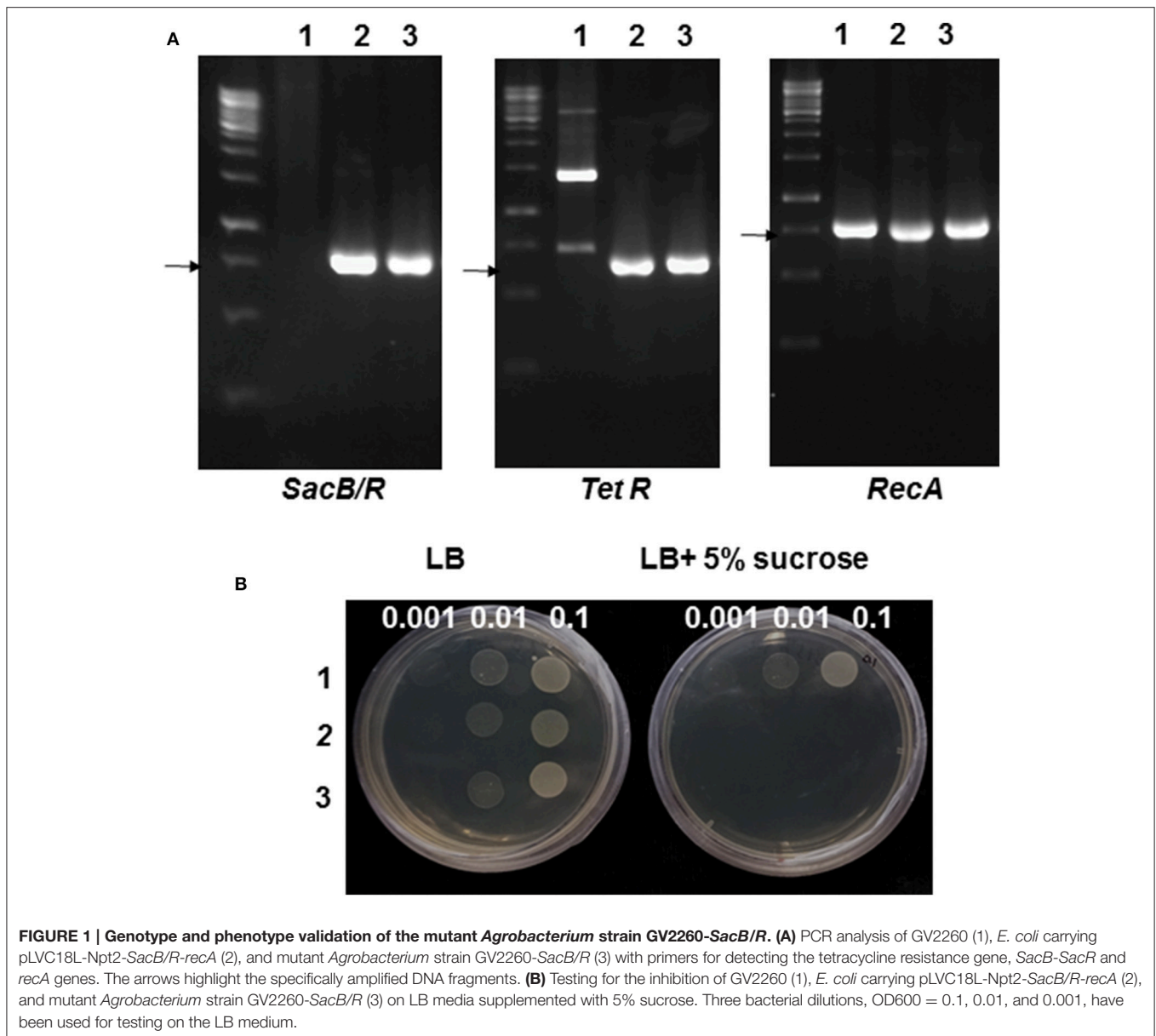
To test the sucrose-sensitivity of GV2260-*SacB/R*, the mutant strain along with the wild type strain GV2260, and *E. coli* strain DH5 α carrying pLVC18L-*Npt2-SacB/R-recA* were grown on LB agar medium supplemented with or without 5% sucrose. The wild type *A. tumefaciens* strain GV2260 grew equally well on LB medium with or without 5% sucrose, which suggests that sucrose in LB agar medium has no inhibitory effect on *A. tumefaciens* GV2260 (**Figure 1B**). In contrast, the *E. coli* strain DH5 α carrying pLVC18L-*Npt2-SacB/R-recA* and GV2260-*SacB/R* grew well on LB agar medium without 5% sucrose, but showed almost no growth on LB agar medium with 5% sucrose. This result suggests that *E. coli* carrying pLVC18L-*Npt2-SacB/R-recA* and GV2260-*SacB/R* containing the *SacB-SacR* gene cassette can be effectively inhibited by the 5% sucrose presented in the culture medium.

Mutant *Agrobacterium* Strain GV2260-*SacB/R* Maintains Its Ability of Transforming Tobacco Plant Cells

To examine if GV2260-*SacB/R* can be used for plant cell transformation, the GV2260 and GV2260-*SacB/R* strains carrying plasmid construct pEarleygate101-YFP-HA were infiltrated into the leaves of *N. benthamiana*. In this construct, the YFP gene was cloned behind the CaMV 35S promoter, and it can be expressed in the transformed tobacco plant cells. Strong YFP fluorescence signals were detected from leaves inoculated with either GV2260 or GV2260-*SacB/R* carrying the YFP gene (**Figure 2**), which suggests that both strains can successfully transform the *N. benthamiana* plant cells.

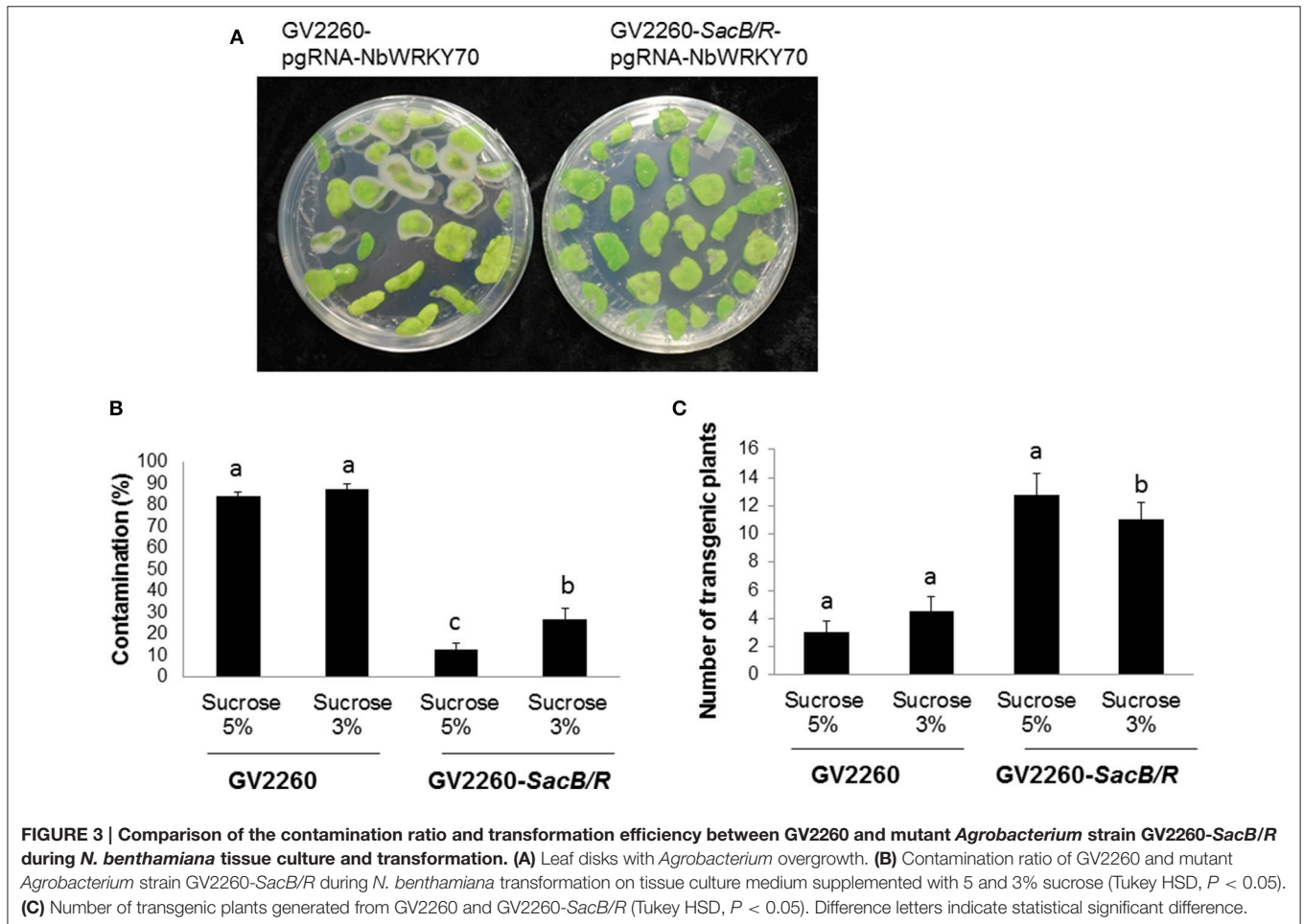
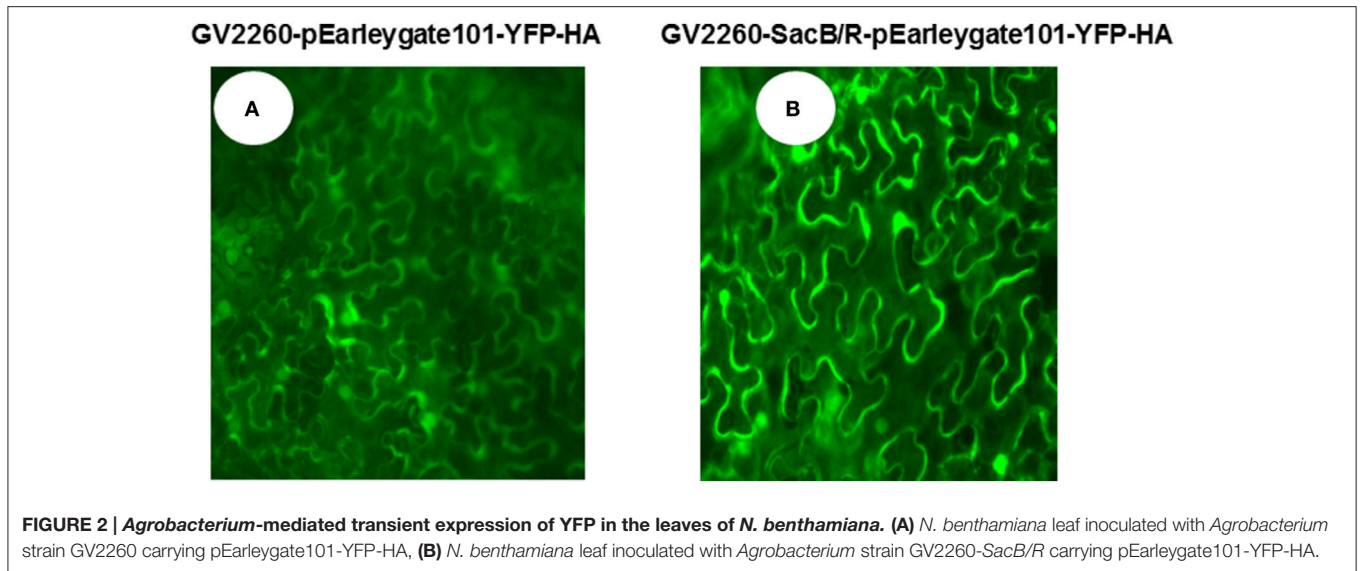
Generation of Stable Transgenic *N. benthamiana* Plants Using the Mutant *Agrobacterium* Strain GV2260-*SacB/R*

To test the transformation efficiency of GV2260-*SacB/R*, *N. benthamiana* tissue culture and transformation was conducted with *Agrobacterium* strain GV2260 or GV2260-*SacB/R* carrying plasmid pgRNA-*NbWRKY70*. The construct pgRNA-*NbWRKY70* carries a synthesized *Cas9* gene driven by the *Arabidopsis* Ubiquitin 10 promoter. The expression of a guiding RNA targeting on the *N. benthamiana* WRKY70 gene was driven by the *Arabidopsis* U6 promoter. We modified the *N. benthamiana* leaf disk transformation protocol (An, 1985), where the leaf disks infected with *Agrobacterium* strains were only slightly washed, which usually can cause *Agrobacterium* overgrowth problems during the selection of transformed plant cells. The infected *N. benthamiana* leaf disks were cultured on selection medium supplemented with either 3 or 5% sucrose. The leaf-disk contamination caused by the overgrowth of *Agrobacterium* was recorded after 4 weeks of culture on the selection medium (**Figure 3A**). Under the test conditions, the contamination rates caused by GV2260-*SacB/R*



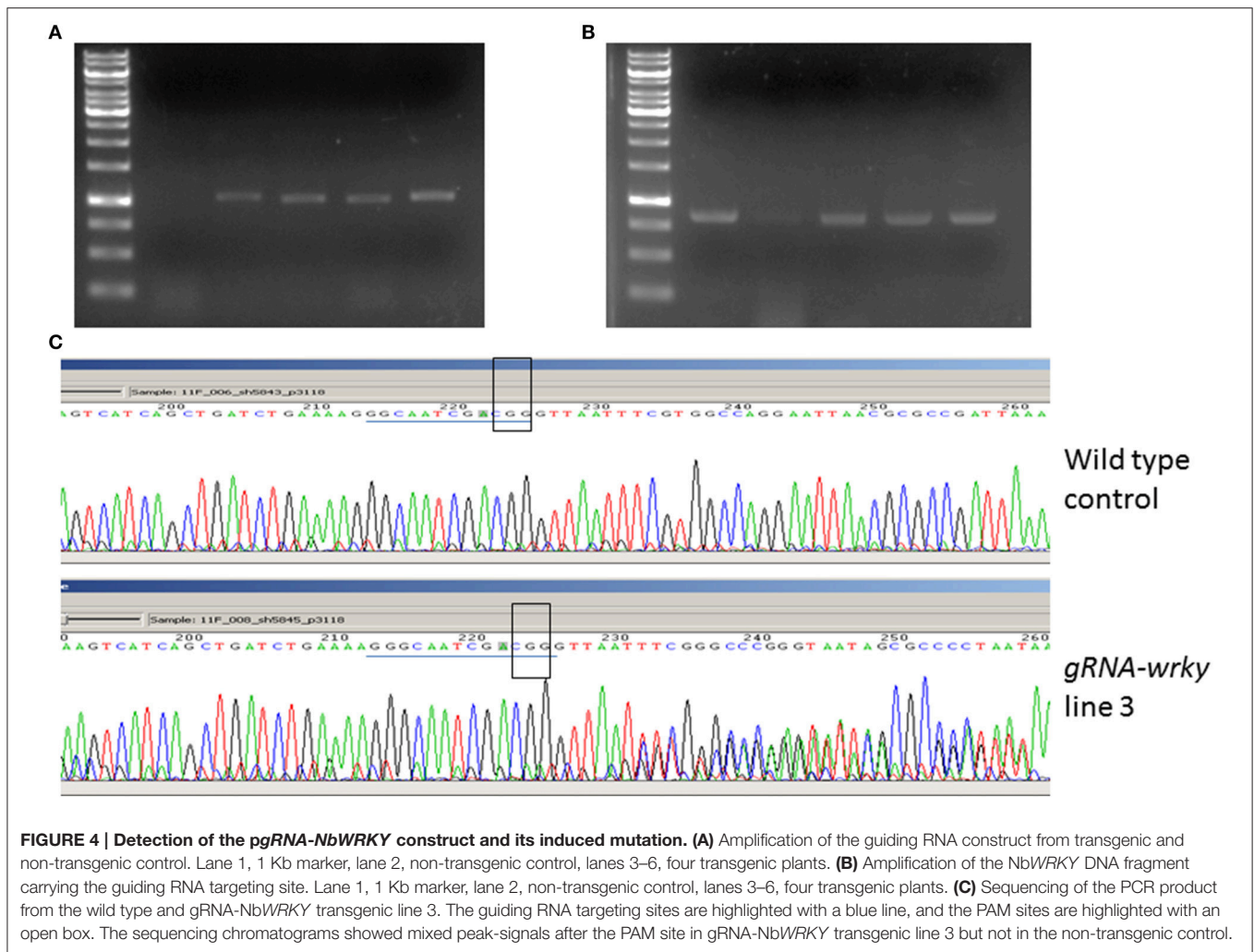
on medium supplemented with 5 and 3% sucrose were 13.0 and 26.9% respectively, which are significant lower than the contamination rate of >80% caused by GV2260 (Figure 3B). Therefore, GV2260-*SacB/R* can be efficiently inhibited by the sucrose presented in the *N. benthamiana* tissue culture medium. However, under our testing conditions, there were still quite high numbers of contaminated leaf disks when in infections with GV2260-*SacB/R*. We speculate that the slightly rinsed leaf disks carried relatively high numbers of *Agrobacterium* cells, which may develop mutations on the *SacB-SacR* genes during the tissue culture process. It will be interesting to further test with different wash conditions, which may reduce the carry-on bacterium cells, and allow for further reduction of the contamination ratio. It will also be interesting to test if we can reduce or even eliminate antibiotic during the tissue culture process.

After 2 months of culture selection, putative transgenic *N. benthamiana* plants were generated. The number of transgenic plants generated from GV2260-*SacB/R* was significantly higher than those generated from GV2260 (Figure 3C) because of the lower contamination rate caused by the GV2260-*SacB/R* strain. Four transgenic plants generated by GV2260-*SacB/R* were genotyped, showing the presence of the *Cas9* gene (Figure 4A). To examine if the p*gRNA-NbWRKY70* transgenic plants carry mutations in *WRKY70*, we amplified an *NbWRKY70* DNA fragment carrying the guiding RNA targeting site. All four putative transgenic lines amplified an *NbWRKY70* DNA fragment with similar size (Figure 4B). The PCR products were gel purified and sequenced. As shown in the chromatogram, the PCR product amplified from the wild type plants yields a clean sequence, while the PCR product from a transgenic



plant (line 3) yields double peaks near the PAM site (CGG) (Figure 4C) (Li et al., 2011; Nekrasov et al., 2013). The double peak near the PAM site indicates the heterozygosity of template

DNAs, which suggests there are *Cas9* induced mutations at the *WRKY70* gene. The PCR products were also cloned and individual clones were sequenced, which confirmed the presence



of mutants (data not shown). The phenotype of transgenic plants will be further characterized in the future. Nevertheless, our result demonstrated that GV2260-*SacB/R* could successfully transform *N. benthamiana* plant cells to generate stable transgenic plants. It will be interesting to test the transformation capacity of GV2260-*SacB/R* in other plant species. In this study, we also confirmed that the CRISPR-*Cas9* system is a powerful tool for introducing mutations on target genes in *N. benthamiana* (Nekrasov et al., 2013; Belhaj et al., 2015).

CONCLUSIONS

We generated a mutant *Agrobacterium* strain GV2260-*SacB/R* that is sensitive to sucrose. The mutant strain can be used for plant cell transformation as demonstrated by *Agrobacterium*-mediated transient assays and stable transformation. The overgrowth of mutant strain GV2260-*SacB/R* can be inhibited by 3–5% sucrose, a common carbon source used in plant tissue mediums. Therefore, GV2260-*SacB/R* can be a valuable tool for plant transformation research.

AUTHOR CONTRIBUTIONS

BZ conceived the project and the cloning strategy. YML and JM performed the experiments. ST, DK, YL, ZN, and XZ contributed vectors and other reagents. YML, JM, ST, DK, YL, ZN, XZ, ZL, and BZ analyzed the data and wrote the draft manuscript. YML and BZ wrote the final manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Guofu Hu for his technical assistance. The study was supported by Binational Agricultural Research and Development Fund (US-4216-09 to BZ), US National Science Foundation (IOS-0845283 to BZ), and the Virginia Agricultural Experiment Station (VA135872). The project was also partially supported by a grant from the program of Plant Feedstock Genomics for Bioenergy of the US Department of Energy (DE-SC0008338 to Kevin L. Childs, XZ, and BZ).

REFERENCES

- An, G. (1985). High efficiency transformation of cultured tobacco cells. *Plant Physiol.* 79, 568–570. doi: 10.1104/pp.79.2.568
- Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N. J., and Nekrasov, V. (2015). Editing plant genomes with CRISPR/Cas9. *Curr. Opin. Biotechnol.* 32, 76–84. doi: 10.1016/j.copbio.2014.11.007
- Bhau, B. S., and Wakhlu, A. K. (2001). Effect of some antibiotics on the *in vitro* morphogenetic response from callus cultures of coryphantha elephantidens. *Biol. Plant.* 44, 19–24. doi: 10.1023/A:1017905917971
- Bi, X., and Liu, L. F. (1994). RecA-independent and RecA-dependent intramolecular plasmid recombination: differential homology requirement and distance effect. *J. Mol. Biol.* 235, 414–423. doi: 10.1006/jmbi.1994.1002
- Brendel, V., Brocchieri, L., Sandler, S. J., Clark, A. J., and Karlin, S. (1997). Evolutionary comparisons of RecA-like proteins across all major kingdoms of living organisms. *J. Mol. Evol.* 44, 528–541. doi: 10.1007/PL00006177
- Chambert, R., and Petitglatron, M. (1989). Study of the effect of organic solvents on the synthesis of levan and the hydrolysis of sucrose by *Bacillus subtilis* levansucrase. *Carbohydr. Res.* 191, 117–123. doi: 10.1016/0008-6215(89)85051-7
- Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., et al. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol.* 115, 971–980. doi: 10.1104/pp.115.9.971
- Clark, A. J., and Margulies, A. D. (1965). Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K12. *Proc. Natl. Acad. Sci. U.S.A.* 53, 451–459. doi: 10.1073/pnas.53.2.451
- Earley, K. W., Haag, J. R., Pontes, O., Opper, K., Juehne, T., Song, K., et al. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* 45, 616–629. doi: 10.1111/j.1365-313X.2005.02617.x
- Ellis, D. D., Lazaroff, W. R., Roberts, D. R., Flinn, B. S., and Webb, D. T. (1989). The effect of antibiotics on elongation and callus and bud formation from embryonic tissue of *Piceaglauca*. *Can. J. For. Res.* 19, 1343–1346. doi: 10.1139/x89-207
- Farrand, S. K., O'Morchoe, S. P., and McCutchan, J. (1989). Construction of an *Agrobacterium tumefaciens* C58 recA mutant. *J. Bacteriol.* 171, 5314–5321.
- Gay, P., Le coq, D., Steinmetz, M., Berkelman, T., and Kado, C. I. (1985). Positive selection procedure for entrapment of insertion-sequence elements in gram-negative bacteria. *J. Bacteriol.* 164, 918–921.
- Gelvin, S. B. (2000). *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 223–256. doi: 10.1146/annurev.arplant.51.1.223
- Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6, 271–282. doi: 10.1046/j.1365-313X.1994.6020271.x
- Horsch, R., Fry, J., Hoffmann, N., Neidermeyer, J., Rogers, S., and Fraley, R. (1989). "Leaf disc transformation," in *Plant Molecular Biology Manual*, eds S. Gelvin, R. Schilperoord, and D. Verma (Dordrecht: Springer Netherlands), 63–71.
- Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., and Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat. Biotechnol.* 14, 745–750. doi: 10.1038/nbt0696-745
- Jones, H. D., Doherty, A., and Wu, H. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. *Plant Methods* 1, 1–9. doi: 10.1186/1746-4811-1-5
- Kurnit, D. M. (1989). *Escherichia coli* recA deletion strains that are highly competent for transformation and for *in vivo* phage packaging. *Gene* 82, 313–315. doi: 10.1016/0378-1119(89)90056-5
- Li, R., and Qu, R. (2011). High throughput *Agrobacterium*-mediated switchgrass transformation. *Biomass Bioenergy* 35, 1046–1054. doi: 10.1016/j.biombioe.2010.11.025
- Li, Y., Mendiratta, S., Ehrhardt, K., Kashyap, N., White, M. A., and Bleris, L. (2011). Exploiting the CRISPR/Cas9 PAM constraint for single-nucleotide resolution interventions. *PLoS ONE* 11:e0144970. doi: 10.1371/journal.pone.0144970
- Lovett, S. T., Drapkin, P. T., Sutera, V. A. Jr., and Gluckman-Peskind, T. J. (1993). A sister-strand exchange mechanism for recA-independent deletion of repeated DNA sequences in *Escherichia coli*. *Genetics* 135, 631–642.
- Matthysse, A. G., Stretton, S., Dandie, C., McClure, N. C., and Goodman, A. E. (1996). Construction of GFP vectors for use in gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiol. Lett.* 145, 87–94. doi: 10.1111/j.1574-6968.1996.tb08561.x
- Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013). Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655
- Quandt, J., and Hynes, M. (1993). Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* 127, 15–21. doi: 10.1016/0378-1119(93)90611-6
- Ren, Y., Bang, H., Curtis, I., Gould, J., Patil, B., and Crosby, K. (2012). *Agrobacterium*-mediated transformation and shoot regeneration in elite breeding lines of western shipper cantaloupe and honeydew melons (*Cucumis melo* L.). *Plant Cell Tissue Organ Cult. (PCTOC)* 108, 147–158. doi: 10.1007/s11240-011-0024-6
- Ried, J. L., and Collmer, A. (1987). An NptI-SacB-SacR cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-*eviction* mutagenesis. *Gene* 57, 239–246. doi: 10.1016/0378-1119(87)90127-2
- Schweizer, H. (1992). Alielic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable *Bacillus subtilis* sacB marker. *Mol. Microbiol.* 6, 1195–1204. doi: 10.1111/j.1365-2958.1992.tb01558.x
- Silva, J. A. T. D. and Fukai, S. (2001). The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. *J. Appl. Horticult.* 35, 71–77. Available online at: http://www.horticultureresearch.net/journal_pdf/20013-12.pdf
- Song, J., Bradeen, J. M., Naess, S. K., Helgeson, J. P., and Jiang, J. (2003). BIBAC and TAC clones containing potato genomic DNA fragments larger than 100 kb are not stable in *Agrobacterium*. *Theor. Appl. Genet.* 107, 958–964. doi: 10.1007/s00122-003-1334-9
- Tereso, S., Miguel, C., Maroco, J., and Oliveira, M. M. (2006). Susceptibility of embryogenic and organogenic tissues of maritime pine (*Pinus pinaster*) to antibiotics used in *Agrobacterium*-mediated genetic transformation. *Plant Cell Tissue Organ Cult.* 87, 33–40. doi: 10.1007/s11240-006-9130-2
- Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., et al. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J.* 11, 1369–1376. doi: 10.1046/j.1365-313X.1997.11061369.x
- Traore, S., and Zhao, B. (2011). A novel Gateway(R)-compatible binary vector allows direct selection of recombinant clones in *Agrobacterium tumefaciens*. *Plant Methods* 7:42. doi: 10.1186/1746-4811-7-42
- Tsuda, K., Qi, Y., Nguyen, L. V., Bethke, G., Tsuda, Y., Glazebrook, J., et al. (2012). An efficient *Agrobacterium*-mediated transient transformation of *Arabidopsis*. *Plant J.* 69, 713–719. doi: 10.1111/j.1365-313X.2011.04819.x
- Tzfira, T., and Citovsky, V. (2006). *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr. Opin. Biotechnol.* 17, 147–154. doi: 10.1016/j.copbio.2006.01.009
- West, S. C. (1992). Enzymes and molecular mechanisms of genetic recombination. *Annu. Rev. Biochem.* 61, 603–640. doi: 10.1146/annurev.bi.61.070192.003131
- Wydro, M., Kozubek, E., and Lehmann, P. (2006). Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamiana*. *Acta Biochim. Pol.* 53, 289–298. Available online at: http://www.actabp.pl/pdf/2_2006/289.pdf
- Yaseen, M., Ahmad, T., Sablok, G., Standardi, A., and Hafiz, I. A. (2013). Review: role of carbon sources for *in vitro* plant growth and development. *Mol. Biol. Rep.* 40, 2837–2849. doi: 10.1007/s11033-012-2299-z
- Yu, T.-A., Yeh, S.-D., and Yang, J.-S. (2001). Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. *Bot. Bull. Acad. Sin.* 42, 281–286.
- Zang, N., Zhai, H., Gao, S., Chen, W., He, S., and Liu, Q. (2009). Efficient production of transgenic plants using the bar gene for herbicide resistance in sweetpotato. *Sci. Hortic.* 122, 649–653. doi: 10.1016/j.scienta.2009.06.023

- Zhang, X., Wang, L., and Shou, L. (2013). Modified CTAB method for extracting genomic DNA from wheat leaf. *Agric. Sci. Technol.* 14, 946–949. Available online at: <http://search.proquest.com/openview/748d315d04b0db8ed3779e4549788799/1?pq-origsite=gscholar>
- Zhao, B., Dahlbeck, D., Krasileva, K. V., Fong, R. W., and Staskawicz, B. J. (2011). Computational and biochemical analysis of the *Xanthomonas* effector AvrBs2 and its role in the modulation of *Xanthomonas* type three effector delivery. *PLoS Pathog* 7:e1002408. doi: 10.1371/journal.ppat.1002408
- Zhao, Z.-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., et al. (2000). *Agrobacterium*-mediated sorghum transformation. *Plant Mol. Biol.* 44, 789–798. doi: 10.1023/A:1026507517182

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Liu, Miao, Traore, Kong, Liu, Zhang, Nimchuk, Liu and Zhao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.