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Transformation of *Lesquerella Fendleri* with the New Binary Vector pGPro4-35S

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Abstract: Problem statement: Crop genetic engineering requires the use of various promoters to control the expression of introduced transgenes. Some of the binary vectors currently available for promoter characterization in dicotyledonous plants have pitfalls due to their construction, such as containing a selectable marker cassette with enhancer sequences that can potentially interfere with the expression specificity of nearby promoters. Also, many binary vectors are quite large in size and contain few useful restriction sites making their *in vitro* manipulation technically challenging. **Approach:** A small (7698 bp) and flexible binary vector named pGPro4 was constructed to possess unique features favorable for promoter analysis in dicot plants. A *nopaline synthase* (*nos*) promoter was used to control the expression of the selectable marker of pGPro4 to prevent the problem of interference with the neighboring promoter-reporter fusion. In pGPro4, the nos promoter and *hygromycin phosphotransferase II* (*hptII*) sequences are flanked by *loxP* sites, which allow for Cre recombinase-mediated removal when hygromycin resistance is no longer desired. pGPro4 also contains a bifunctional *ß-glucuronidase-enhanced Green Fluorescent Protein* (*gusA-eGFP*) reporter gene that provides visual detection of reporter gene expression using either fluorescence in live cells or histochemical detection of β-glucuronidase activity. **Results and Conclusion:** To demonstrate the usefulness of the pGPro4 vector, a CaMV35S promoter was fused to *gusA-eGFP* and the resulting plasmid, pGPro4-35S, was used to transform *Lesquerella fendleri*. Primary shoots were generated from explants at an expected frequency of 10-27.5%, indicating that the nos promoter drove sufficient *hptII* expression to generate hygromycin resistant plants. Six independent transgenic *L. fendleri* lines were grown to maturity and generated T_1 seeds. The bifunctionality of the *gusA-eGFP* reporter gene was verified by detecting both green fluorescence and β-glucuronidase activity in multiple T_1 *L. fendleri* seedlings from 5 of the 6 the independent transgenic lines.

Key words: Promoter, hygromycin phosphotransferase II, β-glucuronidase, green fluorescent protein, *Agrobacterium*-mediated transformation

INTRODUCTION

Lesquerella fendleri (A. Gray) S. Wats. (Brassicaceae), under development as a new industrial oilseed crop in the southwestern region of the U.S., is valued for its unusual Hydroxy Fatty Acid (HFA). HFAs and derivatives are used as raw materials for numerous industrial products, such as lubricants, plasticizers and surfactants (Gunstone and Padley, 1997). *L. fendleri* is amenable to *Agrobacterium*-mediated transformation (Chen, 2011; Skarjinskaia *et al*., 2003; Wang *et al*., 2008), thus genetic transformation provides an alternative means to improve this crop. One of the essential components of *Agrobacterium*-mediated transformation system is a binary vector that consists of a plasmid backbone and a Transfer-DNA (T-DNA). The T-DNA typically carries a selectable marker and a gene of interest flanked by Right Border and Left Border sequences. For more than two decades, considerable efforts have been made to create improved binary vectors that facilitate the generation of transgenic plants. These efforts have typically included minimizing the plasmid backbone size for ease of genetic manipulation, improving the properties of the T-DNA region by including more unique restriction endonuclease sites facilitating gene of interest insertion,

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incorporating a variety of plant selectable marker genes and/or integrating novel reporters or sequence tags to generate proteins for tissue/cellular localization or protein-protein interaction studies (Lee *et al*., 2008).

One sometimes overlooked problem in many popular vectors such as pCAMBIA (www.cambia.org) and pGreen (Hellens *et al*., 2000) is the use of the CaMV35S promoter with one or more enhancer sequences to drive selectable marker gene expression. When an organ-specific promoter-target gene casette is placed within a vector, the enhancer can bidirectionally interfere with the transcription of the target gene as well as nearby genes, affecting the fidelity of the organ-specific gene expression and/or causing unintended misexpression of nearby genes (So *et al*., 2005; Xie *et al*., 2001). To avoid this problem, we describe the construction of two new binary vectors, pGPro4 and its derivative pGPro4-35S. The pGPro4-35S vector carries the double enhanced version of the CaMV 35S promoter fused to the bifunctional β-*glucuronidase-enhanced Green Fluorescent Protein* (*gusA-eGFP*) reporter gene. We examine the utility of this novel vector by genetic transformation of *L. fendleri* and investigate its functionality by visualizing reporter gene activity in the transgenic plants produced.

MATERIALS AND METHODS

Construction of the pGPro4 and pGPro4-35S binary vectors: The pGPro4 binary vector is a derivative of the previously described pGPro1 plasmid (Thilmony *et al*., 2006). The pGPro1 T-DNA carries a selectable marker expression cassette that consists of the rice *Actin1* (*Act1*) promoter controlling the expression of a *hptII* gene and is well suited for *Agrobacterium*-mediated transformation of monocot plants (Thilmony *et al*., 2006). Although the *Act1* promoter is a strong constitutive promoter in monocots, it does not confer strong expression in dicotyledonous plants. To generate a comparable construct for use in *L. fendleri* and other dicots, the rice *Act1* promoter was removed from a pGPro2 precursor vector that contained only the selectable marker cassette (and not the *gusA-eGFP* reporter gene). The pGPro2 vector (Thilmony *et al*., 2009) is very similar to pGPro1, the annotated pGPro2 sequence is available in GenBank accession EU147786. A 290bp *Nopaline Synthase* (*nos*) promoter fragment was amplified with the nosPFor 5'-gcatgcCGATACATGAGAATTAAGGGAG-3′ and nosPRev 5'-actagtGAGTTGAGAGTGAATATGAGAC-3′ primers, digested with SphI and SpeI and inserted into the SphI and NheI restriction sites replacing the *Act1* promoter.

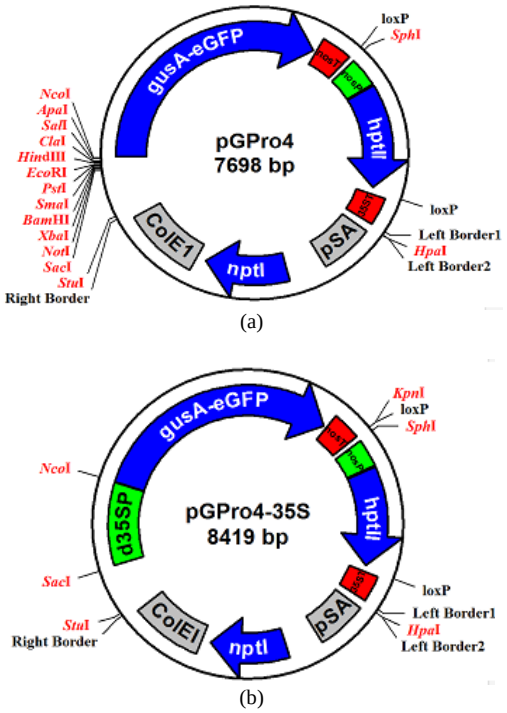

Fig. 1: Plasmid maps of the pGPro4 and pGPro4-35S binary vectors. (A) Schematic diagram of the pGPro4 plasmid. This vector contains a promoterless *gusA-eGFP* (*β-glurcuronidase-enhanced Green Fluorescent Protein*) reporter gene. (B) Map of the pGPro4-35S binary vector containing the double enhanced CaMV 35S promoter (d35SP) fused to the bifunctional reporter gene. Unique restriction sites, Right and Left Border regions and loxP sites are marked with tick marks. Gene coding sequences are drawn as blue arrows, promoters as green boxes, 3′ terminators/poly adenylation signals as red boxes and the vector origins of replication as gray boxes. 35ST (CaMV 35S terminator), hptII (*hygromycin phosphotransferase II*), nosT (*nopaline synthase* terminator), nosP (*nopaline sythase* promoter), nptI (*neomycin phosphotransferase I*). The annotated plasmid sequences for these vectors are available from GenBank accessions JN593324 and JN593325.

A promoterless *gusA-eGFP* bifunctional reporter gene and *nos* terminator fragment was subsequently ligated into the KpnI site and a clone containing the insert in the desired orientation was selected and sequence confirmed. The vector map of the pGPro4 vector is shown in Fig. 1A and the annotated sequence is available from Genbank (accession JN593324). The

pGPro4-35S vector was built by directionally inserting a 721bp double enhanced CaMV35S promoter fragment (excised from the pGPro1-35S vector; Thilmony *et al*. 2006) into the SacI and NcoI sites of pGPro4. The map of the sequence confirmed pGPro4-35S vector is shown in Fig. 1B and the annotated sequence is available from GenBank accession JN593325.

Plant material, bacterial strain: The *L. fendleri* seeds, WCL-LY2 (Dierig *et al*., 2001), were kindly provided by Dr. Dave Dierig (USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ). Plants were grown in a greenhouse at temperatures between 28°C (day) and 18°C (night), with supplemental metal halide lighting to provide a 15-h-day length (1000-1250 μmol m^{-2} sec^{-1}). Mature female flowers were hand-pollinated and the seeds were harvested at about 49 days after pollination. An *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) carrying binary vector pGPro4-35S was used for plant transformation. An AGL1/pGPro4-35S culture was started with the inoculation of one clone in 1 mL Luria Broth (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, 1 g L^{-1} glucose, pH 7) supplemented with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin shaken at 200 rpm overnight at 29°C, followed by sub-culturing of 0.1 mL of the overnight culture in 50 mL fresh LB for 24 h.

Tissue culture, transformation and regeneration: The previously described protocol (Chen, 2011) was utilized for *L. fendleri* transformation. In brief, leaves harvested from plants in sterile condition were wounded by scratching slightly on the underside of leaf and then dipped in the half strength MS medium containing AGL1/pGPro4-35S for 5 min. Following the inoculation, leaves were blotted on sterilized filter paper and transferred to Callus and Shoot Induction (CSI) medium composed of basal medium (BM, half strength MS medium plus 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.7) supplemented with 1 mg L^{-1} 6-benzylaminopurine (BA) and 0.1 mg L^{-1} 1-Naphthaleneacetic Acid (NAA). After incubating the infected leaves in the growth chamber for 2 days, the leaves were cut into 5 mm segments and cultured on CSI media plus 25 mg L^{-1} hygromycin (hyg) for transgenic selection and 100 mg L^{-1} timentin for inhibiting the *Agrobaterium* growth. In 6-8 weeks, yellow-greenish hygromycin resistant calli started to appear on the leaf segments. The calli were then transferred to a timentin-free subculture medium CSI+ (CSI with hygromycin increased to 50 mg L^{-1}). Green shoots were developed from the calli on CSI+ medium in about 2-4 weeks after the transfer. To eliminate chimeras, each shoot was cut into small pieces (about 1×1 mm) and placed on the CSI+ medium for shoot-regeneration. After 4 rounds of successive regenerations, shoots were sub-cultured on BM plus 1 mg L^{-1} BA, 1 mg L^{-1} Indole-3-Butyric Acid (IBA) and 50 mg L^{-1} hyg for multiplication. Shoots 10-15 mm in length were transferred to rooting medium (BM plus 1 mg L^{-1} IBA and 50 mg L^{-1} hyg). When a shoot developed 2-3 roots (usually in 3-5 weeks), it was then transferred to a Magenta box (Sigma, St. Louis, Mo) containing sterilized peat-vermiculite growth mixture (Sunshine mix #4, Planet Natural, Bozeman, MT) pre-soaked with 1 mg L^{-1} IBA water solution. After 8-10 weeks in the growth mixture, well-developed primary plants showing 8-12 normal leaves and 2-3 inch height were transferred to a 6-inch pot and placed under a transparent plastic cover for the first 2 weeks for acclimation in the greenhouse.

Detection of β-glucuronidase and green fluorescence reporter gene activity: Mature seeds harvested from primary transgenic plants or wild-type seeds were surface-sterilized in a 0.25% (v/v) sodium hypochlorite solution for 15 min followed by five rinses in sterile deionized water. The seeds were placed on Germination Medium (GM) containing half strength of MS medium supplemented with 0.5% sucrose and 0.6% agar. Etiolated seedlings were germinated in the dark at 25°C for 4 days.

GFP fluorescence and GUS staining images were documented using a Leica dissecting microscope (Leica micro-system Ltd., CH-9435 Heerbrugg, Switzerland) equipped with a digital camera. Microscopic visualization of GFP fluorescence employed an X-cite 12 fluorescence illumination system (EXFO photonic Solution Inc. Quebec, G1M 2K2, CANADA). Histochemical GUS assays were performed using the Jefferson *et al*. (1987) method. The assay solution contained 0.5 mM potassium ferrocyanide, 0.3% (v/v) triton X-100 and 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-gluc) in 50 mM phosphate buffer, pH7.0. Samples were incubated at 37°C overnight in the assay solution and then transferred to 70% ethanol.

RESULTS AND DISCUSSION

Advantages of the pGPro4 and pGPro4-35S vectors: The pGPro4 binary vector was constructed for the characterization of novel promoters in dicot plants. The vector is based upon the pGreen II binary vector

backbone (Hellens *et al*., 2000) and is of a similar structure as the previously published pGPro1 (Thilmony *et al*., 2006) and pGPro2 binary vectors (Thilmony *et al*., 2009), but is designed for transformation of dicotyledonous (rather than monocotyledonous) plants. The plasmid is conveniently small for a binary vector (7698bp; Fig. 1A) and contains a high copy number origin of replication supporting high DNA yields from *E. coli*. Replication in *Agrobacterium tumefaciens* requires the presence of the helper plasmid pSoup (Hellens *et al*., 2000).

The Transfer-DNA (T-DNA) region contains 15 unique restriction sites, most of which are upstream of a *gusA-eGFP* bifunctional reporter gene facilitating the construction of transcriptional or translational fusions with candidate promoters. The bifunctional reporter gene fusion allows the detection of expression in either live tissues via green fluorescence or in histochemically stained tissues by detection of β-glucuronidase activity. Reporter gene activity can also be quantified with a fluorometric β-glucuronidase activity assay. The pGPro4 vector is well-suited for promoter characterization in dicot plants because it avoids promiscuous promoter interactions and/or interference that have been shown to occur between the CaMV35S promoter/enhancer used to control selectable marker expression and nearby promoters in other commonly used binary vectors (Gudynaite-Savitch *et al*., 2009; Hily *et al*., 2009; Singer *et al*., 2010; 2009; So *et al*., 2005; Zheng *et al*., 2007). The *nopaline synthase* (*nos*) promoter has been demonstrated to not alter the expression of nearby organ or tissue-specific promoters (Gudynaite-Savitch *et al*., 2009; So *et al*., 2005; Zheng *et al*., 2007) and thus the nos promoter was utilized to control selectable marker expression in pGPro4 (Figure 1A). The T-DNA also contains *loxP* recombinase recognition sites flanking the nos promoter and *hygromycin phosphotransferase II* (*hptII*) gene, thus enabling Cre recombinase-mediated site-specific excision of the selectable marker gene, to produce marker-free transgenic plants. Finally, the T-DNA is terminated by two tandem copies of the Left Border sequence. Binary vectors containing two or more copies of the Left Border repeats have been shown to reduce the frequency of transfer of non-T-DNA vector sequences into the transgenic plants produced (Kuraya *et al*., 2004).

***L. fendleri* transformation with the pGPro4-35S vector:** To examine the functionality of the pGPro4 binary vector, we constructed the pGPro4-35S plasmid by inserting the double enhanced 35S promoter upstream of the *gusA-eGFP* reporter gene (Fig. 1B) and tested it in *L. fendleri* by *Agrobacterium*-mediated transformation using a previously described protocol (Chen, 2011). In three separate experiments, the percentage of primary hygromycin resistant shoots generated from the explants ranged from 10% to 27.5% (Table 1). The frequency of primary shoot regeneration is lower than that of 22-60% when a pCAMBIA plasmid was used (Chen, 2011), but still gave rise a sufficient number of transformants. Six independent primary transgenic *L. fendleri* plants were grown to maturity in the greenhouse to produce next generation (T_1) seeds.

Verification of transgene activity: Reporter activity conferred by the *gusA-eGFP* gene was examined in the T_1 seedlings from the six primary transgenic plants. Ten mature T_1 seeds from each line as well as a wild-type plant were germinated in the dark to avoid chlorophyll development in cotyledons and the associated auto-fluorescence that can obscure visualization of green fluorescence ((Zhou *et al*., 2005). GFP fluorescence was detected in most of the seedlings of each line, with the exception of line 31, where only one seed germinated and that seedling did not exhibit detectable GFP activity. These same seedlings were then subsequently histochemically stained to detect β-glucuronidase activity. The seedlings exhibiting GFP activity also stained positive for GUS activity, demonstrating the bifunctional reporter gene functions as expected in *L. fendleri*. Representative images of a GFP fluorescent and GUS positive seedling is shown in Fig. 2. Although the population size of the tested progeny was small, we observed one or two seedlings from each population that failed to exhibit reporter gene activity consistent with the expectation of observing null segregants lacking the reporter transgene. We also noticed that little auto-florescence was present in etiolated wild-type seedlings, allowing the easy visualization of the green fluorescence. These results suggest that etiolated *L. fendleri* seedlings will be useful for future studies with fluorescent reporters and demonstrate that the *gusA-eGFP* gene fusion should prove to be a useful bifunctional reporter in *L. fendleri*.

Table 1: Number of calli and shoots produced in leaf segments

Expt No.	No. of segments	No. of calli[a]	No. of shoots[b]	Percentage of shoot
1	40	7	6	15.0
2	40	13	11	27.5
3	40	5	4	10.0

[a]: The number was scored at the 8th week after placing the explants on CSI medium plus 25 mg L^{-1} hygromycin and 100 mg L^{-1} timentin. [b]: The number was scored at the 4th week of subculture on CSI medium plus 50 mg L^{-1} hygromycin

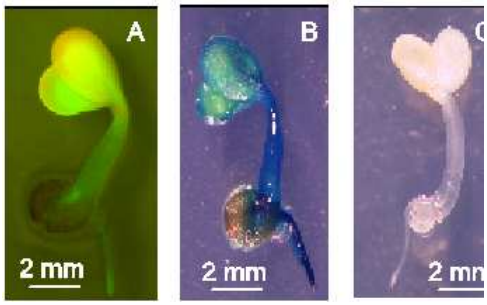

Fig. 2: Reporter gene activity in L. fendleri seedlings. (A) Green fluorescence observed in a T1 transgenic seedling. (B) A representative GUS positive transgenic seedling. (C) A wild-type GUS stained seedling. L. fendleri seed was germinated in the dar

CONCLUSION

We have constructed two new binary vectors, pGPro4 and pGPro4-CaMV35S. The unique features of the T-DNAs of these vectors will facilitate future research in transgenic *L. fendleri* and presumably other dicotyledonous plants. The T-DNA expression cassettes are arranged to avoid potential transcriptional interference and use of the *nos* promoter to control expression of the selectable marker avoids problems that arise from the presence of the CaMV35S promoter/enhancer that is commonly present in other binary vectors. We also documented the bifunctionality of the *gusA-eGFP* reporter gene fusion and demonstrate that it generated both green fluorescence and β-glucuronidase activity in transgenic *L. fendleri* seedlings.

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