Effective Reduction of Chimeric Tissue in Transgenics for the Stable Genetic Transformation of Lesquerella fendleri

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Abstract. To improve the potential of Lesquerella fendleri as a valuable industrial oilseed crop, a stable genetic transformation system was developed. Genetic transformation was performed by inoculating leaf segments with an Agrobacterium tumefaciens strain AGL1 containing binary vector pCAMBIA 1301.1, which contains a ρ-glucuronidase gene as a reporter gene and hygromycin phosphotransferase II as a selection marker gene. Primary shoots were regenerated from the leaf segments on the half-strength Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine, 1-naphthaleneacetic acid, and hygromycin. The frequency of primary shoot generation was between 22.5% and 60%, and 81.1% to 89.3% of these shoots were chimeras. The high frequency of chimeras was probably the result of efficient protection from the hygromycin of non-transformed cells by adjacent transformed ones. The non-transformed cells were removed by multiple rounds of successive shoot regenerations. The purified isogenic shoots were subcultured and roots were induced on the MS medium plus indole-3-butyric acid. Most of the plantlets were able to establish roots and acclimate successfully in the greenhouse. The insertion of the hptII gene was confirmed by segregation analysis in T1 seeds, and the stable inheritance of the transgenes was demonstrated by the characterization transgenic lines through T2 generation. This transformation system can be used to obtain stable transgenic lines for genetic engineering of L. fendleri.

Materials and Methods

Plant material, bacterial strain, and general growth conditions. The L. fendleri seeds, WCL-Y2 (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 to 1250 μmol·m⁻²·s⁻¹). Mature flowers were hand-pollinated and the seeds were harvested at ≈49 d after pollination. An Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) carrying binary vector pCAMBIA 1301.1 (http://www.cambia.org), which contains a GusA gene as a reporter gene and a hygromycin phosphotransferase II (hptII) as a selection marker gene, was used for plant transformation. An AGL1/pCAMBIA1301.1 culture was started with the inoculation of one clone in 1 mL Luria Bertani (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 subculturing of 0.1 mL of the overnight culture in 50 mL fresh LB for 24 h. A control culture of AGL1 without pCAMBIA1301.1 was grown in LB plus 100 mg·L⁻¹ carbenicillin.

Tissue culture, transformation, and regeneration. Agrobacterium cultures were centrifuged at 4000 rpm for 10 min at room temperature (25 °C) and the pellet was suspended to an OD₆₀₀ of 0.5 in half-strength MS liquid medium (Murashige and Skoog, 1962). L. fendleri 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 germinated and grown aseptically on germination medium (GM) containing half-strength MS medium supplemented with 0.5% sucrose and 0.6% agar in a growth chamber at a day/night cycle (16/8 h, 100 μmol·m⁻²·s⁻¹) at 24 °C (day) and 22 °C (night). After 4 to 6 weeks, shoot tips together with four to five true leaves were subcultured and grown aseptically on GM for a continuous supply of leaf material.

To perform the transformation, leaves were harvested from plants after 4 to 6 weeks of subculturing and wounded by scratching slightly on the underside of the leaf and then dipped in the half-strength MS medium containing AGL1/pCAMBIA1301.1 for 5 min. After 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. After incubating the infected leaves in the growth chamber for 2 d,
the leaves were cut into 5-mm segments and cultured on CSI media plus 25 mg L\(^{-1}\) hygromycin for transgenic selection and 100 mg L\(^{-1}\) timentin for inhibiting the Agrobacterium growth. For controls, samples were inoculated with AGL1 and leaf segments were placed either on CSI plus 100 mg L\(^{-1}\) timentin (positive control) or CSI plus 25 mg L\(^{-1}\) hygromycin and 100 mg L\(^{-1}\) timentin (negative control). Positive control samples initiated green calli on the edges of leaf segments in 2 weeks and then vigorously produced multiple shoots from the calli in 4 weeks, whereas negative control leaf segments displayed necrosis in 2 weeks and then died out in 4 weeks. For the transformed samples, yellow–greenish hygromycin-resistant (hyg\(^{\text{R}}\)) calli started to appear in 6 to 8 weeks on 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 \(\approx 2\) to 4 weeks after the transfer.

To eliminate chimeras, each shoot was cut into small pieces (\(\approx 1 \times 1\) mm) and placed on the CSI\(^{+}\) medium for shoot regeneration. After four rounds of successive regenerations, shoots were subcultured on BM plus 1 mg L\(^{-1}\) BAP, 1 mg L\(^{-1}\) indole-3-butyric acid (IBA) and 50 mg L\(^{-1}\) hygromycin for multiplication. Shoots 10 to 15 mm in length were transferred to rooting medium (BM plus 1 mg L\(^{-1}\) BAP and 50 mg L\(^{-1}\) hygromycin). When a shoot developed two to three roots (usually in 3 to 5 weeks), it was transferred to a Magenta box (Stigma, 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 to 10 weeks in the growth mixture, a well-developed plant showing eight to 12 normal leaves and 2- to 3-inch height was transferred to a 6-inch pot and placed under a transparent plastic cover for the first 2 weeks for acclimation in the greenhouse.

**Histochimical β-glucuronidase assay, chimera determination, and T1 and T2 plant verification.** Histochimical β-glucuronidase (GUS) assays were performed using the Jefferson method (Jefferson et al., 1987). The assay solution contained 0.5 mm potassium ferrocyanide, 0.3% (w/v) triton X-100, and 1 mg mL\(^{-1}\) 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc) in 50 mM phosphate buffer, pH 7.0. Samples were incubated at 37 °C overnight in the assay solution and then transferred to 70% ethanol to wash away chlorophyll for observation of blue GUS staining.

For chimera determination, GUS assays were performed on 10 randomly selected shoots derived from each line after 4 weeks of regeneration. A chimeric line was determined if one or more shoots were found to be a mixture of transformed cells (blue) and non-transformed cells (white). To test T1 seeds, T1 seeds were surface-sterilized as stated previously and placed in GM plus 50 mg L\(^{-1}\) hygromycin. Hyg\(^{\text{R}}\) or hygromycin-sensitive (hyg\(^{\text{s}}\)) seedlings were documented after 2 weeks of culture in the growth chamber. Eight to 10 leaf discs (4-mm diameter) from each seedling were pooled and submerged in the GUS assay buffer in an enclosed test tube. The tube was then incubated overnight at 37 °C followed by washing in 70% ethanol. GUS-stained tissue was observed after the chlorophyll was washed away. Because *L. fendleri* requires hand-pollination in the greenhouse to produce seeds, all seeds from greenhouse plants were produced by hand-pollination. Approximately 35 d after pollination, developing T2 seeds were tested for their GUS activities through the procedure described.

**Results**

**Transformation and elimination of chimeras.** In *L. fendleri*, leaf segments are highly prolific in shoot regeneration, and the protocols for tobacco transformation can be easily adapted. In three independent studies, control experiments were performed in the selection medium with (negative control) or without (positive control) 25 mg L\(^{-1}\) hygromycin. All 60 leaf segments from the negative control condition died out in 4 weeks showing complete bleaching (or necrosis), whereas 60 positive control leaf segments all produced green calli on the segment edges in 2 weeks. Approximately 92% of these leaf segments (55 of 60) quickly regenerated shoots (two to five per segment) within 2 to 4 weeks of culture. For leaf segments treated with AGL1/pCAMBIA 1301.1, hyg\(^{\text{R}}\) calli with yellow–greenish color appeared on leaf segments after 4 to 6 weeks of culture. The percentage of hyg\(^{\text{R}}\) calli generated from 80 treated leaf segments varied from 28.8% to 71.3% in three independent experiments (Table 1). To two 4 weeks after transferring the calli to CSI\(^{+}\) medium, most of the calli generated one primary shoot and a few generated zero or two primary shoots. So the overall primary shoot generation to leaf segments ratio was between 22.5% and 60%.

Because chimeras were reported in *L. fendleri* transformation (Skarjinskaia et al., 2003; Wang et al., 2008), a procedure was developed to eliminate the chimeras or to produce isogenic transformants through multiple rounds of shoot regenerations. The percentage of chimeras was evaluated using a GUS assay after each round of regeneration. For example, after the first round, it was estimated that the majority of the primary hyg\(^{\text{R}}\) shoots were chimeras (81.1% to 89.3%; Table 1; Fig. 1C), whereas 8.7% to 16.7% were escapes (non-transgenic) (Fig. 1A) and only 1.7% to 2.2% were pure (or isogenic) transformants (Fig. 1B). The chimeras then went through multiple rounds of regeneration processes until no heterogeneous GUS stain could be found from randomly selected regenerated shoots. After four rounds of successive regenerations, the percentage of chimeras dropped to 1.4% to 2.2% (Table 1).

Pure transgenic shoots were subcultured and roots induced on basal medium plus 1 mg L\(^{-1}\) IBA. Transgenic lines were subsequently established by transferring the plantlets with two to three roots to a sterilized peat–vermiculite growth mixture and cultivating for 8 to 10 weeks. Approximately 81% (152 of 188) of the plantlets reached 2- to 3-inch height and each had eight to 12 normal leaves. By transferring the established plants together with the growth mixture, 151 of 152 of plants representing 82 independent lines acclimated successfully in the greenhouse. Most of the lines were morphologically normal with the exception of one male-sterile line showing no stamens and two bushy lines with compact structure. Using GUS assays on leaf samples of each line, one GUS negative line (escape) was found. Because *L. fendleri* depends on insect pollination in the field, the remaining 78 T0 lines, which were morphologically normal and also GUS-positive, were hand-pollinated. All lines produced hybrid seeds. Among them, 12 lines were also capable of producing self-mate (selfed) seeds, which were further carried on for genetic analysis.

**Genetic analysis in T1 and T2 progenies.** The number of transgene insertions was determined by segregation analyses based on hptII gene activity in the T1 progenies of the 12 selfed T0 plants. Because hptII is a dominant gene, it follows a Mendelian phenotype segregation ratio of 3:1 (hyg\(^{\text{R}}\):hyg\(^{\text{s}}\)) for one locus, 15:1 for two, and 63:1 for three loci. Segregations of hptII were examined by scoring the hyg\(^{\text{R}}\) (Fig. 2A) or hyg\(^{\text{s}}\) (Fig. 2C) phenotype in T1 seedlings. The results are summarized in Table 2. Seven lines, 149, 174, 178, 213, 312, 324, and 344, had a segregation ratio close to 3:1 indicating one hptII insertion locus, whereas Lines 350, 376, and 378 had a ratio close to 15:1 indicating

| Expt. no. | No. of segments | No. of calli | No. of shoots | Percentage of chimera at each round of regeneration\(^\text{a}\) |
|----------|----------------|-------------|---------------|------------------------------------------------|
| 1        | 80             | 23          | 18            | 81.1% 28.9% 4.4% 2.2% |
| 2        | 80             | 57          | 48            | 82.5% 34.2% 6.3% 2.1% |
| 3        | 80             | 35          | 28            | 89.3% 29.3% 4.3% 1.4% |

\(^{a}\)The number was scored at the eighth week after placed on callus and shoot induction medium plus 25 mg L\(^{-1}\) hyg and 100 mg L\(^{-1}\) timentin.

\(^{b}\)The number was scored at the fourth week of subculture on callus and shoot induction medium plus 50 mg L\(^{-1}\) IBA.

\(^{c}\)Ten randomly selected shoots after 4 weeks of each round of regeneration were pooled from each line and tested by β-glucuronidase assay. A chimeric line was determined if one or more shoots were found to be a mixture of transformed cell (in blue) and non-transformed cells (in white).
two hptII insertion loci. In Lines 319 and 322, which had only 24 and 28 seeds germinated, respectively, all showed hygR phenotype; their ratios of hygR:hygS was considered greater than 15:1. Therefore, these two lines were placed in a category of having more than two transgene insertions. However, this classification might not be conclusive, because at least 64 seeds are required for the characterization of three loci.

In addition, a partial hygR phenotype in seven of 35 T1 progenies of Line 378 was observed. The partial hygR seedlings were arrested on the hygromycin medium, showing small green cotyledons and swelled radicle but no true leaf (Fig. 2E). The phenotype could be an indication of hptII gene malfunction. Theoretically, if one of the two unlinked hptII genes lost its full function resulting in a partial phenotype, the segregation ratio of hygR:partial hygR:hygS would be 24:6:2. In the case of Line 378, the observed ratio is 26:7:2, suggesting that it could be the result of a malfunctioning copy of the hptII gene. Detailed sequence and biochemistry analyses on the hptII gene in Line 378 are required to confirm the existing of a malfunctioning copy of the hptII gene.

The numbers of insertion loci of the hptII gene estimated by these genetic segregation analyses in the T1 seedlings of these 12 lines were consistent with the copy numbers of the hptII gene determined by using a quantitative polymerase chain reaction method (Chen and Lin, 2010).

Because the hptII gene is constructed next to a gusA gene in pCAMBIA1301.1, the two genes would most likely segregate together. To confirm the cosegregation, 50% of the normal hygR seedlings from each line (total 149), including the seven partial hygR seedlings from Line 378, were tested by incubating whole seedlings in GUS assay solution. The remaining hygR seedlings were tested for GUS activity by leaf disc assay. Positive GUS staining was observed in all seedlings (Figs. 2B and 2E) and leaf discs tested. The results indicated that at least one copy of the gusA gene cosegregated with the hptII in all lines, including these seven partial hygR seedlings.

The hygR seedlings after leaf disc assay remained healthy and were transplanted to soil for T2 seed production. At the midmaturity stage of T2 seed development (35 d after
pollination) (Chen et al., 2009), the stability of transformation was determined by GUS activity assay. GUS positive (GUS+) T2 seeds were observed in all 136 plants representing 12 T1 lines. Some of the T1 plants were heterozygous and the gusA gene segregated in their developed T2 seeds, showing some GUS+ and some GUS-negative (GUS–). Examples of GUS+ or GUS– T2 seed from Line 344 are shown in Figures 3A and 3B, respectively.

Discussion

In many plant species, in vitro shoot regeneration is a prerequisite and often a limiting step for transformation. In L. fendleri, sufficient shoot regeneration from various tissue types have been reported, including cultured cells (Skarjinskaia et al., 1996), leaf segments (Skarjinskaia et al., 2003), and explants of hypocotyls and cotyledons (Wang et al., 2008). Leaf was chosen for transformation because it is abundant and can be grown aseptically for continued supply of material. When leaf segments were treated with AGL1/pCAMBIA1301.1 and under hygromycin selection, 22.5% and 60.0% of them generated hygR shoots within 8 to 12 weeks (Table 1). This represents improved efficiency compared with a previous published study, which reported an average frequency of hygR shoot regeneration from infected calli of 22.7% (Wang et al., 2008).

Chimeras can give rise to non-transgenic progenies and thus loss of transformants. In L. fendleri, regeneration of chimeras was reported (Skarjinskaia et al., 2003; Wang et al., 2008), but the frequency is not entirely clear. By using gusA as a visual marker, 81.1% to 89.3% of primary shoots were found to be chimeras. To eliminate chimeras, a first approach was tried by increasing hygromycin concentration to 50 mg·L⁻¹ in the CSI medium during the initial culture of leaf segments and the selection of hygR calli. However, it had no effect on reducing the number of chimeras (data not shown). In fact, it dropped the speed and occurrence of hygR calli and subsequent primary shoot production eight- to 10-fold. This phenomenon is probably the result of the inhibitory effect of hygromycin during shoot regeneration. The transformation system presented allowed regeneration of transgenic plants; however, it had the inhibitory effect of hygromycin during shoot regeneration. The phenomenon is probably the result of the inhibitory effect of hygromycin during shoot regeneration.

In some cases, the frequency of chimera was high, 45% in citrus (Dominguez et al., 2004), and 80% in Vigna mungo (Muruganathan et al., 2007). The formation of chimeras has been investigated in detail in tobacco (Schmulling and Schell, 1993), soybean (Christou, 1990), flax (Dong and McHughen, 1993), strawberry (Dominguez et al., 1995), citrus (Dominguez et al., 2004), and Vigna mungo (Muruganathan et al., 2007). In the formation of chimeras, a higher hygromycin concentration to 50 mg·L⁻¹ in the CSI medium is a reasonable length of time for transgenic selection. Under these conditions, most T0 transgenic plants were morphologically normal and capable of producing transgenic seeds (T1).

The phenomenon of chimera production has been reported in many dicotyledonous species in Agrobacterium-mediated transformation and the shoot regeneration process such as cotton (Firoozabady et al., 1987), tobacco (Schmulling and Schell, 1993), soybean (Christou, 1990), flax (Dong and McHughen, 1993), strawberry (Dominguez et al., 1995), citrus (Dominguez et al., 2004), and Vigna mungo (Muruganathan et al., 2007). In some of the cases, the frequency of chimera was high, 45% in citrus (Dominguez et al., 2004) and 80% in Vigna mungo (Muruganathan et al., 2007). The formation of chimeras has been investigated in detail in tobacco (Schmulling and Schell, 1993), soybean (Christou, 1990), and citrus (Dominguez et al., 2004). Several mechanisms have been proposed to explain the generation of chimeras, including 1) a shoot organ originating from a mixture of transformed and untransformed cells; 2) transformation effects in a cell or cells that either cease to divide or divide to daughter cells forming only a sector in a shoot; and 3) transient expression of a transgene occurring in many cells of a shoot. Although the exact reason of chimera formation in L. fendleri remains unknown, patterns of chimeras observed resemble those in soybean and citrus such as sectorial chimeras and spotty single cell chimeras (Fig. 1C–D), which indicates that similar mechanisms might be involved. If a shoot was composed of both transformed and untransformed cells, the untransformed cells could be protected through efficient detoxification of the antibiotic by the transformed cells (Christou, 1990; Dominguez et al., 2004; Schmulling and Schell, 1993). Such cross-protection is certainly possible in L. fendleri, because most escapes seem to arise close to or from chimeric calli (picture not shown). Nevertheless, the success of shoot regeneration process as demonstrated is a feasible strategy to overcome cross-protection by removing chimeric cells or purifying isoegenic transgenic cells.

The transformation system presented allows generation of stable transgenic plants, and it is an effective protocol to engineer L. fendleri as a superior crop for high yield, disease resistance, and other favorable agronomic traits.

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