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GENETIC TRANSFORMATION OF DUCKWEED *LEMNA GIBBA* AND *LEMNA MINOR*

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Summary

We developed efficient genetic transformation protocols for two species of duckweed, *Lemna gibba* (G3) and *Lemna minor* (8627 and 8744), using *Agrobacterium*-mediated gene transfer. Partially differentiated nodules were co-cultivated with *Agrobacterium tumefaciens* harboring a binary vector containing β-glucuronidase and *nptII* expression cassettes. Transformed cells were selected and allowed to grow into nodules in the presence of kanamycin. Transgenic duckweed fronds were regenerated from selected nodules. We demonstrated that transgenic duckweed could be regenerated within 3 mo. after *Agrobacterium*-mediated transformation of nodules. Furthermore, we developed a method for transforming *L. minor* 8627 in 6 wk. These transformation protocols will facilitate genetic engineering of duckweed, ideal plants for bioremediation and large-scale industrial production of biomass and recombinant proteins.

Key words: Agrobacterium-mediated transformation; bioremediation; recombinant protein production; genetic engineering.

Introduction

Duckweed is a group of small, free-floating, fresh-water plants belonging to *Lemnaceae* (Landolt, 1998). Duckweed fronds are floating photosynthetic organs that measure from less than 1 mm to several mm in diameter and multiply primarily by vegetative means. Daughter fronds are generated from meristematic regions of a mother frond, and are abscised as they mature in most species. Duckweed species in *Lemna* and *Spirodela* have one to several roots whose primary function appears to be of support rather than of nutrient uptake while other species do not form any roots (Landolt, 1998). Sexual reproduction of some duckweed species has been observed in nature and can be induced by various laboratory conditions (reviewed in Kandeler, 1985; Tanaka et al., 1994, 1997).

The worldwide *Lemnaceae* Germplasm Collection (Landolt, 1986) housed in our laboratory includes nearly 1000 geographic isolates within the 37 duckweed species from all four genera and provides diverse genotypes for various potential applications of duckweed. Clonal growth habit, ease of propagation, growth on liquid, and small size make duckweed ideal for large-scale production of recombinant proteins and biomass. As the first step in achieving such a production system, our laboratory has developed *in vitro* cultivation and callus induction of *L. gibba* geographic isolates (Moon and Stomp, 1997; Moon et al., 1998). We also demonstrated that certain duckweed geographic isolates grow well in swine lagoon effluent (Bergmann et al., 2000a, b), a potential nutrient source for a large-scale biomass production. In this study we established efficient genetic transformation protocols for two duckweed species: *L. gibba* (geographic isolate G3), *L. minor* (geographic isolates 8627 and 8744).

Particular geographic isolates used in this study were chosen for desirable characteristics of host plants for commercial applications: rapid growth and high protein content. The protein content of *L. gibba* G3 and *L. minor* 8744 was as high as 34% of the dry weight, and up to 38% was recorded for the protein content of *L. minor* 8627 (Monesmith et al., personal communication). Furthermore, *L. minor* 8627 showed promising characteristics for bioremediation of swine lagoon effluent (Bergmann et al., 2000a, b). Establishment of reliable genetic transformation protocols was an important step toward commercial application of duckweed for large-scale production of biomass and recombinant proteins, and bioremediation.

Materials and Methods

Plant material and tissue culture conditions. *L. gibba* G3 [originally provided by J. Slovin, USDA (Beltsville, MD, USA)] and *L. minor* 8627 and 8744 from the *Lemnaceae* Germplasm Collection were maintained in our laboratory in sterile cultures on liquid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) with 10 g l^{-1} sucrose at pH 5.6. The fronds and nodules were maintained in a growth chamber at 23°C under a 16-h photoperiod with approximately 40 μmol m^{-2} s^{-1} light intensity provided by Wide Spectrum fluorescent lights (F40PL/AQ/WS, GE Lighting, Cleveland, OH, USA). Light intensity for the subdued light condition (in a styrofoam box) was approximately 4 μmol m^{-2} s^{-1}.

Callus induction and nodule production. Callus induction from duckweed fronds was obtained through modification of published protocols for *L. gibba* G3 (Moon and Stomp, 1997). Five fronds from approximately 2-wk-old cultures were separated and incubated on a 100×15 mm plate containing 25 ml of callus induction medium: MS (Murashige and Skoog, 1962) basal salts (M-5519, Sigma Chemicals, St. Louis, MO, USA), 30 g l^{-1} sucrose, 4 g l^{-1} Difco Bacto-agar, 1.5 g l^{-1} gelrite, pH 5.6, and either 10 μ*M* 1-naphthaleneacetic acid and 0.5 μ*M* 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron) (for *L. gibba* G3) or 5 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μ*M* thidiazuron (for *L. minor* geographic isolates). Six wk later,

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small white callus pieces were subcultured to nodule production medium (NPM): MS basal medium, 30 g l^{-1} sucrose, 1 μM 2,4-D, 2 μM 6-benzyladenine, 4 g l^{-1} Difco Bacto-agar, 1.5 g l^{-1} gelrite, pH 5.6. Nodules were produced from callus after 2 wk on NPM and were used for transformation or transferred to fresh NPM every 2 wk for future use. We define nodules as partially organized light green cell masses.

Agrobacterium *strain and binary vectors.* *Agrobacterium tumefaciens* C58-z707 (Hepburn et al., 1985) was used as the host strain. pBI121 and pCNL56 (Li et al., 1992), binary vectors containing a bacterial reporter gene encoding β-glucuronidase (GUS) (Jefferson et al., 1987), were used. While the reporter gene in pBI121 is under the control of the CMV35S promoter, the GUS gene in pCNL56 is fused to the modified CMV35S/mannopine synthase promoter, and contains an intron (Vancanneyt et al., 1990) to prevent GUS gene expression in *Agrobacterium*. Both vectors contain kanamycin resistance marker genes for bacterial and plant selection. The plant kanamycin resistance is conferred by the *nptII* gene under the control of the *nos* promoter. Each binary vector was introduced into *Agrobacterium* C58-z707 by tri-parental mating or electroporation and subsequent selection on kanamycin.

Nodule co-cultivation with Agrobacterium. *Agrobacterium* C58-z707 harboring either pBI121 or pCNL56 from a frozen glycerol stock was grown at 28°C overnight on a YEB (1 g l^{-1} Difco Yeast extract, 5 g l^{-1} Difco Beef extract, 5 g l^{-1} Difco Bacto-peptone, 5 g l^{-1} sucrose, 0.5 g l^{-1} $MgSO_4.7H_2O$, 15 g l^{-1} Difco Bacto-agar), or a Difco Potato dextrose agar plate containing 50 mg l^{-1} kanamycin. A small amount of bacteria was then spread on a YEB or a Difco Potato dextrose agar plate containing 50 mg l^{-1} kanamycin and 100 μM acetosyringone and incubated at 28°C overnight. The bacteria were then suspended in bacterial resuspension medium (MS basal salts, 0.6 M mannitol, 100 μM acetosyringone, pH 5.6) at an approximate OD_{595} of 1.0 and incubated for at least 1 h at room temperature. Healthy, rapidly growing nodules approximately 3 mm in diameter were then submerged in the bacterial suspension for 3–5 min. Nodules were then placed on NPM containing 100 μM acetosyringone (10 nodules per 100×15 mm plate) and incubated for 2 d in the dark within the growth chamber at 23°C.

Sequential selection of transformed nodules and frond regeneration on solid media. This protocol applies to all three geographic isolates. After 2 d of co-cultivation without selection, nodules were transferred to solid NPM containing 10–100 mg l^{-1} kanamycin (Agri-Bio, Inc., North Miami, FL, USA) and 500 mg l^{-1} cefotaxime (Agri-Bio, Inc.) (NPM-KC). They were incubated for 4 wk in subdued light achieved by placing cultures in a styrofoam box in the growth chamber. Nodules were transferred weekly to fresh NPM-KC during this time. Nodules were then incubated under full light on NPM-KC for 2 wk or until selected nodules were distinct. Nodules were randomly sampled during selection and stained to monitor GUS expression. When transgenic nodules were selected, healthy nodules were transferred to fresh NPM-KC for another 2 wk. A subset of selected nodules was stained to confirm uniform GUS expression. Frond regeneration was induced by incubating selected nodules on frond regeneration medium (FRM): half-strength SH with 5 g l^{-1} sucrose, 4 g l^{-1} Difco Bacto-agar, 1.5 g l^{-1} gelrite, pH 5.6 for *L. minor* geographic isolates. FRM for *L. gibba* G3 contained only 4 g l^{-1} Difco Bacto-agar and 1.5 g l^{-1} gelrite at pH 5.6 with no nutrients. Although we did not always include kanamycin in FRM in this study, inclusion of kanamycin at 100 mg l^{-1} did not cause detrimental effects on regeneration of transgenic *L. minor* 8627 and was, thus, recommended. Regenerated fronds were proliferated on liquid SH medium for later confirmation of transformation.

Simultaneous selection and regeneration of transgenic fronds for L. minor *8627.* This protocol was used with *L. minor* 8627 only. After 2 d of co-cultivation, each nodule was transferred to a 125-ml flask containing 40 ml of liquid SH medium with 10 g l^{-1} sucrose, 5 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime and incubated on a rotary shaker at 100 rpm in the growth chamber. The medium was changed weekly. Normal fronds were stained for GUS activity as they emerged. In order to avoid establishing multiple transgenic lines from a single transformation event, only one frond from each flask was used to establish an independent transgenic duckweed line.

GUS staining of nodules and fronds. GUS activity in co-cultivated nodules and regenerated fronds was monitored throughout the transformation experiments. GUS histochemical assays were performed at 37°C overnight using X-gluc as a substrate in the presence of a K^+ ferricyanide/ferrocyanide mixture as an oxidation catalyst (Jefferson, 1987). Fronds were cleared in 95% ethanol after staining. Washing fronds for several seconds in 90% acetone followed by a rinse in deionized H_2O prior to staining improved substrate penetration into older fronds.

Analysis of genomic DNA. Genomic DNA was isolated from duckweed fronds expressing GUS activity using the minipreparation method (Dellaporta et al., 1983) and the CTAB method (Doyle and Doyle, 1990). For the DNA gel blot of *L. gibba* G3 and *Agrobacterium* genomic DNA, probes were prepared from a 3 kb *Hin*dIII to *Eco*RI fragment of pBI121 containing the CMV35S promoter, the GUS gene and the *nos* terminator, and a 5.6 kb *Bam*HI to *Hin*dIII fragment of pSL47 (kindly provided by Sharon L. Doty, Washington State University) containing the *virA*, part of *virB* and *tzs* of *Agrobacterium* strain C58. The ^{32}P-labeled probes were prepared using a Prime-a-Gene labeling system (Promega, Madison, WI, USA). DNA transfer, hybridization, and signal detection using ^{32}P-labeled probes were performed according to published protocols (Sambrook et al., 1989).

A 2 kb *Bam*HI to *Eco*RI fragment of pBI121 containing the GUS gene and the *nos* terminator was used as a probe for the DNA gel blot of *L. minor* strains. A DIG-High Prime DNA labeling and detection kit (Roche Molecular Biochemicals USA, Indianapolis, IN, USA) was used for probe preparation, hybridization, and signal detection.

Results and Discussion

Callus induction and nodule production. Several pale yellow or white calluses per each original frond were induced from all three geographic isolates within 6 wk using this protocol. After transfer to NPM, callus morphology changed to form partially organized light green nodules. These nodules proliferated stably on NPM for several months. We have used up to 4-mo.-old nodules of *L. minor* 8627 for transformation. Dark green frond-like structures or white calluses occasionally produced on nodules were not amenable for transformation. Generally, nodules of *L. minor* 8627 and 8744 grew well while *L. gibba* G3 nodules did not always grow as well (data not shown). This indicated that NPM for *L. gibba* G3 requires further optimization.

Sequential selection of transformed nodules and frond regeneration on solid media. We monitored GUS expression of nodules throughout the selection process. Many GUS-expressing cells were present in co-cultivated nodules beginning 2 d after co-cultivation with the pCNL56-harboring *Agrobacterium* (Fig. 1A). Only the selection with 10 mg l^{-1} kanamycin was used for the transformation of *L. gibba* G3 and *L. minor* 8744 while concentrations up to 100 mg l^{-1} were tested for *L. minor* 8627. Nodules grew well for an extended period at low concentrations of kanamycin (e.g. 10 mg l^{-1}) suggesting minimal selection pressure, although some transgenic lines were recovered eventually. Nodules selected at higher concentrations of kanamycin (e.g. 100 mg l^{-1}) exhibited uniform GUS activity when either vector was used, indicating efficient selection. The kanamycin concentration needs to be optimized for each vector because expression of the *nptII* gene can be influenced by a strong adjacent promoter in certain vectors. Surrounding cells often escaped selection when *nptII* expression in transformed cells was high, resulting in non-transgenic plant regeneration.

We observed GUS-positive cells on many nodules that appeared dead. Furthermore, relatively few stable transformants were recovered compared to the large number of GUS-positive cells at early stages. These observations indicate that only a small fraction of cells transiently expressing GUS became stably transformed. While a systematic study of transformation efficiency was not

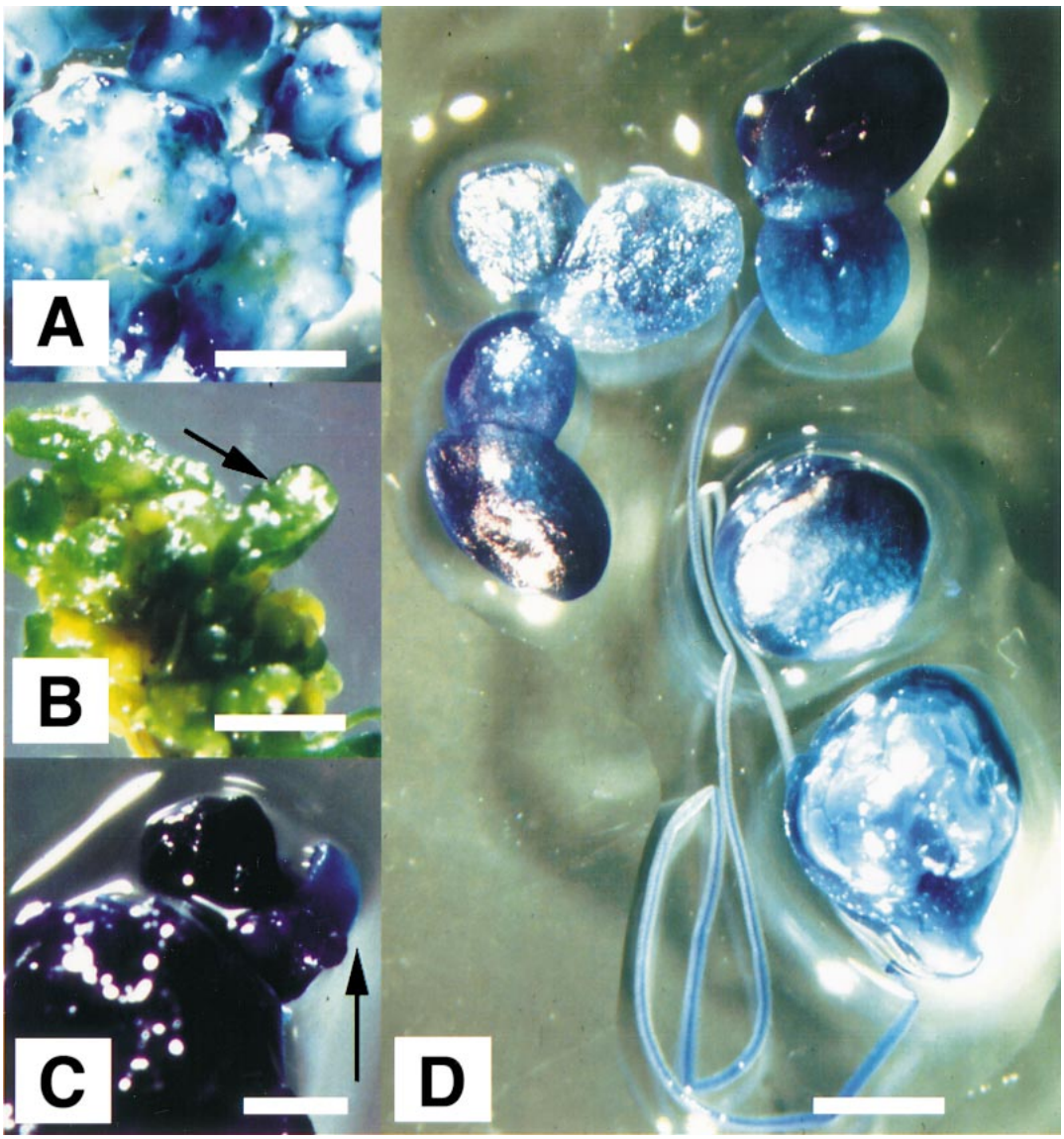

FIG. 1. Various stages of duckweed transformation. A, Typical GUS-staining pattern of *L. minor* 8627 nodules co-cultivated with *Agrobacterium* C58-z707::pCNL56 (*bar* = 2 mm). The nodules were stained with X-gluc 1 wk after co-cultivation. B, Frond regeneration from *L. gibba* G3 nodules (strain D) (*bar* = 2 mm). The arrow represents a regenerating frond. C, A GUS-stained frond regenerating from a selected nodule (*L. gibba* G3-A) (*bar* = 5 mm). The arrow indicates a regenerating frond. D, GUS expression in transgenic lines that have been maintained vegetatively for 2–3 yr (*bar* = 2 mm). Two larger, single fronds and a long root are of *L. gibba* G3-B3 and other fronds are of *L. minor* 8627-H.

conducted, usually multiple independent transformed lines were obtained per plate of 10 original *L. minor* 8627 nodules.

GUS-positive fronds regenerated within 1 mo. after transfer of selected nodules to FRM (Fig. 1B, C). To insure true transgenic regeneration, inclusion of kanamycin in FRM is recommended. Generally, cefotaxime was not necessary during frond regeneration. While regenerating fronds occasionally exhibited abnormal morphology, normal fronds grew from them after they were transferred to liquid medium. Transgenic lines that have been propagated vegetatively for up to 3 yr exhibited uniform GUS activity (Fig. 1D), indicating long-term stability of the GUS expression.

Simultaneous selection and regeneration of transgenic fronds. Variations in the sequential transformation method were tested to improve transformation efficiency. We were particularly interested in facilitating the transformation process by selecting transformants during, rather than prior to, regeneration of fronds and the use of liquid media. *L. minor* 8627 was used for this study because of its consistent nodule production and growth, and it is a preferred strain for swine waste effluent remediation (Bergmann et al., 2000a, b). Using the protocol with simultaneous selection and regeneration, the original nodule grew and often broke up into several nodules, from which frond-like structures began to regenerate within 4–5 wk of selection after the co-cultivation of nodules with *Agrobacterium*. Normal, free-floating fronds were

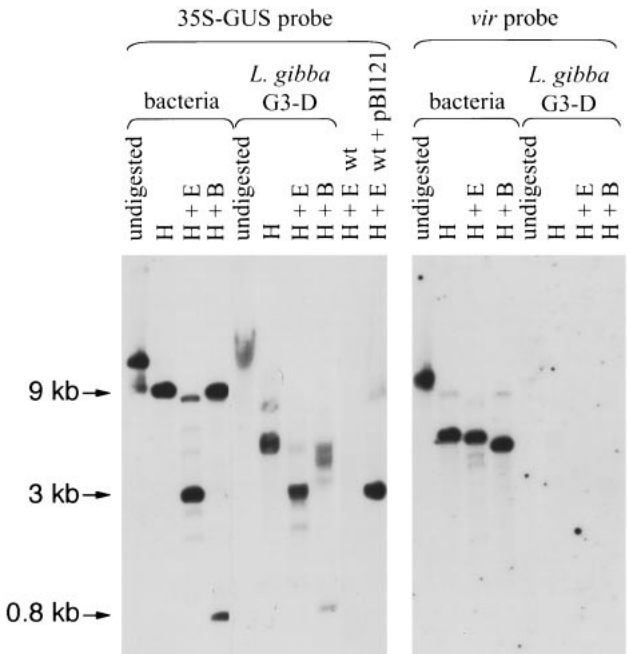

FIG. 2. DNA hybridization analysis of the *L. gibba* G3-D transformant. Total DNA of *L. gibba* G3-D line (10 μg) and C58-z707::pBI121 (10 ng) was digested with a combination of *Hin*dIII (H), *Eco*RI (E) and *Bam*HI (B) and probed first with a 3 kb *Hin*dIII–*Eco*RI fragment of pBI121 containing the CMV35S promoter, GUS gene and the *nos* terminator sequence (left panel). Non-transformed *L. gibba* G3 (wt) with or without pBI121 (10 copies) were included as controls. The blot was stripped and reprobed with a 5.6 kb *Hin*dIII–*Bam*HI fragment of pSL47 containing the *virA*, part of *virB* and *tzs* of the *Agrobacterium* strain C58 (right panel).

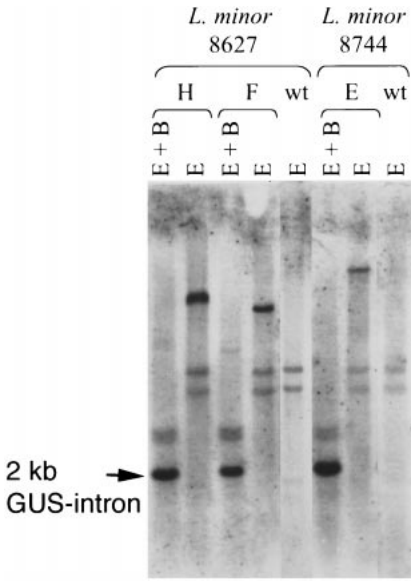

FIG. 3. DNA hybridization analysis of *L. minor* 8627 and 8744 transformed with pCNL56. Ten μg of total DNA from transformed *L. minor* lines H, F (geographic isolate 8627) and E (geographic isolate 8744) were digested with *Eco*RI (E) alone or *Eco*RI and *Bam*HI (B) and fractionated on an agarose gel. *Eco*RI-digested non-transformed *L. minor* 8627 and 8744 (wt) were included as controls. The blot was hybridized with a 2 kb *Eco*RI–*Bam*HI fragment of pBI121 containing GUS gene and the *nos* promoter sequence. The probe hybridizes to a 2 kb *Eco*RI–*Bam*HI fragment of the GUS-intron construct of pCNL56 (arrow). The appearance of a faint doublet in all DNA preparations digested with *Eco*RI may be due to heterologous hybridization of the probe to duckweed genomic DNA.

produced by the fifth to sixth week whereas sequential selection and regeneration using solid NPM and FRM resulted in transgenic fronds 12–16 wk after co-cultivation of nodules with *Agrobacterium*. This improvement was due to the elimination of the slow selection process of transgenic nodules rather than the use of liquid environment. A variation of this protocol in which solid FRM was used to simultaneously select and regenerate fronds has been tried successfully, resulting in transformed fronds within 8 wk. It is not possible to estimate the transformation frequency because we took only one frond from each flask while multiple independent transformation events might have occurred per nodule. Occasionally, a nodule did not generate any transgenic fronds.

Simultaneous selection and regeneration of transgenic fronds was much faster than the original sequential method for transforming *L. minor* 8627. Rapid recovery of transformants and the decreased requirements for kanamycin are both valuable aspects of this procedure. While 40–100 mg l^{-1} kanamycin was needed for efficient selection of transgenic nodules on solid NPM, 5 mg l^{-1} was sufficient using liquid SH medium. This difference was due to altered antibiotic susceptibility in the different media environments. For example, non-transformed *L. minor* 8627 nodules were killed within 3 wk, and no regeneration of fronds occurred in liquid SH medium supplemented with 4 mg l^{-1} kanamycin, while non-transformed nodules grew very well on solid NPM containing 10 mg l^{-1} kanamycin for several weeks. Similar observations have been reported in kanamycin sensitivities of wild-type and transformed mango somatic embryos (Mathews and Litz, 1990; Mathews et al., 1992) and selection of cucumber transformants using G418 and hygromycin (Tabei et al., 1994). Tabei et al. (1994) suggested that the liquid environment allows antibiotics to contact the entire surface of explants, thus ensuring reliable selection. This may also be due to the differences in tissue uptake of kanamycin in different media environments or different availability of kanamycin due to its binding to certain gelling agents (Chauvin et al., 1999). A third advantage of this protocol is the use of liquid medium because it may be suitable for automated handling of large numbers of samples.

Analysis of genomic DNA. Total DNA was extracted from selected transgenic duckweed lines as they were generated and subjected to molecular analyses. DNA from initial transformants (*L. gibba* G3 with pBI121) was tested with a GUS construct probe containing the CMV35S promoter and the *nos* terminator and a probe containing the *vir* region of C58. Figure 2 is the hybridization pattern of *L. gibba* G3-D transformant. The genomic integration of the GUS gene construct was demonstrated by the hybridization signal to the high molecular weight undigested DNA and a *Hin*dIII-digested band of a different size from pBI121 linearized with *Hin*dIII (Fig. 2, left panel). A 3 kb *Hin*dIII to *Eco*RI fragment in both C58-z707::pBI121 and *L. gibba* G3-D indicated the integrity of the GUS construct in the transgenic line. A 0.8 kb *Hin*dIII to *Bam*HI fragment corresponds to the CMV35S promoter. Non-transformed *L. gibba* G3 did not hybridize to these probes. The absence of *Agrobacterium* was demonstrated by the lack of hybridization of the *vir* probe to *L. gibba* G3-D DNA (Fig. 2, right panel).

Figure 3 is the DNA analysis of three transformants of two *L. minor* geographic isolates by hybridization with a 2 kb GUS

probe. These transformants (H, F and E) were generated using pCNL56. The *Eco*RI digestion pattern demonstrated that the GUS gene was inserted at independent genome locations. All strains share a 2 kb *Eco*RI to *Bam*HI fragment containing the GUS gene with an intron.

We have demonstrated an efficient protocol to genetically transform two duckweed species, *L. gibba* (G3) and *L. minor* (8627 and 8744), and a second method to rapidly transform *L. minor* 8627. Duckweed is an excellent vehicle for commercial production of biomass and recombinant proteins. Our rapid transformation protocol will enhance its potential for automation, large-scale screening, and industrial applications.

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References

Bergmann, B. A.; Cheng, J.; Classen, J.; Stomp, A.-M. Nutrient removal from swine lagoon effluent by duckweed. Trans. A.S.A.E. 43:263–269; 2000a.

Bergmann, B. A.; Classen, J.; Cheng, J.; Stomp, A.-M. In vitro selection of duckweed geographical isolates for potential use in swine lagoon effluent renovation. Bioresource Technol. 73:13–20; 2000b.

Chauvin, J.-E.; Marhadour, S.; Cohat, J.; Le Nard, M. Effects of gelling agents on in vitro regeneration and kanamycin efficiency as a selective agent in plant transformation procedures. Plant Cell Tiss. Organ Cult. 58:213–217; 1999.

Dellaporta, S. L.; Wood, J.; Hicks, J. B. Maize DNA minipreps. Maize Genet. Coop. Newsl. 57:26–29; 1983.

Doyle, J. J.; Doyle, J. L. Isolation of plant DNA from fresh tissue. FOCUS® 12:13–15; 1990.

Hepburn, A. G.; White, J.; Pearson, L.; Maunders, M. J.; Clarke, L. E.; Prescott, A. G.; Blundy, K. S. The use of pNJ5000 as an intermediate vector for the genetic manipulation of *Agrobacterium* Ti-plasmids. J. Gen. Microbiol. 131:2961–2969; 1985.

Jefferson, R. A. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5:387–405; 1987.

Jefferson, R. A.; Kavanagh, T. A.; Bevan, M. W. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907; 1987.

Kandeler, R. *Lemnaceae*. In: Halevy, A. H., ed. CRC handbook of flowering. Boca Raton, FL: CRC Press; 1985:251–279.

Landolt, E. The family of *Lemnaceae* — a monographic study. 1. Veröff. Geobot. Inst. ETH. Stiftung Rübel (Zürich) 71:1–566; 1986.

Landolt, E. Anatomy of the *Lemnaceae* (duckweeds). Extreme adaptations in angiospermous hydrophytes. Berlin: Gebrüder Borntraeger; 1998:1–127.

Li, Q. X.; Liu, C. N.; Ritchie, S. W.; Peng, J.; Gelvin, S. B.; Hodges, T. K. Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. Plant Mol. Biol. 20:1037–1048; 1992.

Mathews, H.; Litz, R. E. Kanamycin sensitivity of mango somatic embryos. HortScience 25:965–966; 1990.

Mathews, H.; Litz, R. E.; Wilde, H. D.; Merkle, S. A.; Wetzstein, H. Y. Stable integration and expression of β-glucuronidase and NPT II genes in mango somatic embryos. In Vitro Cell. Dev. Biol. Plant 28:172–178; 1992.

Moon, H. K.; Rajbhandari, N.; Stomp, A. M. Effect of media components and phytohormones on in vitro frond proliferation of *Lemna gibba* G3 and 24 additional *Lemna gibba* strains. Plant Resources 1:98–104; 1998.

Moon, H. K.; Stomp, A. M. Effects of medium components and light on callus induction, growth, and frond regeneration in *Lemna gibba* (duckweed). In Vitro Cell. Dev. Biol. Plant 33:20–25; 1997.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–496; 1962.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning. A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

Schenk, R. U.; Hildebrandt, A. C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199–204; 1972.

Tabei, Y.; Nishio, T.; Kurihara, K.; Kanno, T. Selection of transformed callus in a liquid medium and regeneration of transgenic plants in cucumber (*Cucumis sativus* L.). Breeding Sci. 44:47–51; 1994.

Tanaka, O.; Nakayama, Y.; Emori, K.; Takeba, G.; Beppu, T.; Sugino, M. Flower-inducing activity of exogenous lysine in *Lemna paucicostata* 151 cultured on nitrogen-rich medium. Plant Cell Physiol. 35:73–78; 1994.

Tanaka, O.; Nakayama, Y.; Emori, K.; Takeba, G.; Sato, K.; Sugino, M. Flower-inducing activity of lysine in *Lemna paucicostata* 6746. Plant Cell Physiol. 38:124–128; 1997.

Vancanneyt, G.; Schmidt, R.; O'Connor-Sanchez, A.; Willmitzer, L.; Rocha-Sosa, M. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol. Gen. Genet. 220:245–250; 1990.