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Frond transformation system mediated by *Agrobacterium tumefaciens* for *Lemna minor*

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Abstract

The Lemnaceae, known as duckweed, the smallest flowering aquatic plant, shows promise as a plant bioreactor. For applying this potential plant bioreactor, establishing a stable and efficient genetic transformation system is necessary. The currently favored callus-based method for duckweed transformation is time consuming and genotype limited, as it requires callus culture and regeneration, which is inapplicable to many elite duckweed strains suitable for bioreactor exploitation. In this study, we attempted to establish a simple frond transformation system mediated by *Agrobacterium tumefaciens* for *Lemna minor*, one of the most widespread duckweed species in the world. To evaluate the feasibility of the new transformation system, the gene *CYP710A11* was overexpressed to improve the yield of stigmasterol, which has multiple medicinal purposes. Three *L. minor* strains, ZH0055, D0158 and M0165, were transformed by both a conventional callus transformation system (CTS) and the simple frond transformation system (FTS). GUS staining, PCR, quantitative PCR and stigmasterol content detection showed that FTS can produce stable transgenic lines as well as CTS. Moreover, compared to CTS, FTS can avoid the genotype constraints of callus induction, thus saving at least half of the required processing time (CTS took 8–9 months while FTS took approximately 3 months in this study). Therefore, this transformation system is feasible in producing stable transgenic lines for a wide range of *L. minor* genotypes.

Introduction

Plant bioreactors were exploited after conventional microbial fermentation systems and mammalian cell reactors. In recent years, the use of plants as bioreactors has emerged as an exciting area of research and the significant advances have created new opportunities (Desai et al. 2010; Saveleva et al. 2016). The most attractive point of plant bioreactors is the economical large-scale production and the versatility of the transcription-translation apparatus of plants as eukaryotes (Cox et al. 2006; He et al. 2011). For the efficient production of recombinant product, selection of the host species is very important. The life cycle, biomass yield, containment and scale-up costs are factors should be considered (Sharma and Sharma 2009).

The Lemnaceae, known as duckweed, a monocotyledonous group of flowering aquatic plant, comprises the smallest angiosperms in the plant kingdom and is classified into five genera and 37 species (Appenroth et al. 2013;

Gui-Li Yang and Yang Fang have contributed equally to this work and are co-first authors.

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Wang et al. 2014). The potential application of duckweed in plant bioreactors, in addition to wastewater treatment and energy material use, has drawn increasing attention (Lam et al. 2014; Stomp 2005; Zhao et al. 2012). As a potential expression system, duckweed is a better candidate than most plants presently applied (Stomp 2005), which can be explained by the following reasons: (1) As an aquatic non-crop plant, duckweed suits the current social and resource circumstances, as its use neither competes with human for food needs, nor competes with grain crops for land. (2) Being the smallest angiosperm with the fastest doubling time, duckweed proliferates rapidly in a nearly exponential growth manner analogous to that of microorganisms. It has been documented that an annual yield of 55 t/h/y dry biomass could be realized under appropriate conditions (Oron 1994). Undoubtedly, this higher rate of reproduction will greatly shorten the bioreactor production cycle. (3) Simple and inexpensive culture conditions make duckweed suitable for large-scale production. (4) Laboratory-cultured duckweed undergoes completely asexual reproduction without disturbance by pollen, which allows the research materials to be genetically stable (Stomp 2005).

Lemna minor, a Lemnaceae family member, as one of the most widespread duckweed species in the world, has all the advantage above. Moreover, *L. minor* has been well studied due to its rapid growth and high nutritional value (Ge et al. 2012; Iatrou et al. 2015). Therefore, *L. minor* was chosen as the plant bioreactor material in this study. At present, several proteins have already been expressed in *L. minor* by genetic transformation based on the conventional callus induction approach (hereafter called callus transformation system, CTS) (Bertran et al. 2015; Ko et al. 2011; Popov et al. 2006). However, two main problems with this transformation method are hindering current research. One is that tissue culture, especially callus induction and the subsequent frond regeneration, is time consuming and laborious (Chang and Chiu 1976; Khvatkov et al. 2015; Stefaniak et al. 2002). The other bottleneck is that the tissue culture conditions of duckweed vary among species and even ecotypes; some ecotypes cannot achieve callus induction or regeneration, limiting the transformation of some elite strains (Moon and Stomp 1997). Therefore, it is necessary to establish a prompt and efficient genetic transformation method without the use of callus.

To establish a simple and efficient transformation system for *L. minor*, a frond transformation system mediated by *A. tumefaciens* (hereafter called frond transformation system, FTS) was employed. To evaluate the feasibility of this system, the sterol 22-desaturase gene (*CPY710A11*) of tomato was overexpressed to improve the yield of stigmasterol in *L. minor*. Stigmasterol isolated from plants has been proven to have multiple medicinal values, including antioxidant, antitumor, hypoglycemic, hypocholesterolemic, and anti-inflammatory effects (Batta et al. 2006; Gabay et al. 2010; Ghosh et al. 2011; Jamaluddin et al. 1994; Panda et al. 2009). We hope that the FTS can pave the way for further large-scale production of target products via *L. minor* bioreactor and provide a reference for other species of duckweed or other plants.

Materials and methods

Materials and culture conditions

L. minor strain ZH0055 was originally collected from Ya'an, Sichuan Province, China, then was sent to *Elias Landolt* for species identification. *L. mino*r strain D0158 (6580) was originally collected from Bergen, New Jersey, USA. *L. minor* strain M0165 (6591) was originally collected from Escalon, California, USA. They were conserved in the Duckweed Germplasm Bank, Chengdu Institute of Biology, Chinese Academy of Science. They were preserved on Hoagland solid medium (Hoagland and Arnon 1950) supplemented with 1.5% (w/v) sucrose and 0.7% (w/v) agar at pH 5.8, and grown at 25 ± 1 °C with a light intensity of 40 μmol/m^2/s under a 16 h/day photoperiod. Prior to use, the preserved fronds were transferred to Hoagland liquid medium containing 1.5% (w/v) sucrose to preculture for 7 days at 25 ± 1 °C with a light intensity of 100 μmol/m^2/s under a 16 h/day photoperiod.

Binary vector construction

The open reading frame of *CYP710A11* (GenBank: JN388603.1) behind the CaMV35S (cauliflower mosaic virus 35S) promoter followed by the terminator of the nopaline synthase gene was inserted into the binary vector pCAMBIA2301. The binary vector pCAMBIA2301, which contains a neomycin phosphotransferase gene (*NPTII*) and a β-glucuronidase gene (GUS) gene (*uidA*), both driven by the CaMV35S promoter was obtained from the Center for Application of Molecular Biology to International Agriculture (CAMBIA), Australia. The *uidA* gene contains an intron in the coding region (Supplementary Material Fig. 1) to ensure that the observed GUS activity occurs in the plant cell and is not due to the presence of endogenous residual *A. tumefaciens* (Chhabra et al. 2011). The binary vector pCAMBIA2301-CYP710A11 was transferred into the *A. tumefaciens* strain EHA105 using the freeze–thaw method (R Höfgen 1988).

Determination of the phytotoxic level of the antibiotic

To determine the concentration of antibiotic use, the phytotoxic level of G418 was tested. The concentration gradient was set as 0 mg/L, 5 mg/L, 10 mg/L, and 20 mg/L according

to a preliminary result that *L. minor* tolerance of G418 was mostly lower than 20 mg/L. Sterile *L. minor* was cultured in an artificial climate incubator (GZ-300-GSII, Guangzhi Tech, Shaoguan, China) for two weeks with Hoagland liquid medium at 25 ± 1 °C with a light intensity of 100 μmol/m^2/s under a 16 h/day photoperiod for a preculture period of 7 days. Then, five fronds were inoculated into each well of sterile 12-well plates, which contained 5 mL Hoagland medium with various G418 concentrations. Each concentration treatment was repeated three times. The plates were cultured in an artificial climate incubator under the same conditions as preculture. The culture medium was replaced every 5 days, and the number of fronds per well was recorded 15 days later.

The increased number of fronds was calculated as follows:

$$INF = N_t - N_0$$

INF, increased number of fronds after culture for 15 days; N_t, total number of fronds after culture for 15 days; N_0, initial number of fronds, $N_0 = 5$.

Duckweed transformation

Two approaches were used to transform *L. minor* in this research: one is the CTS, and the other is FTS. The media and solutions used in the transformation processes are shown in Table 1.

For CTS, callus induction and transformation followed the protocol described by Chhabra et al. (2011) with minor modification. Sterile fronds were inoculated on the callus induction medium to obtain embryogenic callus. The callus was rapidly submerged in *A. tumefaciens* suspension ($OD_{600} = 0.5$) and infiltrated (-0.08 MPa) for 10 min at room temperature. Then, the callus was cocultured for 4 days in the dark at 25 °C on coculture medium. Afterward, the callus was transferred to callus selection medium. Callus that remained green was subcultured on fresh callus selection medium every 2 weeks. Finally, frond regeneration from callus was induced on frond regeneration medium. Regenerated fronds were transferred to Hoagland liquid medium containing 15 g/L sucrose to expand the culture. Callus induction efficiency, regeneration efficiency, and frond number per callus were calculated using the methods described by Huang et al. (2016). The minimum diameter of callus accepted was approximately 2 mm.

For FTS, the daughter frond was removed without damaging the underlying meristematic zone of mother frond by a plucking motion using forceps. The wounded mother frond with the meristematic zone exposure was transferred to a new sterile conical flask. Then, 100 mL of pretreatment solution was added. The conical flask was sealed with aseptic breathable film and kept in an ice bath for 20 min. Then,

A. tumefaciens suspension ($OD_{600} = 0.5$) was added to the conical flask and vacuum infiltrated (-0.08 MPa) for 10 min at room temperature. Finally, the fronds were removed and placed on a sterile 90*15 mm petri dish with three pieces of filter paper, and 1.5 mL liquid coculture medium was added to coculture at 22 °C in darkness for 4 days. Filter paper with only 1.5 mL liquid coculture medium was used to avoid the overgrowth of *A. tumefaciens*. After coculture, all fronds were directly inoculated on selection culture without washing. The newly produced fronds were subcultured on fresh selection medium every 2 weeks and selected for approximately 3 months.

GUS (β-glucuronidase) staining and GUS-positive rate

Histochemical GUS staining was conducted and analyzed as described by Jefferson (1987). Fronds or calli after coculture with *A. tumefaciens* for 4 days were stained by GUS-solution. The GUS-positive rate was calculated as follows:

$$GR\,(\%) = \frac{N_s}{N_t}$$

GR, GUS-positive rate (%); N_s, number of blue calli or fronds after GUS staining; N_t, total number of calli or fronds.

The transgenic lines obtained were also identified by the same staining method.

DNA extraction, PCR analysis and Tail-PCR analysis

The genomic DNA of *L. minor* was extracted from GUS-positive and nontransformed control *L. minor* using the Plant Genomic DNA Miniprep Kit (Sigma–Aldrich, St. Louis MO, USA) following the protocol provided by the manufacturer.

PCR analysis of putative transgenic plants was performed using the primer set CYP-F and CYP-R (Supplementary Material Table 1), amplifying gene *CYP710A11* (1506 bp). Meanwhile, the *RNR2B* (ribonucleotide reductase 2B, 895 bp) gene, as a reference gene, was amplified by the primer set RNR2B-F and RNR2B-R (Supplementary Material Table 1). *RNR2B* is the ribonucleotide reductase 2B gene in the genome of *L. minor* (https://genomevolution.org/r/ik6h). Transgenic lines were also tested for the absence of *A. tumefaciens* contamination using the primer set RepA-F and RepA-R (Supplementary Material Table 1) which amplified a specific sequence (1000 bp) of the vector, which is located close to the pVS1-REP (replication origin from *pVS1*) of pCAMBIA2301.

The T-DNA insertion sites were determined by the Tail-PCR method (Liu and Whittier 1995). Primers used were shown in Supplementary Material Table 1. All Tail-PCR reactions were carried out in iCycler iQTM (Bio-Rad,

Hercules, California, USA). The specific PCR products from the tertiary TAIL-PCR reactions were extracted from agarose gel with TAKARA MiniBEST Agarose Gel Extraction Kit Ver. 4.0 (TAKARA, Tokyo, Japan). The DNA fragments were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). *L. minor* genome assembly used in the TAIL PCR analysis was obtained from CoGe database (vs1, genome id40199, https://genomevolution.org/r/ik6). *L. minor* clone 5500 was used in the assembly (Van Hoeck et al. 2015).

RNA extraction and qPCR (quantitative polymerase chain reaction) analysis

The total RNA of *L. minor* was extracted using Total RNA Isolation Solution (Generay, Shanghai, China) following the protocol supplied by the manufacturer. First-strand cDNA synthesis and reverse-transcriptase PCR were conducted by using the Rayscript cDNA Synthesis Kit (Generay, Shanghai, China). For qPCR analysis, the gene-specific primer set CYP-F1 and CYP-R1 (Supplementary Material Table 1) was used to amplify the 841 bp specific sequence of *CYP710A11*. The primer set 18S-F and 18S-R (Supplementary Material Table 1) was used to amplify the 124 bp specific sequence of 18S rRNA, which is stably expressed in *L. minor* tissues, as an internal control gene. All reactions were replicated at least three times. Gene expression was analyzed by qPCR using iQ SYBR-Green (Bio-Rad, Hercules, California, USA). The threshold cycle (Ct) values of the qPCR reactions were obtained from three independent biological replicates with three technical replicates each.

Stigmasterol content determination

Five grams of fresh *L. minor* fronds was dried at 60 °C in a hot-air oven (ZRD-A7230, ZHICHENG, Shanghai, China) to a constant weight. The dried samples were milled using a mortar. One hundred milliliters of methanol was added to extract the stigmasterol for 60 min with 500 W ultrasonic treatment. After extraction, the liquid fraction was collected via filtration using filter paper, and the collected liquid fraction was concentrated to approximately 10 mL at 45–55 °C. Then, methane was added to a final volume of 25 mL to obtain the stigmasterol extract. Prior to determination, the stigmasterol extract was filtered using a 0.45 μm syringe filter. A high-performance liquid chromatograph (HPLC) system (Agilent 1260; Agilent Tech, USA) was used for stigmasterol analysis. Ten milliliters of stigmasterol extract was injected and separated by a C-18 column (Capcell Pak, Shiseido, Japan) at 25 °C using a mixture of 85% acetonitrile and 15% methanol as the mobile phase. Stigmasterol was detected by a DAD detector (G1315B, Agilent Tech, USA) at 240 nm. A stigmasterol solution of 1 mg/mL was used as the external standard, which was prepared by dissolving

1 mg of stigmasterol (Sigma–Aldrich, St. Louis, MO, USA) to 1 mL methanol at 40 °C with 500 W ultrasonic treatment for 5 min. All results were obtained from three independent biological replicates with three technical replicates each.

Statistical analysis

All results are expressed as the mean ± standard error in the figures. Statistical analysis was performed using SPSS software (Version 22.0, SPSS Inc., Chicago, IL, USA). ANOVA followed by Student's *t* test was used to determine significant differences, and $P < 0.05$ was considered statistically significant.

Results

Phytotoxic level of antibiotic

To determine the antibiotic dosage for selection, the concentration of G418 was set as 0 mg/L, 5 mg/L, 10 mg/L, and 20 mg/L. Based on the increase in number of fronds after 15 days of treatment, 20 mg/L G418 was used to ZH0055 selection, 15 mg/L G418 was used for D0158 selection, and 10 mg/L G418 was used for M0165 selection (Fig. 1). The addition of antibiotics to the callus and frond selection media was remarkably effective in this study.

Callus transformation system

Three *L. minor* strains (ZH0055, D0158 and M0165) were transformed using the CTS as controls in this study.
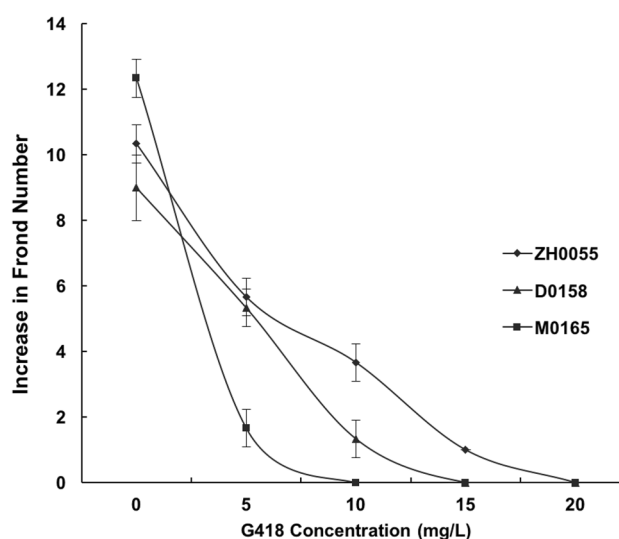
Fig. 1 Resistance to G418. Bars indicate the standard deviation from the mean

Table 1 Media and solutions used in the transformation process

Medium	Basic medium	Supplements									Other
		Sucrose (g/L)	2,4-D (mg/L)	2IL (mg/L)	IAA (µM)	KT (µM)	Gelrite (g/L)	AS (µM)	Timentin (mg/L)	Paromomycin (mg/L)	
CIM	MS medium	30	10	0.5	–	–	3	–	–	–	–
PS	ddH$_2$O	30	–	–	–	–	–	–	–	–	20 mg/L glutamine
ATS	MS medium	–	–	–	–	–	–	200	–	–	A. tumefaciens EHA105
CCM	MS medium	30	10	0.5	–	–	3	200	–	–	–
CSM	MS medium	30	10	0.5	–	5	3	–	100	100	20 mg/L G418
FRIM	MS medium	30	–	–	25	5	3	–	100	100	–
FSM	MS medium	30	–	–	25	5	3	–	100	100	10, 15, 20 mg/L G418

CIM callus induction medium; *PS* pretreatment solution; *ATS Agrobacterium tumefaciens* suspension; *CCM* coculture medium; *CSM* callus selection medium; *FRIM* frond regeneration induction medium; *FSM* frond selection medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2IP, 6- (-γ, -γ-dimethyl allyl amino) purine; *AS* acetosyringone; *IAA* indole-3-acetic acid; *KT* N6-furfuryladenine. "–" indicates not added. The pH of all the solutions and media mentioned above were adjusted to 5.8 using 1 M NaOH

However, only ZH0055 was induced to form callus successfully, indicating that tissue culture of duckweed is limited by genotype. The callus induction and regeneration of ZH0055 are presented in Fig. 2. Yellow fragile callus was induced by repeated subculture every 2 weeks (Fig. 2a). Microscopic observation revealed that the yellow fragile callus was composed of mainly meristematic cells. The cells are approximately 20 µm and always uniform, densely packed, and highly regenerative (Fig. 2b). A part of the meristematic callus of ZH0055 regenerated successfully (Fig. 2c) and formed normal fronds after expanding culture (Fig. 2d). The callus induction efficiency of ZH0055 reached up to 92%, while the regeneration efficiency was relatively low at only 30%. In addition, the frond number per callus was 4.67 ± 1.16. The expression of the GUS gene was detected after callus coculture with *A. tumefaciens* for 4 days (Fig. 2e, f). Nine months later, all transgenic lines of ZH0055 could be passaged stably by vegetative propagation (Fig. 2g, h). The rate of stable transgenic line formation from infected ZH0055 callus was 4%. Twenty-four transgenic lines were obtained from ZH0055 by CTS.

Frond transformation system

Three *L. minor* strains (ZH0055, D0158 and M0165) were transformed successfully by FTS. Individual fronds were separated from one *L. minor* plant, and the meristematic regions of the frond were scratched with a scalpel (Fig. 3a). After coculture with *A. tumefaciens* for 4 days (Supplementary Material Fig. 2d), all ZH0055, D0158 and M0165 showed expression of the GUS gene (Supplementary Material Fig. 2a–c). One plant was separated into three fronds and transferred to a new conical flask, and all wounded fronds were alive and green in the beginning (Fig. 3b). Then, most of the fronds became bleached and died, while new fronds emerged from a few of the meristematic zones of the wounded fronds (Fig. 3c; Supplementary Material Fig. 2e; Supplementary Material Fig. 2f). The new fronds were subcultured on new frond selection medium every 2 weeks. Stable transgenic lines were obtained (Fig. 3d–h) and showed normal phenotypes (Supplementary Material Fig. 2g). The rates of stable transgenic lines formation from the infected fronds of ZH0055, D0158 and M0165 were 2%, 6% and 2%, respectively. The numbers of transgenic lines obtained from ZH0055, D0158 and M0165 by FTS were 12, 35 and 13, respectively. Stable transgenic lines of *L. minor* strains ZH0055, D0158 and M0165 were achieved in 3 months by FTS, much faster than by CTS (Fig. 4).

GUS staining

To compare *A. tumefaciens* infection rate of the CTS and FTS, GUS-positive rates were detected after coculture with
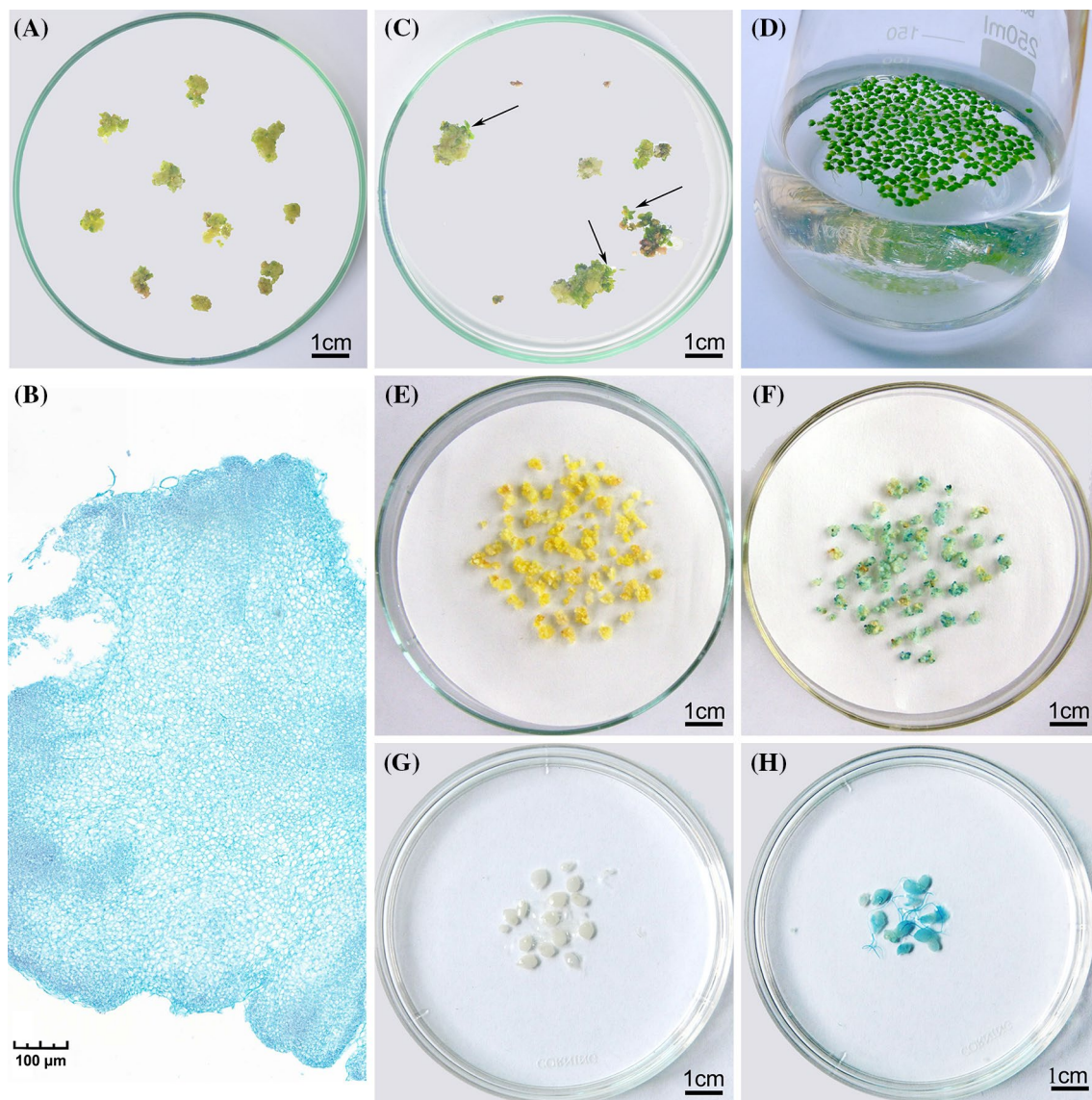
Fig. 2 Tissue culture and callus transformation of *Lemna minor* ZH0055. **a** Yellow fragile callus. **b** Microscopical investigation of the callus, callus was embedded in paraffin and cut into slices, and then stained with safranin and fast green. **c** Fronds regeneration; black arrows indicate the regenerated fronds. **d** Fully regenerated fronds in Hoagland medium. **e** GUS staining of wild-type callus. **f** GUS staining of callus after coculture with *Agrobacterium tumefaciens* for 4 days. **g** GUS staining of the wild type. H GUS staining of a stable transgenic line

A. tumefaciens. The GUS-positive rate of FTS was relatively lower than that of CTS, but it was still between 30% and 40%. Moreover, the GUS-positive rates of ZH0055, D0158 and M0165 were very similar, which indicated that *A. tumefaciens* infection rate is varies little among different genotypes when applied as part of FTS (Fig. 5).

To confirm positive transgenes, GUS was used as a reporter gene. The expression and location of the GUS gene in transgenic lines can be accurately identified by GUS staining because of its high sensitivity. GUS staining showed that the GUS gene was stably expressed in transgenic *L. minor* from both CTS and FTS (Fig. 2g, h; Fig. 3d–g). Microscopic observation revealed that almost every cell of the transgenic *L. minor* expressed the GUS gene (Fig. 3h).

Detection of the *CYP710A11* gene

To determine whether the gene *CYP710A11* was integrated into the genome of *L. minor*, PCR detection was carried out. Genomic DNA isolated from GUS-positive and wild-type *L. minor* was used for PCR amplification. DNA electrophoresis showed that only bands representing *RNR2B* were detected in the wild type, while bands representing both *RNR2B* and *CYP710A11* were clearly detected in the transgenic
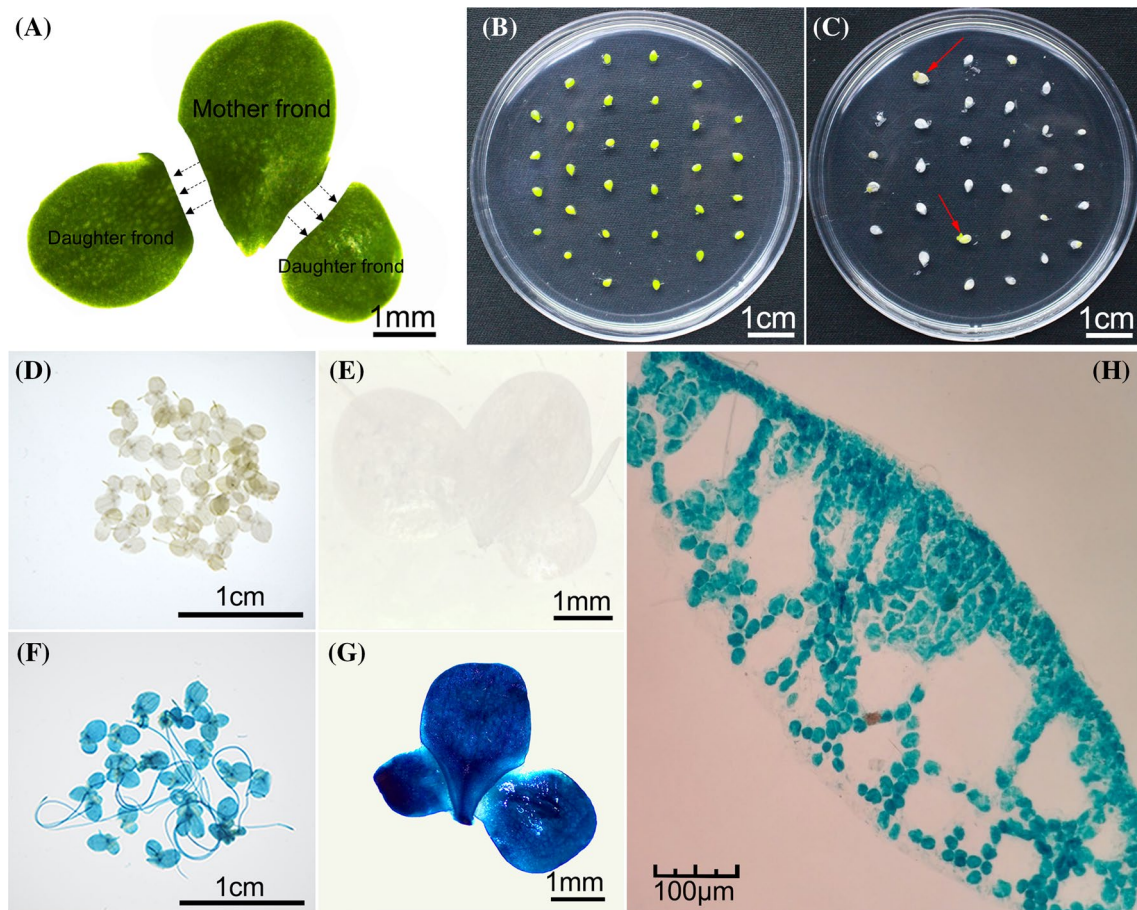
Fig. 3 Frond transformation of *Lemna minor* ZH0055. **a** The daughter frond was removed without damaging the underlying meristematic zone of mother frond by a plucking motion using forceps. **b** At the beginning of selection culture, all fronds were green. **c** During selection culture, red arrows indicate the new fronds that were generated from some fronds, while most of the fronds became bleached. **d, e** GUS staining of the wild type. **f, g** GUS staining of a stable transgenic line. **h** Microscopy of a stable transgenic frond
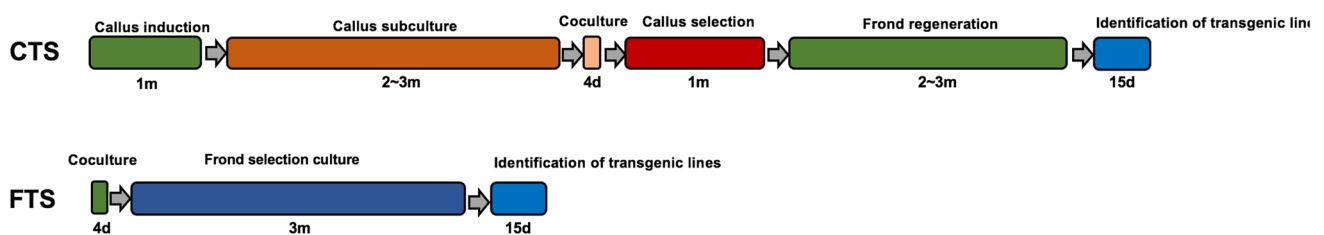
Fig. 4 Timelines of callus transformation system and frond transformation system. *m* months; *d* days

lines, which suggested that all transgenic lines contained *CYP710A11*. In addition, bands representing *CYP710A11* and *RepA* were detected in the plasmid, while no *RepA* band was detected in the transgenic lines, which suggested that contamination by *A. tumefaciens* was not detected in the analyzed samples (Fig. 6). The results of the experiment indicated that *CYP710A11* was successfully transferred into *L. minor* by both CTS and FTS.

To determine the T-DNA insertion sites, *L. minor* genomic DNA flank and the inserted T-DNA left border was cloned by Tail-PCR using T-DNA specific primers together with five arbitrary degenerate primers (Supplementary Material Table 1). Two transgenic lines (D0158-FT3 and M0165-FT3) were chosen to perform Tail-PCR. When the PCR products were sequenced, only one sequence of each transgenic line was obtained,
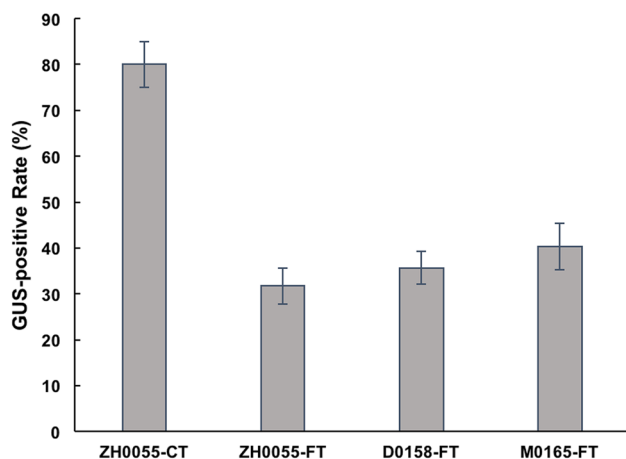
Fig. 5 GUS-positive rates of the two transformation methods. Columns, average transformation efficiency of three repetitions from each duckweed strain; bars, standard deviation in each case

suggesting T-DNA likely integrated into *L. minor* genome in manner of single copy. The T-DNA insertion sites in *L. minor* genome were the 226 bp of contig_5064 and the 10,174 bp of contig_13868 in the transgenic line D0158-FT3 and M0165-FT3, respectively (Fig. 7).

Expression of the *CYP710A11* gene in *Lemna minor*

To study the expression of *CYP710A11*, qPCR for wild-type and transgenic *L. minor* was performed. As shown in Fig. 8, differential expression levels of *CYP710A11* mRNA were detected in all transgenic lines but not in wild types. A paired *t*-test of the relative expression levels of *CYP710A11* indicated that all transgenic lines showed significantly (P < 0.01) higher expression than the wild types did. In addition, the ZH0055 transgenic lines transformed via each system showed no significant (P > 0.05) difference, while the relative expression levels of *CYP710A11* among ZH0055, D0158 and M0165 showed significant (P < 0.05) differences. Among them, M0165 showed the highest expression of *CYP710A11*, D0158 showed an intermediate expression level, and ZH0055 showed the lowest expression of *CYP710A11*.

## Stigmasterol content of transgenic lines

To test the effects of introducing *CYP710A11* into *L. minor*, the content of the downstream product stigmasterol was measured by HPLC. Under the same culture condition, three stable transgenic lines and the wild type of each *L. minor* material were used for HPLC measurement. The results showed that the stigmasterol contents of all transgenic lines were significantly (P < 0.01) higher than those of the wild types, reaching 397.08% higher than that of the wild type in M0165.
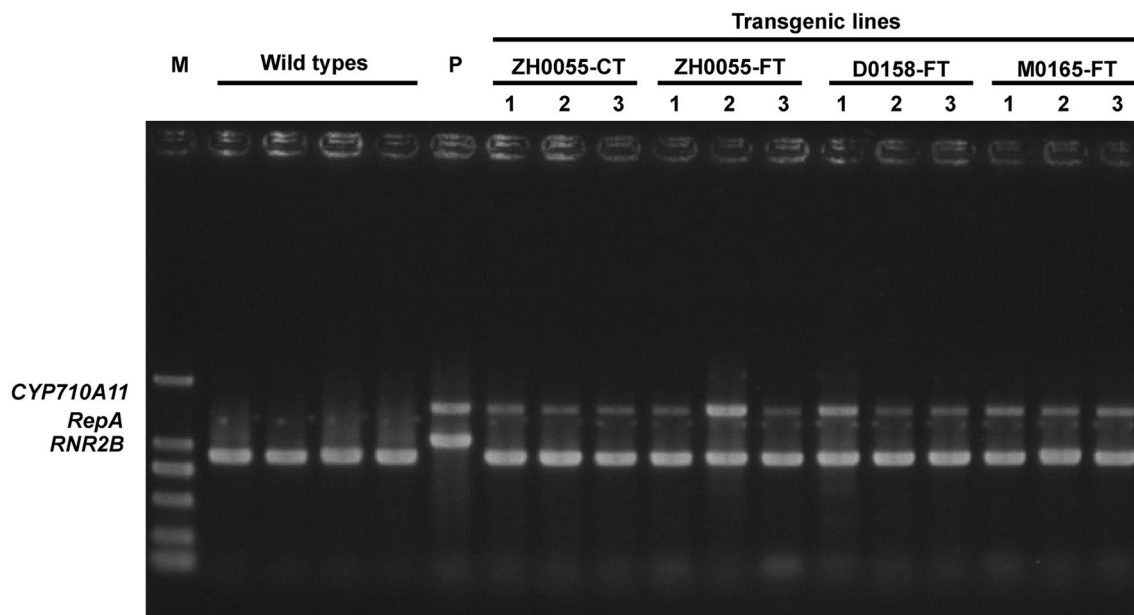
Fig. 6 PCR analysis of *CYP710A11* in the genomes of transgenic duckweed lines. M, 2000 bp marker DNA; Wild types, lanes from left to right represent wild-type *Lemna minor* ZH0055 for callus transformation, wild-type *L. minor* ZH0055, wild-type *L. minor* D0158 and wild-type *L. minor* M0165 for frond transformation, respectively; P, plasmid of *Agrobacterium tumefaciens*; Transgenic lines, stable transgenic duckweed; CT, transgenic duckweed by callus transformation; FT, transgenic duckweed by frond transformation; 1, 2, and 3, three transgenic lines of each duckweed strain
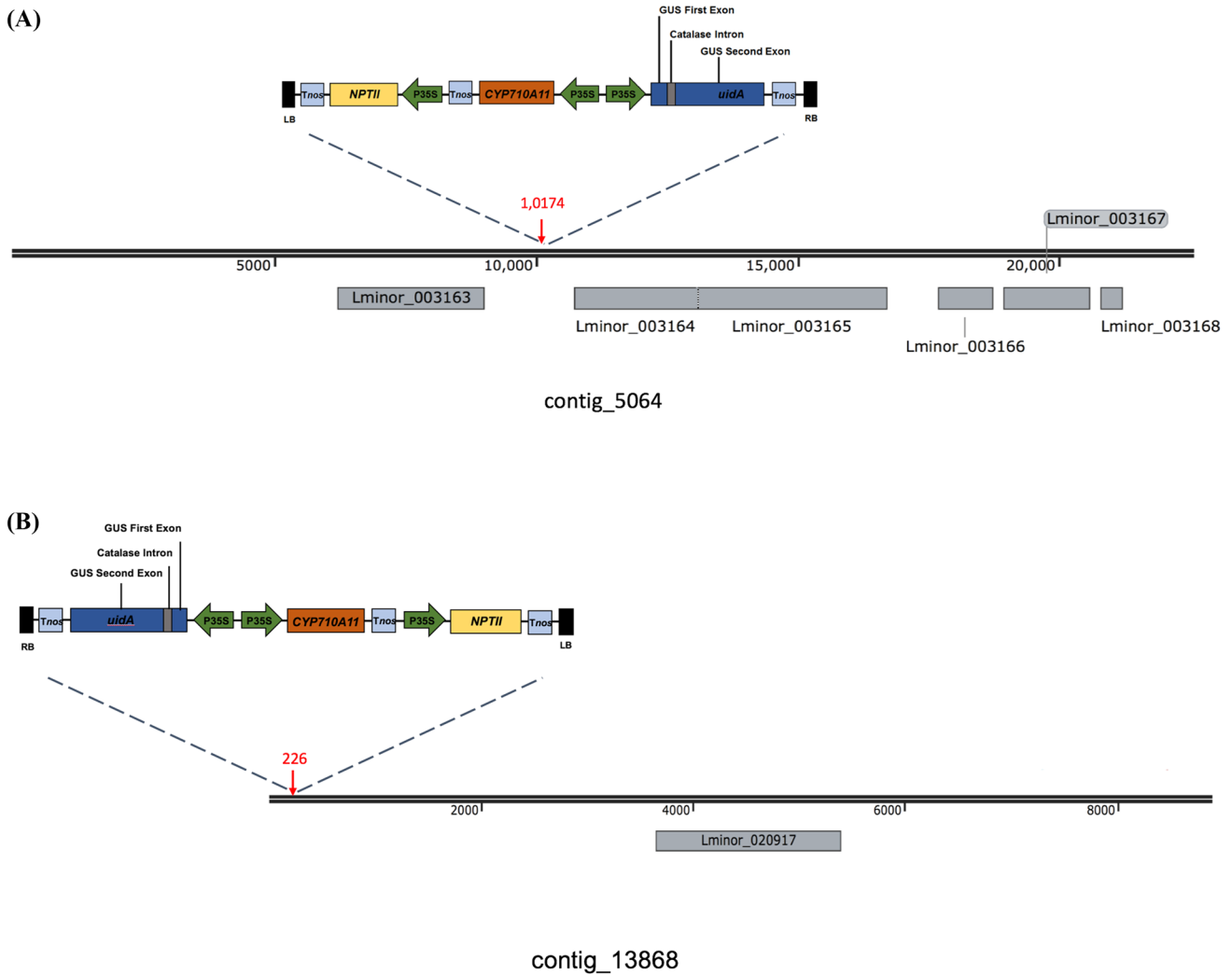
Fig. 7 Distribution of upstream and downstream genes at the insertion sites of transgenic lines D0158-FT3 and M0165-FT3. **a** Distribution of upstream and downstream genes at the insertion sites of transgenic line D0158-FT3. Lminor_020917, DEAD-box ATP-dependent RNA helicase 21. **b** Distribution of upstream and downstream genes at the insertion sites of transgenic lines M0165-FT3. Lminor_003163, Serine/threonine-protein phosphatase PP-X isozyme 2 (PPX2); Lminor_003164, unknown function; Lminor_003165, Fimbrin-5(FIM5); Lminor_003166, Fasciclin-like arabinogalactan protein 7(FLA7); Lminor_003167, Rho GDP-dissociation inhibitor 1 (GDI1); Lminor_003168, Probable calcium-binding protein CML22 (CML22)

Moreover, the content of stigmasterol and the expression of *CYP710A11* showed positive correlations. Interestingly, the stigmasterol content of ZH0055 transgenic lines obtained from the two different transformation systems showed no significant (P > 0.05) difference (Fig. 9) in this study. The results of stigmasterol detection showed that overexpression of *CYP710A11* in *L. minor* can increase the production of stigmasterol.

Discussion

Transformation mediated by *A. tumefaciens* is a standard technique for genetic modification of higher plants, a term that includes both dicotyledonous and monocotyledonous species (Komari and Kubo 1999). Most techniques that introduce foreign genes to generate transgenic plants depend on the dedifferentiation of organ and redifferentiation of transformed cells. Several studies on genetic engineering of duckweed have been performed based on the CTS. However, callus induction and regeneration depend on genotypes, presenting a major bottleneck (Bertran et al. 2015; Ko et al. 2011; Popov et al. 2006; Huang 2016; Moon and Stomp 1997). The Duckweed Germplasm Bank in our laboratory contains all 37 species of duckweed resources and nearly 800 different genotype strains. However, not all of these duckweed strains could be induced to form callus. The FTS, which avoids this restriction will allow better use of these elite duckweed resources. To evaluate the effects of the FTS,

Fig. 8 Quantitative PCR analysis of *CYP710A11* mRNA levels in transgenic duckweed. *W* wild types; *CT* transgenic duckweed by callus transformation; *FT* transgenic duckweed by frond transformation; 1, 2, and 3, three transgenic lines of each duckweed strain. Columns, average relative expression of three repetitions from each RNA sample; bars, standard deviation in each case, asterisks (*$P < 0.05$, **$P < 0.01$) indicate significant differences from the wild type
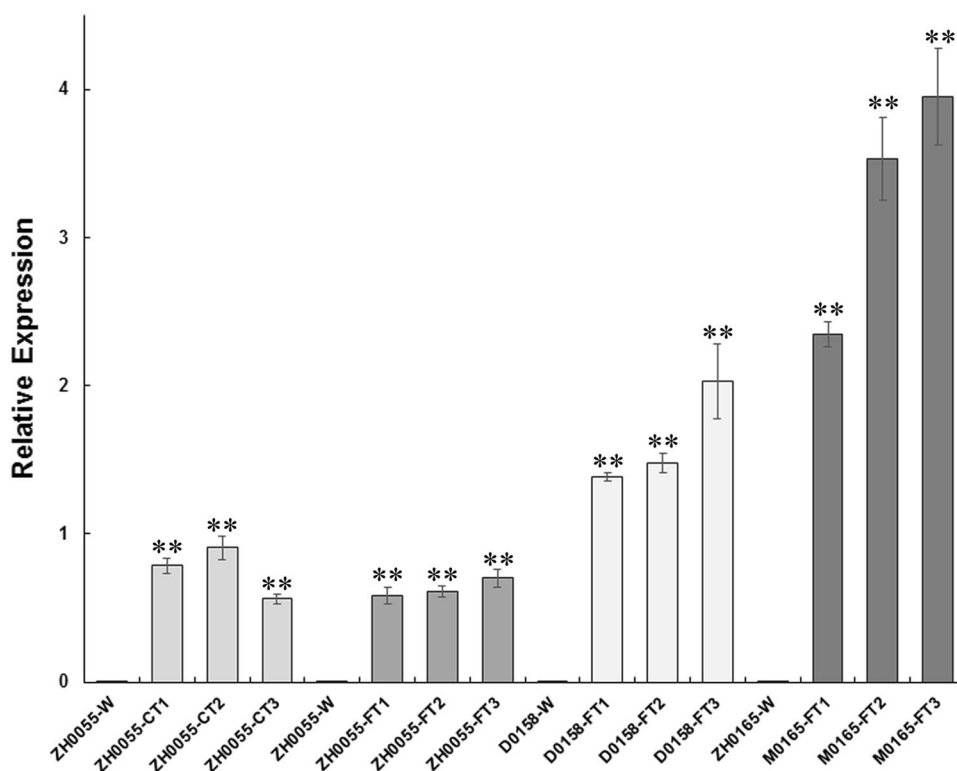
Fig. 9 Stigmasterol content of transgenic duckweed. W, wild types; CT, transgenic duckweed by callus transformation; FT, transgenic duckweed by frond transformation; 1, 2, and 3, tree transgenic lines of each duckweed strain; FW, fresh weight. Columns, average stigmasterol content of three repetitions from each sample; bars, standard errors in each case; asterisks (*$P < 0.05$, **$P < 0.01$) indicate significant differences from the wild type
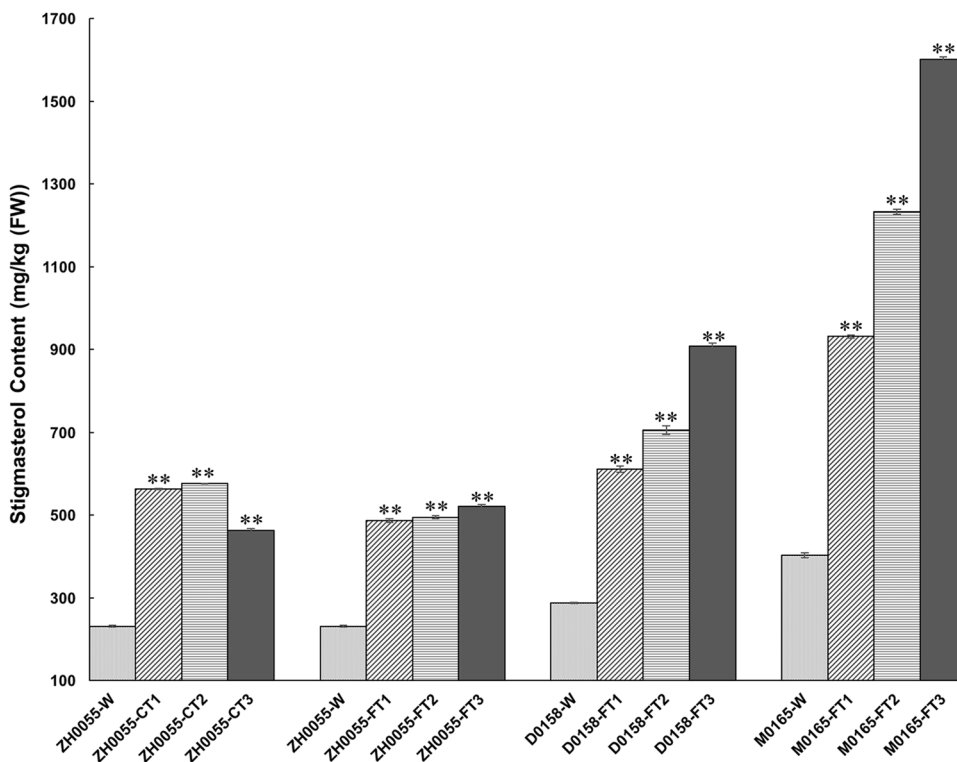
we transformed three different genotypes of the same species *L. minor* (ZH0055, D0158 and M0165). Due to geographical isolation, these strains have different genotypes. In this study, only ZH0055 was induced to form callus, while D0158 and M0165 were failed at callus induction. Furthermore, we compared the results of CTS and FTS. Using the

new system, transgenic plant lines from the three *L. minor* strains were obtained efficiently, and the process was not subject to genotype restriction. GUS staining, PCR, qPCR and stigmasterol detection demonstrated that the function of FTS was as efficient as that of CTS. Although the *A. tumefaciens* infection rate of CTS is higher than that of FTS (Fig. 5), the regeneration rate of ZH0055 was low, at only 30%. In addition, the callus induction and regeneration of ZH0055 took a long time, nearly threefold longer than the time taken by FTS to obtain stable transgenic lines. The latest report showed that callus induction and regeneration still takes 9 weeks and is only suitable for several strains of *Landoltia punctata* (Huang et al. 2016). Therefore, FTS can avoid the genotype restriction of callus induction. Moreover, it saves time.

To determine the selective agent dosages for the different *L. minor* materials, the phytotoxic level of the antibiotic was tested. Kanamycin was the most commonly used selective agent for duckweed in previous studies. However, high level of kanamycin was used during selection (Ko et al. 2011; Vunsh et al. 2007). This lower sensitivity may allow the regeneration of untransformed plant cells on kanamycin-containing medium. Generally, kanamycin is very effective for transgenic dicot species but not for many monocots (Wilmink and Dons 1993), while the antibiotic G418 was tested as an alternative selectable marker shown to be more effective in various monocots, such as *Gramineae* (Dekeyser et al. 1989; Ozias-Akins et al. 1988), perhaps due to more effective binding to ribosomes. Therefore, G418 was chosen as the selective agent for *L. minor*, a monocotyledon. Finally, the G418 dosage for the lines ZH0055, D0158 and M0165 were 20 mg/L, 15 mg/L and 10 mg/L in selection culture, respectively. The results of cellular GUS staining of *L. minor* cells showed that G418 is remarkably effective for *L. minor* selection.

The existence of chimerism may be the main problem to be worried about when applying this simple FTS. The bother of chimerism seems to be more frequent than originally thought and it has been reported in several plants, including tobacco (Schmülling and Schell 1993), soybean (Christou 1990), potato (Rakosy et al. 2007), rice (Christou and Ford 1995), and strawberry (Mathews et al. 1995). In this study, the problem of chimerism can be settled by repeatedly subculturing on frond selection medium containing the selective agent. This issue is also the reason we selected the more effective antibiotic (G418) and its concentration. Low sensitivity to antibiotics may lead to the formation of chimeras (Dominguez et al. 2004). After selection for 3 months, stable transgenic lines were generated, and almost all cells of the transgenic lines were found to show the expression of the GUS reporter gene (Fig. 3h). One possible explanation is that duckweed usually reproduces asexually. The leaves of duckweed are commonly termed as fronds and are not considered leaves by the strict botanical definition. Unlike the ordinary leaves of most plants, new fronds emerge from meristematic zones of their parent fronds and generate a whole plant (Les et al. 2002). The FTS is effective because the meristematic cells were transformed when the meristems were wounded, and then the transgenic daughter fronds arose on the selective culture medium. The expression of the GUS gene was observed at the wounded meristems by GUS staining after 4 days of coculture with *A. tumefaciens* (Supplementary Material Fig. 2a–c). After multiple passages, stable transgenic lines were obtained (Fig. 3d–h). This phenomenon is similar to that of stable wheat transformation based on shoot apical meristems (SAMs) which avoiding the problems arise in tissue culture (Hamada et al. 2017).

Plants as bioreactors are the most attractive objects in the biotechnology field, and a major advantage of plants is their autotrophic type of nutrition (Saveleva et al. 2016). In contrast, most heterologous expression systems based on bacteria, yeast, and cell cultures require high-cost propagators and media for growth (Itakura et al. 1977; Moreira 2007; Wildt and Gerngross 2005). The utilization of transgenic *L. minor* as a bioreactor makes it possible to produce large amounts of biomass with low labor costs and little capital investment, which benefits commercial-scale production. Phytosterols, secondary metabolites including stigmasterol, possess many therapeutic properties. To date, stigmasterol isolated from plants has been proved to have antioxidant, antitumor, hypoglycemic, hypocholesterolemic, and anti-inflammatory effects (Batta et al. 2006; Gabay et al. 2010; Ghosh et al. 2011; Jamaluddin et al. 1994; Khanna et al. 2007; Panda et al. 2009; Pandith et al. 2013). It also serves as a precursor for the synthesis of synthetic progesterone, adrenocortical hormone and ecdysteroids (Kovganko and Survilo 2000; Sundararaman and Djerassi 1977). Fortunately, the biosynthetic pathway of stigmasterol is very well understood. In brief, β-sitosterol is catalyzed by sterol 22-desaturase to produce stigmasterol. Previously, overexpressing the sterol 22-desaturase gene had been reported to enhance stigmasterol production in tomato (Raksha et al. 2016; Whitaker and Gapper 2008). Similarly, stigmasterol production in all transgenic lines significantly increased after the sterol 22-desaturase gene (*CYP710A11*) was transferred into *L. minor* in this study (Fig. 9). Therefore, FTS will allow rapid exploitation of the *L. minor* bioreactor system, because it is not restricted by genotypes amenable to callus induction. In the future, *L. minor* bioreactor can be used to express additional useful products, such as monoclonal antibodies and cytokines.

Conclusion

In summary, we established a rapid, simple, efficient and stable frond transformation system mediated by *A. tumefaciens* for *L. minor*. This method has advantages over the CTS. The FTS overcomes the genotype restriction of *L. minor* callus

induction, and shortens the time required for genetic transformation via simplify the complex regeneration progress. By frond transformation, the foreign gene was expressed effectively and the product yield increased significantly in transgenic *L. minor*. This study could be utilized to transform a wide range of *L. minor* genotypes and will pave the way for the exploitation of *L. minor* as a bioreactor.

Author contributions HZ, XRM, GLY and YF conceived the research. GLY and XRM performed experiments. GLY and XRM analyzed the data and wrote the manuscript. YLX, LT, QL, YL, FL, YLJ, APD, and KZH contributed partial experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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