Genetic transformation of Indian isolate of *Lemna minor* mediated by *Agrobacterium tumefaciens* and recovery of transgenic plants

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Abstract Transgenic plants of an Indian isolate of *Lemna minor* have been developed for the first time using *Agrobacterium tumefaciens* and hard nodular cell masses ‘nodular calli’ developed on the BAP - pretreated daughter frond explants in B₅ medium containing sucrose (1.0 %) with 2,4-D (5.0 μM) and 2-iP (50.0 μM) or 2,4-D (50.0 μM) and TDZ (5.0 μM) under light conditions. These calli were co-cultured with *A. tumefaciens* strain EHA105 harboring a binary vector that contained genes for β-glucuronidase with intron and neomycin phosphotransferase. Transformed cells selected on kanamycin selection medium were regenerated into fronds whose transgenic nature was confirmed by histochemical assay for GUS activity, PCR analysis and Southern hybridization. The frequency of transformation obtained was 3.8 % and a period of 11–13 weeks was required from initiation of cultures from explants to fully grown transgenic fronds. The pretreatment of daughter fronds with BAP, use of non-ionic surfactant, presence of acetosyringone in co-cultivation medium, co-culture duration of 3 d and 16 h photoperiod during culture were found crucial for callus induction, frond regeneration and transformation of *L. minor*. This transformation system can be used for the production of pharmaceutically important protein and in bioremediation.

Keywords *Lemna minor* · Tissue culture · *Agrobacterium*-mediated transformation · Transgenic plants · Pharmaceutical proteins

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	6-(-γ,-γ-dimethyl allyl amino) purine
BAP	6-benzylaminopurine
IAA	Indole-3-acetic acid
Kin	Kinetin
TDZ	Thidiazuron
CaMV	Cauliflower mosaic virus

Introduction

Lemna minor belongs to a group of small floating aquatic plants commonly called duckweeds, found worldwide and often seen growing in thick, blanket-like mats on still, nutrient-rich fresh and brackish waters. Duckweeds are monocotyledons belonging to the family *Lemnaceae* and are classified as higher plants, or macrophytes (Landolt 1986; Les et al. 2002). However, Duckweeds are now considered to be a branch of the arum or aroid family (*Araceae*), the name ‘*Lemnaceae*’ is rapidly falling out of use among taxonomists, who treat it as a subfamily called *Lemnoideae* (Cabrera et al. 2008). The growth response of *L. minor* is similar to microbial cultures. The plant rapidly proliferates through vegetative budding from the meristematic regions, present at the ventro-lateral positions away from the midrib of the frond. These regions are protected by tissue flaps, making a pocket like structure. Fronds bud alternately from these pockets. Doubling times varies from 36 to 72 h depending upon the culture conditions including availability of nutrients and water temperature (Landolt 1986; Brain and Solomon 2007). Duckweed grown under ideal conditions will commonly have a crude fiber content of 5–15 % and a protein content of 35–45 % with a better

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array of amino acids similar to animal protein (Skillicorn et al. 1993; Yilmaz et al. 2005). The duckweeds have been commercially used mainly as a feed for animals, poultry and fish, as human food additives (Haustein et al. 1987; Porath 1993; Men et al. 1995, 1997) and as a bioindicator for environmental pollution (OECD guidelines 2002; Cheng and Stomp 2009; Khellaf and Zerdaoui 2009; Hagner et al. 2010; Leblebici and Aksoy 2010). The high protein content makes the duckweed an ideal choice to develop as an efficient, plant-based system for the production of recombinant proteins. For commercial production of valuable polypeptides, a duckweed-based system has a number of advantages over existing microbial or cell culture systems. Plants demonstrate post-translational processing that is similar to mammalian cells, overcoming one major problem associated with the microbial cell production of biologically active mammalian polypeptides, and it has been shown by others (Cox et al. 2006) that plant systems have the ability to assemble multi-subunit proteins, an ability often lacking in microbial systems. The duckweed can be grown in fully contained and controlled biomass production vessels, making the system's integration into existing protein production industrial infrastructure far easier. The commercial application of duckweeds for large scale production of recombinant proteins and bioremediation requires a reliable and efficient genetic transformation protocol (Yamamoto et al. 2001). However, attempts to transform *Lemnaceae* species have been only partially successful because of limited availability of efficient callus induction and regeneration protocols (Li et al. 2004; Chhabra et al. 2007). The gene transfer in duckweed in general and in *L. minor* in particular has, so far, been difficult and challenging because of their inherent recalcitrance to *in vitro* culture and regeneration from cells and calli. Genetic transformation via *Agrobacterium* in *L. gibba* G3 and *L. minor* 8627 and 8744 (Yamamoto et al. 2001) and via particle bombardment in *Wolffia columbiana* (Kruse et al. 2002) have been attempted, although stable transformation still awaits optimization (Bog et al. 2010).

The only available protocol reporting the transfer of *nptII* and *gusA* genes to *L. minor* (Yamamoto et al. 2001) relied solely on the natural gene transfer ability of the *Agrobacterium tumefaciens*. The conditions influencing host tissue competence and *Agrobacterium* virulence, and in consequence the transformation frequency were not optimized. It is known that different duckweed isolates may differ in physiological properties including regeneration potential, hormonal requirements and susceptibility to *A. tumefaciens*. Consequently, the protocols of regeneration and transformation should be adapted to the peculiarities of used duckweed isolates. Accordingly, the present investigation provides methods and compositions that increase the

efficiency of the duckweed gene expression system as a tool to produce biologically active polypeptides.

Materials and methods

Plant material and preparation of explant

A local Indian isolate of *L. minor* collected from the place near to New Delhi, India and maintained in our laboratory from the last six years, was used in the present study. The young daughter fronds manually separated from the 7-d-old uniform sized fully expanded mother fronds (pre-cultured on Hoagland's medium containing 10 μM BAP) were used as explants. The roots of the fronds were either removed completely or shortened with the help of a sterile surgical blade.

Callus induction

The hard nodular cell masses termed as 'nodular calli' were induced on the BAP preconditioned explants cultured on the Gamborg's B₅ medium (Gamborg et al. 1968) containing 1 % sucrose (B₅S1.0) supplemented with filter sterilized phytohormones either in a combination of 2,4-D (5.0 μM) and 2-iP (50.0 μM) or 2,4-D (50.0 μM) and TDZ (5.0 μM) and 0.35 % phytigel (Sigma, USA), pH 5.7. Ten to sixteen explants per Petri dish were cultured with their adaxial surfaces in contact with the medium dispensed in 90 mm $\times$ 15 mm glass Petri dishes (approximately 20 ml per Petri dish). The explants were periodically checked for callusing under a light microscope. All the cultures were maintained at 25 ± 2 °C under 16 h photoperiod of cool-white fluorescent light of 80 $\mu\text{Em}^{-2} \text{ s}^{-1}$ intensity.

Binary vector and *Agrobacterium* strain

The binary vector pCambia2301 that contains a neomycin phospho-transferase gene (*nptII*) and a β - glucuronidase (GUS) gene (*uidA*), both driven by CaMV35S promoter was obtained from the Center for Application of Molecular Biology to International Agriculture (Cambia), Australia. It was mobilized into the disarmed *Agrobacterium* strain EHA105 by triparental mating. The mobilization was confirmed by colony PCR and Southern hybridization analysis.

The *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCambia2301 was used for transformation studies. The *uidA* gene contains an intron in the coding region to ensure that the observed GUS activity occurred in the plant cell and not due to the presence of endogenous residual agrobacterial cells (Fig. 1).

Fig. 1 Schematic representation of the T-DNA (5.3 kb) of pCAMBIA2301 containing the β -glucuronidase (GUS) gene (*uidA*) with intron (3.2 kb) and neomycin phosphotransferase (*nptII*) gene (2.1 kb) each with CaMV35S promoter sequences. Bar line represent the

region of the probe prepared for southern hybridization. The position of the *HindIII* is indicated on the T-DNA. No other *HindIII* sites are present on pCAMBIA2301 (total size: 11.6 kb). LB/RB left and right T-DNA border sequences

Optimization of transformation protocol

To establish a gene transfer protocol for *L. minor*, various parameters influencing transformation efficiency were optimized. Two days prior to the transformation experiment, *A. tumefaciens* (pCAMBIA2301) from a freshly streaked plate, was inoculated in 5 ml of liquid YEM medium (yeast extract - 1.0 g l⁻¹, mannitol - 10.0 g l⁻¹, NaCl - 0.1 g l⁻¹, MgSO₄·7H₂O - 0.2 g l⁻¹, K₂HPO₄ - 0.5 g l⁻¹, pH-7.2) containing rifampicin (50 mg l⁻¹) and kanamycin (50 mg l⁻¹) and grown overnight on a rotary shaker (200 rpm) at 28 °C. Next day, 0.5 ml of this primary culture was inoculated in 20 ml liquid YEM medium containing the same concentrations of rifampicin and kanamycin (50 mg l⁻¹ each) to raise a rapidly proliferating secondary culture of *Agrobacterium*. The bacteria were centrifuged at 5,000 rpm for 10 min at RT and resuspended in liquid co-cultivation medium (B₅S1.0). Approximate concentration of the bacteria ranged between 10⁵ and 10⁹ cells ml⁻¹ was measured by O.D. at 600 nm. The nodular calli obtained from the preconditioned explants on callogenic medium as stated in earlier section, were dipped in an aqueous solution of a nonionic surfactant, Tween 20 (0–0.4 %) and were inoculated by immersing in bacterial suspension for 5–30 min with gentle shaking at 80 rpm. These nodular calli were blotted dry on sterile filter paper and co-cultivated on filter paper discs soaked in liquid B₅S1.0 co-cultivation medium adjusted to pH (5.2–6.2), for 1–3 days at 22–28 °C under light or dark conditions. The effect of phenolic-acetosyringone (50–100 μ M) during co-cultivation was also tested. After co-cultivation, nodular calli were washed with sterile distilled water for five to six times, blotted dry on sterile paper towel, and incubated in X-gluc solution at 37 °C for histochemical GUS assay (Jefferson et al. 1987). For each variable in an experiment,

35 nodular calli, each weighing 0.025 g, were taken and each experiment was repeated thrice. The data were subjected to analysis of variance and significant differences were selected by Newman-Keul's multiple range test (Brunning and Kintz 1977). The frequency of transient GUS expression was calculated as the number of nodular calli showing blue spots out of the total number of nodular calli treated.

Selection and regeneration of transformants

After 3 - d of co-cultivation, the nodular calli were washed several times with sterile distilled water containing 500 mg l⁻¹ cefotaxime to kill the *Agrobacterium* cells and were transferred to the semisolid selection medium (B₅S1.0 medium containing kinetin (5.0 μ M), IAA (25.0 μ M), kanamycin (125 mg l⁻¹) and cefotaxime (500 mg l⁻¹) for the selection of transformed fronds.

The cultures were maintained initially for 10–12 days on the selection medium with 125 mg l⁻¹ of kanamycin but subsequently the kanamycin concentration was reduced to 75 mg l⁻¹ until the regeneration of fronds. The cultures were examined visually every day. Regenerated fronds from nodular calli were transferred to the Erlenmeyer's flask containing B₅S1.0 liquid medium with kanamycin (50 mg l⁻¹) and cefotaxime (500 mg l⁻¹). Each regenerated frond was propagated in the separate flask to establish independent transformant line. The cultures were aerated occasionally by shaking or by changing the culture medium.

Enzyme assay

The GUS activity was localized histochemically in nodular calli immediately after co-culture with *Agrobacterium*

according to Jefferson et al. (1987). Stable GUS activity was checked in the fronds regenerated from the nodular calli co-cultivated with *Agrobacterium*. GUS assay was performed by incubating the nodular calli and fronds in histochemical buffer (0.1 M sodium phosphate buffer, pH-7.0, 50 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1 % Triton-X-100 and 1 mg ml⁻¹ X-gluc (5-bromo-4-chloro-3-indolyl- β -glucuronidase) (Duchefa) for overnight at 37 °C. The next day, nodular calli and fronds were washed with deionized water and cleared with 70 % ethanol prior to observations using a stereomicroscope (Nikon, SMZU).

Molecular analysis of putative transgenic fronds

PCR analysis

Total genomic DNA was extracted from nontransformed (control) and putative transformed *Lemna* fronds using a modified CTAB (cetyl trimethyl ammonium bromide) method (Roger and Bendich 1988) and used as template to amplify *nptII* and *uidA* genes by using primers specific to their coding regions. The polymerase chain reaction (PCR) was carried out with *Taq* DNA polymerase (Sigma, USA) in 25 μ l reaction volume. Each reaction mixture consisted of approximately 100 ng of template DNA, 1 μ M of each primer (forward and reverse of *nptII* or *uidA*), 50 μ M of dNTP, 1X PCR buffer with 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase. PCR was carried out in a thermal cycler (Bio-Rad, USA) for the amplification reaction with the following cycle set up: Initial 7 min denaturation at 95 °C, followed by 30 cycles comprising - 60 s denaturation at 95 °C, 60 s annealing at 58 °C and 120 s extension at 72 °C with the final extension of 10 min at 72 °C. The 540 bp segment of *nptII* was amplified by using 20 bp oligonucleotide primers (5'-CCA CCA TGA TAT TCG GCA AC-3'/5'-GTG GAG AGG CTA TTC GGC TA-3' and the 250 bp of *uidA* was amplified using 24 bp oligonucleotide primers (5'-TAA CCT TCA CCC GGT TGC CAG AGG-3'/5'-CCT TAA CTA AGC CGG AAT CCA TCG-3'). DNA amplified fragments were detected on a 1 % (w/v) agarose gel and photographed at 302 nm wavelength.

Southern hybridization of PCR positive *Lemna* plants

Ten microgram of DNA was restricted with *HindIII* (10 U/ μ l) overnight at 37 °C, separated on 1 % agarose gel and transferred onto a positively charged nylon membrane (Amersham Pharmacia, USA) (Sambrook et al. 1989). DNA was bound to the membrane by UV cross linking and probed with dCT-[P³²] - labeled PCR amplified *nptII* gene fragment of 750 bp following supplier's instructions (Bio-Rad USA). Another blot of randomly picked GUS

positive plants was hybridized with dCT-[P³²] - labeled PCR amplified *uidA* gene fragment of 280 bp. Hybridization was performed overnight at 65 °C followed by washes in 2X SSC and 1X SSC + 1 % SDS for 30 min each. The membranes were then exposed to X-ray film at -80 °C with two intensifying screens (Kodak) for 3–4 d.

Results and discussion

Callus induction

The explants preconditioned on BAP induced the small whitish green callus, from the meristematic regions on the ventral surface of the frond on CIM, (B₅S1.0 containing 2,4-D (5.0 μ M) + 2-iP (50.0 μ M) or 2,4-D (50.0 μ M) + TDZ (5.0 μ M)) which subsequently, organized into hard nodular cell masses 'nodular calli' (Fig. 2a). The maximum number of nodular calli per explant was observed on the medium containing 2,4-D (5.0 μ M) and 2-iP (50.0 μ M) (data not shown).

Optimization of transformation protocol

Nonionic surfactants improved the efficiency of transient *Agrobacterium* mediated transformation in wheat (Cheng et al. 1997; Wu et al. 2003) and Arabidopsis (Chung et al. 2000; Kim et al. 2009). In the present study also similar results on the effects of a surfactant, Tween 20 on transient GUS expression were obtained. Tween 20 applied at low concentration, i.e. 0.2 % significantly elevated the transient GUS expression. However, concentration higher than 0.2 % caused a drastic reduction in transient expression. Improvement in transformation efficiency may be due to the better bacterial penetration in the plant tissue.

Bacterial concentration greatly affected the transformation efficiency. Of the five different bacterial concentrations tested (10⁵–10⁹ cell ml⁻¹) *Agrobacterium* used at 10⁷ cell ml⁻¹ concentration gave the highest percentage of calli showing GUS activity (Table 1). Agro concentrations higher than 10⁷ cell ml⁻¹ significantly decreased the number of calli expressing GUS activity due to the hypersensitive response of calli and also difficulties in the elimination of bacteria after co-culture.

The significant differences on transient GUS expression were observed when nodular calli inoculated with bacterial suspension for 20 to 60 min. Maximum transformation efficiency was observed in nodular calli inoculated for 60 min.

Addition of acetosyringone and low pH of co-cultivation medium induced *vir* operons and enhanced T-DNA transfer to the plant cells (Saini and Jaiwal 2007). The low pH (5.6)

Fig. 2 (a) Hard nodular callus developed on the explant (b) Frond regeneration from the nodular callus (c) Transformed nodular calli showing typical GUS sectors (arrow mark represent new frond arising) (d) Transformed fronds showing GUS activity

and the presence of acetosyringone (100 μM) gave the maximum frequency of transient GUS expression (Table 1).

Maximum transformation frequencies were observed in nodular calli co-cultured for 3-d at 25 °C under 16 h light / 8 h dark photoperiod (Table 1).

Regeneration of transformants

The partially differentiated cell masses (nodular calli) immediately after co-culture with *A. tumefaciens* under optimized conditions showed GUS activity in the surface cells of nodular calli in 63 % of the cultures.

The co-cultured nodular calli were transferred to the selection medium ($\text{B}_5\text{S1.0} + \text{Kin } 5.0 \mu\text{M} + \text{IAA } 25.0 \mu\text{M} + \text{kanamycin } 125 \text{ mg l}^{-1} + \text{cefotaxime } 500 \text{ mg l}^{-1}$) for frond regeneration. Prolonged incubation of calli at 125 mg l^{-1} of kanamycin was detrimental for their growth and frond regeneration. Therefore, kanamycin concentration was gradually reduced to 100 and then 75 mg l^{-1} at subsequent sub-cultures of 10 days interval. The fronds regenerated from the nodular calli (Fig. 2c) were transferred to liquid medium ($\text{B}_5\text{S1.0}$) containing kanamycin (50 mg l^{-1}). Each regenerated frond may represent a single transformation event. Kanamycin in liquid medium seems to be the most effective selection as the entire surface of the fronds in liquid environment remains in contact with antibiotics. However, in an earlier study, kanamycin at 10 mg l^{-1} was

used for transformation of *L. gibba* G3 and *L. minor* 8744 while concentrations up to 100 mg l^{-1} were needed for *L. minor* 8627 (Yamamoto et al. 2001). The transgenic fronds regenerated from different transformed nodular calli showed no phenotypic aberrations. The transgenic fronds vegetatively proliferated for several generations (up to 6-months) showed uniform GUS activity indicating stable inheritance of transgene through mitotic divisions (Fig. 2d). The transgenic fronds did not flower during their maintenance and proliferation on $\text{B}_5\text{S1.0}$ medium. In the four different experiments, a total of 338 nodular calli co-cultured with *Agrobacterium* produced a total of 37 fronds on kanamycin selection medium (Table 2), of which 13 fronds showed stable intense GUS activity throughout the fronds, with an overall transformation frequency of 3.8 % (13 fronds GUS positive out of 338 nodular calli). As compared to Yamamoto et al. 2001, in our case a systematic study of the transformation efficiency was possible because we transferred the each regenerated frond in the separate flask for clonal propagation (Fig. 4).

Molecular analysis of transformants

PCR analysis

Out of the 13 GUS positive transformants selected on kanamycin containing medium six randomly analyzed trans-

Table 1 Effect of different transformation parameters on transient *gus* expression in nodules of *L. minor* L. co-cultivated with *Agrobacterium tumefaciens* strain EHA 105 harboring binary vector pCAMBIA-2301

Factor	Variable	Gus expression (%) ^a
Bacterial concentrations	10 ⁵ cells ml ⁻¹	47 ^a
	10 ⁶ cells ml ⁻¹	49 ^a
	10 ⁷ cells ml ⁻¹	68 ^b
	10 ⁸ cells ml ⁻¹	37 ^a
	10 ⁹ cells ml ⁻¹	29 ^c
Inoculation time	20 min.	49 ^a
	40 min.	58 ^b
	60 min.	65 ^b
Acetosyringone	0.0	46 ^a
	50.0 μM	60 ^b
	100.0 μM	80 ^c
pH	5.2	33 ^a
	5.6	87 ^c
	5.8	63 ^b
	6.2	46 ^d
Co-cultivation time	1 d	20 ^a
	2 d	65 ^b
	3 d	88 ^c
Co-cultivation temperature	22 °C	52 ^a
	25 °C	68 ^b
	28 °C	43 ^c
Photoperiod	16 h light/ 8 h dark	89 ^a
	24 h dark	14 ^b
Prewashing with tween-20	0.0	46 ^a
	0.1 %	46 ^a
	0.2 %	73 ^b
	0.4 %	29 ^c

Data based on 35 nodules per treatment and each experiment repeated thrice

^a Mean value within columns followed by same letter are not significantly different according to Newman Keul's multiple range test ($P=0.05$)

formants showed the amplifications of 0.54 kb and 0.25 kb fragments corresponding to the coding regions of *nptII* and *uidA* genes, respectively in PCR analysis indicating the presence of transgenes in all of them (Fig. 3a, b).

Southern hybridization

The T-DNA (5.3 kb) of pCAMBIA2301 contained the selectable marker 35S-*nptII*-35S and reported 35S-*uidA*-

nos located on left and right side of a single unique *HindIII* site present at multiple cloning sites in the *lacZ* alpha region (Fig. 1). Southern analysis of genomic DNA of 4–5 randomly selected PCR positive transgenic fronds digested with *HindIII* and hybridized with *nptII* and *uidA* probes, revealed different patterns of junctions fragments between the T-DNA and the plant genome, depending upon the integration site. This confirmed that the plants are derived from independent transformation events. The sizes of band

Table 2 Transformation efficiency following inoculation of nodules of *L. minor* L. with *Agrobacterium tumefaciens* EHA 105 harbouring the binary vector pCAMBIA 2301

Total no. of nodules inoculated with <i>Agrobacterium</i>	No. of nodule regenerating on kanamycin containing medium	No. of fronds recovered on kanamycin containing medium	GUS + ve lines	PCR positive transformed lines		Southern Positive Lines		Transform-ation frequency (%) [#]
				With <i>uidA</i> primers	With <i>nptII</i> primers	With <i>uidA</i> probe	With <i>nptII</i> probe	
338	34	37	13	6 (6)*	6 (6)*	5(5)*	4(4)*	3.8 %

*Number in parenthesis indicates the transformed lines analyzed

[#]Transformation frequency is calculated by - number of GUS positive fronds / total no. nodules inoculated X 100

Fig. 3 Molecular analysis of the transgenic *Lemna minor* plants. PCR analysis of primary transformants (a) using *nptII* primers. Lane M marker DNA, lane C DNA from untransformed control, Lane P positive control, lanes 1–6 transformed plants. (b) using *uidA* primers. Lane M marker DNA, Lane P positive control, lane C DNA from untransformed control, lanes 1–6 transformed plants (c) Southern blot analysis of genomic DNA of independent transformed and non-transformed control plants. The DNA was digested with *HindIII*, and

the blot was probed with the PCR amplified fragment (750 bp) of *nptII* gene. Lane M marker DNA, lane C DNA from untransformed control, lanes 1–4 transformed plants. (d) Southern blot analysis of genomic DNA of independent transformed and non-transformed control plants. The DNA was digested with *HindIII*, and the blot was probed with the PCR amplified fragment (280 bp) of GUS gene. Lane M marker DNA, lane C DNA from untransformed control, lanes 1–5 transformed plants

detected were greater than that of *HindIII* digested fragments (2.1 kb) of T-DNA left border containing *nptII* gene, identified with *nptII* probe and confirmed the integration of *nptII* gene into plant nuclear genome (Fig. 3c). Similarly the sizes of bands detected with *uidA* probe were also greater than that of *HindIII* digested fragment (3.2 kb) of T-DNA right border containing *uidA* gene (Fig. 3d). This confirmed the integration of the *uidA* gene into plant nuclear genome. The number of hybridization signals indicated that the copy number of the transgenes integrated into the genome of transformed plants. Plants with a single copy and the two copies of T-DNA integrated into their genome were detected (Figs. 3c and d). DNA from untransformed (control) plants failed to hybridize to the *nptII* and *uidA* gene probes.

In conclusion, we developed efficient and reproducible protocols for genetic transformation of an Indian isolate of *Lemna minor*. The nodular calli were used for genetic transformation with *A. tumefaciens*. The factors such as the use of a surfactant (Tween 20), presence of acetosyringone during inoculation and in co-cultivation medium and co-cultivation period of 3 d were found critical for transformation of *L. minor*. Using the optimized genetic transformation protocol, a stable inheritance of transgenes in the clonal progenies of transgenic plants with a transformation

frequency of 3.8 % was achieved within a short period of 11–13 weeks. This protocol can be further used for the introduction of pharmaceutically important proteins genes into the Indian isolates of *L. minor*.

Fig. 4 Flow chart of the transformation protocol

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