Note

Agrobacterium-Mediated Transformation of Euphorbia tirucalli Callus

Hidenobu UCHIDA,1,2,3 Hirofumi YAMASHITA,3 Toyoaki ANAI,4 Toshiya MURANAKA,5,6 and Kanji OHYAMA2

1School of Environmental Science and Engineering, Kochi University of Technology, Tosayamada, Kochi 782-8502, Japan
2Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan
3Graduate School of Human and Environmental Sciences, Kyoto Prefectural University, Nakaragi, Shimogamo, Sakyo, Kyoto 606-8522, Japan
4Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Honjo, Saga 840-8502, Japan
5RIKEN Plant Science Center, Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan
6Kihara Institute for Biological Research, Yokohama City University, Maiotsu, Totsuka, Yokohama, Kanagawa 244-0813, Japan

Received October 21, 2009; Accepted January 5, 2010; Online Publication, April 23, 2010
[doi:10.1271/bbb.90783]

In order to establish a basis for transformation technology in the petroleum plant Euphorbia tirucalli, the callus of the plant was infected with Agrobacterium, washed with distilled water, sterilized with distilled water containing 100 mg/l of carbenicillin, selected on solidified B5 medium containing 13 mg/l of G418 and 100 mg/l of carbenicillin, and then on solidified B5 medium containing 25 mg/l of G418 and 100 mg/l of carbenicillin for the transgenic calli, and then the callus lines were subcultured successively on solidified B5 medium containing 50 mg/l of G418. We performed PCR analysis of sterilized G418-resistant callus line DNA and concluded that the gene introduced was integrated into the callus genome.

Key words: Agrobacterium; callus; Euphorbia tirucalli; petroleum plant; transformation

Euphorbiaceae plants store abundant amount of latex,1–3 the major constituents of which are sterols and triterpenoids.4–6 Callus formation in E. tirucalli explants accompanies oil body formation7 and substantial decreases in free triterpenoids as compared with total free sterols.8 A previous analysis indicated that overexpression of an introduced E. tirucalli squalene synthase gene (EtSS) in E. tirucalli callus enhanced the accumulation of phytosterols,9 but the method of transformation of E. tirucalli callus has not yet been described. Here, we present a detail protocol of Agrobacterium-mediated gene transfer to the E. tirucalli callus. This report might contribute to future plant biotechnology using E. tirucalli callus, which would contribute to sterol production.

Modified B5 medium includes iron at 27.8 mg/l of FeSO4·7H2O and 36 mg/l of EDTA-Na2Fe(III) salt, hormones at 1 mg/l of 2,4-D, 2 mg/l of NAA, and 0.22 mg/l of BA, the other components of the original B5 medium,10 and 1 g/l of casamino acid.11,12 The pH was adjusted to 7.0 with KOH solution. This modified B5 medium is referred to as B5 medium below. Solidified B5 medium includes 0.8% agar (Wako, Osaka, Japan). Carbenicillin, hygromycin, and G418 were purchased from Nacalai Tesque (Kyoto, Japan). The culture conditions for E. tirucalli callus,8 the construction of binary vector pEtSS/121 that included an EtSS open reading frame (ORF), which was replaced from gus ORF in pBI121,8) extraction of DNA13 from the callus were described elsewhere. Agrobacterium LBA4404 was used in this study. PCR was carried out as described previously.14 In order to detect the introduced gene, primers of IGF (5'-TGAATGATATGGTTACTAATG-CTTGGA-3') and IGR (5'-ATTTGTTTTCTATCGCG-TATTAATG-3') were used. To detect contaminating Agrobacterium DNA, primers of VF (5'-CTGGCAAC-TTACTGATTTAGTGTATGA-3') and VR (5'-CTAA-TATATCTATAAGGCGA-3') were used. Primers IGF and IGR annealed with the EtSS ORF and NOS terminator respectively, while primers VF and VR annealed with the pBI121 vector sequences outside the left and right borders (Fig. 3A).

In search of suitable drugs for transgenic callus selection, the sensitivity of wild-type callus to antibiotics was checked. Wild-type callus was excised into pieces about 5 mm in diameter, which were transferred onto solidified B5 medium containing appropriate concentrations of G418 or hygromycin, cultured for 24 d (Fig. 1). The addition of 25 and 50 mg/l of G418 or 25 and 50 mg/l of hygromycin to the medium inhibited the growth of the callus, while no drug addition resulted in 3–4 fold callus growth in diameter.

Agrobacterium bearing binary vector of chimeric EtSS was precultured on solidified YEP medium.

1 To whom correspondence should be addressed. Tel: +81-887-53-1050; Fax: +81-887-57-2520; E-mail: uchida.hidenobu@kochi-tech.ac.jp

Abbreviations: BA, 6-benzyladenine; NAA, naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; ORF, open reading frame; EtSS, Euphorbia tirucalli squalene synthase gene
containing kanamycin and subcultured in liquid AB\textsuperscript{15}) medium containing kanamycin for 3 h at 25 °C. Wild-type E. tirucalli callus was cut into 5-mm pieces and immersed in Agrobacterium suspension culture, and the callus was blotted onto sterilized paper to remove excess Agrobacterium and incubated on solidified B5 medium for 2 d. Agrobacterium-infected calli were washed for 2 h with distilled water within vessels that were set on a rotary shaker, and then blotted onto the paper. This process was repeated once. The callus was further washed for 1 d with distilled water that included 100 mg/l of carbenicillin, and then blotted onto the paper. This step was repeated twice. The callus was transferred onto solidified B5 medium containing 100 mg/l of carbenicillin (Fig. 2A) and subcultured for 4–6 weeks on the medium. Subsequent culturing of the calli onto solidified B5 media containing 25 mg/l of G418 and 100 mg/l of carbenicillin led to scarce growth of calli, due to the stress of Agrobacterium infection and sterilization by distilled water including carbenicillin. Then the sterilized calli were transferred onto solidified B5 medium containing 100 mg/l of carbenicillin (Fig. 2B) and subcultured 2 more times on the medium every 2–3 weeks. Growing callus (inset in Fig. 2B) was subcultured 3 times successively on solidified B5 medium containing 100 mg of carbenicillin.

Fig. 1. Sensitivity of Callus Growth to Antibiotics.
Euphorbia tirucalli callus was cultured for 24 d on solidified B5 medium containing various concentrations of G418 (A) or hygromycin (B).

Fig. 2. Growth of Agrobacterium-Infected Calli on Media Containing G418.
Calli were infected with Agrobacterium, washed with sterilization solutions, and inoculated onto solidified B5 medium containing 100 mg/l of carbenicillin (A), and subcultured on B5 medium containing 13 mg/l of G418 and 100 mg/l of carbenicillin (B). The inset shows a magnified view of panel B, showing a callus with newly proliferating G418-resistant cells attached. G418-resistant callus was subcultured in a medium containing 25 mg/l of G418 and 100 mg/l of carbenicillin (C). Proliferating callus was transferred onto a medium containing 50 mg/l of G418 and subcultured repeatedly.

Fig. 3. PCR Detection of Introduced DNA in G418-Resistant Calli.
Using a primer set of IGF and IGR (A) and equal amounts of DNAs from wild-type callus and G418-resistant calli 1 and 2, PCR amplification was performed (B). To check for contaminating Agrobacterium within the sterilized callus, PCR amplification was performed as described above (C) using a primer set of VF and VR (A). On PCR, plasmid and DNA from wild-type callus were used as positive and negative control templates respectively.
and 25 mg/l of G418 (Fig. 2C). DNA was extracted from G418-resistant calli, and was checked by PCR to detect a transgene using primers IGF and IGR. In G418-resistant callus lines 1 and 2, the introduced chimeric gene was detected on PCR amplification (Fig. 3B). This suggests that the introduced EtSS gene was integrated into the callus genome.

In order to check the existence of contaminating Agrobacterium in these two calli, the G418-resistant callus lines were transferred onto a medium containing no carbenicillin and 50 mg/l of G418, subcultured successively on the medium, and subjected to PCR analysis using primers VF and VR. The corresponding fragment was not detected using DNAs from the two callus lines, in contrast to plasmid control (Fig. 3C).

In Agrobacterium infection, about 180 wild-type callus pieces were fragmented onto six solidified B5 medium, subjected to Agrobacterium infection, sterilized, selected, subcultured on solidified B5 media containing 50 mg/l of G418, and then 1–5 sterilized transgenic callus lines were obtained. Sterilized calli were subcultured successively for more than 1 year. Phenotype analysis of the sterilized callus lines indicated that overexpression of the introduced sterol-synthesis gene (EtSS) resulted in upregulation of end products in the metabolic pathway.29

Transgenic callus lines were also obtained by infection with Agrobacterium bearing a binary vector that included the hptII gene, wash with the distilled water, sterilization with the distilled water containing 100 mg/l of carbenicillin, selection on solidified B5 medium containing 6 mg/l of hygromycin and 100 mg/l of carbenicillin, and then on solidified B5 medium containing 13 mg/l of hygromycin and 100 mg/l of carbenicillin. Then they were subcultured successively on solidified B5 medium containing 25 mg/l of hygromycin (data not shown).

A previous report described the regeneration technology of E. tirucalli plantlets from internode segments.16 Isolation of the transgenic plants after Agrobacterium-transfection can be performed in the future using the protocol described here.

Acknowledgments

We thank Dr. Takeshi Ohama and Dr. Masafumi Taniwaki of Kochi University of Technology (KUT), and Dr. Tatsuro Hamada and Dr. Miho Takemura of Ishikawa Prefectural University for encouragement throughout this study. It was partly supported by the KUT Post Doctoral Fellow (Research Associate) Research Promotion Fund (code 2121302) to H.U. (code 2000119). It was partly performed as one of the technology development projects of the “Green Biotechnology Program,” and was supported by a NEDO (New Energy and Industrial Technology Development Organization) grant to K.O.

References