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First Report of *Agrobacterium tumefaciens* mediated genetic transformation of aquatic Rice paddy herb (*Limnophila aromatica*)

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ABSTRACT

The study presents first report of Agrobacterium mediated genetic transformation in Rice paddy herb (*Linmophila aromatica*). A. *tumefaciens* strain C58C1 harboring pBin19 Plasmid containing β-glucuronidase (GUS) and neomycin phosphotransferase II (*nptII*) gene, under the control of 35S promoter and NOS terminator was used. Shoot tip explants were inoculated for 30 min followed by co-cultivation for 72 h and selected on agar semi solidified MS medium containing 100 mg/l Kanamycin and 1.0 mg/l BA; whereas total number of 78 putative transgenic shoots were obtained. The shoots were rooted on MS medium containing 1.0 mg/l IBA and 100 mg/l Kanamycin where 43 plants survived and rooted. Expression of GUS gene in the putative transgenics was confirmed by histochemical GUS assay. Visible localised gus expression was noted in a few cells and callus tissues of 4 plantlets that were photographed using compound light microscope.

Introduction

Aquatic plants have great importance in remediation of water ecosystem. They have ability to increase or decrease the efficiency of an ecosystem based on their position as primary and secondary producers (Chi et al., 1998). Aquatic plants are the primary source of oxygen in water bodies (Gorai et al., 2014), source of feed, shelter and providing excellent environment for the safety of fish, their egg laying and hatching (Brahmachari, 2008). Most of the aquatic plants/weeds are also used for ornamental purpose in aquariums all over the world owing to their attractive green, pink and red colours. Many recent studies report use of aquatic plants for phytoremediation of municipal wastes (Kukongviriyapan et al., 2003; Bui et al., 2004) and as pollution biomonitors of land locked water bodies around the world (Sribusarakum et al., 2004).

Rice paddy herb (*Linmophila aromatica*), family Scrophulariaceae is an important perennial ornamental and medicinal aquatic or semi-aquatic plant of tropical origin. People in South East Asian countries cultivate *Limnophila* (Gorai et al., 2014) as spice and medicinal herb (Chi et al., 1998). It contains important flavonoids (Bui et al., 2004) that also exert antioxidant activities (Kukongviriyapan et al., 2003; Sribusarakum et al., 2004). The leaves of Limnophila contain 0.1% essential oil (Brahmachari, 2008) with limonene, perillaldehyde and ketone, cis-4caranone and others as main constituents (Katzer, 2014). Moreover, it is also commonly used to treat dysentery, elephantiasis, indigestion and menstrual problems (Bhuiyan et al., 2010).

Advancement in genetic engineering and biotechnology have enabled the scientists to engineer crops with desired traits. *Agrobacterium* mediated genetic transformation studies in aquatic plants started late and first report on genetic transformation was reported in *Lemna gibba* and *Lemna minor* in 2001 (Yamamoto et al., 2001). To date, a number of optimization of *Agrobacterium* mediated genetic transformation studies in aquatic plants has been reported including *Ipomea aquatica* (Khamwanı et al., 2003; Meerak et al., 2006), *Typha latifolia* (Nandakumar et al., 2005), *Cryptocoryne willisii* (Wong et al., 2013), *L. minor* (Chhabra et al., 2011), *Mentha aquatica* (Hajian et al., 2011) and *Bacopa monnieri* (Nisha et al., 2004; Ramesh et al., 2011; Mahender et al., 2012; Yadav et al., 2014; Kumari et al., 2015).

To date no effort has been made for optimization of genetic transformation studies in *L. aromatica*. The present study is the first report on the *Agrobacterium* mediated gentic transformation studies on *L. aromatica* with aim to develop protocol for future aquatic plant biotechnlogy studies.

Material and Methods

The plants were taken from Karamanoglu Mehmetbey University, Department of Biology, Biotechnology Laboratory and multiplied following Karataş and Aasim (2015) to obtain sufficient plant material (explants) for genetic transformation studies. The shoot tip explants were cultured on MS (Murashige and Skoog 1962) medium containing 1.0 mg/l BA for 6 weeks under 16 h light photoperiod using White LEDs at 23°C. All mediums used in this study were prepared by adding 30 g/L (w/v) sucrose and 0.65% (w/v) agar to distilled water and pH was adjusted to 5.8. Therafter, media were autoclaved at 104 kPa atmospheric pressure and 120°C for 21 min.

This study used *A. tumefaciens* strain C58C1 harboring pBin19 containing β-glucuronidase (GUS) and neomycin phosphotransferase II (*nptII*) gene, driven by 35S promoter and *nos* terminator (Figure-1). GUS gene was interrupted by an intronic region to induce expression of GUS only from eukaryotic genes. A single colony of *Agrobacterium* was inoculated in nutrient broth (NB) containing 50 mg/l kanamycin and rifampicin each followed by overnight incubation at 28°C. Thereafter, the bacterial suspension was diluted with liquid MS medium to optimum optical density (OD_{600}) to 0.6-0.8. Shoot tip explants were isolated and inoculated for 30 min. It was followed by co-cultivation for 72 h on MS medium containing 1.0 mg/l BA. Thereafter, the explants were shifted to selection medium supplemted with 100 mg/l Kanamycin, 500 mg/l broad spectrum antibiotic (Duocid) and 1.0 mg/l BA. Duocid was used to suppress growth of Agrobacterium. After 6 weeks of culture, in vitro regenerated kanamycin resistant plants were excised and aseptically transferred to rooting medium containing 1.0 mg/l IBA and 100 mg/l Kanamycin.

Leaf samples were taken from in vitro regenerated plantlets and subjected to GUS analysis (Jefferson et al., 1987). All samples were dipped in X-GLUC solution (100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and incubated at 37°C for 1–2 h. The solution was decanted and the tissues were dipped in ethanol for 24 h to destroy chlorophyl and visualise GUS positive cells/tissues. Similar procedure was also employed for 2-4 weeks old callus of *Limnophila* to identify the genetically transformed tissues. All samples were also checked under compund light microscope for confirmation of GUS expression cells.

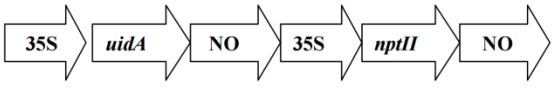

Figure 1 Schematic diagram of T-DNA region with *uid and nptII* genes with 35S promotor and NOS terminator. Kanamycin was used at concentration of 100 mg/l as plant selectable marker.

Results and Discussion

Agrobacterium strains and explants types play an important role in the transformation process, as they are responsible both for infectivity but also for the efficiency of gene transfer. The suitability of C58C1 strain harbouring various plasmids for the transformation of many economic plants has already been reported (Bakhsh et al. 2014). This study used shoot tip explants taken from in vitro grown stock material multiplied on MS medium containing 1.0 mg/l BA for 6 weeks following Karataş and Aasim (2015). All explants proliferated and multiple shoot induction after 6 weeks of culture. Thereafter, in vitro regenerated shoot tip explants were taken for genetic transformation studies. Shoot tip explants are potent explants used for genetic transformation studies in other economically important plants like corn (Cao et al., 2014; Zhong and Sticklen, 2000), cotton (Gould et al., 1998; Lei et al., 2012), soybean (Loganathan et al., 2010), jute (Saha et al., 2014), eucalyptus (Silva et al., 2011) etc. showing their suitability for genotype independent genetic transformation.

The explants were inoculated for 30 min to achieve genetic transformation from shoot tip explants. Longer exposure of explants in the incoulation medium tended to excessive *Agrobacterium* growth in the co-cultivation medium. After inoculation, shoot tip explants were shifted to co-cultivation medium for 72 h followed by transfer to selection medium for 6 weeks using Kanamycin as selection agent. Shoot prolifeartion with callus induction started within two weeks with clear multiple shoots (Figure 2a) was recorded after 4 weeks of culture. A total of 78 putative transgenic shoots were produced on kanamycin containing selection medium. These shoots were shifted to rooting medium containing 100 mg/l Kanamycin resulting in selection of 43 putative transgenic plantlets that were subjected to histochemical GUS analysis for staining. 43 out of 78 plants (55.13%) plants showing regeneration on MS medium supplemented with Kanamycin were speculated a escapes that were further confirmed by GUS histochemical analysis. Although Kanamycin is believed to be a good selection agent for putative plants, still escapes have been documented in various genetic transformation studies (Bakhsh et al., 2014; McCormick et al., 1986).

GUS histochemical assays revealed blue staining of leaf samples under light microscope (Figure 2a). A total of 4 samples (plants) showed GUS expression out of selected 43 plants. A GUS activity revealed a heterogeneous pattern of staining with the substrate 5-bromo-4-chloro-3-indoyl-β-d-glucuronic acid in some transformed cell cultures. Blue-staining was often observed on few cells among unstained callus masses. Results clearly revealed low genetic transformation efficiency for GUS gene with low and localised expression. In order to find out the reasons for low GUS expression, a new experiment was performed and 2-4 weeks old explants (with shoot buds or calli) subjected to GUS analysis. Explants (calli with shoot buds) showed localised GUS expression with blue staining at specific sites under microscope (Figure 2c, d).

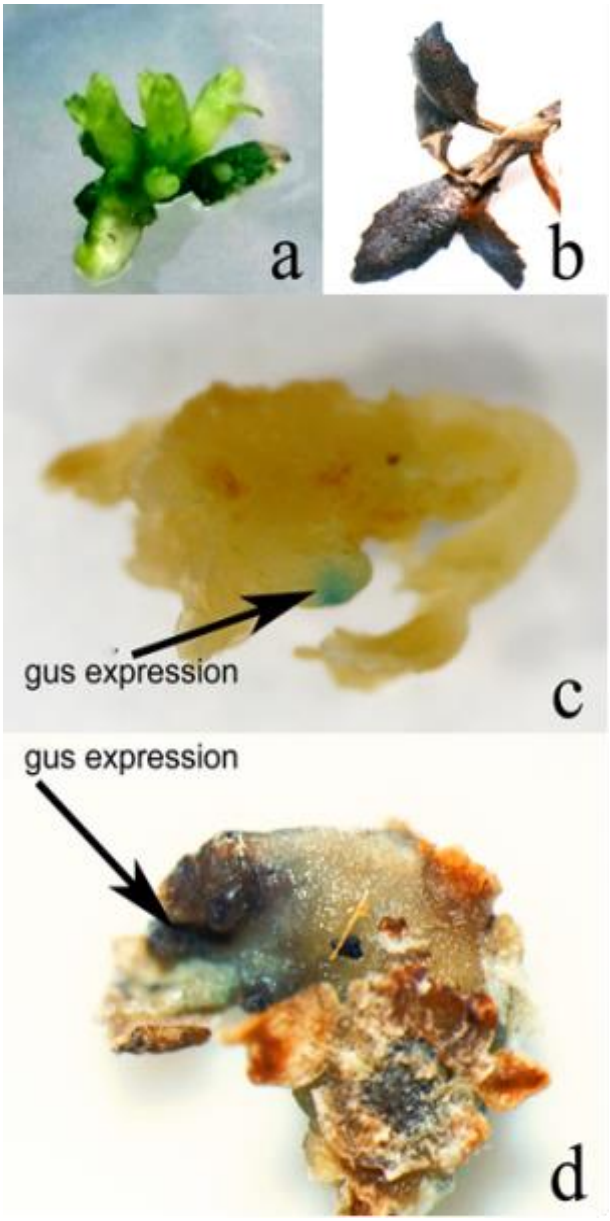

Figure 2 *Agrobacterium* mediated genetic transformation on *L. aromatica* (a) shoot proliferated using shoot tip explants (b) Histochemical Gus expression in transformed leaves (c) localised Gus expression in transformed cells and (d) Gus expression on callus cells

The methodology to generate transformation system is a key factor in transformation. Overall transformation efficiency as reported in this paper is very low, the establishment of efficient transformation system for rice paddy herb represents a significant achievement as it constitute first transformation report using this plant species. It is evident that this report will increase possibility of transgenic rice paddy herb making a definite contribution to the advancement of studies at molecular and biochemical levels. The results revealed that *Limnophila* is recalcitrant plant to *Agrobacterium* mediated genetic transfotmation. However, there is need to carry out further studies to see the mechanism of genetic transformation including behaviour of plant cell walls, their structural and chemical complexity in *Agrobacterium* mediated transformation. Genetic transformation is a powerful vehicle in studying gene function in plants and can be used for generation of new knowledge pathways involving the secondary metabolites production. This study may result in enhancement of production of important metabolites beneficial for living beings and may lead to development of important applications that will further enhance pharmacological significance of this important medicinal plant.

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