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In planta Agrobacterium-Mediated Transformation of Rice

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Abstract: The floral-dip transformation, the simplest technique, is no requirement of tissue culture procedure, and can directly transfer the interest gene into plant reproductive cells. It has been successfully applied to various plant species. In this study, the optimal conditions of a floral-dip method for production of transgenic rice variety RD41 were explored. The simple and effective inoculation medium was composed of Murashige and Skoog (MS) medium, 5% sucrose, 44 nmol/L benzylaminopurine, and 0.075% surfactant Tween-20 with pH 5.7. The transformation efficiencies of *Agrobacterium tumefaciens* strains AGL1 and EHA105 were compared with the *Agrobacterium* density at OD_{600} = 0.8–1.0 and the co-cultivation at 25 ºC for 48 h. *A. tumefaciens* strain EHA105 gave slightly higher transformation efficiency than AGL1, with statistically non-significant difference. The floral-drop transformation using the optimal floral-dip conditions showed higher transformation efficiency than the floral-dip method, but the dropped flowers turned brown and died within 2 d. Production of transgenic rice variety RD41 by the floral-dip method was achieved using *A. tumefaciens* strain EHA105 with the optimal conditions. Screening for the *gusA* gene by PCR using the *gusA* specific primers in the T_0 lines, there were 4 transgenic lines from 286 T_0 lines (1.4% transformation efficiency). However, histochemical glucuronidase (GUS) assay demonstrated that only three of four transgenic lines exhibited *gusA* expression. These results indicated that floral-dip transformation is a potential tool for production of the transgenic rice, which can be used for molecular breeding via genetic engineering in the future.

Key words: *Agrobacterium*-mediated transformation; floral-dip; floral-drop; transformation efficiency; *in planta*; rice

Rice transformation has been successfully developed via several techniques including electroporation, polyethylene glycol treatment, particle bombardment and *Agrobacterium*-mediated method since the late 1980s (Chen et al, 2009). *Agrobacterium*-mediated gene transfer, which is one of the most common rice transformation methods, has been extensively used for developing transgenic rice to study gene function and gain improving agricultural traits, for example, resistance to disease and insect pest, tolerance to drought and salt, and higher quality and yield. In general, *Agrobacterium*-mediated transformation is a tissue culture-based method, which usually requires aseptic condition and a long process to regenerate shoots and roots from the transformed tissues. Moreover, a tissue-culture technique frequently generates transformed plants harbouring undesirable mutations such as somaclonal variation (Clough and Bent, 1998; Bent, 2000). To avoid tissue culture, *in planta* transformation can directly transfer the interest gene into plant tissues or organs and can be a potential alternative for plant transformation experiments. *In planta* transformation techniques have been developed in various types including vacuum infiltration, agroinfiltration, sonication, spraying, floral drop and floral dip.

The floral-dip transformation is the simplest and the most convenient method with no requirement of any equipment and tissue-culture procedures. The first successful *Agrobacterium*-mediated floral-dip transformation was demonstrated in flowering *Arabidopsis* plants (Clough and Bent, 1998). This offers a simple, convenient and inexpensive transformation method, which eliminates the risk of unnecessary microbial contamination commonly observed in tissue culture. However, the floral-dip method has low transformation efficiency and requires many flowers and seeds because it has no specific target site of organ transformation. Apart from *Arabidopsis*, this method has been successfully applied to various crops such as

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radish (Curtis and Nam, 2001), tomato (Yasmeen et al, 2009), wheat (Zale et al, 2009), rapeseed (Li et al, 2010), white sweet clover (Hirsch et al, 2010), camelina (Liu et al, 2012), corn (Mu et al, 2012) and flax (Bastaki and Cullis, 2014).

The *in planta* rice transformation was firstly established by Supartana et al (2005) for japonica varieties by inoculating the embryonic apical meristems of soaked seeds with *A. tumefaciens*. Lin et al (2009) produced transgenic indica rice by vacuum infiltration of soaked mature seeds pierced by a needle with *A. tumefaciens*. Recently, Rod-in et al (2014) reported that anthers are a major target of rice transformation using a modified floral-dip method of *Arabidopsis* (Clough and Bent, 1998) and pollen is also transformed. Here, we report the achievement of transgenic rice production using a modified floral-dip method of Rod-in et al (2014), which can be used to study the role and function of rice genes, and for molecular breeding of rice via genetic engineering.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa* L.) variety RD41, obtained from the Phitsanulok Rice Research Center, Thailand, was grown in pots with natural light until flowering in a greenhouse. The inflorescences at the growth stage (the beginning of panicle emergence and tip of inflorescence emerged from sheath) (Lancashire et al, 1991) were used for transformation.

Agrobacterium-mediated transformation

A single colony of *A. tumefaciens* strains AGL1 or EHA105 harbouring pCAMBIA1304 carrying a *gusA* gene (pCAMBIA1304 sequence and map are available at http://www.cambia.org/daisy/cambia/585.html) was cultured in Luria Bertini (LB) broth medium supplemented with kanamycin (50 mg/L) and rifampicin (40 mg/L) at 28 ºC and 250 r/min shaking for 2 d. One millilitre of the *Agrobacterium* suspension culture was inoculated in 250 mL LB broth medium without antibiotics at 28 ºC and 250 r/min shaking overnight until reaching the stationary phase (OD_{600} = 0.8–1.0). *Agrobacterium* cells were collected by centrifugation at 5 600 r/min for 3 min. The cell pellets were resuspended in 200 mL standard inoculation medium (SIM) containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 5% sucrose, 44 nmol/L benzylaminopurine (BAP) and 0.075% Tween-20, modified from Clough and Bent (1998), and Das and Joshi (2011). The pH of the inoculation medium was adjusted to 5.7.

In planta rice transformation

The floral-dip method was modified from Rod-in et al (2014). Rice inflorescences were prepared by clipping the tips of the pre-anthesis spikelet located at the middle of the inflorescences. Non-selected spikelet for clipping was removed from the inflorescences, which was subsequently dipped into the SIM containing *Agrobacterium* for 1 min. For the floral-drop technique, 100 mL SIM containing *Agrobacterium* were directly dropped into the individual spikelet cup (clipped spikelet) using the 1 mL syringe with a needle. The inoculated inflorescences were covered with plastic bags to maintain humidity and co-cultivated at 25 ºC for 48 h. The *Agrobacterium*-dipped or *Agrobacterium*-dropped spikelets were then covered with paper bags until seed collection.

Screening for transgenic rice by PCR analysis

Genomic DNA was extracted from T_0 transgenic rice leaves using the cetyltrimethylammonium bromide (CTAB) method according to Ahmadikhah (2009). The transgenic lines were verified using specific primers designed from the *gusA* sequence of pCAMBIA1304 binary vector (GenBank: AF234300). The PCR reaction was performed using a pair of specific primers, gusA-F1: 5′-CAACGAACTGAACTGGCAGA-3′ and gusA-R1: 5′-TCTCTTTGATGTGCTGTGCC-3′, to amplify the 989 bp fragment, and a pair of specific primers, gusA-F2: 5′-TGCG TCACAGCCAAAAGC-3′ and gusA-R2: 5′-CTCGCATTACC CTTACGC-3′, to amplify the 361 bp fragment. The PCR cycling conditions were pre-denaturation at 95 ºC for 5 min, 35 cycles of 95 ºC for 30 s, 55 ºC (gusA-F1/gusA-R1) or 51 ºC (gusA-F2/gusA-R2) for 30 s, 72 ºC for 30–60 s, followed by a final extension at 72 ºC for 5 min.

Histochemical glucuronidase (GUS) assay

The transformed rice tissues were evaluated through *gusA* expression according to the method of Jefferson et al (1987). Inoculated spikelet and T_0 leaves were immersed in 90% acetone for 1 h and incubated at 37 ºC overnight in glucuronidase (GUS) staining solution containing 50 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D glucuronidase (X-gluc), 0.5 mmol/L potassium ferrocyanide, 0.5 mmol/L potassium ferricyanide, 1 mol/L Na_2PO_4, 0.5 mol/L EDTA (pH 7.0), 0.5% Triton X-100 and 20% methanol. The stained tissues were washed once with methanol:acetic acid (3:1) at room temperature (25 ºC–28 ºC) overnight and then once with 70% ethanol, and subsequently stored at 4 ºC for at least 2 h. Transformation efficiency of spikelet was calculated from percentage of spikelet with GUS-stained floral tissues. Transformation efficiency of anthers was reckoned from percentage of GUS-stained anthers in GUS-positive spikelet.

Statistical analysis

All experiments were performed in a completely randomized design with three biological replicates (three inflorescences per replicate). Statistical significance was analysed using one-way ANOVA (analysis of variance) followed by Duncan's multiple range tests and *t*-test for mean comparison using SPSS 17.0 software. Differences in $P < 0.05$ were considered significant.