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The floral-dip method for rice (*Oryza sativa*) transformation

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We developed an effective floral dip, *Agrobacterium*-mediated transformation method for rice (*Oryza sativa* L.) cultivar RD41. *A. tumefaciens* strain AGL1 harboring the binary vector pCAMBIA1304 carrying the *gusA* gene (AGL1-1304) was used to infect rice spikelets via the floral-dip method. The tip-cut spikelets of the rice inflorescence stage 51 (beginning of panicle emergence: tip of inflorescence emerged from sheath) were dipped in the *Agrobacterium* AGL1-1304 suspension and co-cultivated at 25 °C for 3 d. The target sites of transformation were detected by histochemical GUS assay. Eighty three of six hundred and fifty two, 12.73%, of rice spikelets dipped were positive for GUS activity in various tissues. Tissues positive for GUS staining included female organs (ovary, stigma, style and lodicule), male organs (anther and filament), and lemma. The primary target of the floral-dip transformation in rice was the anther which had the highest transformation efficiency (89.16%) whereas ovary transformation was rare (7.23%). In addition, we demonstrated that most of the pollen in the GUS-stained anthers expressed GUS activity. This result indicated that the germ-line cells were transformed and the transgene can be expressed. Our results suggest that floral-dip transformation is a simple potential tool for production of transgenic rice with no requirement of tissue culture.

Keywords: *Agrobacterium*, Floral dip, GUS, Rice, Transformation

Introduction

The first *in planta* flora-dip, *Agrobacterium*-mediated transformation, was successfully developed for *Arabidopsis* (Clough and Bent, 1998). This method is easy, convenient and economical and eliminates the microbial contamination which is commonly found in the tissue culture required for plant regeneration (Li et al., 2010). The disadvantages of the floral-dip method include low transformation efficiency, requirement of many flowers and seeds, no specific target site of organ transformation, and limitations in the application of this method to all plant species. At present, this method has been successfully applied to various plant species, e.g., *Arabidopsis thaliana*

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(Clough and Bent, 1998; Das and Joshi, 2011), *Medicago truncatula* (Trieu *et al*. 2000), *Raphanus sativus* (Curtis and Nam, 2001), *Solanum lycopersicum* (Yasmeen *et al*., 2009), *Triticum aestivum* (Zale *et al*., 2009), *Brassica Napus* (Li *et al*., 2010), *Melilotus alba* (Hirsch *et al*., 2010), *Camelina sativa* (Liu *et al*., 2012), and *Zea Mays* (Mu *et al*., 2012). However, there has not been reported on the floral-dip transformation in rice (*Oryza sativa* L.) yet.

Rice is one of the most versatile cereal crops and staple foods in the world, but its quality and yield have been decreased from the destruction of diseases and insect pests, and suboptimal environmental conditions. Genetic modification has been used in rice for studying gene function and improving agricultural traits to gain resistance to both biotic and abiotic stresses, for example, the improvement of rice tolerant to drought and salt stresses (Rohila *et al*., 2002; Zhang *et al*., 2010), the engineering of insect resistant rice (Ye *et al*., 2009), and the production of rice resistant to the sheath blight disease (Molla *et al*., 2013).

As tissue culture is labor intensive and slow, and frequently generates transformed plants harboring undesirable mutations from somaclonal variation (Bent, 2000; Clough and Bent, 1998), floral-dip transformation has no requirement of tissue culture and would be an advantageous method for rice improvement.

In this study, we evaluated the use of the floral-dip transformation method to introduce a model gene, *gusA*, driven by the CaMV35S promoter into rice. The primary goal of this study was to identify the sites of productive transformation in the floral-dip procedure for *Agrobacterium*-mediated transformation of rice. It was our hope to identify a potential tool for production of the transgenic rice via the floral-dip transformation method.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* L.) cultivar RD41 obtained from the Phitsanulok Rice Research Center, Thailand was used in this study. The RD41 seeds were soaked in water for 2 d and then placed in a moist filter cloth until germination. The germinated seeds were planted in a pot containing fertile and wet clay. The 1-2 week-old seedlings were moved to a new pot, three plants per pot. The rice plants were grown in the greenhouse at Naresuan University, Phitsanulok province, Thailand.

The inflorescences at stage 51 (the beginning of panicle emergence: tip of inflorescence emerged from sheath) according to the BBCH-scale (Lancashire et al., 1991) were used for the floral-dip transformation.

Agrobacterium strain and plasmid

A. tumefaciens strain AGL1 harboring pCAMBIA1304 was used for the floral-dip transformation. The binary vector pCAMBIA1304 contains the *hygromycin phosphotransferase (hptII*) gene, and the reporter genes, *green fluorescent protein* (*gfp*) and *β-glucuronidase* (*gusA*), under the control of the CaMV35S promoter (Figure 1).

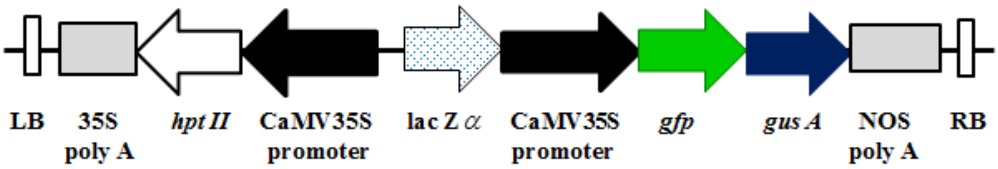

Fig. 1. Schematic diagram of the pCAMBIA1304 T-DNA region. The *hptII*, *gfp* and *gusA* genes are under the control of the CaMV35S promoter

Agrobacterium culture and inoculum

A. tumefaciens strain AGL1 harboring pCAMBIA1304 was grown in 5 ml of Luria broth (LB) medium supplemented with kanamycin (50 mg/l) and rifampicin (40 mg/l) at 28°C and 250 rpm for 2 d. One milliliter of the *Agrobacterium* suspension culture was inoculated in 250 ml of LB medium. The culture was grown at 28°C and 250 rpm until reaching the stationary phase OD_{600} 0.8–1.0. Bacterial cells were collected by centrifugation at 5600xg for 3 min. The cell pellets were resuspended in the inoculation medium which was modified from Clough and Bent (1998). The inoculation medium was composed of basal medium Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 5% (v/v) sucrose, 44 nM benzylaminopurine, and 0.075% (v/v) of the surfactant Tween-20 (Das and Joshi, 2011). The pH of the inoculation medium was adjusted to 5.7 for dipping (Clough and Bent, 1998).

Floral dip transformation

Rice inflorescences at stage 51 as mentioned above were used for transformation. The tips of selected rice spikelets were cut off before the inflorescence was dipped in the *Agrobacterium* inoculation medium. Dipping was carried out for 1 min. Each dipped inflorescence was covered with a plastic bag (to maintain humidity) at 25°C for 3 d.

GUS Assay

The achievement of transformation was evaluated by the GUS assay in the dipped spikelets according to the method of Jefferson *et al*. (1987). The dipped rice spikelets were immersed in GUS staining solution containing 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D glucuronidase (X-gluc), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 M Na_2PO_4, 0.5 M EDTA (pH 7.0), 0.5% Triton X-100 and 20% Methanol. The GUS staining spikelets were incubated in the dark overnight at 37°C. The stained spikelets were immersed in methanol: acetic acid (3:1) and 70% ethanol overnight at room temperature to remove the chlorophyll. The transient GUS activity of spikelets was recorded as blue spots (irrespective of size) using a microscope and photographed.

Results and discussions

GUS expression in transformed tissues of rice flowers

The floral-dip transformation for rice was investigated based on the floral-dip method for *Arabidopsis* (Clough and Bent, 1998) with some modifications as mentioned above. We examined the target tissues for the *gusA* transformation by the GUS assay.

Eighty-three of six hundred and fifty-two dipped spikelets (12.73%) showed GUS expression. GUS activity was detected in various tissues of the GUS-stained spikelets, i.e., lemma, male organs (anther and filament), and female organs (ovary, stigma, style and lodicule) (Figure 2). Among these tissues, the anther, a major target, was commonly transformed (89.16%) while transformation of the ovary, another important target, was rare (7.23%) (Table 1). To generate the transgenic plants, the target tissues of the floral-dip transformation are the sexual organs, the ovary and anther which carry the germ-line cells, ovules and pollen, respectively. The results indicate that the anthers are the primary site of the floral-dip transformation in rice. Our results differed from those of previous research with floral-dip transformation in *Arabidopsis* which found that the female germ line was the primary site of transformation whereas the male germ-line transformation was rare (Bechtold *et al*., 2000; Desfeux *et al*., 2000; Ye *et al*., 2009).

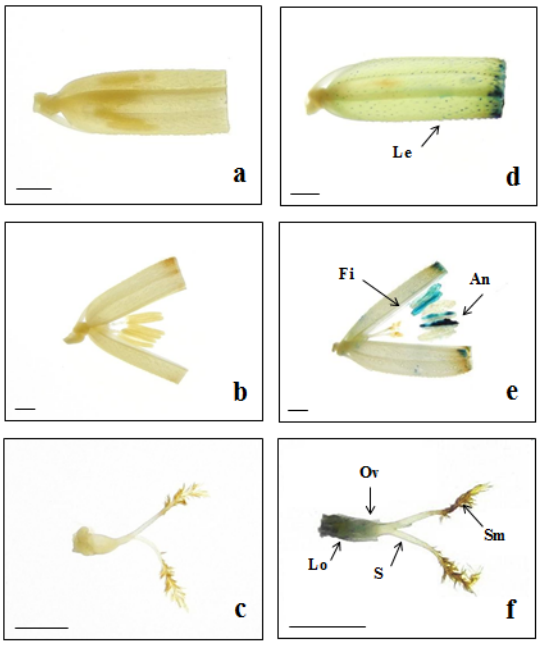

Fig. 2. Analysis of GUS activity in rice spikelets after floral dip transformation: a)-c). The dipped spikelet with no GUS expression in any tissues; d) the dipped spikelets with GUS activity in lemma (Le); e) the dipped spikelets with GUS activity in anthers (An) and filaments (Fi); and f) the dipped spikelets with GUS activity in lodicules (Lo), ovary (Ov), stigma and style (S). Bar = 1 mm.

Table 1. The efficiency of the floral-dip transformation in different tissues of rice spikelets. The data show target sites of transformation from eighty-three GUS-stained spikelets

Floral tissues	Number of GUS-stained spikelets	Transformation efficiency (%)
Ovary	6	7.23
Stigma	5	6.02
Style	7	8.43
Lodicule	9	10.84
Anther	74	89.16
Filament	3	3.61

Determination of GUS expression in the pollens of the GUS-stained anthers

To confirm the male germ-line transformation, pollen from the GUS-stained anthers were examined for staining under a microscope. The result revealed that most of pollen obtained from the GUS-stained anthers showed GUS activity (Figure 3). The staining confirmed that the male germ line was the predominant target of the floral-dip transformation in rice. Our result

suggests that the floral-dip transformation is a potential method to produce transgenic rice. However, determination of the viability and germination efficiency of transformed pollen is an essential step in confirming the usefulness of floral-dip transformation in rice.

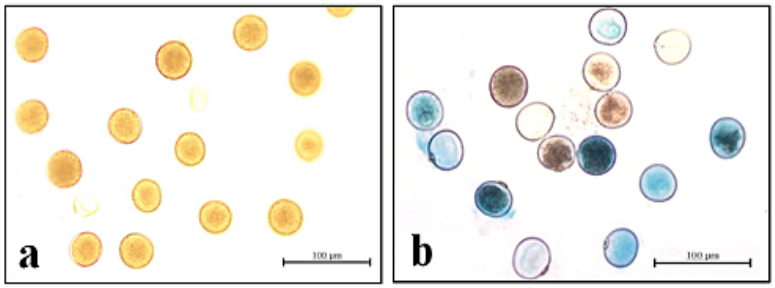

Fig. 3. GUS expression in pollens of the dipped spikelets: (a), pollens from the GUS-negative anthers; (b), pollens from the GUS-positive anthers. The photographs were taken under a compound microscope at 200x magnification. Blue-stained pollens indicate GUS expression. Bar = 100 µm.

Conclusion

In this study, we developed a floral-dip method for rice transformation by modifying the method used for *Arabidopsis* (Clough and Bent, 1998). GUS assays of the rice spikelets dipped in the suspension of *A. tumefaciens* strain AGL1 harboring pCAMBIA1304 showed that 12.73% of the treated spikelets exhibited GUS activity in various floral tissues. The anther, a major target of the floral-dip transformation in rice, was transformed at a rate of 89.16%. In contrast, ovary transformation was rare. We also demonstrated that most of the pollen in the GUS-stained anthers showed GUS activity. Our results indicate that floral-dip transformation is a simple, potentially useful method for production of transgenic rice.

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References

Bechtold, N., Jaudeau, B., Jolivet, S., Maba, B., Vezon, D., Voisin, R. and Pelletier, G. (2000). The Maternal Chromosome Set Is the Target of the T-DNA in the in Planta Transformation of *Arabidopsis thaliana*. Genetics 155:875-1887.

Bent, A.F. (2000). *Arabidopsis* in Planta Transformation. Uses, Mechanisms, and Prospects for Transformation of Other Species. Plant Physiology 124:1540-1547.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant Journal 16:735-743.

Curtis, I.S. and Nam, H.G. (2001). Transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) by floral-dip method – plant development and surfactant are important in optimizing transformation efficiency. Transgenic Research 10:363–371.

Das, P. and Joshi, N.C. (2011). Minor modifications in obtainable *Arabidopsis* floral dip method enhances transformation efficiency and production of homozygous transgenic lines harboring a single copy of transgene. Advances in Bioscience and Biotechnology 2:59-67.

Desfeux, C., Clough, S.J. and Bent, A.F. (2000). Female Reproductive Tissues Are the Primary Target of *Agrobacterium*-Mediated Transformation by the *Arabidopsis* Floral-Dip Method. Plant Physiology 123:895-904.

Hirsch, A.M., Lee, A., Deng, W. and Tucker, S.C. (2010). An open-flower mutant of *Melilotus alba*: Potential for floral-dip transformation of a papilionoid legume with a short life cycle? American Journal of Botany 97:395-404.

Hoque, M.E., Mansfield ,J. and Bennett ,M. (2005). *Agrobacterium*-mediate transformation of Indica rice genotypes: an assessment of factors affecting the transformation efficiency. Plant Cell Tiss Organ Cult 82:45-55.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusions: ,B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal 6:3901-3907.

Lancashire, P.D., Bleiholder, H. and Boom, T.V.D., Langel, Ü.D.,Deke, P., Stauss, R., Weber, E. and Witzenberger, A. (1991). A uniform decimal code for growth stages of crops and weeds. Annals of Applied Biology 119:561-601

Li, J., Tan, X., Zhu, F. and Guo, J. (2010). A rapid and simple method for *brassica napus* floral-dip transformation and selection of transgenic plantlets. International Journal of Biology 2:127-131.

Liu, X., Brost, J., Hutcheon, C., Guilfoil, R., Wilson, A., Leung, S., Shewmaker, C., Rooke, S., Nguyen, T., Kiser, J. and Rocher, J. (2012). Transformation of the oilseed crop *Camelina sativa* by *Agrobacterium*-mediated floral dip and simple large-scale screening of transformants. In Vitro Cell.Dev.Biol.-Plant 48: 462-468.

Molla, K.A., Karmakar, S., Chanda, P.K., Ghosh, S., Sarkar, S.N., Datta, S.K. and Datta, K. (2013). Rice *oxalate oxidase* gene driven by green tissue-specific promoter increases tolerance to sheath blight pathogen (*Rhizoctonia solani*) in transgenic rice. Molecular Plant Pathology: n/a-n/a.

Mu, G., Chang, N., Xiang, K., Sheng, Y., Zhang, Z. and Pan, G. (2012). Genetic Transformation of Maize Female Inflorescence Following Floral Dip Method Mediated by *Agrobacterium* Biotechnology 11: 178-183.

Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15:473-497.

Rohila, J.S., Jain, R.K. and Wu, R. (2002). Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley Hva1 cDNA. Plant Science 163: 525-532

Trieu, A.T., Burleigh, S.H., Kardailsky, I.V., Maldonado-Mendoza, I.E., Versaw, W.K., Blaylock, L.A., Shin, H., Chiou, T.J., Katagi,. H., Dewbre, G.R., Weigel, D. and Harrison, M.J. (2000). Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. The Plant Journal 22: 531-541.

Yasmeen, A., Mirza, B., Inayatullah, S., Safdar, N., Jamil, M., Ali, S. and Choudhry, M.F. (2009). In Planta Transformation of Tomato. Plant Mol Biol Rep 27:20-28.

Ye, R., Huang, H., Yang, Z., Chen, T., Liu, L., Li, X., Chen, H. and Lin, Y. (2009). Development of insect-resistant transgenic rice with Cry1C*-free endosperm. Pest Management Science 65: 1015-1020.

Zale, J.M., Agarwal, S., Loar, S. and Steber, C.M. (2009). Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant Cell Reports 28:903–913.

Zhang, H., Liu, W., Wan, L., Li, F., Dai, L., Li, D., Zhang, Z. and Huang, R. (2010). Functional analyses of ethylene response factor JERF3 with the aim of improving tolerance to drought and osmotic stress in transgenic rice. Transgenic Research 19:809-81.

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