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Genetic Transformation of Maize Female Inflorescence Following Floral Dip Method Mediated by *Agrobacterium*

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Abstract: As a simple and high-efficient genetic transformation approach, floral dip method has been widely used for the studies of gene function in *Arabidopsis* and other plant species. However, there is no report about its applications in maize. In this study, the floral-dip method of transformation by immersed maize female inflorescences in *Agrobacterium* suspension was firstly applied in the maize transformation. We found that the best transformation period of corn silk size is 5-10 cm and the optimal cell concentration of *Agrobacterium* transformation is OD_{600} = 1.0. A large number of green phosphor dots by Green Fluorescent Protein (GFP) were observed in the T_0 transformed seeds indicating that the T-DNA-carried *gfp* gene integrated and expressed in the receipt plant genome. The hygromycin selection and PCR assay of exogenous gene *hpt* displayed that 3.3% produced plantlet were positive. Therefore, we concluded that the floral dip method mediated by *Agrobacterium* can be used for the genetic transformation in maize.

Key words: Maize, female inflorescence, *Agrobacterium*-mediated floral dip method

INTRODUCTION

The plant genetic transformation is not only the significant approach to the hereditary modification but also the indispensable technique for study of gene and its function (Martin, 1998; Ramachandran and Sundaresan, 2001; Bennetzen, 2002). However, conventional transformation methods has some defects such as low transformation rate, difficulty for regeneration and identification of transgenic plant and time-consuming in practical application (Birch, 1997; Bent, 2000). Almost twenty-five years ago, Feldmann and Mark (1987) developed a plant transformation method which was described as in 'planta' transformation, because such a method excludes the use of tissue culture and plant regeneration. Through this method, they got stable transgenic lines, but the transformation rate was very low. Several years later, Bechtold *et al.* (1993) reported that vacuum-aided infiltration of *Arabidopsis* inflorescence with *Agrobacterium* could dramatically improve the transformation efficiency which was frequently named as the *Agrobacterium* vacuum infiltration method. The transformation procedures were also further modified and substantially improved by using a surfactant (Silwet L-77) to substitute vacuum-aided infiltration (Clough and Bent, 1998). The *in planta Agrobacterium*-mediated gene transfer method was known as the floral dip method which directly produces transformed seed and completely negates the tissue culture procedures. At present, it has became common transformation method for functional genome research of *Arabidopsis* (Lawrence and Pikaard, 2003; Gaspar *et al.*, 2004). Similar protocols have been successfully used in some dicotyledon such as *Medicago truncatula* (Trieu *et al.*, 2000), *Brassica napus* (Fu *et al.*, 2004), *Raphanus sativus* (Curtis and Nam, 2001) and *soybean* (Wang *et al.*, 2010).

Since Grave and Goldman (1987) found that the *Agrobacterium tumefaciens*-mediated transformation worked well for the monocot genus *Gladiolus*. the *Agrobacterium*-mediated transgenic method had been used for gene transformation in cereal crop (Christie *et al.*, 1988; Chan *et al.*, 1992; Raineri *et al.*, 1993). But genetic transformation of cereal plant using floral dip method was just at the initial stage and there was no report about its application in maize. Therefore, the objectives of present study were to probe the floral dip method in maize female inflorescence transformation, to develop a rapid and simple genetic transformation procedure for maize.

MATERIALS AND METHODS

Plant materials: Elite maize inbred line 18-599, obtained from Maize Research Institute of Sichuan Agricultural University, was used as transformation recipient plant. Healthy and strong plants were chosen for transformation at flowering stage.

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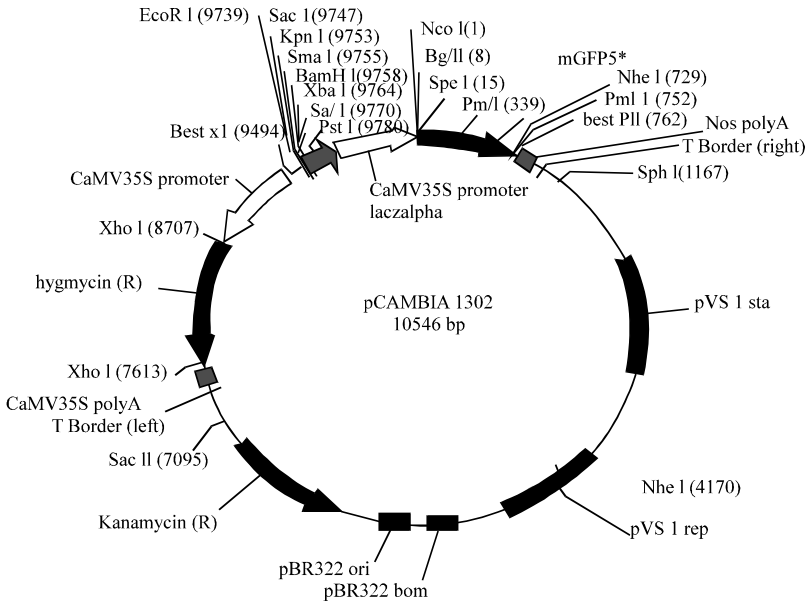

Fig. 1: Cleavage map of pCAMBIA 1302

Bacterium and plasmid: *Agrobacterium tumefaciens* strain C58 (nopaline utilizer) was obtained from the Agricultural culture collection of China. The binary plasmid pCAMBIA 1302 (Cambia, Australia) contains green fluorescence protein reporter gene (*gfp)*, hygromycin resistance gene (*hpt)* and kanamycin resistance gene (*nptII)* for expression in plants (Fig. 1). It was offered by Triticeae Research Institute of Sichuan Agricultural University.

Methods

***Agrobacterium* culture and inoculum preparation:** In the primary culture, *Agrobacterium* strains harboring gene of interest in binary plasmid pCAMBIA 1302 were inoculated into 5 mL of YEB liquid medium with kanamycin and hygromycin of each 50 mg L^{-1} incubated at 28°C and 220 rpm rotation for 12-16 h. Secondary culture was prepared by inoculating 2 mL of primary culture into 100 mL of YEB medium and allowed to grow up to 2.0 OD at 600 nm, in same condition as primary culture. Bacteria cells were collected by centrifugation at 5000 rpm for 10~15 min and the pellet was re-suspended in infiltration medium (liquid N6 medium) which is composed of 100 μL L^{-1} CSO40, 100 μL L^{-1} Triton X-100, 20 mg L^{-1} Acetosyringone (AS), 5 μg L^{-1} GA, 2 μg L^{-1} 6-BA, 1 mg L^{-1} IAA, 500 mg L^{-1} hydrolysis casein, 2 mg L^{-1} L-proline, 5% (w/v) sugar and PH adjusted to 5.8. Finally, four levels of concentration of the bacterial culture (OD_{600} = 0.5 OD_{600} = 1.0 OD_{600} = 1.5 OD_{600} = 2.0) were used to check their effect on the transformation rate.

Transformation of maize female inflorescence using floral dip method: In the spring of 2010, maize inbred line 18-599 was planted in the field of Sichuan agricultural university. When the length of corn silk was 5~10 cm, the corn silk were immersed in *Agrobacterium* suspension for 60-90 sec, or injected *Agrobacterium* into the leaf ear and let the liquid overflowed from the top of the ear. The procedure was applied once a day for three days.

GFP detection for T_0 transgenic corn silk and seeds: After transformation procedure in field, presence of GFP was visualized in corn silk using the fluorescence microscope.

Transformed plants were performed by artificial pollination. The ears were taken back from field after thirty days. Randomly selected seeds were used to make broken and paraffin section. Tissue and cells were visualized for the presence of GFP using the fluorescence microscope.

Screening of T_0 transformed seeds: T_0 transformed seeds were used for hygromycin-resistant selection. Seeds were soaked in warm water for 12 h and then sown into the abluent sand. When seeds germinated, cotyledons were daubed with hygromycin solution at a concentration of 20 mg L^{-1}. Transformed seedlings were not susceptible to hygromycin due to hygromycin

resistance of the *hpt* gene. Resistance plants were chosen for next detection after two weeks.

Transgene detection using PCR: Genomic DNA from the leaf of hygromycin-resistant plants were extracted by CTAB method (Wang *et al*., 2010). Primers (forward primer: 5' TCGGCGAGTACTTCTACACAGC 3' and reverse primer: 5' CTGGCAAACTGTGATGGACGAC 3) were designed according to the *hpt* gene sequence. PCR amplification was carried out with a denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 20 sec, 55 for 30 sec, 72°C for 1 min and terminated at 72°C for 10 min. All PCR products were separated by agarose gel and visualized under UV light.

RESULTS

Transformation protocol: The length of corn silk were vital for the Maize floral- dip methods, different length of corn silk were tested and we found stage of corn silk of 5-10 cm was suitable for *Agrobacterium*-mediated transformation based on the observation under fluorescence microscope (Fig. 2). Moreover, four levels of concentration of the *Agrobacterium* culture (OD_{600} = 0.5′ OD_{600} = 1.0 OD_{600} = 1.5 OD_{600} = 2.0) were also tested to corn silk transformation, we found that *Agrobacterium* suspension at the concentration of OD_{600} = 2.0 will cause the serious infection by plant diseases and insect which resulted in ear rot and no seed reaped. The plant transformed by the other concentrations could grow and harvest grain and were used to further detect the transformation rate.

GFP expression of T_0 transformed seeds: As shown in Fig. 3 of the paraffin section after the corn silk were transformed with different concentrations of bacteria liquid, the strong green fluorescence points were observed in the T_0 seeds and the cell edge. Appearance of the fluorescence points indicated that the T-DNA carried exogenous green fluorescent protein gene expressed in the plant cell nucleus genome. Moreover, the fluorescence signal appeared in the edge of the cell revealed that some GFP had expressed on the plant cell membrane. The GFP expression in different site was related to the degree of seeds maturity. The fluorescence expression quantity on photos exposed that transformed by *Agrobacterium* liquid at concentration of OD_{600} = 1.0 could go to the optimal efficiency (Fig. 3b).

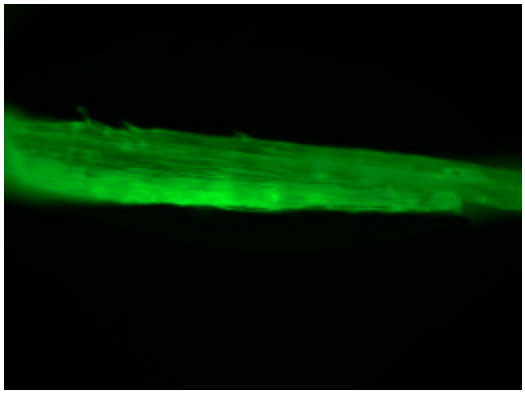

Fig. 2: Fluorescence observation of corn silk

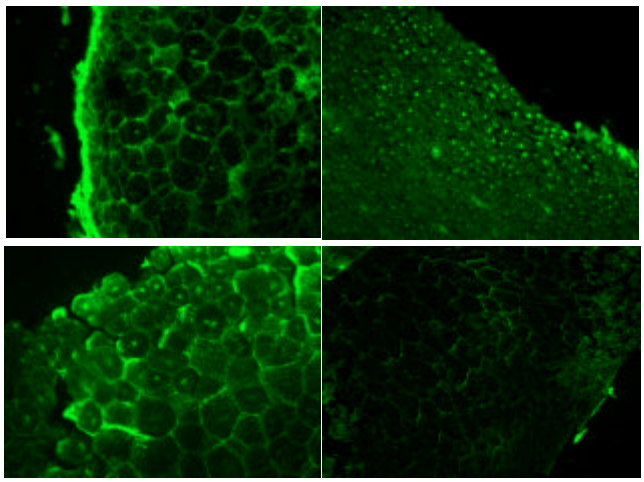

Fig. 3 (a-d): The GFP observation of paraffin sections treated by different concentration of *Agrobacterium* medium , (a) OD_{600} = 0.5, (b) OD_{600} = 1.0, (c) OD_{600} = 1.5 and (d) CK

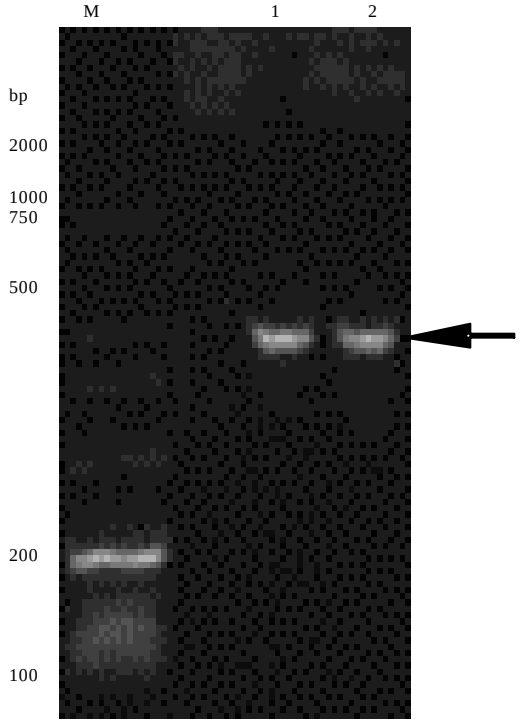

Fig. 4: Amplification of *hyp* gene positive plant, M: Marker DL 2000; 1,2: Fragment of positive plant, arrow indicating the target band

Selection of hygromycin-resistant seeds: Seventy-nine T_0 seeds were used to screen hygromycin resistant. The results showed that only thirty of T_0 kernels germinated, with the germination rate of 38%. There were three possibilities for the low germination rate, (1) the seeds maturity were not enough to germinate, (2) pathogen led to seed mildew or rot and failed to germinate and (3) possibility of lethal mutations might exist. Through the screening experiment, hygromycin-resistant seedlings were easily distinguished from the others. The resistant seedlings showed green expanded cotyledons whereas non-resistant seedlings displayed cotyledons with yellowish-brown necrotic spots. Finally, one hygromycin-resistant seedling was acquired and the positive rate was about 3.3%.

PCR detection of T_1 positive plant: The T_1 positive transgenic plants were used for detection of PCR using HYP primers. About 910 bp band of exogenous *hyp* gene was amplified (Fig. 4), indicating a T-DNA carried exogenous *hpt* gene had integrated into the plants genome.

DISCUSSION

Previous studies used dicotyledonous model plant *Arabidopsis thaliana* as the object to research the floral dip process. Based on the studies of the *gus* gene expression and other results from pollination experiment, ovules was considered as direct and effective transformation target using *Agrobacterium*-mediated vacuum infiltration or impregnation method (Rakousky *et al*., 1997; Ye *et al*., 1999; Desfeux *et al*., 2000). Maize is the typical dicliny plant which is extremely helpful for the occurrence location of transgenes in different tissues and cells using floral-dip method. Therefore, the maize female inflorescence was chose for transformation in the present study. In the meantime, the sensitive degree of plant materials for *Agrobacterium* was related to its developmental and physiological state (Escudero *et al*., 1996). It is thus very important to select the suitable development state of plant for increasing transformation efficiency in floral dip method. When maize corn silk elongate to 20~30 cm which has lignified and is close to maturity, it is not suitable for transformation. In the present study, the maize inflorescences whose corn silk size is 5~10 cm was selected for transformation, because this stage lignification of corn silks was about to begin and cells were at the mitosis period. Present results confirmed that this stage of corn silk was suitable for *Agrobacterium*-mediated transformation.

The *Agrobacterium*-mediated floral dip method was firstly used on transformation of maize female inflorescence. The concentration of *Agrobacterium* culture will also be important for the transformation efficiency, since the medium with high sugar content in transformed-maize ears usually make it easy to be invaded by diseases and pests. We designed four levels of concentration of the bacterial culture to check the effects on transformation rate. Transformed by bacteria liquid at concentration of OD_{600} = 1.0 went to optimal efficiency. Wherever, transformed by bacteria liquid at the concentration of OD_{600} = 2.0 has ear rot and no seed reaped. The reason might be that concentration of OD_{600}= 2.0 was too high for maize *Agrobacterium*-mediated transformation, leading to maize grain decay. Our result was different from the report of floral dip transformation for soybean (Wang *et al*., 2010), it is maybe resulted from different types of flower organs between corn and soybean.

The detection result of GFP expression with strong fluorescence spots were visualized at the embryo of seeds, indicating *gfp* gene has been successfully integrated into the plant nucleus genome. Furthermore,

green fluorescent spots occurred not only in part of the cells but also in almost every cell. Thus, it could be deduced the seed developed from a zygote that came from fertilization of the female gametophyte cells carried exogenous gene and male gametophyte cells, consequently every cell divided by this zygote cell would carry the exogenous gene, so green fluorescent spots existed in almost every cell. There were no fluorescent spots in a few cells, probably because the nucleus was just section to another connected slice of paraffin section. Previous finding exposed that maize female inflorescence could be used as receptor of the floral dip method transformation and female gametophyte may be the target cells. This result is accordance with the viewpoint of Desfeux *et al.* (2000). In this study maize tassel were not transformed which will be probed in further research.

According to expression of reporter genes GFP, hygromycin-resistance screening experiment and PCR detection of transgenic plant, maize female inflorescence was sensitive for *Agrobacterium* inoculation. and one hygromycin-resistant seedling was acquired which demonstrates that as a cereal crops, maize can be infected by *Agrobacterium* and the *Agrobacterium*-mediated floral dip method also can be used for maize gene transfer. This findings confirmed the previous points of view (Chan *et al*., 1992; Raineri *et al*., 1993). Successful application of floral dip method on maize represents a considerable breakthrough compared with traditional maize transgenic methods, as it negates complicated tissue culturing procedures and can produce transgenic plants in a relatively short period of time (Clough and Bent, 1998).

CONCLUSION

In this study, the *Agrobacterium*-mediated floral dip method was successfully used in maize female inflorescence transformation in the fields. The result of GFP detection for T_0 transformed corn silk and seeds showed that the female inflorescence of maize was sensitive to *Agrobacterium*. Through hygromycin selection, a transgenic plant with hygromycin-resistance had been acquired successfully. The period when the corn silk hold out 5-10 cm was suitable for maize floral dip method transformation. The optimal cell concentration of *Agrobacterium* transformation was $OD_{600} = 1.0$.

Agrobacterium-mediated floral dip can be used for the genetic transformation of maize. The application of new method of genetic transformation for maize will greatly improve the maize gene transformation efficiency which is significant for maize function genome research and molecular breeding.

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