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Full Length Article

Maize Genetic Transformation by *Agrobacterium tumefaciens* Assisted with Emery and Sonication Treatment

Xue-lian Liang[1], Kun Chu[1], Xiao-xiong Nie[1], Hua-jie Feng[1], Gui-li Han[1], Wen-bin Yi[1], Qi-jing Wu[1] and Yi Sun[2*]
[1]*College of Life Science, Zhongkai University of Agriculture and Engineer, Guangzhou 510225, PR China*
[2]*Biotechnology Research Center, Shanxi Academy of Agricultural Sciences/Key Laboratory of Crop Gene Resources and Germplasm Enhancement on Loess Plateau, Ministry of Agriculture, Taiyuan 030031, PR China*
[*]For correspondence: sunyi692003@163.com

Abstract

In the past three decades, *Agrobacterium*-mediated genetic transformation has been widely used in breeding and molecular biology research. However, the classical *Agrobacterium* transformation method must employ tissue culture procedures, which are considered tedious, lengthy, and expensive as well as being genotype dependent. In this study, the ultrasonication-assisted *Agrobacterium* treatment method was modified with the addition of emery to increase the treatment intensity to the apical meristem cells of germinating seeds. In this study, the feasibility of emery grits assisted *Agrobacterium* transformation for maize was preliminary evaluated by glyphosate screening and PCR detection, which suggested that *epsps* gene was introduced into the maize cultivar. The transformation rate was enhanced by the addition of the emery and sonication treatment. The method has the advantages of circumventing the tedious tissue culture procedures and being easy to apply.

Keywords: *Agrobacterium*; Maize; Corundum powder; Ultrasonic; Germinated seeds; *epsps* gene

Introduction

Over the past three decades, the value of introducing foreign genes into the important arable crops has been well documented (Malik and Sanadhya, 2018; Kauscha *et al.,* 2019). Various genetic transformation techniques have been employed to introduce potentially useful agronomic traits, e.g. insect, disease and herbicide resistances and abiotic stress tolerance (Liang and Skinner, 2004; Rivera *et al.,* 2012; Kauscha *et al.,* 2019). Since the first transgenic plant by *Agrobacterium* transformation was reported (Bevan, 1984), the technique has been widely-used and transformation mechanism of *Agrobacterium* has been gradually upgraded. *Agrobacterium* transformation method has advantages in transferring low copy numbers of foreign DNA, as well as large DNA fragments (Zhang *et al.,* 2001; 2017). Since then, a number of *Agrobacterium* transformation systems have been established for maize (*Zea mays*), rice (*Oryza sativa*) and other important cereal crops (Kumar *et al.,* 2019; Wu and Sui, 2019). However, tissue-culture procedures were still employed in their protocols, which are considered to be tedious, lengthy, and often cause somaclonal variation. Therefore, it is desirable to have plant transformation methods circumventing tissue culture procedures. Transformation techniques not involving tissue culture are also desirable for crop species in which many cultivars or lines do not respond to tissue culture (Liang and Skinner, 2004). New transformation approaches have been innovated, including pollen-mediated transformation of maize (Wang *et al.*, 2001; Liu *et al.*, 2016; Yang *et al.*, 2017), sorghum (*Sorghum bicolor*) (Wang *et al.*, 2007a) and *Brassica juncea* (Wang *et al.*, 2008), *Agrobacterium* transformation of maize germinating embryos (Wang *et al.*, 2007b; Liang *et al.*, 2016; Wang *et al.*, 2017), and pollen transformation with magnetic nanoparticles as gene carriers (Zhao *et al.*, 2017). The acetosyringone (AS) is often used for enhancing the transformation efficiency in the studies of transforming monocotyledonous plants (Hiei *et al.,* 1994; Smith and Hood, 1995).

Ultrasonication treatments of plant tissues are also being used by some researchers to improve transformation frequencies. Trick and Finer (1998) used it in transforming soybean suspension tissue culture and Santarém *et al.* (1998) used it to assist transforming soybean immature cotyledons. A method called Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT) was developed to transform cells in the target tissue, which subjects the maize tissue to brief periods of ultrasound in the presence of *Agrobacterium* (Vasconcelos*et al.*, 2008). SAAT treatment produces small and uniform fissures and channels throughout the tissue, allowing the *Agrobacterium* easier access to internal plant tissues. Comparing with other

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methods, ultrasonic transformation approach has the following advantages: simple equipment, convenient operation, large sample processing capacity, and low cost.

Wang *et al.* (2007) reported an *Agrobacterium*-mediated transformation method using plant germinating seeds as recipients, in which embryos of the germinating seeds are scarified, and co-cultivated with *Agrobacterium* Ti plasmid as gene carrier, then detect transformants by bioassay, PCR amplification and Southern hybridization.

Therefore, we assumed that that micro particles of emeries added in the ultrasonic treatment would further facilitate the *Agrobacteriu*m to deliver exotic DNA into recipient plant cells. The wounds made by ultrasonication and emeries on the plant cell walls might enhance the probability of foreign DNA to enter the cells, thus to further improve the transformation efficiency and reduce the formation of chimera. In this study, the former ultrasonic assisted *Agrobacterium* treatment method of maize germinating seeds was used with the addition of emery assisted treatment to increase the treatment intensity to the growth point of germinating seeds, as well as reducing the time of ultrasonic treatment of seeds. In this paper, the feasibility of emery grit assisted *Agrobacterium* for maize gene transformation was preliminary studied and reported.

Materials and Methods

Experimental Material

The test material was a waxy maize cultivar, Zhongnuo No. 1 and the gene expression vectors used in this study was *Agrobacterium tumefaciens,* strain: EHA105 (Fig. 1). The PCR primers for detecting the presence of the 1227 bp foreign gene were designed within the CP4-*epsps* gene sequence as follows: Forward primer: CGC AAA TCC TCT GGC CTTTC; Reverse primer: GAC AGG GTT TTC CGA CAC G.

Germination and emery ultrasonic treatment of maize seeds: A total 1000 mature maize seeds were soaked in 30℃ water bath for 24 hours, and were divided into the experimental group (800 seeds) and the control group (200 seeds). Then for the seeds in the experimental group wounds were made in approximate the apical meristem region by scratching both longitudinally and transversely at the emerging embryos using a scalpel. The cutting depth was about 0.5 mm, and care was taken not to damage the embryos. To further wounding the apical meristem cells and increase the opportunity for the *Agrobacterim* to invade the wounded cells, the wounded maize seeds were put into a beaker with emery powder and water, and then the ultrasonic treatment (Fig. 2) was applied to the solution by using JY92-II Ultrasonicator from Ningbo Xinzhi Biotech Company. The parameters for sonication treatment were: sonic intensity of 800 W, treatments for 3 times each for 10 s with 3 s intervals. The seeds in the control group were made to germinate normally without any special treatment.

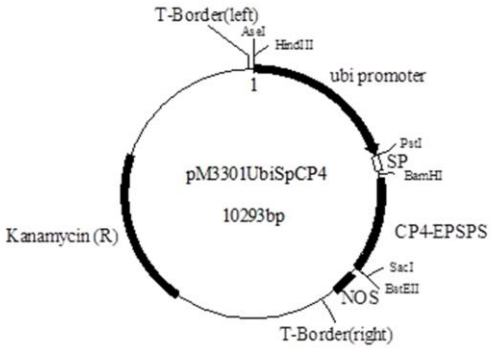

Fig. 1: Gene expression vector

Fig. 2: Ultrasonic treatment of wounded maize seeds with the addition of emery grits

Co-cultivation of wounded seeds with the *Agrobacterium*: The co-cultivation methods were following Wang *et al* (2007). Cultures of the *A. tumefaciens* strain were initiated from glycerol stocks and inoculated in liquid Luria–Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) containing 50 mg/l kanamycin and allowed to grow overnight at 27–28℃ with shaking (150 rev./min) to the mid-exponential phase with attenuance (D_{600}) 0.7. The bacterial cells were collected by centrifugation and re-suspended in sterilized water containing 100 μmol/l acetosyringone (AS). The *A. tumefaciens* cell concentration was adjusted to give a D_{600} of 0.2–0.4 for co-cultivation.

Inoculation and co-cultivation: The treated seeds were put into jars (25 seeds/jar) and cultured in a shaker after adding the cell suspension of *A. tumefaciens* with acetosyringone. The control group was also put into jars, but added with tap water instead of bacterial suspension. The experimental group and the control group were placed on a shaker and

agitated (about 150 rev./min) for about 12 h at 25–26℃.

Sowing and screening after the co-cultivation: After the co-cultivation the seeds were washed with running tap water, and then sown to an experimental field. Fields were fertilized and watered at the five leaf stage. Plants were sprayed with glyphosate at the following stages and concentrations: A concentration of 0.15% glyphosate was sprayed on the seedlings at the end of the week 2; the 2nd spray of 0.3% glyphosate was applied at the week 4; and the 3rd spray of 0.3% was at the week 8. There were 27 plants in the experimental group survived of the glyphosat screening, which were denoted from a to z and A, and the plants in the control group were denoted from 1 to 7.

Five plants were randomly selected from each of the treatment and control groups to measure the plant height, and Student's *t*-test was employed to compare the significance of the difference between the two groups (Table 1).

DNA extraction and PCR amplification: Total DNA of putative transgenic plants was extracted with SDS (Sodium dodecyl sulfate) reagent. According to the sequence of the CP4-*epsps* gene, a pair of primers was designed. The primers were synthesized by the Invitrogen. The fragment size between the two primers was 1227 bp. PCR amplification was completed using the Dongshen Biotech TaqTM Kit and a EPPENDORF Master cycler gradient 5331. The PCR reaction system was shown in Table 2 and the reaction procedures were shown in Table 3. The PCR products were fractioned by agarose electrophoresis, and viewed by a Tannon-4100 Gel Imaging Analyzer from Shanghai Tianneng Biotech Company.

Results

Field Screening and DNA extraction

Field statistics: Emergence rate of untreated maize seeds = number of seedlings in the control group/ number of seeds sown in the control group = 147/200 = 73.50%.

Emergence rate of treated maize seeds = number of seedlings in the experimental group/number of seeds sown in the experimental group = 93/800 = 11.63%.

The emerged 93 plants were sprayed three times with above glyphosate solution, and 27 of them survived (Fig. 3), their leaves were taken with a scissors for DNA extraction at the week 10. Total DNA of both putative **T_0** and control maize plants were extracted as shown in Fig. 4.

The plant height of the transgenic plants was significantly lower than those of the control plants (Table 1; $P < 0.05$).

PCR Amplification and Electrophoresis of T_0 Generation

Most of the plants from the experimental group showed a 1200 bp band in PCR amplification (Fig. 5) implying that cp4-*epsps* gene was successfully transferred into them.

The transformation rate = number of the PCR positive plants / number of emerged seedlings = 23/93 = 24.7%; but the rate would be 85.19% if we use the number of total survived putative T_0 plants as the denominator.

Table 1: Effect of treatment on maize plant height

Treatments	Plant height (CM)					
	1	2	3	4	5	Mean
Treated plants	148	173	156	164	168	161.8 a
Control plants	185	180	190	178	188	184.2 b

Note: Student`s (T) test for significance of difference , a, b ($P < 0.05$)

Table 2: The PCR system for detecting the existence of the *epsps* gene

PCR ingredients	Volume (μl)
ddH_2O	13
10 × Taq buffer	2.0
dNTPs	0.5
Primer 1	1.0
Primer 2	1.0
Template DNA	2.0
TaqDNA polymerase	0.5
The total volume	20.0

Table 3: The PCR reaction procedures for detecting the existence of the *epsps* gene

Steps	Temperature	Time	Process
Step 1	94℃	5 min	Pre denaturation
Step 2	94℃	1 min	Step 2 to 4 for 30 circles
Step 3	55℃	45 s	
Step 4	72℃	1 min	
Step 5	72℃	10 min	Extension
Step 6	4℃	Hold	End

Discussion

The above results showed that the transformation efficiency of *Agrobacterium* could be improved by treating germinating maize seeds with emery and sonication. In the previous report (Wang *et al*., 2007), the efficiency of the *Agrobacterium*-mediated transformation targeting maize seeds is relatively low. The overall transformation rate was only 0.6% when total treated seeds were taken into account, and about 29% of putative T_0 seedlings were found to be transgenic. A similar experiment by scratching the apical meristematic region and followed co-cultivation with *Agrobacterium* was about 1.8% (Liang *et al.*, 2005) and 2% (Wang *et al.*, 2017), respectively. In this study, with the addition of emery and ultrasonic treatment together with scratching germinating seeds the overall transformation rate was enhanced to about 24.7% and more than 85% of the glyphosate-survived plants were detected being PCR positive. The shock of ultrasonic wave strongly pushed the movement of emery particles, which further rubs the apical meristematic region of maize seeds and enlarge the fissures made by scratching. This would give the bacteria better

Fig. 3: Glyphosat screening for transgenic maize plants
A: Treatment rows (27 glyphosat resistant plants); B: Control rows

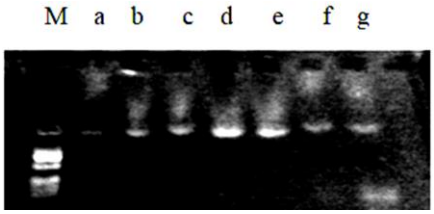

Fig. 4: DNA extraction of partial putative T_0 plants
Note: T_0: Plants of first transformed generation; M: Marker; a-g: partial putative T_0 plants

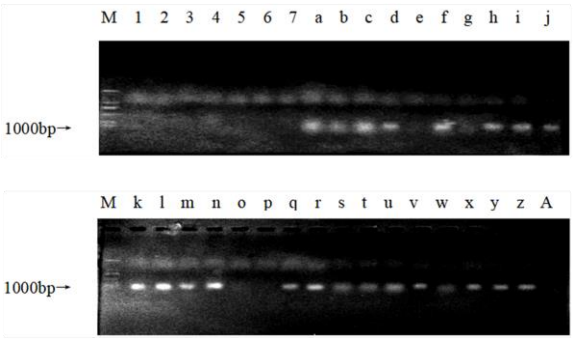

Fig. 5: Agarose gel electrophoresis of the PCR products for detecting T_0 plants
Note: T_0: Plants of first transformed generation; M: Marker; 1-7: Plants of control; a-A: Transgenic plants

chance to invade the meristematic cells and deliver T-DNA harboring exotic DNA to the cells.

After the treatment with scratching, emery addition and sonication, the emergence rate of treated seeds was 11.63%, and the higher transformation rate was achieved compared with co-cultivating germinating seeds alone. Heat would be generated continuously during ultrasonic treatment, which would increase the temperature of the solution and produce certain thermalization effect on seeds. Prolonged treatment might cause greater damage to the seeds. With the addition of emery, the treatment strength on the growth point of seeds was enhanced, and the processing time of ultrasonication could be reduced to avoid the damage from the thermal effect on the seeds. The plants obtained from the treated germinating seeds had lower plant height than control plants (Table 1). The reason behind is that these plants were suffered from the wounding and herbicide application on the T_0 generation. The effect of the transgene to the plants of later generations needs to be investigated.

We have here reported an easy-applicating *Agrobacterium*-mediated genetic transformation approach using sonication at the presence of emery particles. This technique is easy to apply and ready to be integrated into conventional breeding program. It might also be applicable to other plants, especially those small seed plants, which are not feasible to use embryo scratching by hands. Facilities used for this approach is relatively simple and cost less. The study on inheritance of the transgene is undergoing.

Conclusion

The addition of emery together with the ultrasonic treatment promoted the introduction of *epsps* gene into maize. The study led to the conclusion that the feasibility of emery assisted maize gene transformation was preliminary confirmed through field screening and PCR detection.

Acknowledgments

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