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Full Length Article

Generation of Transgenic Maize by Two Germinating Seed Transformation Methods

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Abstract

Two germinating seed transformation methods i.e., scarifying and puncturing methods, were used to transfer the bar gene into an inbred maize line. We generated transgenic glufosinate-tolerant maize plants, and conducted a comparison of the two transformation methods. We used the plasmid pCAMBAR.CHI.11 as the donor and germinating maize seeds as the recipients. The germinating seeds were subjected to puncture or scarifying treatment after being soaked in water for 12 h. The injured seeds were co-cultivated with *Agrobacterium* for 24 h and then sown in the experimental plots. Putative transgenic maize plants were selected by basta tolerance screening and PCR detection, and were further confirmed by Southern blotting. Following glufosinate tolerance assay and molecular detection of transgenic lines of the transgenic seedling (T_0), first generation of transgenic seedling (T_1) and second generation of transgenic seedling (T_2), we have confirmed that the bar gene was stably inherited and expressed. The PCR amplification result for T_2 transgenic plants showed that the genetic segregation ratio of the bar gene followed the 3:1 ratio of a single dominant Mendelian factor. The study proved that both the germinating seed treatment approaches are rapid and simple plant transformation methods. In particular, the application of the puncture method can expand the tissue culture free transformation to dicotyledonous plants. © 2016 Friends Science Publishers

Keywords: Maize; Genetic transformation; Germinating seeds; Scarify; Puncture; Bar gene

Introduction

The global acreage of transgenic crops has expanded rapidly since 1996 and exceeded 181 million hm^2 in 2014, which is the 18th year of successive increase (Clive, 2015; http://www.isaaa.org/resources/publications/briefs/49/default.asp). Currently, widely used methods for plant transformation are *Agrobacterium*-mediation and particle bombardment (Bo *et al.*, 2015; Vijaya *et al.*, 2015). Both methods use calli as recipients, and require tissue culture processes, but the common shortcomings of plant transformation methods using tissue culture are lengthy, tedious, and expensive with relatively low efficiency (Ozeki *et al.*, 1997; Wang *et al.*, 2007). Furthermore, they are also genotype dependent and prone to produce somaclonal mutations (Gao *et al.*, 2009; Frame *et al.*, 2011; Omer *et al.*, 2013).

Using particle bombardment, although there are no limits on the hosts, there may be some bad effect such as mutations. Using agrobacteria is a natural mechanism however, maize is not a natural host for *Agrobacterium* (Jackson *et al.*, 2013). Researchers have attempted various means to improve transformation efficiency, such as adding acetosyringone (AS) during the infection process (Shahla and Donald, 1987), screening for virulent strains and using a binary vector, etc. (Miller *et al.*, 2002; Vega *et al.*, 2008; Deeba *et al.*, 2014). Glufosinate is a broad-spectrum herbicide used to control certain important weeds. It is applied to young plants during early development for full effectiveness. It is typically used for directed sprays for weed control, including in genetically modified crops (https://en.wikipedia.org/wiki/Glufosinate).

To circumvent tissue culture processes, researchers have been attempting tissue-culture-free plant transformation methods for decades. The pollen tube pathway, which was first proposed by Zhou *et al.* (1983) as a plant genetic transformation method using the plant reproductive system as the pathway and zygots as recipients, allowed the successful transformation of cotton (Vijaya *et al.,* 2015). The method was confirmed by Luo and Wu

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(1989) on rice, but failed on soybean (Shou *et al*., 2002). Wang *et al*. (2001) recovered transgenic maize plants using a pollen-mediated transformation method, in which they collected fresh pollen in a sucrose solution and conducted ultrasonic treatment of the pollen together with a plasmid DNA harboring a target gene. This process is more suitable for plants with large amount of pollen like maize (Eapen, 2011).

In their efforts to improve plant transformation techniques, the researchers continued to attempt transformation studies using various *in vivo* (*on planta*) tissues and organs, such as the shoot apex, apical meristems and mature seeds (Chen and Dale, 1992; Sticklen and Oraby, 2005; Al-Abed *et al.,* 2006; Risacher and Craze, 2012). These strategies are simple, time- and labor-effective and genotype-independent since they do not depend on tissue culture procedures. Liang *et al.* (2005) and Wang *et al*. (2007) hypothesized that the injured germinating embryos are ideal natural competent statuses for transformation, *Agrobacterium* could take advantage of the natural imbibition process of seeds and penetrate through wounded embryos, thus achieving the transformation to the meristematic cells. Therefore, they reported a method for transforming embryos of germinating seeds, in which they made wounds longitudinally along the embryos on the growing point region of germinating seeds, then the wounded seeds were cultivated in an *Agrobacterium* suspension on a shaker. Transformants were obtained by followed herbicide screening and molecular verification of seedlings from the treated seeds. Also transformation has been achieved in rice, tomato and *Morinda citrifolia* with this and similar methods (Liu *et al.*, 1999; Lee *et al.*, 2013; Vinoth *et al*., 2013). Electron microscopy analysis of rice embryos has revealed that the internal structure and cell surface of apical growing points go through a series of microscopic and submicroscopic structural changes during soaking; these structural changes were highly favorable for invasion by *Agrobacterium* (Liu *et al.*, 1999).

We attempted to enhance the efficiency of the *Agrobaterium*-mediated transformation method. It is hypothesized that making wounds is a key step in this method, and being able to injure the meristematic region precisely thus allowing agrobacteria to invade the cells is vital. In this article, we have compared two transformation methods using germinating seeds, the puncture method on embryos with puncture needles and the scarifying germinating seed method.

Materials and Methods

Maize Materials

The inbred maize line Zheng 58 was provided by the Shanxi Qiangsheng Seed Co Ltd. The study was conducted at the experimental plots of Shanxi Academy of Agricultural Sciences, China between 2010 ~ 2014.

Treatments of Germinating Seeds using the Puncture or Scarifying Method

After soaking the maize seeds in water at 25ºC for 12 h, they were divided into two groups. In one group, the growing point region of the embryos were scarified with a scalpel, and in the other group, the growing point region of the embryos were punctured with a cluster of needles (three acupuncture needles were fixed together with a rubber band into a small triangle with a side length of approximately 1 ~ 1.5 mm, and puncture depth should be 1 ~ 1.5 mm). The *Agrobacterium* tumefaciens strain (LBA4404, harbouring the binary vector pCAMBAR.CHI.11) were initiated from glycerol stocks and inoculated in liquid Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) with 50 mg/L kanamycin. When the *Agrobacterium* culture were at the logarithmic phase (OD_{600} = 0.6), 100 mM acetosyringone (AS) was added in (Ishida *et al*., 2007; Eva *et al*., 2011). Three hundred wounded seeds were submerged in a mixture of 135 mL of water and 15 mL of above *Agrobacterium* culture, and then cultivated on a shaker at a speed of 200 rpm for 24 h. Following the co-cultivation, the treated seeds were rinsed with tap water and sown in the experimental plots, and the top soil was kept moist until seedlings emerged.

Agrobacterium Strain and Plasmid

The vector used in the transformation was pCAMBAR.CHI.11, carrying a hygromycin resistance gene and a *bar* gene from *Streptomyces hygroscopicus* (hereafter referred to as the bar gene or the target gene); the *Agrobacterium* strain was LBA4404, which was provided by Prof Muthkrishnan of Biochemistry Department, Kansas State University, USA. The promoter for the *bar* gene is CaMV35S and the terminator is CaMV35SPoly (A). The physical map of the plasmid is shown in Fig. 1A.

Screening Tolerant Maize Plants in the Field

The treated germinating seeds were sown directly in the plots; when the seedlings grew up 3 to 5 leaf stage, glufosinate (1.5‰, Phosphinothricin PPT) was uniformly sprayed on the leaves, and a follow-up spray was applied one week later. Fourteen days after the second spray, seedlings were examined for survival rate, plant height and injured leaf area (an estimated value). Tolerance evaluation was done following the method of Mannerlöf *et al.* (1997). T_1 generation seeds were sown into an experimental plot and seedling leaves were painted with PPT (1.5‰) solution at 3-5 leaf stage, and the number of survived seedlings was counted after 10 days. The surviving seedlings were then investigated for tolerance levels. The specific criteria used for the classification of tolerance were as follows (Mannerlöf *et al*., 1997):

(a) Highly tolerant (H): entire leaves of the plant were upright with normal green color. There was no or only single yellow spots on the leaf surface (equivalent to 8–10 levels and the plant growth was not affected.

(b) Moderately tolerant (M): the majority of leaves had normal green color but drooped slightly, their growth was retarded, and yellow spots appeared on a few leaves (equivalent to 6–8 levels).

(c) Lowly tolerant (L): some leaves were yellow and wilted with apparent symptoms of infection. Their growth and development were affected, but the plants were able to restore their growth afterwards; the plants showed tolerance to some extent (equivalent to 3–5 levels).

(d) Sensitive (S): all leaves turned yellow and wilted and never returned to growth again (equivalent to 1–2 levels).

Molecular Detection

Total plant DNA was extracted with the method of Wang *et al.* (2007); the PCR was performed in the volume of 25 µL, and the amplification primers of bar gene were 5'-ACCATCGTCAACCACTACAT-3' (forward) and 5'-AGTCCAGCTGCCAGAAACCC-3' (reverse). The primers were synthesized by Shanghai Sangon Co. and the size of the amplified product was 438 bp. The amplification reaction program was: initial denaturation at 94ºC for 300 s, 30 cycles (denaturation at 94ºC for 40 s, annealing at 58ºC for 45 s, extension at 72ºC for 60 s). The PCR products were separated by electrophoresis on a 1.2% agarose gel; the results were observed on the SYN gel imaging system (Syngene, Cambridge, England) and the specificity of the amplification product was analyzed, and then photographed.

For Southern blotting hybridization, the probe of bar gene from pCAMBAR.CHI.11 was labeled using the PCR DIG Probe Synthesis Kit,then 20 µL genomic DNA was digested by *Bam*HI, and were fractioned by 0.7% (w/v) agarose gel electrophoresis, transferred to a nitrocellulose (NC) filter membrane, the membrane was hybridized with bar probe at 68ºC for 12 h according to the DIG DNA Labeling and Detection Kit, finally detected with CSPD (disodium 3-{4-methoxyspiro [1,2-dioxetane-3,2′-(5′-chloro) tricyclo(3.3.1.1^{3,7}) decan]-4-yl} phenyl phosphate) florescence stain, and exposed to an X-ray film (Liang *et al.*, 2005; Wang *et al.*, 2007). The PCR Dig Probe synthesis Kit, Dig DNA Labeling and Detection Kit, and CSPD were purchased from Boehringer Mannheim, Mannheim, Germany.

The T_1 plants containing a single copy transgene revealed by Southern blotting were advanced to T_2 generation, and the T_2 plants were subjected to PCR for the genetic segregation analysis of the target gene.

Statistical Analysis

Chi square (χ^2) test was used to analyze the significance of differences between the scarifying and puncturing methods and validity of the target gene segregation among the T2 population.

Results

Screening of the Transformed Seeds

A total of 3580 seeds were treated, among them, 1580 seeds were treated using the scarifying, and 2000 seeds were treated by puncturing. The treated seeds were sown directly in an experimental plot. Among the 3580 seeds, 802 seedlings emerged including 324 from scarifying treatment and 478 from puncture treatment. The emergence rates were 20.5% (324/1580) and 23.9% (478/2000) for the scarifying- and puncturing-treated seeds, respectively. In the 3 to 5-leaf stage, glufosinate was sprayed twice for screening purposes and 76 seedlings survived (31 scarifying-treated and 45 puncturing-treated, see Table 1). Based on the χ^2-test, highly significant differences existed between the two treatments for seedling emergence, but no significant difference was detected for PPT tolerance rate and PCR positive lines.

The total DNA of fresh leaves of all survived plants was extracted, and 23 putative transformed plants were obtained using PCR amplification (9 scarifying-treated and 14 puncturing-treated). The results of herbicide screening revealed that there was a significant difference between the putative transformed plants and the control plants. All control and non-transgenic plants died after herbicide spraying, and putative transgenic plants showed certain tolerance to glufosinate, although there were variations among the individuals (Fig. 2A).

T_0 Generation Transgenic Plants

The 438-bp band was detected (Fig. 1B) with PCR amplification in all of the 23 T_0 tolerant seedlings, which were self-pollinated and 23 putative transgenic ears were obtained.

Glufosinate Tolerance Screening of T_1 Seeds

Seeds of all 23 putative transformed plants of the T_0 generation were sown in the experimental plot; and 1.5 ‰ PPT solution was painted on young leaves at the 5-leaf stage. After 10 days, the painted plants were evaluated for PPT tolerance (Fig. 2B). At this stage, there was little effect of the glufosinate on most seedlings; only pale yellow traces appeared where the glufosinate was painted. However, most control plant leaves were wilted, and even if the seedling did not die, the entire plant wilted and was unable to return to normal growth.

Tolerance screening was performed on the seeds of 23 T_1 ears (Table 2). A total of nine ears and 134 seeds were obtained from the scarifying-treatment of which 54 seedlings survived after herbicide screening, which were both PPT painting-tolerant and PCR-positive. A total of

Table 1: Information on T_0 generation plants obtained using the two germinating seed transformation methods

Treatment	Number of treated germinating seeds	Germination rates	PPT-tolerance rate	PCR-positive lines
Scarifying	1580	324(20.5%) **	31 (9.5%) NS	9 NS
Puncture	2000	478(23.9%) **	45 (9.4%) NS	14 NS
Total	3580	802(22.4%)	76 (9.4%)	23

**denote the highly significant differences, and NS means the differences were not significant

14 ears and 350 seeds were obtained from the puncture treatment, of which 75 seedlings survived after herbicide screening, which were both PPT painting-tolerant and PCR-positive. The PCR-positive rates of the PPT painting tolerant seedlings were 100% and 98.7% for the scarifying and puncture treatments, respectively. Combined with subsequent Southern blotting results, 10 positive lines (3 from the scarifying treatment and 7 from the puncture treatment) were obtained. Transgenic lines were evaluated for glufosinate tolerance, which showed that three lines were highly tolerant, four had moderately tolerance, and three had low tolerance. For the growth robustness, apparent differences existed among various transgenic lines, in addition to herbicide tolerance activity; but the performances of plants within lines were relatively uniform.

Southern Blotting of T_1 Plants

Southern blotting of the T_1 generation (Fig. 1C) proved that the target bar gene was integrated into the genome with both single and multiple copy insertions (Lanes 2, 4). No specific hybridizing band was observed in the Lane 5, a PCR-positive plant, which might be due to the reason that the target gene only existed freely in the plant cell and was not integrated into the genome.

We finally obtained 3 Southern hybridization-confirmed transgenic lines from scarifying treatment, with an overall transformation rate of 0.19% (3/1580). Seven Southern hybridization- confirmed transgenic lines from the puncture treatment, with an overall transformation rate of 0.35% (7/2000). However, if the number of herbicide tolerant plants was used as the base to calculate the transformation rate, the rate for scarify treatment was 9.6% (number of Southern-positive seedlings, i.e., 3 per number of tolerant seedlings that were scarifying-treated, i.e., 31); and the rate for the puncture treatment was 15.5% (the number of Southern positive seedlings, i.e., seven per the number of tolerant seedlings that were puncturing-treated, i.e., 45).

Segregation of Target Gene in T_2 generation

A total of 1260 seedlings were subjected to PCR and 932 were positive. The χ^2 analysis suggested that the segregation of all the T_2 lines followed the 3:1 ratio of Mendelian genetic segregation law (P>0.05), implying that the exotic bar gene exhibited as a single dominant genetic factor in segregation of the T_2 generation (Table 3).

Table 2: Tolerance screening of the T_1 generation

Number of ear	Method	Number of seeds	Painting tolerant seedlings	PCR-positive seedlings	Southern positive seedlings	Tolerance level
1	Scarify	27	8	8	1	H
2		25	0	0	0	
3		29	7	7	0	
4		21	8	8	2	MM
5		18	5	5	0	
6		20	6	6	0	
7		22	0	0	0	
8		22	7	7	0	
9		20	5	5	0	
10	Puncture	19	0	0	0	
11		26	9	9	1	L
12		20	4	4	0	
13		31	7	7	0	
14		24	7	7	1	M
15		26	7	7	0	
16		18	6	6	0	
17		19	5	5	0	
18		27	6	6	1	H
19		29	8	8	2	MH
20		27	8	8	0	
21		28	7	7	1	L
22		29	5	5	1	L
23		30	3	3	0	
Total		487	128	128	10	

H: Highly tolerant; M: Moderately tolerant; L: Lowly tolerant

Table 3: PCR amplification and statistical analysis of the T_2 generation

No. of T_2 line*	No. of PCR-positive plants	No. of PCR-negative plants	Expected ratio	Probability	χ^2
T_2-1	138	49	3:1	0.703	0.144
T_2-4	130	54	3:1	0.173	1.855
T_2-5	122	53	3:1	0.106	2.607
T_2-6	140	53	3:1	0.429	0.623
T_2-7	149	40	3:1	0.223	1.483
T_2-9	125	49	3:1	0.335	0.927
T_2-10	119	39	3:1	0.926	0.008
Totle1260	932	328	3:1	0. 715	6. 635

*T_2 line: Second generation of transgenic seedling

Discussion

When treating germinating embryos by *Agrobacterium* transformation, we should primarily consider the impact of the depth of cutting or puncturing on each embryo because too deep cut would make the entire embryo to die; and too shallow will not be exposed the meristematic cells of embryos and thus will not achieve transformation. An optimal depth should be penetrating through the seed coat until the growing point of the embryo was slightly wounded (Al-Abed *et al.*, 2006) whilst minimizing the

damage to other tissues. When puncturing an embryo, the three puncture needles were fixed by a piece of rubber and mini holes were made in the growing point region, which might increase the likelihood of exposing the embryo meristematic cells and improve transformation efficiency. The wounded seeds should be co-cultivated with the *Agrobacterium* for about 24 h; extended co-cultivation period will cause the seeds to rot, while the too short time may not give *Agrobacterium* enough time to invade the seed meristematic cells. A higher bacterial concentration was not always better; the *Agrobacterium* concentration we used was at a logarithmic growth phase and was diluted to the optical density of OD_{600} = 0.6 and the AS concentration was 100 mM (Ishida *et al.*, 2007; Eva *et al.*, 2011). To summarize, the optimization of these factors should be investigated further such as whether ultrasonic treatment should be added before or after the embryos are injured, the effects of various seed shapes and locations of embryos, and the effects of seed coat thickness on the puncture and scarifying methods should also be considered. In addition, shallow furrow should be used for sowing the treated germinating seeds, which were to be covered with a thin layer of soil and should be kept moist, because the seeds were injured and their shoots are weak.

It was observed that some positive plants of T_0 became negative at the T_1 generation and vice versa. This could likely be attributed to the DNA samples were collected from different leaves of T_0 plants. Because we made the injury treatment on seeds to make transformation, but the seed embryos had already formed four to six leaf primodia (McDaniel and Poethig, 1988; Chen and Fang, 2003) before maturing; thus the transgenic plants we obtained might be chimeric, which means that the first several leaves were transformed, while the apical meristematic cells were not, and vice versa. If T_0 generation samples were collected at the 3-4 leaf stage for PCR detection, we might get some pseudo-transgenic plants, since they could not produce transgenic seeds. Similarly, we might lose some true transgenic plants, too. Therefore, samples for DNA extraction should be collected at the seven-leaf stage or later. Although no significant difference was detected for PPT tolerance and PCR positive lines between the two treatments, highly significant differences existed at seedling emergence, which implied that we could get more viable seedlings from the puncturing method. Although the transformation rate for the puncture method was higher than that of the scarifying method, both rates were still low; however, both transformation procedures were simple, rapid and genotype independent.

The principles of the two transformation methods are similar. Making wounds on the meristematic region of a germinating seed can break the cover of the seed coat over the embryo, and provide a greater opportunity for *Agrobacterium* to invade its meristematic cells. In comparison with the scarifying method however, the puncture method causes less damage to the seed embryo,

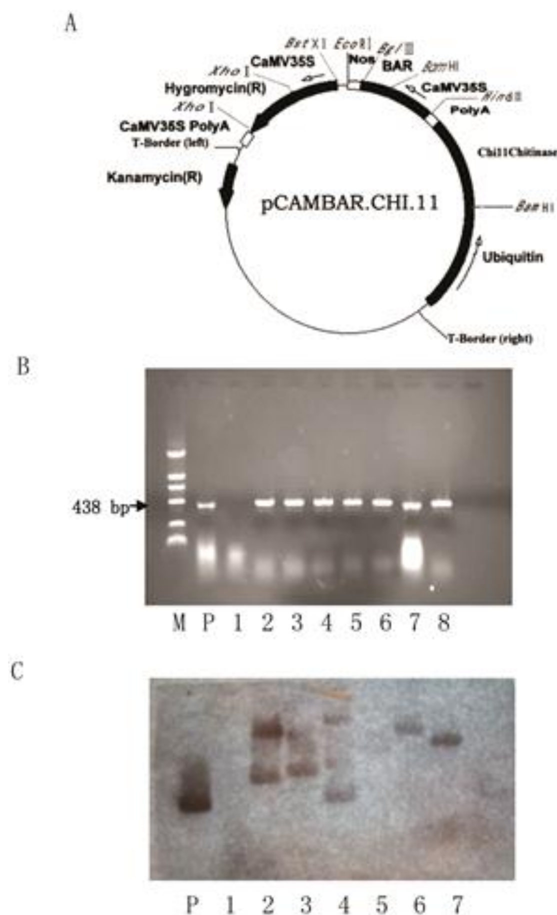

Fig. 1: The vector plasmid and molecular detection (A) physical map of plasmid pCAMBAR.CHI.11. (B) PCR results of some T_0 generation putative transgenic plants. M: Marker; P: plasmid; lane 1: negative control; lanes 2-8: transgenic plants. (C) Southern blotting of partial T_1 transgenic plants (Zheng 58). P: plasmid; lane 1, negative control; lanes 2-7: transgenic plants

and the target area could be more focused. In addition, the puncture method is more suitable for dicotyledonous seeds such as soybean and peanut whose embryos are difficult to locate and treat. In this study, we obtained transgenic plants using the puncture and scarifying methods, which were confirmed by molecular evidence. Biological assay in the field also proved that the herbicide-tolerance of transgenic plants had been improved.

The data confirmed that germinating seed embryo transformation methods could obviate the lengthy and tedious process of tissue culture and can achieve transformation rates greater than or equal to those of the particle bombardment, normally 10^{-3} to 10^{-2} (Sun, 2011). Therefore, the scope of these transformation methods can be extended to other crops. For the mechanism analysis, the embryo has a natural morphogenetic ability. The enzyme activities of embryonic cells of water-imbibing

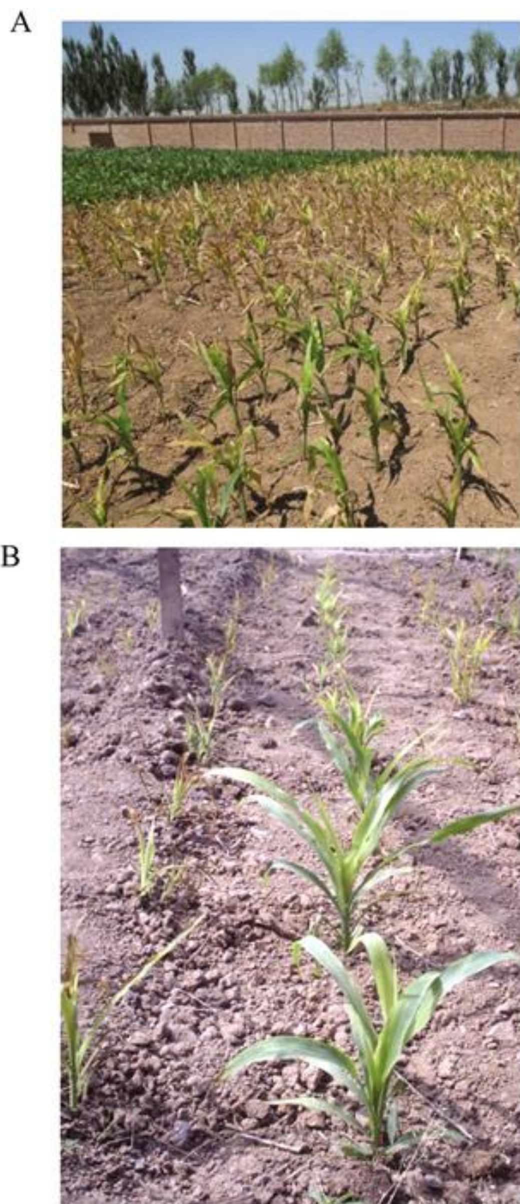

Fig. 2: Screening tolerant maize plants in the field. (A) glufosinate screening of T_0 plants. (B) Responses of tolerant seedlings of the T_1 generation to glufosinate, the plants on the left showed low tolerance; the plants on the right showed highly tolerant. Left：control plants; Right：T_1 plants

seeds are enhanced, the respiration of germinating seeds increases abruptly, carbohydrates, proteins and nucleic acids in the embryos are converted and changed rapidly, which are important for the success of *Agrobacterium* transformation. And at this stage, the germinating embryo would be highly sensitive to external factors. When the embryonic cells begin to divide, and the wrapping of embryo by the cotyledons is loosened, all these indicate a competent state of growth point for invading agrobacteria. Therefore, infection of the germinating embryos using a highly virulent *Agrobacterium* for co-cultivation would be beneficial for increasing transformation rate. In addition, the micro-injury treatment of germinating embryos can ensure an optimum competent state, stimulating cell division in the injured area, which will facilitate agrobacteria to enter the host cells. The AS signaling molecule can induce gene activation of the VIR region on a Ti plasmid (Shahla and Donald, 1987). In brief, higher transformation rate could be achieved through the combination of *Agrobacterium* co-cultivation, AS induction, and the competent state of the micro-injured germinating embryos, as well as the subsequent herbicide screening.

Conclusion

The two germinating seed transformation methods could be used for generating transformants, especially, the puncture method can expand the germinating embryo treatment method to dicotyledonous plants such as soybean and peanut, whose embryos are wrapped by the cotyledons and are not easily located. In addition, these transformation methods are superior to those that need to transform the callus through tissue culture technology, and are ready to be integrated into conventional plant breeding programs.

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