RESEARCH ARTICLE

Co-culturing on dry filter paper significantly increased the efficiency of *Agrobacterium*-mediated transformations of maize immature embryos

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Abstract In the *Agrobacterium tumefaciens*-mediated transformations of maize immature embryos (IEs), the common co-culturing media used are MS or N6-based (MC). Here, we used a novel co-culturing method in which maize ‘Qi319’ IEs inoculated with *Agrobacterium*-harboring target vector were placed on dry filter paper (DC) in a petri dish. To compare the effects of the DC and MC co-culturing methods on transformation efficiency, we designed three experiments: (1) *A. tumefaciens* strain AGL1 independently carrying two plasmids, pXQD12 and pXQD70; (2) two *A. tumefaciens* strains, AGL1 and EHA105, carrying pXQD12; and (3) strains AGL1 and EHA105 each independently inoculated with pXQD12 and pXQD70 for different infiltration periods, 5, 10, 15, 20 and 25 min. We used *A. tumefaciens* to inoculate IEs derived from maize ears 9–15 d after pollination, and then IEs were placed in petri dishes for co-culturing. The DC treatment significantly increased the percentage of IEs expressing green fluorescence protein (%GFP), indicating positive transformants. DC-treated IEs had ~ 3 to 4 times the %GFP compared with MC-treated IEs at 8 d after inoculation (3 d co-culture and 5 d restoration). The average regeneration frequency (%GFP positive regenerated calli

of infected IEs) and stable transformation frequency (%GFP positive T₀ plants of infected IEs) significantly increased with the DC treatment. Thus, the DC method may be used to develop a more efficient *Agrobacterium*-mediated transformation method for maize IEs.

Keywords Dry filter paper · Co-culturing · Transformation efficiency · *Agrobacterium* · Maize

Introduction

Maize is an economically important food and forage crop worldwide. In 2013, the Food and Agriculture Organization of the United Nations investigated the global production of several cereal crops, and found that maize production had reached ~ 1 billion metric tons (<http://faostat3.fao.org/>). Since the first *Bacillus thuringiensis*-modified maize was launched in the mid-1990s, the transgenic approach has been widely used in maize breeding (Que et al. 2014). Thus, it is fundamentally important to develop various transformation systems to support maize molecular breeding and the newly emerging CRISPR/Cas9-based genome editing (Yin et al. 2017).

Several highly efficient and reproducible transformation systems for maize have been developed. Maize has been transformed using protoplasts (Fromm et al. 1986; Rhodes et al. 1988a, b), electroporation (D’Halluin et al. 1992), PEG (Golovkin et al. 1993), silicon carbide whiskers (Frame et al. 1994) and particle bombardment (Fromm et al. 1990; Gordon-Kamm et al. 1990; Komari 1990; Kozziel et al. 1993). However, these strategies do not always result in fertile transgenic plants. Ishida et al. (1996) first reported that fertile transgenic plants were obtained using *Agrobacterium*-mediated transformations of

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immature embryos (IEs) of the inbred maize line A188. Since then, *Agrobacterium*-mediated transformation has been the standard method of genetic maize transformation because it tends to produce a greater percentage of single-copy transgenic events (Zhao et al. 2002; Ishida et al. 2003; Hiei et al. 2006; Que et al. 2014; Li et al. 2015; Nahampun et al. 2016).

Despite the great progress that has been made in maize, *Agrobacterium*-mediated transformations are still not efficient enough to support high-throughput analyses required for maize functional genomics studies. In previous studies, the dry filter paper (DC) co-culturing method has been effective in the *Agrobacterium*-mediated transformations of recalcitrant millet (Ceasar and Ignacimuthu 2011; Ceasar et al. 2017) and wheat (Cheng et al. 2003). In *Agrobacterium*-mediated maize transformations, although IEs and seedling-derived calli have been transformed using an MS or N6-based (MC) only or DC only co-culturing method, no study has directly compared the efficiencies of MC and DC co-culturing methods (Cheng et al. 2003; Sidorov et al. 2006; Sidorov & Duncan 2009).

Here, we compared the effects of MC and DC co-culturing methods on *Agrobacterium*-mediated maize transformations of IEs and provide clear evidence that DC is superior to MC, being almost more efficient, at least in transient assays. In the *Agrobacterium*-mediated maize transformations of the inbred maize line Qi319 IEs, the DC co-culturing method led to the uniform high transient expression of the synthetic green fluorescence protein (*GFP*) marker gene (Qu et al. 2008) when using different vectors, *Agrobacterium* strains and inoculation durations. Furthermore, compared with the MC method, the DC method also resulted in the production of more regenerated calli and maize seedlings harboring *GFP* in *Agrobacterium*-mediated transformations of maize IEs. Thus, the DC co-culturing method is more effective than the MC method and should be a standard method for the *Agrobacterium*-mediated transformation of maize.

Materials and methods

Plant materials

Maize inbred line Qi319 was used throughout the experiments. Plants were grown in a greenhouse or field depending on the growing seasons. IEs were isolated from ears pollinated 9–15 d, which ranged between 1 and 1.5 mm in length, and the male or female parent of the IEs derived from the wild type (WT). Freshly isolated IEs were used directly for inoculation with *Agrobacterium*.

Agrobacterium strains and transformation constructs

Agrobacterium strains EHA105 (Hood et al. 1984) and AGL1 (Petti et al. 2009) were used in this work. Construct pXQD12 contained a pCambia1300 backbone and a hygromycin phosphotransferase (*HPT*) marker gene driven by a double CaMV35S promoter. Construct pXQD70 contained a pZP211 backbone and a neomycin phosphotransferase (*NPTII*) marker gene driven by a double CaMV35S promoter. In both constructs, a *GFP* gene (Qu et al. 2008) was driven by the maize ubiquitin 1 promoter (Christensen and Quail 1996; Fig. 1).

Media formulations

The types and components of media used at various stages were as previously described by Ishida et al. (2007). The critical modification in our protocol was the use of DC instead of the MS-based co-culture medium.

Transformation procedures

Our established standard protocol, which was modified from the previous protocols (Frame et al. 2011; Ishida et al. 2007) is described below.

Preparation of the inoculum, embryo dissection and inoculation process

A. tumefaciens was cultured on solid MGL medium (2.5 g/l yeast extract, 5 g/l tryptone, 5.2 g/l NaCl, 10 g/l D-mannitol, 0.2 g/l MgSO₄·7H₂O, 0.5 g/l KH₂PO₄, 15 g/l L-glutamic acid and 15 g/l agar at pH 7.2) supplemented with 0.1 mg/ml spectinomycin or kanamycin and 200 mM acetosyringone in the dark at 28 °C for 2 d (Sambrook et al. 1989). For infection, the concentration of the *Agrobacterium* suspension in the inoculation medium, supplemented with 200 µM acetosyringone, was adjusted to an OD₆₀₀ of 0.5–0.6. The inoculum was freshly prepared. The method of IE dissection was as previously described by Ishida et al. (2007). Unless otherwise stated, all embryos were immersed in 2–3 ml of inoculation medium containing *Agrobacterium* suspension in a 10-ml microcentrifuge tube for 5 min at 23 °C without shaking.

Co-culturing and transient expression analysis of GFP

After infection, the excess inoculum was removed using a pipette with a fine tip. The IEs inoculated with *Agrobacterium* harboring pXQD12 or pXQD70 were placed on DC in a petri dish (9 cm in diameter) or MC, and then sealed

Fig. 1 Schematic diagram of transformation vectors pXQD12 and pXQD70. LB, T-DNA left border; RB, T-DNA right border; 35S, CaMV 35S promoter; ZmUbi1, maize Ubiquitin 1 promoter; *sGFP*, a synthetic green fluorescence protein; *HPT*, hygromycin

phosphotransferase gene under the control of 2X CaMV 35S promoter; *NPTII*, neomycin phosphotransferase gene under the control of 2X CaMV 35S promoter. The backbone of pXQD12 and pXQD70 derive from pCambia1300 and pPZP211, respectively

with parafilm (VWR International Inc., Earth City, MO, USA). Plates were kept in a growth incubator (28 °C, dark) for 3 d. Then, the colonies were transferred to the restoration medium without antibiotics. The transient expression of *GFP* in calli could be tested using a GFP3 filter under a stereomicroscope (Leica DC200) after 5 d on the restoration medium. The transient transformation efficiency (% of transformed IEs against total number of IEs used) was scored as the percentage of calli with GFP against the total number of IEs used (8 d after inoculation: 3 d for co-culture and 5 d for restoration). In this study, more than 100 IEs were used in each experiment. Five independent biological replicates were performed for each treatment.

The selection and regeneration of transgenic plants

After the restoration treatment, the calli were transferred onto the first selection medium (Ishida et al. 2007) supplemented with 20 mg/l hygromycin (for pXQD12) or 40 mg/l G418 (for pXQD70). The plates were maintained in a growth incubator (28 °C, dark) for 10–15 d. Then, callus pieces were transferred onto the second selection medium supplemented with the same antibiotics for an additional 10–15 d. The resistant calli were analyzed using the GFP signal. The regeneration efficiency was scored as the percentage of regenerated calli showing GFP expression against the total number of IEs used (32 d after inoculation: 3 d for co-culture, 5 d for restoration and 24 d for the two selections). Regenerated GFP⁺ calli were transferred to regeneration medium supplemented with the same antibiotics. Plates were maintained in a growth chamber at 16 h: 8 h light: dark at 26 °C. After 2–3 weeks, healthy shoots appeared and were transferred onto the rooting medium. Plants with healthy roots were analyzed

for GFP signals and transplanted into soil to produce seeds. A stable transformation frequency was scored as the percentage of plants expressing GFP against the total number of IEs used. Because the transgenic plants grew weakly, a cross with WT plants was performed to ensure seed production. If the transgenic plants produced stamens with fertile pollen, then the fertile pollen of the transgenic plants was scattered to the female reproductive part of the wild type plants through artificial pollination or, if the transgenic plants produced pistils, then the fertile pollen of the wild type plants was scattered to the pistil of the transgenic plants through artificial pollination.

PCR and RT-PCR

DNA and RNA from leaves of 10 putative T₀ transgenic maize plants were subjected to molecular analyses to demonstrate the presence and transcriptional levels of the *GFP* and *NPTII* genes. First, total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987) and analyzed by PCR to confirm the presence of *GFP* and *NPTII*. Second, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and analyzed by RT-PCR to evaluate the expression levels of *GFP* and *NPTII*. For PCR, we used 1 µl of DNA solution and primers at a final reaction concentration of 0.4 µM. For reverse transcription PCR (RT-PCR), cDNA was synthesized from 1 µg of total RNA using a reverse transcription kit (Biotek, Beijing, China). In a total volume of 20 µl, 1 µl cDNA solution was used with primers at a final reaction concentration of 0.4 µM. The primers used to amplify the *GFP* (715 bp), *NPTII* (525 bp) and *Actin* (500 bp) genes are listed in Table S1. The annealing temperature for both genes was 58 °C, and

the PCR and RT-PCR products were analyzed by agarose gel electrophoresis.

Southern hybridization

The total DNA of the 10 T₀ transgenic plants was also extracted using the CTAB method (Doyle and Doyle 1987) and analyzed by southern blotting to detect the integration patterns of the foreign genes. Southern hybridization was performed as previously described (Sambrook et al. 1989). In total, 30 µg genomic DNA per sample was digested with *Bam*HI at 37 °C overnight and separated on a 0.8% (w/v) agarose gel. Digoxigenin (DIG)-labelled probe for *GFP* (from pXQD70) was generated using a DIG High Prime DNA Labeling Detection Starter Kit I (Roche, Shanghai, China). The primers for *GFP* are described in Table S1.

Inheritance of the transgene in progeny

To ensure that seeds from the putative T₀ transgenic plants were produced, crosses were performed between T₀ and wild type plants. Then, the F₁ progeny of 10 independent T₀ transgenic plants were chosen to evaluate the inheritance patterns of the *GFP* transgene. The F₁ seeds were placed in petri dishes with 5 ml water for 3 d, and then the *GFP* expression was determined under a stereomicroscope using a GFP3 filter. F₁ seedlings showing GFP fluorescence were identified, and the segregation of the GFP transgene in the F₁ population was statistically analyzed for the expected Mendelian ratio of 1:1 for a single locus using the Chi square (χ^2) test.

Statistical analysis

In this study, at least three technical replicates were performed for each experiment. The statistical methods in Wu et al. (2014) were used to determine the *p* values of the data.

Results

Comparison of two co-culturing methods (DC and MC) on the transient transformation efficiency under different conditions

IEs infected with *Agrobacterium* are routinely cultured on MS or N6-based co-culture media. In the genetic transformation of maize, DC has only been used in the transformation of seedling-derived calli (Sidorov et al. 2006; Sidorov and Duncan 2009). To explore whether DC co-culturing effects the transformation frequency positively under different conditions, in this study, two co-culturing

methods, DC and MC, were compared using *Agrobacterium* strain AGL1 independently carrying plasmids pXQD12 and pXQD70, strains AGL1 and EHA105 harboring the pXQD12 plasmid and strains AGL1 and EHA105 independently carrying the selected plasmids pXQD12 and pXQD70 for different infection periods (5, 10, 15, 20 and 25 min).

The initial ‘pXQD70’ experiments showed that the co-culturing method had a pronounced effect on the transient transformation efficiency. The %GFP positive calli obtained by DC (26.29%) was nearly four times greater than that obtained by MC (6.71%) after 3 d of co-culturing and 5 d of restoration (Fig. 2a, b). The ‘pXQD12’ experiments were also performed, and similar results were obtained (Fig. 2b).

To confirm the above phenomenon, we reproduced transformation experiments side-by-side with DC and MC co-culturing methods with both strains AGL1 and EHA105 harboring the plasmid pXQD12 under the same experimental conditions. Both the ‘AGL1’ and ‘EHA105’ experiments confirmed that the DC significantly increased the %GFP positive calli in IEs infected with *Agrobacterium* by nearly two times in comparison with the MC. There was no significant difference in the transient transformation efficiencies between the two *Agrobacterium* strains (Fig. 2c).

To further demonstrate the reproducibility of the co-culturing methods, we also compared the two co-culturing methods (DC and MC) in strains AGL1 and EHA105 independently carrying the selected plasmids pXQD12 and pXQD70 for different infection periods: 5, 10, 15, 20 and 25 min. The transient transformation efficiency was significantly increased nearly twofold by the use of DC compared with MC at the different infection periods (Fig. 2d–g). In this experiment, a 15 min infection period combined with the DC method provided the optimal results (Fig. 2d–g).

Thus, the DC method could significantly increase the transient transformation efficiency of *Agrobacterium*-mediated transformations of maize IEs.

Regeneration and preliminary analyses of T₀ transgenic plants

In this study, more than 10,000 maize IEs derived from ears harvested 9–15 d after pollination were inoculated with *Agrobacterium* suspensions. After co-culturing, restoration, selection, the regeneration of shoots and rooting, many resistant seedlings were produced (Fig. 3a–g). Under a stereomicroscope, seedlings with GFP fluorescence were identified and then successfully established in soil (Fig. 3h–n). However, some of the GFP positive T₀ transgenic plants were sterile and needed to be crossed with

Fig. 2 Effect of different co-culture methods on the efficiency of *Agrobacterium*-mediated transformation of maize immature embryos (IEs). **a** Transient expression of GFP in *Agrobacterium*-infected maize calli (8 d after inoculation, 3 d for co-culture and 5 d for restoration; left, white light; right, GFP3 filter; bar = 0.6 mm). **b–g** Comparison of the two co-culture methods (MC medium; DC dry filter paper) under different conditions in terms of percentage of GFP positive calli (**b** plasmids pXQD12 and pXQD70 in *Agrobacterium*

strain AGL1; **c** *Agrobacterium* strains AGL1 and EHA105 carrying pXQD12; **d–g** inoculation durations of 5 min, 10 min, 15 min, 20 min and 25 min of AGL1/pXQD12, AGL1/pXQD70, EHA105/pXQD12 or EHA105/pXQD70. Data in **b–g** are shown as mean $\pm$ S.D. Student's *t*-tests were used to generate the *p* values: *t* test: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Five independent biological replicates were done for each treatment and more than 100 embryos were used in each experiment

wild type plants to produce viable seeds, while others were fertile and produced viable seeds (Fig. 3o–q).

To determine the presence, expression and integration patterns of the foreign genes, PCR and RT-PCR were

Fig. 3 Procedure of *Agrobacterium*-mediated transformation of maize immature embryos (IEs). **a** Immature embryo isolated from the ear (bar = 2 mm). **b** immature embryo under high resolution (bar = 0.14 mm). **c** Individual calli infected with *Agrobacterium* (bar = 0.95 mm). **d** Calli infected and selected for 1 month. **e** Callus in **d** under high resolution (bar = 0.46 mm). **f** Regeneration of resistant plants. **g** Rooting in tubes. **h, i** GFP fluorescence assay

(**h** white light; **i** GFP3 filter; left: WT; right: transgenic plant; bar = 0.5 mm). **j, k** GFP fluorescence assay (**j** white light; **k** GFP3 filter; left: WT; right: transgenic plant; bar = 0.5 mm). **l–n** transgenic plant survived in soil and in field. **o** ear after cross with WT. **p, q** GFP fluorescence assay (**p** white light; **q** GFP3 filter; left: WT; right: transgenic plant; bar = 0.2 mm)

performed. The two tested genes were integrated into the genome of putative transgenic plants and stably expressed (Fig. 4). Furthermore, the southern hybridization pattern suggested that low copy numbers of the target genes were

integrated into the genome of transgenic plants. However, some transgenic events (T_0) were undetectable by Southern blotting, which might be attributed to loading an insufficient amount of sample genomic DNA. Thus, the events

Fig. 4 PCR and RT-PCR analysis of transgenic plants. **a** PCR assay of DNA extracted from the leaves of the wild type (WT) and transgenic lines. PCR products of *GFP* and *NPTII* are 715 bp and 525 bp in length. **b** RT-PCR assay of RNA extracted from the leaves

of the WT and transgenic lines. RT-PCR products of *GFP*, *NPTII* and *Actin* are 715 bp, 525 bp and 500 bp in length. Three technical replications were performed and the results were similar

might still carry the target element but failed to exhibit the signal (Fig. S1). In general, the foreign genes were integrated into the maize genome and stably expressed in the transgenic seedlings.

Impacts of two co-culturing methods (DC and MC) on the regeneration and stable transformation efficiencies

In our study, the regeneration efficiency and stable transformation efficiency were compared between the two co-culturing methods. The average regeneration and stable transformation frequencies significantly increased using the DC method. In the transformation experiments of pXQD12, using the DC method, the regeneration efficiency increased from an average 1.46% up to 4.63%, and the stable transformation frequency increased from an average 0.52% to 1.44%. Similarly, in the transformation experiments of pXQD70, the regeneration efficiency increased from an average 1.98% up to 5.90% using the DC method, which also resulted in the substantial increase in the stable transformation efficiency from an average 0.47% to

1.55%, almost threefold more than in MC experiments (Table 1). Although the number of positive transgenic maize plants per each experiment were relatively small, the results suggested that the DC method led to a greater stable transformation efficiency than the MC method in maize.

Segregation analysis of the foreign *GFP* gene in F_1 progeny

To further determine the integration patterns of the foreign genes, a segregation analysis of the foreign *GFP* gene in F_1 progeny was performed. If the T_0 transgenic plants were crossed with wild type and harbored only one *GFP* transgene copy, then the expected segregation ratio would be 1:1 ($GFP^+ : GFP^-$). However, only a few progeny presented the Mendelian segregation ratio, while the others presented a large bias ($\chi^2 \geq 4.80$, Table 2), which suggested the existence of multiple transgene copies in the maize genome.

Table 1 Transformation efficiency in terms of GFP positive seedlings of *Agrobacterium*-mediated transformation of maize immature embryos

Plasmid	Co-culture media	Experiment No.	NO. of IEs (A)	No. of transformed IEs (B) ^a	No. of regenerated GFP ⁺ calli (C) ^b	No. of GFP ⁺ Plants (D)	Transient transformation efficiency (B/A, %)	Regeneration efficiency (C/A, %)	Transformation frequency (D/A, %)
pXQD12	MC	1	151	7	3	1	4.64	1.99	0.66
		2	173	14	3	1	8.09	1.73	0.58
		3	212	19	2	1	8.96	0.94	0.47
		4	250	12	3	1	4.80	1.20	0.40
		5	174	10	3	1	5.75	1.72	0.57
		Total	960	62	14	5	6.46	1.46	0.52
pXQD12	DC	1	202	50	4	1	24.75	1.98	0.50
		2	150	49	6	2	32.67	4.00	1.33
		3	133	29	7	3	21.8	5.26	2.26
		4	210	41	9	1	19.52	4.29	0.48
		5	102	31	7	2	30.39	6.86	1.96
		6	75	34	5	2	45.33	6.67	2.67
		7	161	52	4	2	32.30	2.48	1.24
		8	112	46	10	3	41.07	8.93	2.68
		9	108	39	6	2	36.11	5.56	1.85
		Total	1253	371	58	18	29.61	4.63	1.44
pXQD70	MC	1	154	16	5	1	10.39	3.25	0.65
		2	131	12	5	1	9.16	3.82	0.76
		3	321	25	3	1	7.79	0.93	0.31
		4	251	22	4	1	8.76	1.59	0.40
		Total	857	75	17	4	8.75	1.98	0.47
pXQD70	DC	1	120	37	4	1	30.83	3.33	0.83
		2	100	31	6	2	31.00	6.00	2.00
		3	156	41	8	2	26.28	5.13	1.28
		4	121	29	5	1	23.97	4.13	0.83
		5	201	95	17	5	47.26	8.46	2.49
		6	101	32	5	1	31.68	4.95	0.99
		7	114	24	6	1	21.05	5.26	0.88
		8	121	61	10	3	50.41	8.26	2.48
		Total	1034	350	61	16	33.85	5.90	1.55

MC and DC are conditions for co-culture after inoculation with *Agrobacterium*. IEs immature embryos; MC medium; DC dry filter paper; *a* no. of calli with GFP (8 d after inoculation: 3 d for co-culture and 5 d for restoration); *b* no. of calli with GFP (32 d after inoculation: 3 d for co-culture, 5 d for restoration and 24 d for two selection)

Discussion

GFP fluorescence is a reliable visual marker for maize genetic transformation

To produce relatively purely resistant maize calli after inoculation with *Agrobacterium*, several rounds of selection were carried out. Although the transient transformation efficiency was high, the regeneration efficiency was low, and few of the resistant calli could produce shoots (Table 1). It appeared that “escape” is a common phenomenon in maize and it was difficult to generate “pure”

transgenic calli even after a very long period of selection using the appropriate antibiotics. In our construct for maize transformation, the *GFP* gene could be used as a visual marker to show the existence of T-DNA in the maize genome (Qu et al. 2008). By using GFP fluorescence as a marker, we found that there was very high percentage of “escape” among the shoots regenerated from the resistant calli (Figs. 2, 3, Table 1). The resistant shoots with GFP fluorescence were confirmed as true transgenics through molecular analysis, which suggested that GFP fluorescence is a reliable visual marker for the identification of true transformants from resistant shoots. However, the

Table 2 Genetic analysis of T₀ transgenic Qi319 maize events

T ₀ lines	Segregation in F ₁ (T ₀ × WT) generation				
	Total	GFP ⁺	GFP [−]	Ratio	χ^2
1	154	70	84	0.83:1	1.27 ^a
2	101	70	31	2.26:1	15.06
3	261	178	83	2.14:1	34.58
4	146	76	70	1.09:1	0.25 ^a
5	188	144	44	3.27:1	53.19
6	30	21	9	2.33:1	4.80
7	95	41	54	0.76:1	1.78 ^a
8	65	39	26	1.50:1	2.60 ^a
9	92	65	27	2.41:1	15.70
10	71	31	40	0.78:1	1.14 ^a

The T₀ lines were crossed with wild type to get F₁ seeds and segregation was scored. GFP⁺ and GFP[−] represent GFP positive and negative

^aIndicates in accordance with Mendel's law

development of new visual markers to identify the true transgenic maize calli and shoots in the induction stages is necessary to reduce this labor-intensive time-consuming work.

The DC method enhanced the maize transformation efficiency

To date, various factors, such as explant types, like protoplast, IEs and calli derived from IEs (Cheng et al. 2004; Al-Abed et al. 2006; Sidorov et al. 2006), the strains of *A. tumefaciens* (Frame et al. 2002; Kant et al. 2012; Cao et al. 2014; Cho et al. 2014; Li et al. 2015), transformation vectors (Frame et al. 2002; Oltmanns et al. 2010; Cao et al. 2014) and the medium's components (Ishida et al. 2003; Frame et al. 2006, 2011; Cho et al. 2014), have been explored and suggested to impact the efficiency level of *Agrobacterium*-mediated maize transformation. To increase the efficiency of the *Agrobacterium*-mediated transformations of maize IEs, two co-culturing methods, DC and MC, were compared using plasmids pXQD12 and pXQD70 transformed into *Agrobacterium* strain AGL1, using strains AGL1 and EHA105 harboring the plasmid pXQD12 and using strains AGL1 and EHA105 independently harboring the selected plasmids pXQD12 and pXQD70 for different infection periods (5, 10, 15, 20 and 25 min). Uniformly, the transient transformation efficiency was increased ~ 2 to 3 times by the DC method under all of the tested conditions (Fig. 2b–g). However, in our experiments, no significant reproducible differences were found for the transient transformation efficiencies between the plasmids or the *Agrobacterium* strains (Fig. 2b, c). This suggested that some factors, like plasmids and

Agrobacterium strains, had limited effects on the efficiency of maize genetic transformations under our experimental conditions. In addition, the optimal combination of a 15-min infection period and the DC method resulted in a significant increase in the efficiencies of strains AGL1 and EHA105 using either of the selected plasmids pXQD12 and pXQD70 separately (Fig. 2d–g). Di et al. (2008) reported that the infection time also affects the transformation efficiency. If the infection time was too short, then the explants absorbed only a small amount of *Agrobacterium*, which prevented the T-DNA from being effectively transferred, resulting in low transformation efficiency. If the infection time was prolonged, then the *Agrobacterium*'s reproduction was excessive, and browning occurred in the explants, which resulted in death and reduced the transformation efficiency (Zhuang et al. 2010; Wang et al. 2011). Sun et al. (2012) also reported that the optimal infection period for embryonic calli was 15 min. Here, the 15-min infection period appeared suitable for the entrance of *A. tumefaciens* into the intercellular spaces of IEs under our experimental conditions. Thus, the DC method is able to significantly increase the transient transformation efficiency of maize.

As far as DC method is concerned, it is well-known that the water content can affect the properties of cell membranes. Simon (1978) reported that low water content might lead to the formation of hydrophilic molecular channels on the cell membrane and that water-soluble substances could be allowed to pass these channels into dry plant cells in the first few seconds of imbibition. The co-cultivation of dry IEs with *A. tumefaciens* suspension could promote the adhesion of *A. tumefaciens* to the cell walls of IEs and the entrance of *A. tumefaciens* into the intercellular spaces of the IEs (Arencibia et al. 1998). In addition, the desiccation during co-culturing may suppress *A. tumefaciens* cell growth. Compared with the MC method, the suppressed *A. tumefaciens* growth subsequently promoted increased cell recovery after co-culturing, which resulted in greater transformation efficiency (Arencibia et al. 1998; Alves et al. 2009; Cheng et al. 2003; Ceasar and Ignacimuthu 2011; Ceasar et al. 2017). Thus, the DC method is able to significantly increase the transient transformation efficiency in maize compared with the MC method.

However, the regeneration and stable transformation efficiency levels in our experiments were not large enough (~ 30% to 40% for transient transformations). Although the DC method showed a high transient GFP expression 8 d after inoculation (3 d for co-culture and 5 d for restoration; Fig. 2), it was not evident after 2 weeks (data not shown). Similar results were reported for the transformations of sugarcane, banana, barley and wheat (Trifonova et al. 2001; Wu et al. 2003; Khanna et al. 2004; Joyce et al.

2010) in which the treatments producing the greatest reporter gene expression produced the least number of stable transformation events. In addition, Ishida et al. (2007) also reported that no stable transformation events were obtained when GUS expression was too low or too high. The reason for this is unclear. This might result from *Agrobacterium* cells being unable to infect plasmolyzed plant cells or the presence of other factors used in the subsequent selection and regeneration stages, such as selectable markers and selective agents. Cheng et al. (2003) also reported that the stable transformation efficiency varied when different selectable markers and selective agents were used. In addition, the genotypes of the transforming material may also influence stable transformation efficiency observed under our experimental conditions. Thus, although the numbers of positive transgenic maize plants generated from each experiment were relatively small under our experimental conditions, the regeneration and stable transformation efficiencies uniformly increased nearly two times using the DC method (Table 2). Thus, the DC method might lead to greater stable transformation efficiency than the MC method in maize.

Efficient transformation systems are still required for maize genetic improvement

As an important forage and food crop worldwide, maize is a main target of breeding using transgenic approaches and molecular biology, which require a reliable maize transformation system. Recently, genome editing tools, like CRISPR/Cas9, were developed and increased the importance of genetic transformation systems for maize (Yin et al. 2017). Although methods like particle bombardment are feasible in maize, the transformation efficiency is relatively low (Fromm et al. 1990; Komari 1990). In addition, particle bombardment tends to produce high copy number insertion events in recipient genomes (Shou et al. 2004). Since the generation of fertile transgenic plants using *Agrobacterium*-mediated transformation of inbred line A188, *Agrobacterium*-mediated transformation has become increasingly popular in maize because it tends to produce single copy inserts in the genome (Ishida et al. 1996; Zhao et al. 1998; Shou et al. 2004). DC methods are also effective in the *Agrobacterium*-mediated transformations of recalcitrant millet (Ceasar and Ignacimuthu 2011; Ceasar et al. 2017) and wheat (Cheng et al. 2003). Like the published data, in our experiments, although we produced a transient transformation efficiency of close to 30%–40%, both the regeneration and the stable transformation efficiencies were relatively low (only 4%–6% and 1%–2%, respectively), and very few true transgenic shoots could be regenerated from a single experiment. Under our experimental conditions, there may be other factors, such as the

maize genotype (Frame et al. 2006) and medium' components (Ishida et al. 2003; Frame et al. 2006, 2011; Cho et al. 2014), that affect the regeneration and stable transformation efficiencies of maize in the DC method. Generally, the current efficiency of the *Agrobacterium*-mediated transformation of maize is not great enough to support the actual requirements of researchers.

Conclusion

Among the factors tested, we found that the DC method increased the transient transformation efficiency of maize ~ 2 to 3 times and had a similar tendency in stable transformation experiments. The data provided clear evidence that the DC method is superior to the MC method and can be used routinely in maize transformations.

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Authors contribution XD contributed all reagents and materials used in the experiments. XD, LZ, JS, WL, WW and HA designed and performed the experiments. HA and XD analyzed the data and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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