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ORIGINAL PAPER

Improvement of *Agrobacterium*-mediated transformation frequency in multiple modern elite commercial maize (*Zea mays* L.) inbreds by media modifications

Myeong-Je Cho · Jenny Banh · Maryanne Yu · Jackie Kwan · Todd J. Jones

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Abstract The current study describes a robust, high-frequency *Agrobacterium*-mediated transformation protocol suitable for multiple recalcitrant modern elite commercial maize inbreds employing media modifications with glucose, cupric sulfate and a cytokinin, 6-benzylaminopurine (BAP). An optimal combination of these three key elements in the co-cultivation, resting, and selection media resulted in 4- to 14-fold improvements in transformation frequencies at the T_0 plant level of 9.7, 31.9, 9.6 and 10.0 % for PH4CN, PH12BN, PHW0V and PH17R8, respectively. Transformation frequency in PH1CP1 was also improved at the T_0 tissue level from 2.5 to 8.3 %. The addition of cupric sulfate and BAP in the co-cultivation medium improved transformation frequency in all inbreds except PH4CN. The use of cupric sulfate and BAP in combination with additional glucose in the selection medium was especially important, significantly improving the transformation frequency in 3 (PH4CN, PHW0V and PH1CP1) out of 5 inbreds by increasing the proliferation of high quality regenerable tissue. It was observed that the amount/ratio of these three components needed to be optimized for each inbred. The results in this study can be applied to optimize the tissue culture response and improve transformation frequency in other recalcitrant elite commercial maize inbreds.

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Keywords Maize elite inbred transformation · *Agrobacterium* · Green regenerative tissue medium · Copper · 6-Benzylaminopurine · Glucose

Abbreviations

BAP	6-Benzylaminopurine
GT	Green regenerative tissue
IE	Immature embryo
moPAT	Maize codon-optimized phosphinothricin acetyltransferase
mPHI-T	Modified PHI-T medium
NSS	Non-stiff stalk
PMI	Phosphomannose isomerase
PPT	Phosphinothricin
QE	Quality event
YFP	Yellow fluorescent protein

Key message

This study describes a high-frequency *Agrobacterium*-mediated transformation protocol for multiple modern elite commercial maize inbreds using media containing 6-benzylaminopurine (BAP), high levels of cupric sulfate and additional glucose.

Introduction

Efficient genetic improvement of major crop species by molecular breeding methods is dependent on the capability to transform commercial elite cultivars without changing their agronomic and quality characteristics. The

establishment of a robust and efficient *Agrobacterium*-mediated transformation method for modern elite commercial maize inbreds is important for biotechnology product development by allowing transgene evaluation in relevant germplasm and facilitating the backcrossing and integration of transgenes across commercial elite genetics. However, development of transformation protocols for elite germplasm is not merely a trivial extension of technologies developed for amenable cultivars that respond optimally to tissue culturing methods.

The initial reports for successful maize transformation were in the early 1990s when transformation was done via microprojectile bombardment (Fromm et al. 1990; Gordon-Kamm et al. 1990; Wan et al. 1995), electroporation (D'Halluin et al. 1992), PEG (Golovkin et al. 1993), and silicon carbide whiskers (Frame et al. 1994). Then in the late 1990s when the first successful *Agrobacterium*-mediated transformation was reported by Ishida et al. (1996) using an amendable inbred, A188, researchers began using *Agrobacterium* as a means of maize transformation (Ishida et al. 1996, 2007; Zhao et al. 1998, 2001; Negrotto et al. 2000; Frame et al. 2002, 2006; Huang and Wei 2005; Vega et al. 2008; Ombori et al. 2013; Cho et al. 2014). In addition to inbred A188, studies have also been done using Hi-II, a hybrid derived from a cross between A188 and B73 (Zhao et al. 1998, 2001; Frame et al. 2002; Vega et al. 2008) and various public inbred lines (Huang and Wei 2005; Frame et al. 2006; Ishida et al. 2007). Improvement has been made and transformation frequencies at the T_0 plant level have ranged from 46.4 to 69.0 % for A188 using constructs, pSB134 and pSB131, respectively (Ishida et al. 2007). Frame et al. (2006) reported a successful *Agrobacterium*-mediated transformation in old inbred lines, B104, B114, and Ky21 with a range of 2.8 to 8.0 % using MS (Murashige and Skoog 1962) salts as the basal tissue culture medium. Sidorov et al. (2006) also described an *Agrobacterium*-mediated transformation method for seedling-derived maize callus from commercial inbred lines using green fluorescent protein and either neomycin phosphotransferase II or glyphosate selection that ranged in efficiency from 2 to 11 %. However, highly efficient *Agrobacterium*-mediated transformation in modern elite inbreds is not routine and remains essential for commercial biotechnology product development.

Green regenerative tissue (GT: highly regenerative, green tissue) technology with the use of high copper and BAP was developed to reduce a genotypic limitation in transformation via microprojectile bombardment in recalcitrant commercial monocot crop varieties such as barley (Cho et al. 1998), wheat (Cho et al. 1999; Kim et al. 1999; Li et al. 2009) and Kentucky bluegrass (Ha et al. 2001). Recently it has been successfully applied for *Agrobacterium*-mediated transformation in sugarcane (Cho et al. 2013) and sorghum (Wu et al. 2014). In addition, we have recently demonstrated a high-frequency *Agrobacterium*-mediated transformation in an elite commercial maize inbred, PHR03, using GT medium components, specifically high cupric sulfate and BAP, in the resting and selection steps (Cho et al. 2014); transformation frequencies at the T_0 plant level have reached up to 57.1 % using *Agrobacterium* strain AGL1 in combination with high glucose in the resting medium. In this study we report a significant improvement in transformation frequency of multiple modern elite commercial maize inbreds which are recalcitrant using the improved transformation protocol for the PHR03 inbred line with additional media modifications in the co-cultivation, resting and selection steps of the transformation protocol.

Materials and methods

Donor maize inbred material

Five elite commercial maize inbred lines, PH4CN, PHW0V, PH12BN, PH1CP1 and PH17R8, were used for tissue culture optimization and transformation experiments. All inbreds tested in this study are proprietary modern elite, commercial non-stiff stalk inbreds of DuPont-Pioneer, with the exception of PH4CN which was coded in 1996. The inbreds have a comparative relative maturity (CRM, Lauer 1998) range of 107–113 days; their brief information is described in Table 1. CRM is the rating of a hybrid based on the duration in days from planting to physiological maturity when the kernel reaches black layer, relative to a set of standard hybrids. Year Coded is the year that an inbred candidate was identified as an official inbred in the DuPont-Pioneer breeding program. Plants from seed were grown and prepared for experiments according to the same specifications as PHR03 (Cho et al. 2014).

Table 1 Elite commercial NSS maize inbred lines used for transformation

Inbred	Comparative relative maturity (CRM) days	Year coded
PH4CN	113	1996
PH12BN	112	2005
PHW0V	111	2004
PH17R8	112	2006
PH1CP1	107	2007

Medium compositions and modifications

The initial media modification test for all 5 inbreds, PH4CN, PHW0V, PH12BN, PH1CP1 and PH17R8, was performed using PI medium and DBC GT medium schemes (Cho et al. 2014). In order to minimize the ear-to-ear variation, immature embryos (IEs) derived from the same ear were evenly distributed per treatment and incubated under the same conditions to test the effect of medium modifications.

Agrobacterium strain and vectors

AGL1 was used for maize transformation. Two binary vectors, PHP26650 (Fig. 1a) and PHP32269 (Fig. 1b; Cho et al. 2014; Wu et al. 2014), were used in maize transformation experiments. PHP26650 contains 2 gene cassettes, maize codon-optimized phosphinothricin acetyltransferase (moPAT) and DsRED from the coral, *Discosoma* sp. (Clontech, Mountain View, CA), both driven by the constitutive maize ubiquitin 1 promoter and its first intron. PHP32269 contains 2 gene cassettes, moPAT translationally fused to yellow fluorescent protein (YFP) and phosphomannose isomerase (PMI), both driven by the constitutive maize ubiquitin 1 promoter and its first intron.

Preparation of *Agrobacterium* suspension for immature embryo infection

Agrobacterium suspensions were prepared for transformation as previously described (Cho et al. 2014). *Agrobacterium* was collected from the YP plate and placed in a solution of PHI-I infection medium (Zhao et al. 2000) with 100 µM acetosyringone. The solution was vigorously shaken and then adjusted to 0.35 at an absorbance of 550 nm (A_{550}) determined by a spectrometer for use in transformation of IEs.

PH4CN transformation

PH4CN ears were collected from donor material described above. IEs from PH4CN, at the optimal size of 1.5–2.2 mm, were aseptically isolated from kernels into a 1.5 mL microfuge tube of PHI-I liquid medium (Zhao et al. 2000) containing 100 µM acetosyringone and allowed to sit for 20 min. The solution was drawn off and the IEs were then infected with 1.2 mL of *Agrobacterium* suspension containing binary vector PHP26650, vortexed at 600–900 RPM for 5 s and allowed to sit for 5 min. AGL1 was used for all experiments in this study. The suspension of *Agrobacterium* and IEs was then poured onto PHI-T co-cultivation medium (Zhao et al. 2000). The *Agrobacterium* solution was drawn off and IEs were then arranged scutellum-side up on the medium. IEs derived from the same ear were evenly distributed per treatment for all transformation experiments to minimize seasonal and ear-to-ear variation of the donor material. The plates were incubated in the dark at 21 °C for 3 days of co-cultivation.

Two culturing schemes were used for bialaphos selection (Fig. 2). Culturing scheme 1 is as follows: IEs were transferred scutellum-side up onto DBC3 medium (Table 2) without a selective agent for resting and incubated at 26 ± 2 °C under dim light (10–30 mE/m^2 s, 16/8-h day and night photoperiod). All media from the resting step contained 100 mg/L cefotaxime (PhytoTechnology Lab., Shawnee Mission, KS). After 4 days, the coleoptiles were removed from the IEs and the tissues were transferred to DBC3 medium containing 3 mg/L bialaphos (Meiji

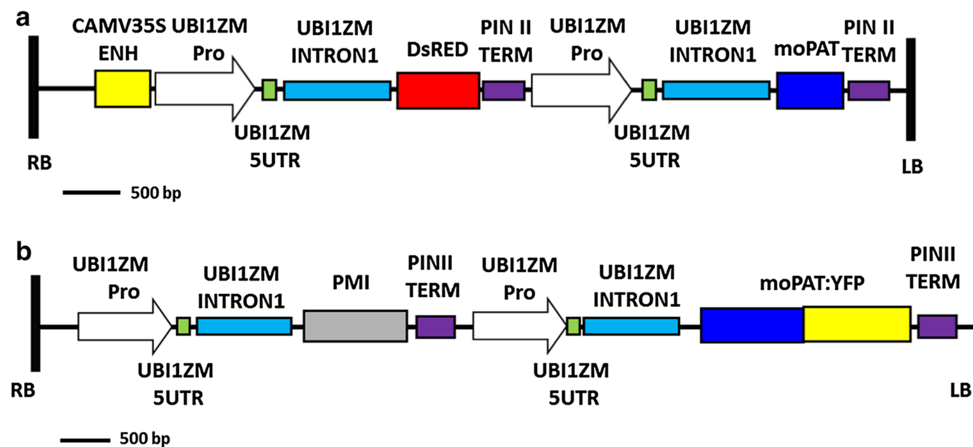

Fig. 1 Schematic diagram of PHP26650 (**a**) and PHP32269 (**b**) used for maize inbred transformation. RB, right border; CAMV35S ENH, CaMV35S enhancer; UBI1ZM Pro, maize ubiquitin 1 promoter; UBI1ZM 5UTR; maize ubiquitin 1 5′ UTR; UBI1ZM INTRON1, maize ubiquitin 1 intron; DsRED: *Discosoma* red fluorescent protein; PINII TERM, potato proteinase inhibitor II terminator; moPAT, maize codon-optimized PAT; LB, left border; PMI, phosphomannose isomerase; moPAT:YFP, moPAT translationally fused to YFP

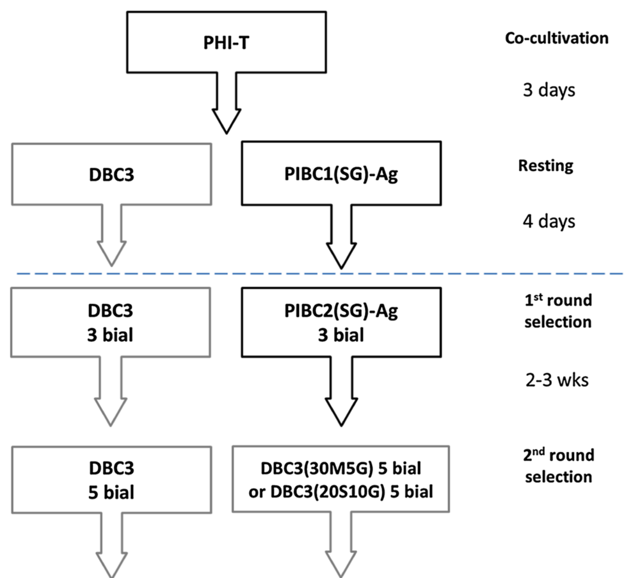

Fig. 2 Culturing schemes to test PH4CN transformation using bialaphos selection. Scheme 1 used DBC3 for 4 days in the resting step and selection steps whereas scheme 2 used PIBC1(SG)-Ag for 4 days in the resting step then PIBC2(SG)-Ag for 1st round and DBC3(30M5G) or DBC3(20S10G) for 2nd round selection with 3 and 5 mg/L bialaphos, respectively

Seika Kaisha, Yokohama, Japan) for *bar/PAT/moPAT* selection. Two to 3 weeks after 1st round selection, tissues were transferred to fresh DBC3 medium supplemented with 5 mg/L bialaphos and sub-cultured at 2–3 week intervals. At the 3rd round of selection, tissues were broken into small pieces and placed on the same medium containing bialaphos for further proliferation until a sufficient amount of tissue was obtained. Culturing scheme 2 is as follows: PIBC1(SG)-Ag, PIBC2(SG)-Ag with 3 mg/L bialaphos and DBC3(30M5G) or DBC3(20S10G) with 5 mg/L bialaphos was used for resting, 1st round selection and 2nd/subsequent selection, respectively; media compositions are described in Table 2. PIBC1(SG)-Ag medium is the same as PIBC2(SG)-Ag but with less BAP, 0.01 mg/L instead of 0.1 mg/L. For both schemes, GTs were then transferred to regeneration medium PHI-X (Zhao et al. 2000) containing 5 mg/L phosphinothricin (PPT) with the addition of 1.22 mg/L $CuSO_4 \cdot 5H_2O$ and incubated at 26 ± 2 °C under dim light for 2–3 weeks. Regenerating shoots were transferred to MSB rooting medium (Cho et al. 2014) containing 3 mg/L bialaphos and exposed to higher light intensity (50–150 mE/m^2 s, 16/8-h day and night photoperiod) for rooting. The plantlets were then transferred to soil.

Table 2 Compositions of media used for maize inbred transformation

Media name	Ingredients	References
PI	4.3 g/L MS basal salts, 2.39 g/L N6 macronutrients 10×, 1.68 g/L KNO, 0.6 mL/L B5H minor salts 1,000×, 6 mL/L 100 × NaFe EDTA for B5H, 0.4 mL/L 1,000 × Erikkson's vitamins, 6 mL/L 100 × Schenk and Hildebrandt vitamin stock, 0.2 mg/L thiamine-HCl, 1.98 g/L L-proline, 0.3 g/L vitamin assay casamino acids, 20 g/L sucrose, 0.6 g/L glucose, 0.8 mg/L 2,4-D, 1.2 mg/L dicamba, 3.5 g/L Phytagel, pH 5.8	Cho et al. (2014)
PIBC1/2/3(SG)-Ag	4.3 g/L MS basal salts, 2.39 g/L N6 macronutrients 10×, 1.68 g/L KNO, 0.6 mL/L B5H minor salts 1,000×, 6 mL/L 100 × NaFe EDTA for B5H, 0.4 mL/L 1,000 × Erikkson's vitamins, 6 mL/L 100×Schenk and Hildebrandt vitamin stock, 0.2 mg/L thiamine-HCl, 1.98 g/L L-proline, 0.3 g/L vitamin assay casamino acids, 20 g/L sucrose, 10 g/L glucose, 0.8 mg/L 2,4-D, 1.2 mg/L dicamba, 0.01, 0.1, and 0.5 mg/L BAP for PIBC1/2/3(SG)-Ag, respectively, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	Cho et al. (2014) for PIBC2(SG)-Ag
DBC3	4.3 g/L MS basal salts, 30 g/L maltose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L L-proline, 1.0 mg/L 2,4-D, 0.5 mg/L BAP, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	Cho et al. (1998)
DBC3(30M5G)	4.3 g/L MS basal salts, 30 g/L maltose, 5 g/L glucose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L L-proline, 1.0 mg/L 2,4-D, 0.5 mg/L BAP, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	
DBC3(20S10G)	4.3 g/L MS basal salts, 20 g/L sucrose, 10 g/L glucose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L L-Proline, 1.0 mg/L 2,4-D, 0.5 mg/L BAP, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	
DBC3 PMI	4.3 g/L MS basal salts, 5 g/L maltose, 12.5 g/L D-mannose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L L-proline, 1.0 mg/L 2,4-D, 0.5 mg/L BAP, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	Cho et al. (2014)
DBC3(30M5G) PMI	4.3 g/L MS basal salts, 5 g/L maltose, 5 g/L glucose, 12.5 g/L D-mannose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L L-proline, 1.0 mg/L 2,4-D, 0.5 mg/L BAP, 1.22 mg/L $CuSO_4 \cdot 5H_2O$, 3.5 g/L Phytagel, pH 5.8	

All media contain 100 mg/L cefotaxime

PHW0V, PH12BN, PH1CP1 and PH17R8 transformation

PHW0V, PH12BN, PH1CP1 and PH17R8 ears were collected from donor material described above. The IEs isolation and transformation process is the same as the one used for PH4CN transformation except the optimal IE size for these inbreds was 1.3–2.0 mm and the *Agrobacterium* suspension contained binary vector PHP32269. Once the 5 min sit in the *Agrobacterium* suspension was over, the suspension of *Agrobacterium* and IEs was poured onto PHI-T or modified PHI-T (mPHI-T) co-cultivation medium for one of 4 culturing schemes (Fig. 3); mPHI-T is PHI-T with addition of 1.22 mg/L $CuSO_4{\cdot}5H_2O$ and 0.1 mg/L BAP. Half of the IEs on PHI-T co-cultivation were put onto culturing scheme 1 which is PIBC2(SG)-Ag resting medium for 4 days, and the tissues were then transferred to DBC3 resting medium for 5–7 days before getting transferred to DBC3 PMI medium for 1st round mannose selection for 3–4 weeks, DBC3 PMI or DBC6 PMI for 2nd round selection and all subsequent selection steps. DBC3 PMI contains 1.0 mg/L 2,4-D and 0.5 mg/L BAP (Table 2) while DBC6 PMI contains 1.0 mg/L 2,4-D and 2.0 mg/L BAP. The remaining half of the IEs on PHI-T co-cultivation were put onto culturing scheme 2 which is PIBC3(SG)-Ag, the same as PIBC3(SG)-Ag but with a higher BAP amount (0.5 mg/L instead of 0.1 mg/). After incubation on resting medium for 4 days, the tissues were transferred to DBC3(30M5G), which is DBC3 medium supplemented with 5 g/L glucose for 5–7 days before getting transferred to DBC3(30M5G) PMI medium for 1st round selection, and DBC3(30M5G) PMI or DBC6(30M5G) PMI for 2nd round selection and all subsequent selection steps. DBC3(30M5G) PMI contains 1.0 mg/L 2,4-D and 0.5 mg/L BAP Table 2) while DBC6(30M5G) PMI contains 1.0 mg/L 2,4-D and 2.0 mg/L BAP. Half of the IEs on mPHI-T were put onto culturing scheme 3 which is PIBC3(SG)-Ag resting medium for 4 days, DBC3 medium for 5–7 days, DBC3 PMI or DBC6 PMI for 1st round selection and all subsequent selection steps. The remaining half of the IEs on mPHI-T were put onto culturing scheme 4 which is PIBC3(SG)-Ag for 4 days, DBC3(M5G) for 5–7 days, and DBC3(30M5G) PMI or DBC6(30M5G) PMI for 1st round selection and all subsequent selection steps. Tissues were sub-cultured at 2–3 week intervals for further proliferation until a sufficient amount of tissues was obtained. Plant regeneration was done as described above using PHI-X (Zhao et al. 2000) with no PPT and the addition of 1.22 mg/L $CuSO_4{\cdot}5H_2O$.

Transformation frequencies calculations and copy number analysis of transgenic plants

Transgenic events were confirmed using YFP expression through fluorescence microscopy and qPCR/PCR.

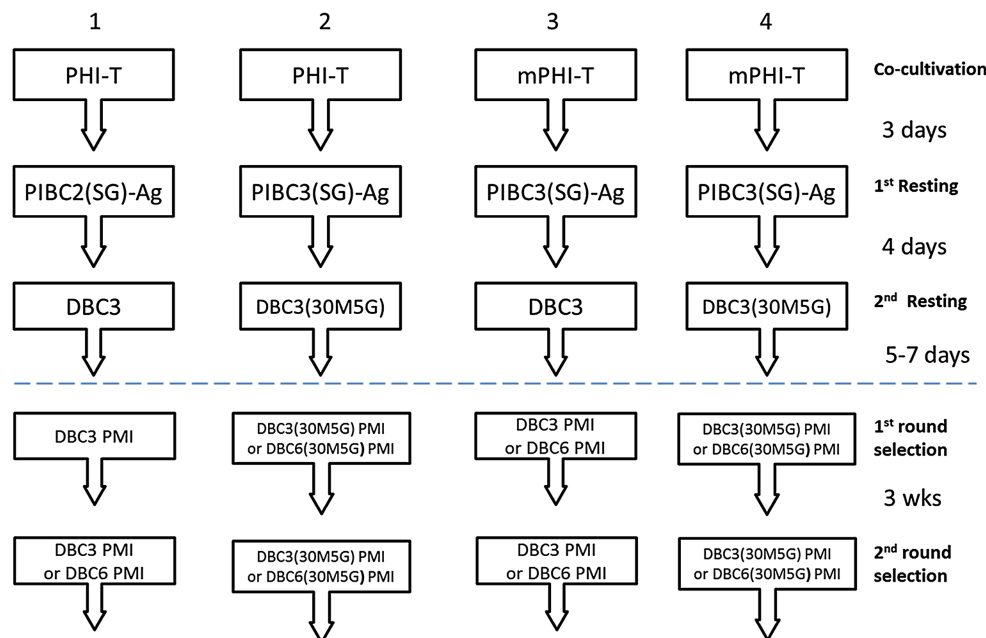

Fig. 3 Culturing schemes to test PH12BN, PHW0V, PH17R8 and PH1CP1 transformation using phosphomannose isomerase (PMI) for mannose selection. Scheme 1–2 starts with PHI-T as the co-cultivation medium then scheme 1 has PIBC2(SG)-Ag and DBC3 as 1st and 2nd round resting respectively before starting selection with DBC3 PMI while scheme 2 has PIBC3(SG)-Ag and DBC3(M5G) as 1st and 2nd round resting respectively before starting selection with DBC3(M5G)PMI or DBC6(M5G)PMI. Scheme 3 starts with modified PHI-T (mPHI-T) for co-cultivation, PIBC3(SG)-Ag for 1st round resting, DBC3 2nd round resting and then follows scheme 1 in selection steps. Scheme 4 starts with mPHI-T for co-cultivation and then follows scheme 2 in remaining steps

Transformation frequency (%) at the T_0 callus tissue level was measured 6 weeks after *Agrobacterium* infection and calculated as (# of IEs with transgenic sectors/# of IEs infected) × 100 %. Transgenic sectors were identified as actively growing tissue exhibiting DsRED or YFP expression. Multiple sectors formed on a single embryo were counted as one event. The transformation frequencies at the T_0 plant level was measured after regenerating T_0 plantlets and was calculated as (# of IEs regenerating plantlets/# of IEs infected) × 100 %. Multiple plantlets formed by a single embryo were counted as one event.

Genomic DNA from leaf tissue was extracted from T_0 plants as well as wild-type negative controls from each inbred by a leaf punch and were submitted for quality event (QE) analysis using qPCR. qPCR for copy number analysis of transgenes and binary vector backbone presence was performed following Wu et al. (2014). QE is defined as an event that is single copy for all transgenes and without *Agrobacterium* T-DNA backbone. For this study, QEs were considered to be *Agrobacterium* backbone-negative and single copy for the DsRed and moPAT genes for PHP26650 and the PMI, moPAT and YFP genes for PHP32269.

Herbicide painting test

In order to determine herbicide resistance (PAT expression) of transgenic plants, a section of leaf blade at the 3- to 5-leaf stage was painted using a cotton swab with a 0.5 % solution (v/v) of Basta® (Bayer CropScience, Monheim, Germany) solution (concentration of 200 g/L PPT) plus 0.1 % Tween 20. Plants were scored 10–14 days after herbicide application.

Results and discussion

Tissue culture medium modifications and optimization for PH4CN

Green regenerative tissues with a high percentage of totipotent cells are capable of sustained cell division and competence for regeneration over long periods. This was seen in a previously recalcitrant commercial elite maize inbred, PHR03 (Cho et al. 2014), as well as other recalcitrant commercial monocot crop varieties such as barley (Cho et al. 1998), wheat (Cho et al. 1999; Kim et al. 1999; Li et al. 2009) and Kentucky bluegrass (Ha et al. 2001). Improvement in shoot meristematic cultures was also observed in maize inbred B73 (Zhang et al. 2002) as well as barley and oat (Zhang et al. 1999) by adding additional copper to BAP-containing medium.

Table 3 Initial callus and green regenerative tissue (GT) induction on different culturing media and tissue quality and growth rate in PH4CN

Medium	PI	PIBC1-Ag	PIBC1 (SG)-Ag	PIBC2-Ag	PIBC2 (SG)-Ag	DBC3	DBC3 (30M5G)	DBC3 (20S10G)
Changes in ingredients	Standard for callus induction	PI + 0.01 mg/L BAP +1.22 mg/L $CuSO_4 \cdot 5H_2O$–$AgNO_3$	Same as PIBC1-Ag except containing 10 g/L glucose	Same as PIBC1-Ag except containing 0.1 mg/L BAP	Same as PIBC2-Ag except containing 10 g/L glucose	Standard for GT induction	Same as DBC3 except containing 5 g/L glucose	DBC3 medium – maltose + 20 g/L sucrose and + 10 g/L glucose
Callus/GT induction frequency[a]	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
Callus/GT induction surface area[b]	20–40 %	20–60 %	20–80 %	20–80 %	20–80 %	5–40 %	10–50 %	5–40 %
Callus/GT quality	+	+(+)	++	++ (+)	++ (+)	++	+++	++
Callus/GT growth rate	++	++ (+)	++++	+++	++++	+	++	++

IEs sized 1.5–2.2 mm derived from the same ear were placed onto each medium and incubated under the same conditions to test the effect of medium modifications. The tissue induction was measured 2 weeks after IE isolation

[a] Callus/GT induction frequency: # IEs inducing callus/GT from scutellum tissue/# IEs plated

[b] Callus/GT induction surface area: % range of induction surface area observed from IEs with callus/GT induction

Table 4 Molecular analysis data from transgenic plants of four elite commercial maize inbreds, PH4CN, PH12BN, PHW0V and PH17RB

	PH4CN[a]		PH12BN[b]		PHW0V[b]		PH17R8[b]	
	# of events	%	# of events	%	# of events	%	# of events	%
SC	7	21.9	32	30.8	12	29.3	3	37.5
SC BB+	0	0.0	5	4.8	2	4.9	1	12.5
SC BB−	7	21.9	27	26.0	10	24.4	2	25.0
MC	25	78.1	72	69.2	29	70.7	5	62.5
MC BB+	13	40.6	29	27.9	6	14.6	2	25.0
MC BB−	12	37.5	43	41.3	23	56.1	3	37.5
Total # of events analyzed	32		104		41		8	

SC single copy event, *MC* multiple copy events, *BB−* *Agrobacterium* backbone-negative, *BB+* *Agrobacterium* backbone-positive

[a] PHP26650 was used

[b] PHP32269 was used

In order to test and optimize the initial callus tissue and GT culture medium compositions for another elite maize inbred, PH4CN, eight media were compared (Table 3). PI medium is appropriate for callus induction due to the absence of BAP while DBC3 containing BAP is efficient for GT induction. Embryos survived with 100 % callus and/or GT induction on all media tested. When BAP and copper were added to PI, better callus/GT induction, quality and growth rate were observed. High glucose containing media, PIBC1(SG)-Ag and PIBC2(SG)-Ag, were best in terms of callus/GT induction surface area, quality and growth rate at the initial tissue induction stage (Table 3; Fig. 4). DBC3 had low callus/GT induction surface area and the initial growth rate was slow (Table 3). However, adding 5 g/L glucose to DBC3, DBC3(30M5G), resulted in good GT maintenance and proliferation after the initial tissue induction. Therefore, additional glucose was required for efficient PH4CN transformation. This differed from what was seen in PHR03 which did not need additional glucose for callus/GT proliferation after initial callus/GT induction (Cho et al. 2014). DBC3(20S10G) was poor for initial callus/GT induction and growth, but it was good for tissue maintenance and proliferation in PH4CN.

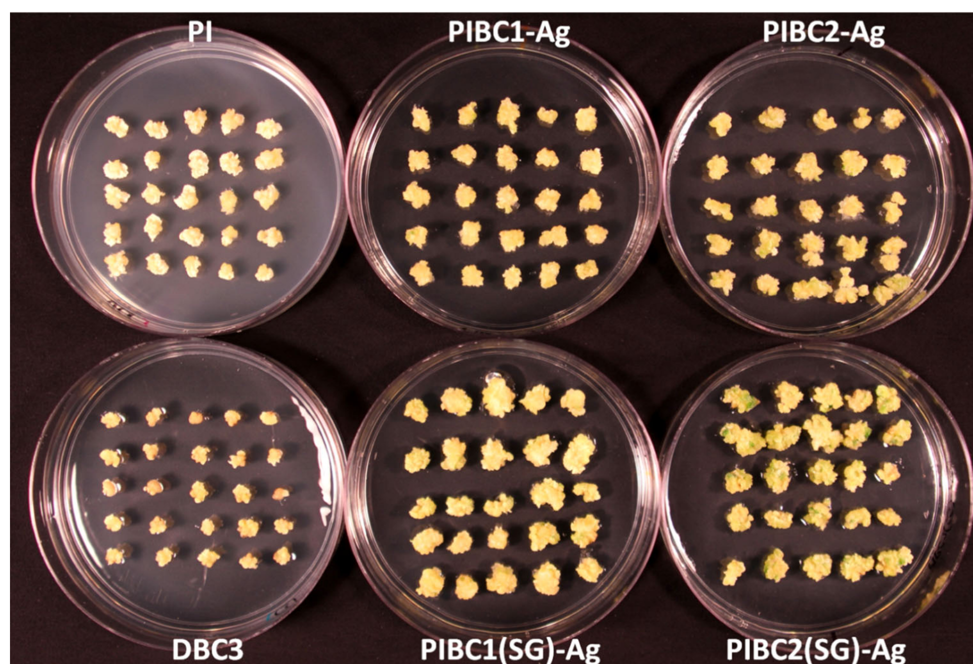

Fig. 4 Initial callus and green regenerative tissue induction from immature embryos of PH4CN on different culturing media. PI, PIBC1-Ag and PIBC2-Ag contain 0.6 g/L glucose while PIBC1(SG)-Ag and PIBC2(SG)-Ag contain 10 g/L glucose. DBC3 does not contain glucose. Photos were taken 3 weeks after immature embryo isolation on six different tissue induction media

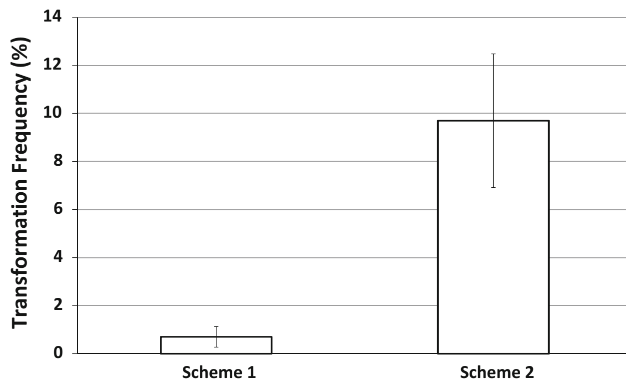

Fig. 5 Transformation frequencies of PH4CN at T_0 plant level using green regenerative tissue medium and bialaphos selection. Culturing schemes are from Fig. 2. Forty-four to 75 immature embryos per each culturing scheme were used for transformation. Each histogram represents the mean level (±standard error) from five replicates per culturing scheme

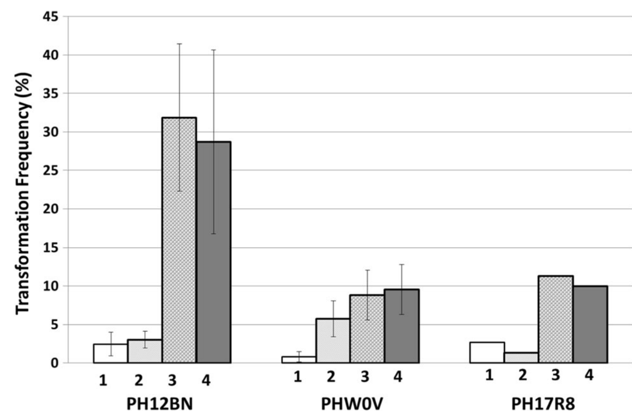

Fig. 6 Transformation frequencies at T_0 plant level in PH12BN, PHW0V and PH17R8 using 4 culturing schemes and phosphomannose isomerase (PMI) for mannose selection. Culturing schemes are from Fig. 3. PH12BN and PHW0V histograms represent the mean levels (±standard error) from 4 and 3 replicates per each culturing scheme, respectively, while PH17R8 histograms represent the mean level from 1 replicate per each culturing scheme

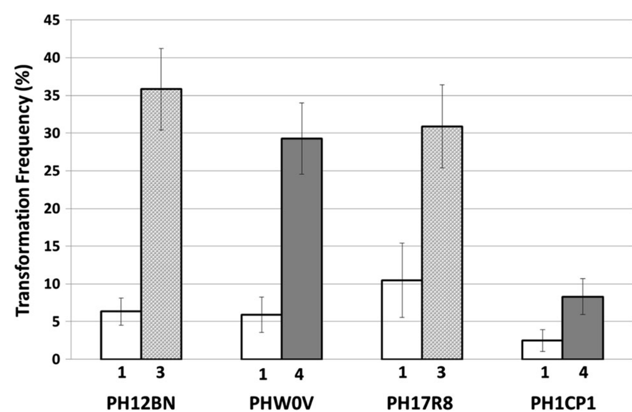

Fig. 7 Transformation frequencies at T_0 tissue level in PH12BN, PHW0V, PH17R8 and PH1CP1 using 2 culturing schemes and phosphomannose isomerase (PMI) for mannose selection. Culturing schemes are from Fig. 3. PH12BN, PHW0V, PH17R8, and PH1CP1 histograms represent the mean levels (±standard error) from 4, 7, 3 and 5 replicates per each culturing scheme, respectively

PH4CN transformation improvement using GT medium with additional glucose in the selection step

Initial transformation frequency of PH4CN with PHP24600 was only 0.7 % (8/310) at the T_0 plant level (Fig. 5) when using the same tissue culturing scheme as PHR03 transformation: DBC3 medium for 4 days in the resting step and bialaphos selection (Fig. 2; Cho et al. 2014). This culturing scheme resulted in a transformation frequency of 25.8 % at the T_0 plant level in PHR03 (Cho et al. 2014). When culturing scheme 2 was used with PIBC1(SG)-Ag for 4 days in the resting step, PIBC2(SG)-Ag for 1st round and DBC3(30M5G) or DBC3(20S10G) for 2nd round selection, transformation frequency was significantly improved to 9.7 % (29/299). A similar improvement in transformation frequency was also noted for PHR03, up to 57.1 % at the T_0 plant level, when PIBC2(SG)-Ag medium containing additional glucose was used as the resting medium (Cho et al. 2014). The addition of 5–10 g glucose per liter in selection steps was required for PH4CN because tissues did not proliferate well without the high glucose levels (Table 3; Fig. 4).

Improvement of PH12BN, PHW0V, PH17R8 and PH1CP1 transformation frequencies using modified co-cultivation medium and/or glucose-containing selection medium

Four different culturing schemes were used to improve transformation frequencies in 4 additional modern elite commercial maize inbreds, PH12BN, PHW0V, PH17R8, and PH1CP1 (Figs. 3; 6). Culturing scheme 1 is the culturing scheme described in Cho et al. (2014) that produced a transformation frequency up to 57.1 % at T_0 plant level for PHR03. However, this scheme produced only 2.4 % (5/205), 0.8 % (1/124) and 2.7 % (2/75) transformation frequencies at the T_0 plant level in PH12BN, PHW0V, and PH17R8, respectively (Fig. 6), and 0 % frequency was obtained in PH1CP1. The current study shows that the addition of cupric sulfate and BAP in the co-cultivation medium resulted in a dramatic increase in the T_0 plant level transformation frequencies to 31.9 % (73/229), 9.6 % (13/136) and 11.3 % (9/80) in all 3 inbreds, PH12BN, PHW0V and PH17R8, respectively, when compared to PHI-T which does not have BAP and high cupric sulfate (Fig. 6). Especially critical was the use of cupric sulfate and BAP in combination with high glucose in the selection medium

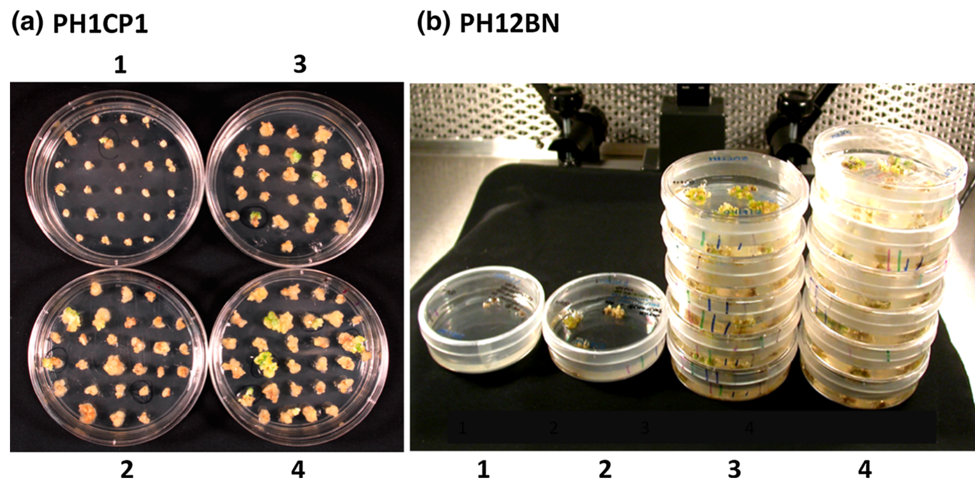

Fig. 8 Comparison of PH1CP1 (**a**) and PH12BN (**b**) tissue growth with phosphomannose isomerase (PMI) for mannose selection on 4 different culturing schemes. Culturing schemes are from Fig. 3. **a** The photo was taken on 2nd round selection (6 weeks after *Agrobacterium* infection) for PH1CP1. **b** About fifty-five IEs of PH12BN per each culturing scheme were initially infected with AGL1 containing PHP32269 and the surviving tissues on selection medium were maintained and proliferated. The photo was taken on 3rd round selection (8 weeks after *Agrobacterium* infection)

Fig. 9 Transgenic green regenerative tissue (GT), shoots, roots and seeds derived from PH12BN. **a** Transgenic GT expressing YFP, **b** regenerating transgenic plantlets, **c** transgenic root and shoot expressing YFP, **d** phenotype of T_0 plants at 2 different stages, **e** T_1 ear showing good seed set, **f** T_1 seed expressing YFP and **g** Two leaves on the *top* represent the wild-type after Basta® treatment while two leaves on the *bottom* from the *transgenic line* PH12BN showed Basta® resistance 10 days after Basta® painting. *Bar* = 2 mm

which resulted in the highest transformation frequency for PHW0V (Fig. 6) and PH1CP1. This combination increased the proliferation of high quality, regenerable tissue (Figs. 7; 8) similar to PH4CN (Fig. 4). PH12BN and PH17R8 did not require additional glucose in the selection medium to improve transformation frequency (Fig. 6). Culturing scheme 1 was further compared to the best culturing scheme for that specific inbred to compare transformation frequency at the T_0 tissue level (Fig. 7). Consistently all 4 inbreds showed significantly higher transformation frequencies at the T_0 tissue level with culturing scheme 1 versus 3 or 4: 6.3 % (13/205) versus 35.8 % (82/229), 5.9 % (20/339) versus 29.3 % (99/338), 10.5 % (20/191) versus 30.9 % (59/191), 2.5 % (9/360) versus 8.3 % (30/360) in PH12BN, PHW0V, PH17R8 and PH1CP1, respectively. It was observed that the addition of high cupric sulfate and BAP in the co-cultivation medium improved formation of more transgenic sectors from YFP-expressing foci in all 4 inbreds than without BAP and additional copper. PH12BN, PHW0V and PH1CP1 T_0 plants transformed with PHP32269 had the gene properly inserted and actively expressed as confirmed by YFP visual marker expression (Fig. 9) and had normal phenotype. Plants that were successfully transformed had normal height, good tassel quality, and excellent seed set comparable to wild-type equivalents. YFP expression and bialaphos resistance was stably transmitted to T_1 progeny plants (Fig. 9).

In PH4CN 22 % (7/32) of the T_0 events were QEs for PHP26650 (Table 4). For the three other inbreds, PH12BN, PHW0V and PH17R8, the QE ratios were 26 % (27/104), 24 % (10/41) and 25 % (2/8), respectively (Table 4). This was similar to the result from PHR03 using AGL1 which was 26 % (Cho et al. 2014). Multi-copy event ratios in the 4 inbreds were in the range of 63–78 % and *Agrobacterium* backbone-positive ratios were 20–41 % (Table 4).

In conclusion, the present study demonstrates a significant improvement in the transformation of multiple modern elite commercial maize inbreds by modifying three key elements, glucose, cupric sulfate and BAP, in the co-cultivation, resting and selection steps. We observed that the amount/ratio of these three components needed to be optimized for each inbred. The results in this study can be applied to other recalcitrant elite commercial maize inbreds.

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

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