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Full Length Research Paper

Establishment of a high-efficiency plant regeneration and transformation system for the elite maize inbred lines from three heterotic groups

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Maize (*Zea mays* L.) is one of the most important crops in the world and its agronomic traits could be improved by genetic transformation with desirable genes. A successful transformation must depend on a high-efficiency *in vitro* plant regeneration and genetic transformation system. In our studies, six media compositions were used to induce callus from the immature zygotic embryos of seven maize inbred lines and LM6 was proved the best callus induction media, with high callus induction percentage (CIP) and callus quality. Furthermore, four phytohormones were analyzed on their effects on callus induction, the results indicated that 2,4-dichlorophenoxyacetic acid (2,4-D) played an important role in callus initiation, but, 3.3 mg/l Dicamba could provide higher embryogenic callus induction percentage (EIP) than 2 mg/l 2,4-D; both 6-benzyl aminopurine (6-BA) and kinetin (KT) could decrease CIP, EIP and callus quality at the concentration level of 0.2 mg/l. The experimental result also revealed that media LM6-CI was preferable to LM6 in callus induction. The selected LM6-CI media, together with other two media (LM6-EI and LM6-PR), were used to identify 18 elite maize inbred lines from three heterotic groups on their tissue culture characteristics, as a result, the eight materials, including 3189/4380, 4380/Sanzong5, 8103, Xianzao17, 18-599(red), 501, 178 and Ji53 belonging to group Reid or Compound Germplasm, presented higher CIP, EIP and plant regeneration percentage (PRP) than others; the four materials, including Huangzao4, Huangye4, Jing24 and Ji853 from Tangsipingtou group, were not easy to be differentiated into plants, in spite of high CIP. Maize inbred line 18-599(red) as a representative was further used to establish genetic transformation system, its embryogenic calli, initiated from immature zygotic embryos, were transformed with the plasmid p35SBarNos harboring *Bar* gene by microprojectile bombardment, after selection and differentiation culture, partial bombarded calli were regenerated into green plantlets and further fertile plants. The results of molecular identification for fertile regenerated plants showed that *Bar* gene had been integrated into maize genome and the transformation frequency was high up to 66.7%. All these results were beneficial for the studies on *in vitro* plant regeneration and genetic transformation in plant.

Key words: Maize (*Zea may* L.), tissue culture, *in vitro* plant regeneration, genetic transformation.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important crops in the world and its agronomic traits could be improved by genetic transformation with desirable genes, but a successful transformation must depend on a high-efficiency *in vitro* plant regeneration and genetic transformation system (Frame et al., 2006; Binott et al., 2008). With the rapid development of tissue culture techniques, many types of explants, including gametic embryo, immature zygotic embryo, mature zygotic embryo and leaf tissue had been successfully regenerated into plants by tissue culture (Aulinger et al., 2003; Huang and Wei, 2004;

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Table 1. Culture media and their components (mg/l).

Media	Components[a]														
	MaIC	MiIC	MOC	2,4-D	Dic	6-BA	KT	NAA	PPT	Pro	CH	Ino	SN	Suc	AP
LM1	N6	N6	N6	2						690	500	100	10	30,000	7,000
LM2	N6	B5	B5	2						690	500	100	10	30,000	7,000
LM3	MS	MS	MS	2						690	500	100	10	30,000	7,000
LM4	8114	B5	B5	2						690	500	100	10	30,000	7,000
LM5	MS	B5	B5	2						690	500	100	10	30,000	7,000
LM6	N6	B5	RTU	2						690	500	100	10	30,000	7,000
LM7	N6	B5	RTU	2		0.2				690	500	100	10	30,000	7,000
LM8	N6	B5	RTU	2			0.2			690	500	100	10	30,000	7,000
LM6-CI	N6	B5	RTU		3.3					690	500	100	10	30,000	7,000
LM6-EI	N6	B5	RTU			0.5				690	500	100		60,000	7,000
LM6-PR	N6	B5	RTU							690	500	100		30,000	7,000
LM6-RI	1/2 MS							0.5						20,000	6,000
LM6-SC1	N6	B5	RTU		3.3				6	690	500	100	10	30,000	7,000
LM6-SC2	N6	B5	RTU		3.3				10	690	500	100	10	30,000	7,000
LM6-SC3	N6	B5	RTU		3.3				15	690	500	100	10	30,000	7,000

[a]MaIC, macro-inorganic components; MiIC, micro-inorganic components; MOC, macro-organic components; Dic, dicamba; 6-BA, 6-benzyl aminopurine; KT, Kinetin; NAA, 1-naphthalene acetic acid; PPT, phosphinothricin; Pro, L-Proline; CH, casein hydrolysate; Ino, Inositol; SN, silver nitrate; Suc, sucrose; AP, agar powder.

Ahmadabadi et al., 2007). But at present, the most popular explant is still immature zygotic embryo in maize transformation, owing to simple inoculation operation and facile callus induction (Binott et al., 2008).

Many factors can affect callus induction and differentiation, of which genotype and media composition are the two most important factors (Binott et al., 2008). For example, Hodges et al. (1986) reported that maize inbred line A188 had better regeneration ability than the others under same tissue culture conditions. Thus, screening of genotypes for *in vitro* plant regeneration is always a very important research task. Regarding the optimization of media composition, there have been many elite media compositions in literatures (Du et al., 2007; Binott et al., 2008; Zhang et al., 2008), but to be mentioned, any media containing specific reagents was only suitable for limited materials. Thus, to develop a new media for given materials is always necessary and significant.

One of the most important motives for tissue culture was to introduce desirable genes into acceptor cells for improving some characters of plant. At present, many methods have been successfully used for introduction of foreign genes (Aulinger et al., 2003; Frame et al., 2006) and microprojectile bombardment is one of the most efficient methods. It has been widely used for establishing genetic transformation system in maize, due to high transformation frequency and simple operation (Theodore et al., 1989; Elumalai et al., 2009).

Chinese maize germplasm could be classified into five herterotic groups, including Reid, Lancaster, Tangsipingtou, Ludahonggu and Compound Germplasm. Many inbred lines within the five groups have been used to produce elite hybrids in maize production, but, most of them had not been studied on the characteristics of tissue culture and transformation.

In this present study, 15 media compositions were used to evaluate 18 maize elite inbred lines from three heterotic groups on their tissue culture characteristics and 18-599 (red) was transformed with plasmid p35SBarNos harboring *Bar* gene by microprojectile bombardment. The objectives were to (1) obtain an elite media composition used for callus induction, (2) evaluate different elite inbred lines on their tissue culture characteristics and (3) establish a high-efficiency genetic transformation system.

MATERIALS AND METHODS

Plant materials, culture media and vector

The 18 elite maize inbred lines involved in our experiments were from three heterotic groups. Huangzao4, Jing24, Huangye4 and Ji853 belonging to Tangsipingtou group; 3189/4380, 478, 66478 and A632 from Reid group; Han21, 4380/sanzong5, 8103, Xiangzao17, 18-599(red), 178, 501, Ji53, Hai92-1 and C123 belonging to Compound Germplasm group.

15 media were designed and applied in our studies and their components were shown in Table 1. LM1, LM2, LM3, LM4, LM5, LM6, LM7, LM8 and LM6-CI were used for callus induction; LM6-EI, LM6-PR and LM6-RI were employed in embryoid initiation, plant regeneration and rootage induction, respectively; LM6-SC1, LM6-SC2 and LM6-SC3 were applied during selection culture of transformed calli.

The plasmid vector p35SBarNos was used for delivery of *Bar* gene into 18-599(red), under control of *CaMV35S* promoter and *Nos* terminator (Figure 1).

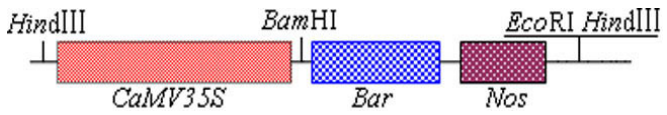

Figure 1. Line diagram of plasmid p35SBarNos (4.4 kb).

Callus induction

All the maize inbred lines were planted at the farm of Beijing Agro-biotechnology Research Center, Beijing City, P. R. China. 11 d after self pollination, their ears were removed and surfacely sterilized with 70% ethanol for 10 min, then, the bracts were got rid of. Subsequently, the immature zygotic embryos, possessing 1 - 2 mm in diameter, were aseptically excised with fine forceps and placed on callus initiation media, with scutellum side up. According to our demands, 150 immature zygotic embryos for each experimental treatment were averagely inoculated on 10 petri plates with 90 mm in diameter.

Screening of callus induction media

To select an elite callus induction media, six media compositions (LM1, LM2, LM3, LM4, LM5 and LM6) were used to analyze seven inbred lines (Huangzao4, Han21, 8103, Xianzao17, 18-599(red), 501 and J53). The immature zygotic embryos of these materials were inoculated to callus induction media, one month later, callus induction percentage (CIP) for the 42 experimental treatments was calculated and the obtained data were further analyzed by multiple comparisons using LSD method of SPSS11.5 software (www.SPSS.com).

Analysis of phytohormones on their effects in callus induction

To realize the effects of 2,4-dichlorophenoxyacetic acid (2,4-D), Dicamba, 6-benzyl aminopurine (6-BA) and kinetin (KT) on callus initiation, six inbred lines including Huangzao4, Han21, 8103, Xianzao17, 18-599(red) and 501 were inoculated onto four callus induction media (LM6, LM7 LM8 and LM6-CI), 30 d later, the CIP and embryogenic callus induction percentage (EIP) of each experimental treatment were calculated and analyzed.

Identification of different genotypes on their tissue culture characteristics

To identify tissue culture characteristics of different maize materials, all the 18 inbred lines involved in our studies were inoculated onto LM6-CI media, 30 d later, their CIP and EIP were calculated and the shape and color of calli were investigated carefully. After one-month subculture on new LM6-CI media, the embryogenic calli were selected and transferred onto LM6-EI media for embryoid induction, one week later, transferred onto LM6-PR media to be differentiated into plantlets and their plant regeneration percentage (PRP) was calculated and evaluated.

Transformation, selection and plant regeneration of 18-599(red)

The elite inbred line 18-599(red) was further used to establish genetic transformation system. Its embryogenic calli, obtained through above procedure, were placed on LM6-CI media appended 0.4 mol/l mannitol for hypertonic treatment for 4 h, then, bombarded with plasmid p35SBarNos harboring *Bar* gene by microprojectile bombardment. Microcarriers (1.1 µm gold particles) were prepared and coated with plasmid DNA according to the protocol by Frame et al. (2000), the embryogenic calli were bombarded by employing the Biolistic PDS-1000/He Particle Delivering System (Biorad, USA) as described in the manufacturer's instructions, with 1100 psi helium pressure and a target distance of 6 cm. 24 h after bombardment, the bombarded calli were transferred onto LM6-CI media for renewing livability, 7 d later, transferred to selection media for obtaining resistant calli against phosphinothricin (PPT). According to the lethal concentration (6 mg/l PPT) determined by the primary experiment using the embryogenic calli of untransformed 18-599(red), the transformed calli were successively cultured on LM6-SC1, LM6-SC2 and LM6-SC3 media, each for 28 d. Subsequently, the resistant calli were placed on LM6-EI media for inducing embryoids, 7 d later, transferred to LM6-PR media to generate green plantlets. The generated green plantlets were placed on LM6-RI media for shooting for about two weeks, well developed plantlets were transferred to flowerpots containing mixture of 3:1 vermiculate and sterile soil for acclimatization, further two weeks later, the live seedlings were transplanted into field.

Molecular identification of transgenic plants

Polymerase chain reaction (PCR) analysis was used to confirm the presence of *Bar* gene in the plants generated from transformed calli. Total DNA was extracted from maize leaves using CTAB method (Allen et al., 2006) and the primers, used to amplify *Bar* gene, were designed as follows:

The forward primer: 5'-ATGAGCCCAGAACGACGCCC-3';
The reverse primer: 5'-CTAAATCTCGGTGACGGGCAG-3'.

PCR reaction was designed as follows: 50 µl mixture contains 5 µl 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH8.3), 4 µl dNTP (2.5 mM each), 1 µl *Taq* DNA polymerase from Takara Biotechnology Co., LTD (5 U/µl), 1µl forward primer (20 µM), 1 µl reverse primer (20 µM), 1 µl plate DNA (100 ng/µl) and 37 µl sterilized ddH_2O. Thermal-cycle parameters were set according to the following procedure: commencing with the initial denaturation step at 94℃ for 4 min. Followed by 25 cycles consisting of denaturation at 94℃ for 1 min, annealing temperature at 55℃ for 0.5 min and extending at 72℃ for 1 min. And then, 10 min at 72℃ was used for final extension. The PCR products were separated on 1.0% agarose gel contained 0.05% ethidium bromide (EtBr) in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH8.0). The untransformed 18-599(red) was used as control.

RESULTS

Screening of callus induction media

Six induction media were used to induce callus from seven genotypes, the results showed that the average CIP on LM6 media was highest (Table 2), followed by LM1 media, the calli on LM1 and LM6 media displayed straw yellow, friable and fast-growing. While, the calli induced on LM2, LM3, LM4 and LM5 media behaved brown, humid and compact, even, some white roots occurred on the surface of partial calli. These results revealed that LM6 was the best media in maize callus induction.

Furthermore, it could be found that different genotypes had different CIP on same induction media. Based on the

Table 2. The CIP of seven genotypes on six media (%).

Genotypes	Media					
	LM1	LM2	LM3	LM4	LM5	LM6
Huangzao4	9.5	8.9	7.3	7.5	8.6	10.0
Han21	100	100	100	100	100	100
8103	60.3	57.4	59.7	49.8	52.1	65.0
Xianzao17	74.7	69.3	57.8	61.9	67.3	76.6
18-599(red)	71.1	77.0	73.9	75.2	76.8	71.5
501	69.7	70.3	53.3	60.9	57.1	71.1
Ji53	91.1	73.4	70.5	60.9	100	90.3
mean	68.1	65.2	60.4	59.5	66.0	69.2

Table 3. The multiple comparisons between seven genotypes.

Genotypes	Average CIP (%)	Significance level	
		0.05	0.01
Han21	100.0	a	A
Ji53	81.0	b	B
18-599(red)	74.3	b	BC
Xianzao17	67.9	c	C
501	63.7	cd	C
8103	57.4	d	C
Huangzao4	8.6	e	D

data in Table 2, the seven genotypes were further analyzed on their CIP by multiple comparisons. The results showed that Han21 had the highest average CIP (Table 3), all its immature zygotic embryos could be induced on all the six media. While, Huangzao4 had only 8.6% of average CIP, lower than the other six genotypes at the 0.01 of significance level. These results suggested that genotype was an important influencing factor on callus initiation.

Analysis of phytohormones on their effects in callus induction

The four media, including LM6, LM6-CI, LM7 and LM8, were used to study phytohormone effects in callus initiation and the results were listed in Table 4. LM6-CI media provided the highest average CIP and EIP, followed by LM6. The calli induced via LM6-CI and LM6 media behaved straw yellow, friable and fast-growing. While, the two media LM7 and LM8 provided lower average CIP and EIP, besides, some white shoots could be found in partial calli on the two media. These results demonstrated that 2,4-D played an important role in callus initiation, but, 3.3 mg/l Dicamba was better than 2 mg/l 2,4-D, with higher CIP and EIP. Both 6-BA and KT could decrease CIP, EIP and callus quality at the concentration level of 0.2 mg/l. From the experiment, LM6-CI was proved better than LM6 in callus induction.

Identification of different genotypes on their tissue culture characteristics

The selected LM6-CI media was further used to evaluate 18 maize inbred lines from three heterotic groups on their tissue culture characteristics, as a result, all the materials but Huangzao4 could easily be induced (Table 5). For most materials, high EIP determined high PRP, for example, 4380/Sangzong5 possessed over 90% of EIP, its PRP was also high up to 60.0%. Few materials possessed quite high CIP and low EIP, for instance, Jing24 had over 90% of CIP, while its EIP was less than 9.0%. To be noticed, these materials, including 66478, A632, Ji853, Huangye4, Jing24, Hai92-1 and C123, could not be differentiated into plants, in spite of high CIP (over 80%). Han21 was a special genotype, although its EIP was only 19.6%, all embryogenic calli could be regenerated into plants.

Transformation and regeneration of 18-599(red)

18-599(red) was used to establish transformation system. In this experiment, total 500 embryogenic calli were transformed by microprojectile bombardment. After three-month selection culture, 295 calli, showing resistance to PPT, were transferred to LM6-EI media for inducing embryoids and further transferred to LM6-PR to be differentiated into green plantlets. As a result, 180 of 295 calli could be regenerated into green plantlets, the PRP value was over 60%. After further rooting and acclimatization culture, 34 young plants with healthy roots were transplanted to field, finally, 9 plants grown up and bore seeds.

Molecular identification of transgenic plants

The results of PCR identification for the 9 fertile regenerated plants were showed in Figure 2, only 6 presented the target fragment, about 500 bp in length. The other three, same with the untransformed 18-599(red) as control, did not displayed bands at all. These results revealed that *Bar* gene has been integrated into maize genome and the transformation frequency was high up to 66.7%.

Table 4. The influence of phytohormones on callus induction.

Genotypes	LM6		LM6-Cl		LM7		LM8	
	CIP (%)	EIP (%)	CIP (%)	EIP (%)	CIP (%)	EIP (%)	CIP (%)	EIP (%)
Huangzao4	9.0	1.0	7.9	1.0	6.7	0	7.8	0
Han21	100	19.0	100	20.0	100	0	100	0
8103	65.8	40.0	75.0	45.0	66.7	17.8	59.9	13.3
Xianzao17	75.0	56.2	87.7	59.0	63.6	2.0	68.2	37.9
18-599(red)	70.5	33.3	83.9	48.5	56.1	3.5	47.8	5.1
501	69.8	46.0	81.8	51.1	57.3	6.1	60.9	17.9
Mean	65.0	29.4	72.7	37.4	58.4	4.9	57.4	12.4

Table 5. The characteristics of callus induction and plant regeneration of different genotypes.

Genotypes	Frequency of callus, embryogenic callus and plant regeneration (%)			Genotypes	Frequency of callus, embryogenic callus and plant regeneration (%)		
	CIP	EIP	PRP		CIP	EIP	PRP
3189/4380	86.0	63.9	59.8	4380/Sanzong5	100	93.8	60.0
478	58.5	52.3	9.0	8103	75.0	45.0	57.0
66478	86.2	30.0	0	Xianzao17	87.7	59.0	50.0
A632	83.3	16.7	0	18-599(red)	83.9	48.5	30.4
Huangzao4	7.9	1.0	0.5	501	81.8	51.1	30.1
Ji853	100	60.0	0	178	83.9	43.7	29.8
Huangye4	91.3	51.6	0	Ji53	91.9	71.8	29.5
Jing24	94.8	9.0	0	Hai92-1	68.2	50.0	0
Han21	100	19.6	100	C123	86.2	12.1	0

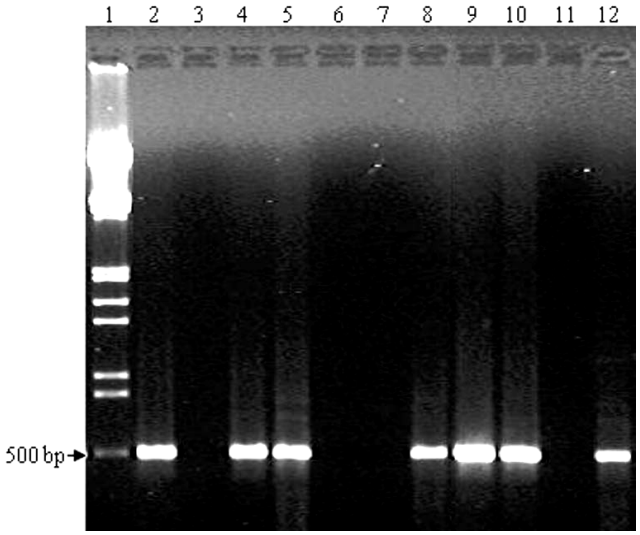

Figure 2. PCR analysis of transgenic lines. Lane 1, DNA marker; lane 2, plasmid p35SBarNos; lane 3, untransformed 18-599(red); lanes 4-12: transformed 18-599(red).

DISCUSSION

Media composition is one of the most important factors affecting maize tissue culture (Frame et al., 2006; Binott et al., 2008). N6, B5, MS, 8114 and RTU were several classical media, at present, most of media applied widely were derived from them (Li et al., 1999; Sairam et al., 2003). Although many elite media were documented, the application scope of each media was limited (Binott et al., 2008). Thus, to develop a special media, suitable for given genotypes, was very necessary and significant. In our experiment, six media (LM1, LM2, LM3, LM4, LM5 and LM6) were designed and used to induce callus from immature zygotic embryos of seven genotypes and LM6 was proved the best, with the highest CIP and best callus phenotypes.

Phytohormones were the key reagents within media, for example, 2,4-D, a plant hormone applied widely, has an important function in callus induction (Wang et al., 2001; Yuan et al., 2001). In our study, besides 2,4-D, Dicamba was also used for callus induction. The results showed that 2,4-D played an important role in callus initiation, but, 3.3 mg/l Dicamba was better than 2 mg/l 2,4-D, with higher CIP and EIP. 6-BA and KT were two important plant growth regulators and often applied at the stage of plant regeneration in tissue culture, due to promoting callus differentiation (Ma, 2007). Some studies also showed that they could increase callus induction frequency (Hongchang et al., 2006; Li et al., 2007). But, in our results, both of them decreased CIP and callus quality and restrained callus growth. In addition, the experiment

Table 6. Maize inbred lines possessing elite tissue culture characteristics.

Heterotic groups	Genotypes	References
Reid	3189/4380, A188, B73, 7922 , 4112	Hodges et al. 1986; Yuan et al. 2001; Wang et al. 2001.
Lancaster	Oh43, C103, Mo17, Zi330	Du et al. 2002; Wang et al. 1989.
Ludahonggu	E28, Zheng22, Lujiukuan, 340	Wang et al. 2001; Du et al. 2002; Li et al. 1999.
Compound Germplasm	18-599(red), 18-599(white), 4380/sangzong5, 8103, Xianzao17, 501, 178, Ji53 and Qi319	Wang et al. 1989; Xia et al. 2001; Sun et al. 2007.

results revealed that LM6-CI was a preferable callus induction media to LM6.

Genotype is another important influence factor in tissue culture, different genotypes showed variation on same media (Yuan et al., 2001; Huang and Wei, 2004). To evaluate the tissue culture characteristics of different genotypes, LM6-CI, selected from nine callus induction media, was used to analyze 18 inbred lines from three herterotic groups. As a result, the eight genotypes, including 3189/4380, 4380/Sanzong5, 8103, Xianzao17, 18-599(red), 501, 178 and Ji53, had high CIP, EIP and PRP. According to this and previous results, the 22 genotypes were regarded as better materials, suitable for *in vitro* plant regeneration and genetic transformation (Table 6). Whereas, the four materials from Tangsipingtou group, including Huangye4, Huangzao4, Jing24 and Ji853, were not easy to be differentiated into plants, in spite of high CIP.

One of the most important objectives for plant tissue culture was to establish a transformation system (Cho et al., 2004; Shirgurkar et al., 2006), to this date, many related studies had been reported in maize (Aulinger et al., 2003; Zhao and Ranch, 2006; Ahmadabadi et al., 2007). But, to be noticed, any transformation system was only suitable for limited genotypes. Thus, the inbred line 18-599(red) as a representative was used to establish genetic transformation system. PCR results demonstrated that foreign *Bar* gene has been integrated into maize genome, this suggested that a genetic transformation system had been successfully established. Here stepwise protocol reported was beneficial for other transgenic study in plant.

In summary, six different media were used to analyze seven genotypes and LM6 was proved the best induction media in callus induction. Four phytohormones were evaluated on their functions in callus initiation, the results showed that 2,4-D played an important role in callus initiation, but, 3.3 mg/l Dicamba was better than 2 mg/l 2,4-D, with higher CIP and EIP. Both 6-BA and KT could decrease CIP, EIP and callus quality at the concentration of 0.2 mg/l. 18 elite inbred lines from three heterotic groups were studied on their tissue culture characteristics, as a result, 8 belonging to group Reid or Compound Germplasm presented high CIP, EIP and PRP; while, the four genotypes belonging to Tangsipingtou group could hardly be differentiated into plants, in spite of high CIP. 18-599(red) was used for transformation, PCR identification for fertile regenerated plants demonstrated that foreign *Bar* gene had been integrated into maize genome. All these results were beneficial for the studies on *in vitro* plant regeneration and genetic transformation in plant.

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Abbreviations: CIP, callus induction percentage; **EIP,** embryogenic callus induction percentage; **PRP,** plant regeneration percentage; **PPT,** phosphinothricin; **PCR,** polymerase chain reaction; **6-BA,** 6-benzyl aminopurine; **KT,** kinetin; **NAA,** 1-naphthalene acetic acid.

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